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Human milk, nutrition and infant development

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Human milk, nutrition and infant development

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Editorial: Human milk, nutrition and infant development

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Editorial on the Research Topic
Human milk, nutrition and infant development

Breast milk is the gold standard for infant nutrition and feeding. Human milk is composed of a thousand components with nutritional functions and some of them as additionally bioactive properties. Breast milk composition differs between mothers due to maternal background, immunity, nutrition, lifestyle, and other confounding factors. In addition, the same mother's milk composition varies over time; colostrum contains the highest level of active proteins compared to transitional and mature breast milk to provide maximal immunity to the newborn. Indeed, the levels of bioactive proteins and macronutrients are higher in preterm milk than in full-term milk to promote their development and compensate for their immaturity. Breast milk composition is also affected by the mother's diet. Although milk proteins and carbohydrates are only slightly influenced, there is a strong correlation between dietary lipids and breast milk, as well as minerals, vitamins, and trace elements. Besides these factors, other situations, globally considered as the "exposome," can have also an influence on human milk composition. The discovery of new components in the mother's milk remains challenging due to the determination of the specific and accurate roles in infant growth and protection. The composition of human milk is even more complex since the evolution of different metabolomic techniques, which provide a very complete and up-to-date nutrient-by-nutrient record of breast milk.

This Research Topic compile compendium of 26 up-to-date original research and review articles focused on several properties of breast milk, including nutrition, immunity, and child development. In addition, some articles describe the implications of incorporating constituents to design breast milk substitutes as well as the use of donor human milk to compensate for the lack of breast milk/breastfeeding, especially for vulnerable premature neonates.

First of all, three interesting articles highlight the importance of breastfeeding and how their practices can vary widely across different cultures, with traditions and beliefs. These distinct cultures lead to shaping how, when, and for how long mothers breastfeed their infants. Whereas, the research done by Rodrigues et al. shows how relevant are the demographic, health, and economic factors in the feeding practices in three Latin American countries in three decades, Negesse et al. describes the influence of living or not in a secure

area, among other factors, on the exclusive breastfeeding practice. Finally, the relationship between breastfeeding duration and Body Mass Index (BMI) has been the subject of several studies, particularly in relation to maternal and child health. Sun et al. describe in this Research Topic their findings in a 9-year population-based study that highlight the importance of breastfeeding to reduce childhood overweight/obesity and prevent diseases, both in clinical and public health settings.

Another important group of articles summarizes the wide variety of bioactive components present in breast milk and their role in both maternal and infant health.

Fat in human milk primarily consists of triglycerides, which make up about 98% of the total fat content and are forming the Fat Globule Core. The milk fat globule membrane (MFGM) contributes to 0.2–2% of total fat in breast milk and has evidenced potential benefits for brain development. For that reason, Zhang et al. studied its effects in piglets, demonstrating its role in enhancing the connection of white matter fiber trace and improving neonatal piglets' learning and memory abilities. In this subject but at human level, another article within this Research Topic describes a randomized, double-blind, controlled clinical trial that showed that the combination of phospholipids and long chain polyunsaturated fatty acids supports neurodevelopmental outcomes in infants (Ren et al.). The fat necessity for this and other effects has led to the development and large-scale production of human milk fat analogs, as is the case of the study performed by Zhou et al. based on fermentation of microalgae. In addition, in breast milk, as in other natural sources, unsaturated fatty acids can have either cis or *trans* configuration double bond in their chain with distinct physiological effects. Hatem et al. revise in their article that trans isomeric fatty acids in human milk could impact infant health and development; but since WHO rules and improvement of Hydrogenated oils the *TFAs* human milk levels is <1% of fatty acids, insuring safety and positive outcome. Overall, the relationship between fat milk composition and infant's development is guaranteed. Omega 3 and particularly EPA, DHA reduces inflammatory, obesity, cancers, but also infant neurodevelopment and visual function. Maternal docosahexaenoic acid supplementation during lactation improves exercise performance, enhances intestinal glucose absorption and modulates gut microbiota in weaning offspring mice (Lu et al.). These results are in line with several longitudinal studies on pre-gestational, gestational, and lactation periods in which it has been observed that the maternal fish and omega 3 consumption influence the outcomes in 17 years old infants. The omega 3 maternal intake decreases CpG sites numbers on DNA methylation and the biological age, so improves the infant and adult programing outcome.

After fat and lactose, human milk oligosaccharides (HMO) are the most abundant solid component in human milk and their composition varies during lactation and exert a broad list of effects on the infant's health. Not all mothers have same HMO composition or are functionally the same. However, Mainardi et al. showed how the different HMO could act synergistically in influencing infant's growth. 2′-FL concentration cut off of 200 mg/L separated secretors and non-secretors with 100% accuracy: <200 mg/L in the milk of non-secretor women (see article Liu et al.), while it is the most abundant HMO in the milk of secretors. Two articles in this compendium bring evidences of their effects. On the one hand, it is showed the influence of microbially fermented 2'-fucosyllactose on neuronal-like cell activity in an *in vitro* co-culture system (Kuntz et al.), and also its effects in influencing systemic immune development and function in piglets when it is combined with *Bifidobacterium longum* subspecies infantis (Daniels et al.). Overall, HMO, as undigestible sugars who are necessary to reach the gut intact, are able to influence the gut microbiome, and at the same time some factors influence its HMO composition, such as maternal geographic, environment, genetics, childbirth (caesarian vs. vaginal), antibiotics treatment but also the maternak diet and microbiome (Ajeeb et al.).

In the last years, the presence of human milk extracellular vesicles (HM-EVs) have been also a matter of study. These are lipid bilayer membrane vesicles (50–200 nm) containing myriad signaling molecules including proteins, lipids, microRNAs, mRNAs, and other biomolecules protected from degradation. Gómez-Ferrer et al. demonstrated in their study that the omega-3 oxylipins in HM-EVs can have pro-resolutive actions in gastrointestinal inflammation. In addition, another study showed the associations between the miRNAs present in HM-EVs and oligosaccharide concentrations in human milk (Holzhausen et al.).

Globally, breast milk is known to modulate infant intestinal gut barrier. A comparative analysis between raw and pasteurized breast milk was conducted by Rodríguez-Camejo et al. to understand the adverse effects of heat-treatment on cellular functions associated with the gut epithelial barrier and responses to inflammatory stimuli. These authors reveal that all milk types stimulated epithelial cell proliferation, but raw colostrum increased cell migration and interfered with the attachment of *E. coli* on epithelial cells.

In addition, components from other milk species have shown bioactivity comparable to that from human origin. As example, an expert panel reviewed the safety data and physiological role of dietary bovine osteopontin in infancy. They concluded that osteopontins from human milk and bovine milk had comparable plasma absorption and properties on cognition and immunity (Fleming et al.).

As mentioned before, human milk is a dynamic fluid which composition changes due to many internal and external factor such as the lactational period, geography, diet, and environment. For instance, it is shown that lactational and geographical aspects have an influence on the variation in the concentration of six oligosaccharides in Chinese breast milk. These results, from a multicenter study over 13 months postpartum developed by Liu et al. demonstrated that, compared with other studies, the variation among different geographical sites within China is smaller than the variation observed between different countries. Specifically, it is showed that that 79% of Chinese mothers have the secretor phenotype and 21% express a non-secretor phenotype respectively with high or low 2′FL level in the human milk; conversely, 3′-FL was ubiquitous and increased as in others countries all over the lactation. Human milk microbiome, the microogranisms who can also use these oligosaccharides, also differs depending on several factors, and it seems to be associated with maternal diet and infant growth, among others (Ajeeb et al.).

On the other hand, the breast milk immune composition also varies during the lactation period. The transition stage of lactation, which is the short period of days between colostrum

and mature milk, is the one with less information regarding its immunoglobulin composition or immunoglobulinome. The article of Rio-Aige et al. characterizes two different clusters of milk types in basis of immunoglobulins and cytokines presente from the first to the last day of transitional milk, which have been called immunotypes. For that 75 mother-child pairs from the MAMI cohort. Following with the immune composition of human milk, it has been also a matter of study during the COVID-19 pandemic. It has been demonstrated the presence of specific Ig against the SARS-CoV-2 both after infection and vaccination. In their article, de Graaf et al. explores the human milk polyclonal IgA1 response to repeated SARS-CoV-2 vaccinations by using LC–MS based fab profiling.

Maternal factors also can influence the milk hormone concentrations, as is explored in the systematic review by Qureshi et al.. The revised studies consistently revealed the presence and varying concentrations of adiponectin, leptin, insulin, cortisol, and ghrelin in breast milk. Some of them change in relation to maternal factors such as BMI, weight and other health indicators (environment, lifestyle, and smoking). To date, maternal pregestational BMI increases milk leptin level and maternal diabetes increases milk insulin levels and increase also fat body mass of their infants. For the others hormones there is no evidence link between their milk content and maternal factors.

Prematurity is defined as a birth that occurs before 37 completed weeks of gestation, resulting in infants who may face a higher risk of health complications and developmental challenges due to their early arrival. Breast milk in this early period could have a critical role. This Research Topic, includes an article by Pütz et al. showing the association of different types of human milk with bronchopulmonary dysplasia in preterm infants. A lower prevalence of bronchopulmonary dysplasia was found in neonates fed with mother's own milk compared to those fed with donor human milk as well as when breast milk was used "fresh" compared to "frozen/thawed." These results underline the importance of mother's own milk and its proportion administered on the prevention of bronchopulmonary dysplasia. In addition, human milk needs to be fortified in some situations, as in low birth weight neonates, such as prematures. Biasini et al. reported the long-term advantages of protein-fortified human milk in this population. Specifically, the article shows that neonatal enteral protein supplementation in very low birth weight preterm infants leads to no positive nor adverse consequences in long-term assessment, suggesting that benefits are restricted to the neonatal term and 1st years of age.

In the absence of the mother's own milk, Donor Human Milk (DHM) is essential for the nutrition of premature babies. Human milk banks (HMB) are essential for collecting, processing, storing, and redistributing DHM. There are around 900 HMBs around the world, unevenly distributed, including 250 in Europe and many in South and North America. The creation of an HMB in a developing country is crucial and poses problems of organization and funding, as it is shared in the article of Nakibuuka et al. which explains the setting up of the first human milk bank in Uganda. Its novelty contraposes to the HMB of Vietnam (Thi Tran et al.), which has been in existence for 5 years, collects 10,000 L of milk in 5 years and analyses the factors influencing the volume of donations (14 L/donor), but also faces the financial difficulties for poor people ($60/L).

A critical aspect for the HMB is to ensure the preservation quality of the components of raw breast milk and its microbiological safety. For that reason, although pasteurization (Holder) is the most used technique for ensuring preservation of DHM, new alternatives need to be implemented to better preserve its quality (Moro et al.).

In summary, the Research Topic includes interesting articles focusing on the human breast milk composition in terms of nutrients, biological compounds, microbiome, HMO, and extracellular vesicles, among others. All of them participate boosting the immune and metabolic system development and support the short and long-term health of newborns. The critical aspects of maternal exposome in milk composition and the milk banks function is also of importance in this Research Topic.

Further studies are encouraged to expand the knowledge of breast milk and complete this fist volume, especially those focused on proteins, their role in nutrition and their bioactive properties in immunity, the microbiome and neurodevelopment; minerals (phosphorus-calcium metabolism), trace elements and vitamins and the different supplements to be administered to the mother before, during gestation and during lactation; antioxidants in breast milk such as phenolic components; maternal factors in the perinatal environment (toxins, pollutants, exercise, smoking, alcohol, over- or under-nutrition, pathologies, and nutrition) that can affect the medium- and long-term outcome of the child. Finally, research into the genetic and epigenetic regulation of the volume and composition of breast milk, metabolism and growth of the fetus, newborn, and child should be analyzed in depth.

Author contributions

FP-C: Writing – review & editing, Conceptualization. VD-M: Writing – review & editing. CB: Writing – review & editing.

Conflict of interest

VD-M was employed by Janssen Pharmaceutical Companies of Johnson & Johnson.

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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Lactational and geographical variation in the concentration of six oligosaccharides in Chinese breast milk: a multicenter study over 13 months postpartum

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Introduction: Understanding the variations of oligosaccharide in breast milk contribute to better study how human milk oligosaccharides (HMOs) play a role in health-promoting benefits in infants.

Methods: Six abundant HMOs, 2′-fucosyllactose (2′-FL), 3-fucosyllactose (3-FL), Lacto-N-tetraose (LNT), Lacto-N-neotetraose (LNnT), 3′-sialyllactose (3′-SL) and 6′-sialyllactose (6′-SL), in breast milk collected at 0–5 days, 10–15 days, 40–45 days, 200–240 days, and 300–400 days postpartum from six locations across China were analyzed using high-performance anion-exchange chromatography-pulsed amperometric detector.

Results: The concentration of individual HMO fluctuated dynamically during lactational stages. The median ranges of 2′-FL, 3-FL, LNT, LNnT, 3′-SL, and 6′-SL across the five lactational stages were 935–2865 mg/L, 206–1325 mg/L, 300–1473 mg/L, 32–317 mg/L, 106–228 mg/L, and 20–616 mg/L, respectively. The prominent variation was observed in the content of 6′-SL, which demonstrates a pattern of initial increase followed by a subsequent decrease. Among the five lactational stages, the transitional milk has the highest concentration, which was 31 times greater than the concentration in mature milk at 300–400 days postpartum, where the content is the lowest. Geographical location also influenced the content of HMOs. LNT and LNnT were the highest in mature milk of mothers from Lanzhou among the six sites at 40–240 days postpartum. Breast milks were categorized into two groups base on the abundance of 2′-FL (high and low). There was no significant difference in the proportions of high and low 2′-FL phenotypes among the six sites, and the percentages of high and low 2′-FL phenotypes were 79% and 21%, respectively, across all sites in China.

Discussion: This study provided a comprehensive dataset on 6 HMOs concentrations in Chinese breast milk during the extended postpartum period across a wide geographic range and stratified by high and low 2′-FL phenotypes.

KEYWORDS

human milk, oligosaccharides, variation, lactation, geography

1. Introduction

WHO recommend exclusive breastfeeding for the first 0–6 months and continued breastfeeding up to 2 years and beyond with appropriate complementary foods (1). Breast milk is associated with a lower incidence of diarrhea, obesity and infectious disease in infants by the combined action of macronutrients, micronutrients, and bioactive components (2–5). Human milk oligosaccharides (HMOs) are the third-largest solid component of human milk after lactose and fat (6). At least 150 HMOs have been identified in human milk using advanced detection technology (4). Fucosylated HMOs represent 45–83% of total HMO content, acetylated HMOs represent 6–35% of total HMO content, and sialylated HMOs represent 6–21% of the total HMO content (7–13). 2′-fucosyllactose (2′-FL), 3-fucosyllactose (3-FL), Lacto-N-tetraose (LNT), Lacto-N-neotetraose (LNnT), 3′-sialyllactose (3′-SL), and 6′-sialyllactose (6′-SL) are representative of the three major categories of HMOs and are among the most abundant and extensively studied individual structures. An increasing number of preclinical studies revealed that HMOs have health-promoting benefits, including the selective proliferation of intestinal microbiota such as Bifidobacterium and Lactobacillus (14–17), inhibiting pathogen binding to receptors on intestinal epithelial cells by mimicking structural features of histo-blood group antigens (HBGAs) (18–20), modulating immune function by binding to pattern recognition receptors, cytokine receptors, and other types of receptors (21–23), and promoting brain and cognitive development by participating in the formation of gangliosides and polysialic acid chains in the brain through a long-term potentiation effect mediated by the vagus nerve and/or systemic distribution via the circulation (24, 25).

Multiple previous studies have indicated a wide range of HMO variation in breastmilk. For example, fucosylated HMOs in breastmilk ranges from 0.02 to 6 g/L (4). Lactational stage and maternal phenotype may contribute to variations in HMO content and composition. The HMO content is highest in colostrum, about 20–25 g/L, and tends to decrease over the course of lactation to about 5–15 g/L in mature milk (6, 9, 12, 26, 27). Maternal HMO phenotype is determined in part by two alleles of the secretor (Se) and Lewis (Le) genes. The presence of α1-2-fucosyltransferase (FUT2) in secretor-positive mothers can link fucose to the core structure through α1-2 linkage, resulting in HMOs with an α1-2 linkage structure in expressed breast milk, such as 2′-FL and Lacto-N-fucopentaose I (LNFP I). Lewis-positive mothers have α1-3-fucosyltransferase (FUT3) that can link fucose to the core structure through α1–3/4 linkage, resulting in HMOs with α1–3/4 linkage such as 3-FL and Lacto-N-fucopentaose II (LNFP II) are abundantly present in breast milk (28–30). In a study by Lefebvre et al. (31), the proportion of Se+ and Se− mothers in Germany was 88%: 12% and the proportion of Le+ and Le− mothers was 94%: 6%.

In addition to lactational stage and maternal phenotype, other evidence has shown that the concentration of HMOs varies by geographical location. An international cohort study demonstrated that the average concentration of LNT in breast milk collected in European (Spanish and Swedish) and African (Ethiopian, Ghanaian, and Kenyan) was higher than in a South American (Peruvian) population (13). Breast milk from a cohort in Spain was shown to have a higher mean concentration of 6′-SL relative to a cohort from Sweden. Even within the same country, study subjects from California expressed higher levels of Lacto-N-fucopentaose III (LNFP III) relative to a comparable study cohort from Washington (13). Geographic variation in the proportion of secretors and non-secretors has also been observed. A recent study reported that the percentage of secretor-positive mothers from South America was higher than in Africa (32). These data indicate that there are differences in HMO concentration even within the same geographical region, and together with the variation in the frequency of maternal phenotypes between populations, suggest the need for a large-scale multicenter study to determine the range and variation in HMO concentration in breast milk.

Currently, several important HMOs including 2′-FL, 3-FL, LNT, LNnT, 3′-SL and 6′-SL are safe for use in infant formula according to the European Food Safety Authority and the Food and Drug Administration, and are available in large-scale commercial production (33–36). Hence a comprehensive dataset on HMOs profile in Chinese breast milk is useful to fortify Chinese infant formula products with HMOs. Several previous studies have reported on the content of HMOs in Chinese breast milk (10, 26, 28, 37–40). The MING study reported the content of 10 HMOs in 446 breast milk samples from 0–240 days postpartum in three cities in China (10). In a study by Ma L et al., the concentration of HMOs in 140 breast milk samples over 30–240 days postpartum was reported (26). In a large cross-sectional study conducted in eight provinces of China ($n = 6,481$), Ren et al. (39). randomly analyzed 481 breast milk samples to determine the concentrations of 24 HMOs and the distribution of the Lewis phenotype. Additionally, this study investigated the relationship between various clinical data of mothers and infants and the levels of HMOs. Our previous study investigated the variation in HMO concentration in breast milk collected from one city over a prolonged breastfeeding period (37). However, to the best of our knowledge, there is limited data available on HMO concentration over a prolonged period of lactation in Chinese populations. In addition, the HMO profile of milk from mothers with different Se phenotypes has not been determined in China with a larger-size number of participants. Hence, the primary aim of this study was to conduct a comprehensive study of the abundance of six of the major HMOs, representative oligosaccharides for each of the three categories, in breast milk collected from different regions of China over a prolonged breastfeeding period. In this study, we analyzed HMOs in 2,616 breast milk samples from 1,758 healthy mothers at 0–13 months postpartum collected across six representative sites in China using high-performance anion-exchange chromatography-pulsed amperometric detector (HPAEC-PAD). This study expanded our knowledge of HMO concentration in Chinese breast milk and analyzed HMO composition in high 2′-FL and low 2′-FL phenotypic subgroups. This data is useful as a scientific basis for HMO fortification of infant formula and ongoing evaluation of HMO composition in China.

2. Materials and methods

2.1. Study design and participants

The results presented in this study are a part of the Maternal Nutrition and Infant Investigation (MUAI) study aimed to explore the relationship between nutrient levels in breast milk and infant growth outcomes. The purpose of this study was to investigate geographic

variation in HMOs concentrations and the frequency of the secretor phenotype across multiple geographic locations in China. Breast milks were collected from mothers in six cities, including Northeast (Changchun), Northwest (Lanzhou), Southwest (Chengdu), East (Tianjin), South (Guangzhou), and a metropolitan city (Shanghai). Five stages of lactation were chosen to explore the content of HMOs in colostrum's period (0–5 days postpartum), transitional milk's period (10–15 days postpartum), mature milk's period (40–45 days postpartum), the period during complementary food introduced (6–8 months postpartum) and one year after delivery (11–13 months postpartum). The recruitment and exclusion criteria for mother-infant pairs were consistent with our previously published literature (37). Healthy mother-infant pairs meeting the following criteria were recruited: mothers aged between 20 and 35 years old, living in the local area for more than 2 years, and single childbirth. The baby's gestational age is between 37–42 weeks and Apgar score over 8. All subjects gave their informed consent for inclusion before they participated in this study. This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Tianjin hospital of ITCWM Nankai hospital. This study was registered in the China Clinical Trial Center (ChiCTR1800015387).

2.2. Breast milk sample collection

The volumes of breast milk samples collected at 0–5 days, 10–15 days, 40–45 days, 200–240 days, and 300–400 days postpartum were 8–10 mL, 10–15 mL, 50–100 mL, 50–100 mL, and 50–100 mL, respectively. Samples from lactating mothers were collected by extracting total milk from one side of the breast using either a manual or an electric breast pump between 8: 00–11: 00 AM on the designated collection day. After collecting the breast milk, it was gently inverted 10 times before transferring the specified volume into a foil-wrapped tubes. The remaining milk was then returned to the lactating mother. The breast milk samples in foil-wrapped tubes were delivered to the local laboratory in each city as soon as possible at 4°C. Upon arrival at the local laboratory, the samples underwent a 20-min incubation in a water bath set at 25°C, followed by a 2-min vortexing process to ensure complete homogenization. This step ensured that the top layer of breast milk was free from any solid or semi-solid greasy components, and there were no small particle attachments on the inner surfaces of the tubes. The breast milk samples were then divided into 1.2 mL sub-packages and the resulting aliquoted samples were subsequently stored at a constant temperature of −80°C. All subpackaged samples were transported every 6 months through a cold-chain transportation at-80°C to a central laboratory in Shanghai. The analysis of HMOs was carried out at the central laboratory.

2.3. HMOs analysis

The determination of 6 HMOs in breast milk samples was conducted using a well-established method, employing high-performance anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD), as previously described (37). For colostrum and transitional milk samples, 0.1 milliliter of breast milk was mixed with 1.9 milliliters of laboratory water. For breast milk collected at 40–400 days postpartum, 0.2 milliliter of breast milk was mixed with 1.8 milliliters of laboratory water. Filter through a 0.22 μm nylon filter into an autosampler vial, aiming to remove a fraction of the high-molecular-weight proteins and fats to reduce the load on the chromatography column. The HPAEC-PAD analyses were performed on a Thermo Fisher HPAEC ICS 5000 series instrument (Thermo, USA), utilizing a CarboPacTM PA1 Analytical column (4 × 150 mm, Thermo) connected to a CarboPacTM PA1 Guard column (4 × 50 mm, Thermo). The separation of the 6 types of HMOs was achieved using elution gradients at a flow rate of 1.0 mL/min and a temperature of 25°C. For the separation of 2′-FL, 3-FL, LNT, and LNnT, the elution gradients were as follows: 0.00–12.00 min, 60 mM NaOH; 12.00–20.00 min, 60–155 mM NaOH; 20.00–30.00 min, 155 mM NaOH; 30.01–43.00 min, 125 mM NaOH/18 mM NaOAc; 43.01–48.00 min, 100 mM NaOH/240 mM NaOAc; 48.01–63.00 min, 60 mM NaOH. For the separation of 3′-SL and 6′-SL, the elution gradients were as follows: 0.00–19.00 min, 100 mM NaOH/60 mM NaOAc; 19.01–23.00 min, 100 mM NaOH/240 mM NaOAc; 23.01–30.00 min, 100 mM NaOH/60 mM NaOAc. The quantification of HMOs was performed using the external standard method.

2.4. Statistical analysis

All exploratory and descriptive statistical analyses were performed with SPSS 25.0 (SPSS, Inc., Chicago, IL, USA). The statistical analysis method was selected based on normality plots with tests and homogeneity of variance testing of the data. HMO concentrations were reported as the median (p25, p75) by Weighted Average (Definition 1) since the data presented a non-normal distribution. Statistical analyses were considered significant at $p < 0.05$ using two-sided tests. The difference in continuous variables according to maternal characteristics was evaluated by the Kruskal Wallis Test, and distributions across groups were compared by Kruskal-Wallis One-way ANOVA (multiple comparisons, all pairwise, adjusted p value using the Bonferroni correction for multiple tests). The difference of categorical variables in maternal characteristics was compared by Chi_square Test, and the proportions across groups were compared by z-test with adjusted p-values (Bonferroni method). The variation of HMO concentrations among lactational stages and geographical sites was explored with Independent Nonparametric Test (Kruskal-Wallis One-way ANOVA, all pairwise, adjusted p value by the Bonferroni correction for multiple tests). The proportions of secretor and non-secretor were compared by Chi-square Test.

3. Results

3.1. Basic characteristics

A total of 1,758 healthy mothers participated in the study, including 244 from Changchun, 299 from Lanzhou, 281 from Chengdu, 229 from Tianjin, 342 from Guangzhou, and 363 from Shanghai. The description of the sample size within each lactational period in each city was presented in Supplementary Figure S1. Overall, the MEAN (SD) age at delivery of the participant mothers was 29.9 (3.5) years. The pre-pregnancy BMI and pre-delivery BMI of the participant mothers was 21.1 (2.9) kg/m^2 and 26.6 (3.3) kg/m^2, respectively. The weight gain during pregnancy was 14.6 (5.6) kg. The gestational age of the participant mothers was 39.2 (1.6) weeks. The rate of vaginal delivery and primipara was 59 and 71%, respectively.

49% of infants were female. The mean infant birth-weight and birth-length were 3,397.6 (637.9) g and 49.9 (2.1) kg, respectively. The characteristics of participant mothers in each city are listed in Table 1. Except for the maternal age at delivery and the infant's sex, the characteristics of mothers and infants showed significant differences across cities.

3.2. Concentrations of six HMOs over lactational stage and geographical sites

The median (p25, p75) values of six HMOs in 2,616 breast milk samples collected at 0–13 months postpartum were 2′-FL 1,466 (624, 2,410) mg/L, 3-FL 665 (259, 1,310) mg/L, LNT 555 (299, 1,072) mg/L, LNnT 97 (39, 199) mg/L, 3′-SL 125 (100, 165) mg/L, and 6′-SL 190 (29, 461) mg/L. The median (p25, p75) values of total six HMOs was 3,467 (2,879, 4,672) mg/L.

Our data showed that in general, lactational stage and geographical location influenced the content of HMOs. The variation in all six HMOs over 0–13 months postpartum is shown in Figure 1 and Supplementary Table S1. The six HMOs varied in notable ways. For example, the dominant fucosylated HMO changed from 2′-FL to 3-FL, and the predominant sialylated HMOs changed from 6′-SL to 3′-SL due to differences in concentration over time. The prominent variation was observed in the content of 6′-SL, which demonstrates a pattern of initial increase followed by a subsequent decrease. Among the five lactational stages, the transitional milk has the highest concentration, which was 31 times greater than the concentration in mature milk at 300–400 days postpartum, where the content is the lowest.

Geographical location was associated with differences in the concentrations of HMOs (Figure 2; Supplementary Table S2). The content of 2′-FL in milk collected at 40–45 days postpartum in Lanzhou was about 1.3 times higher than in samples collected in Chengdu. At 40–45 days postpartum, the concentration of 3-FL in breast milk from Changchun was significantly higher compared to Shanghai, with levels of 526 mg/L and 350 mg/L, respectively. The breast milk from Lanzhou showed the significantly highest concentration of LNT and LNnT among the six sites at 40–240 days postpartum. Breast milk from coastal cities (Guangzhou and Shanghai) contained significantly higher concentrations of 3′-SL compared to inland cities (Lanzhou) at 200–400 days postpartum. The concentration of 6′-SL in breast milk from Chengdu is significantly higher compared to that in breast milk from Guangzhou in colostrum, with an increase of 21%.

3.3. The proportion of high and low 2′-FL level subjects

2′-FL was detected in 99.8% (2,611/2,616) of the samples and a clear separation was found in the distribution of 2′-FL levels (Supplementary Figure S2). Milk samples with 2′-FL concentration greater than or equal to 200 mg/L were assigned to the high 2′-FL level group while those with 2′-FL concentration below 200 mg/L were assigned to the low 2′-FL level group. The distribution of high and low 2′-FL subjects in China was determined by testing samples from six geographical regions. The proportion of high and low 2′-FL level subjects at the 6 geographical sites and at each sampling time is shown in Figures 3A–E. The fraction of low 2′-FL level subjects was lower than the proportion of high 2′-FL level subjects at every geographical site. There was no significant difference in the proportion of high and low 2′-FL level subjects among the six regions at all five sampling time points ($p = 0.697$, 0–5 days postpartum; $p = 0.655$, 10–15 days postpartum; $p = 0.598$, 40–45 days postpartum; $p = 0.116$, 200–240 days postpartum; $p = 0.136$, 300–400 days postpartum). The percentage of high and low 2′-FL level subjects in China is 79%: 21% (Figure 3F).

3.4. Variations in HMO concentration over lactational stage and geographical sites stratified by high and low 2′-FL level

The median (p25, p75) values of six HMOs in the high and low 2′-FL level groups were 2′-FL 1,849 (1,166, 2,656) mg/L and 22 (11, 40) mg/L, 3-FL 458 (217, 1,078) mg/L and 1,401 (826, 2,033) mg/L,

TABLE 1 Characteristics of participant mothers in each city (MEAN ± SD or %).

Characteristic	Changchun (*n* = 244)	Lanzhou (*n* = 299)	Chengdu (*n* = 281)	Tianjin (*n* = 229)	Guangzhou (*n* = 342)	Shanghai (*n* = 363)	*P* value
Maternal							
Age at delivery, years	29.9 ± 3.0	29.8 ± 4.3	29.5 ± 3.3	30.3 ± 3.2	29.7 ± 3.7	30.1 ± 3.0	0.054
Pre-pregnancy BMI, kg/m^2	21.3 ± 3.0 ^{a,b}	21.3 ± 2.8 ^{a,b}	20.7 ± 2.5 ^{b}	21.8 ± 3.4 ^{a}	20.3 ± 2.5 ^{b}	21.2 ± 2.8 ^{a,b}	<0.001
Pre-delivery BMI, kg/m^2	27.4 ± 3.8 ^{a}	26.8 ± 3.0 ^{a,b}	26.2 ± 2.8 ^{b}	27.3 ± 3.4 ^{a}	25.5 ± 3.1 ^{b}	26.7 ± 3.6 ^{a,b}	<0.001
Gestational weight gain, Kg	16.5 ± 5.5 ^{a}	14.3 ± 5.3 ^{b}	14.2 ± 4.6 ^{b}	14.6 ± 7.6 ^{b}	13.5 ± 4.9 ^{b}	14.7 ± 5.5 ^{b}	<0.001
Gestational age, weeks	39.0 ± 1.1 ^{b}	39.0 ± 2.1 ^{a,b}	39.5 ± 1.6 ^{a}	39.2 ± 1.7 ^{a,b}	38.9 ± 1.4 ^{b}	39.6 ± 1.0 ^{a}	<0.001
Vaginal delivery, %	42% ^{a}	59% ^{b}	43% ^{a}	68% ^{b,c}	77% ^{c}	61% ^{b}	<0.001
Primipara, %	95% ^{a}	50% ^{b}	73% ^{c,d}	77% ^{d}	64% ^{c}	74% ^{c,d}	<0.001
Infant							
Birthweight, g	3,380.1 ± 401.1 ^{a,b}	3,277.8 ± 445.1 ^{b}	3,409.5 ± 624.7 ^{a,b}	3,396.7 ± 490.8 ^{a}	3,542.6 ± 1,004.9 ^{a,b}	3,332.3 ± 368.2 ^{a,b}	0.025
Birth-length, cm	50.5 ± 1.7 ^{a}	49.0 ± 2.5 ^{c}	49.8 ± 1.5 ^{b,c}	50.4 ± 2.6 ^{a,b}	50.3 ± 2.3 ^{b}	49.4 ± 1.3 ^{c}	<0.001
Female infant, %	49%	47%	43%	51%	48%	53%	0.159

$^{a–d}$Values within a row in individual cities with different superscript letters were significantly different (adjusted $p < 0.05$).

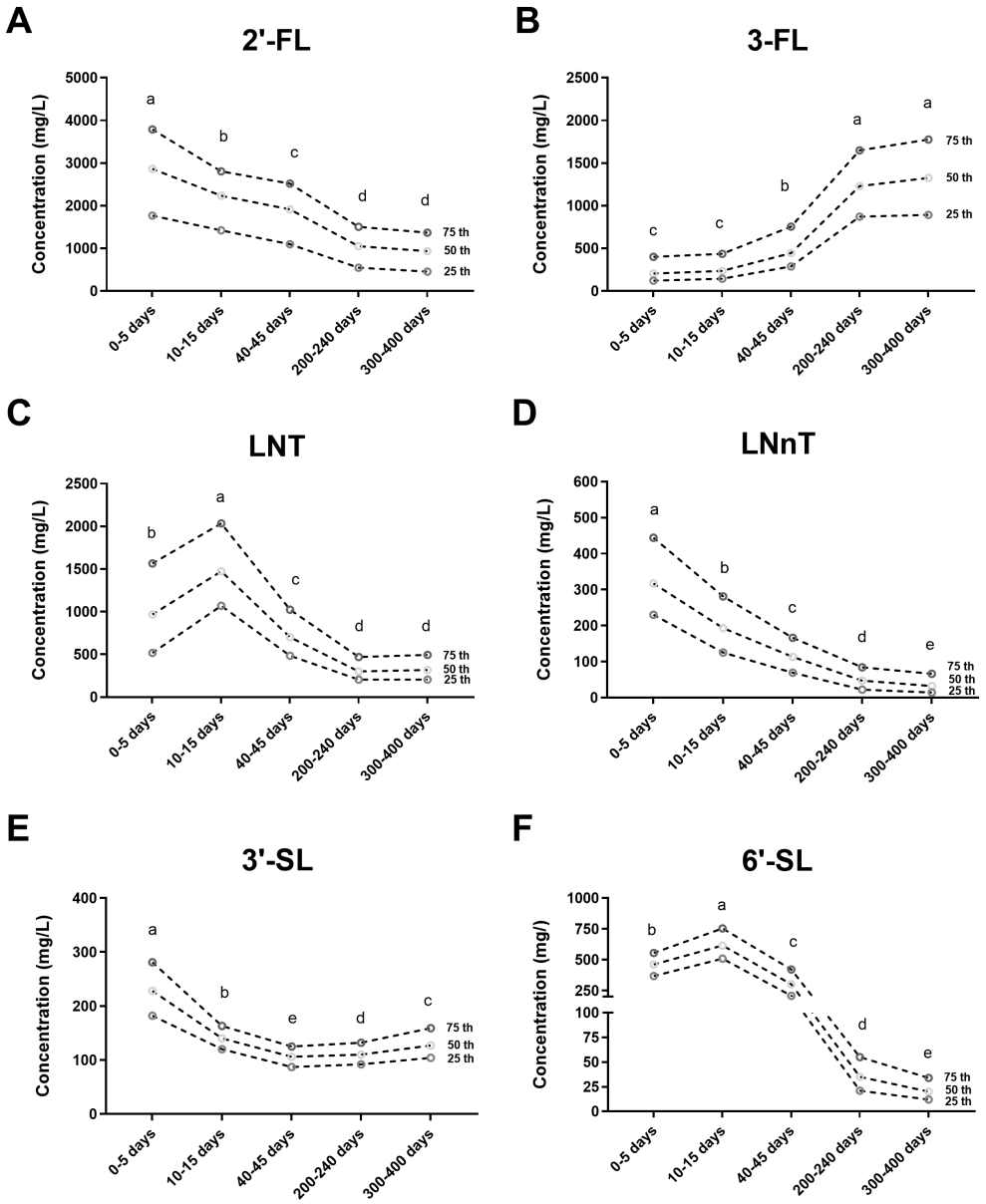

FIGURE 1
The concentration and variation of six human milk oligosaccharides (HMOs) at 0–13 months postpartum **(A–F)**. 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; LNT, Lacto-N-tetraose; LNnT, Lacto-N-neotetraose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose. Different superscript letters (a–e) indicated significant differences (adjusted $p < 0.05$ by the Bonferroni correction for multiple tests) according to an independent nonparametric test (Kruskal–Wallis one-way ANOVA, all pairwise).

LNT 514 (285, 921) mg/L and 882 (403, 1,759) mg/L, LNnT 114 (52, 222) mg/L and 42 (16, 108) mg/L,3′-SL 126 (101, 166) mg/L and 123 (97, 161) mg/L, 6′-SL 187 (29, 460) mg/L and 195 (29, 463) mg/L. The median (p25, p75) values of total six HMOs in the high and low 2′-FL level groups were 3,639 (2,947, 4,917) mg/L and 3,062 (2,566, 3,695) mg/L, respectively.

The proportion of individual HMOs and the variation in each of the six HMOs from 0–13 months postpartum are separated by high and low 2′-FL level categorization and shown in Figure 4. Although the concentration of fucosylated and acetylated HMOs differed significantly between the high and low 2′-FL level groups, the lactational variation of each HMO in both groups were consistent with those before grouping. And the effect of geographical variation on HMO concentration was also consistent with before grouping, except for LNT and LNnT, which were not influenced by geographic factors in the low 2′-FL level group (Tables 2, 3).

4. Discussion

In this study, we analyzed the concentration of six major HMOs in 2,616 human milk samples collected from six geographical sites in China, including the northeast, northwest, southwest, east, south, and a metropolitan city, spanning from the initiation of lactation up to 13 months postpartum. Despite the samples being collected from

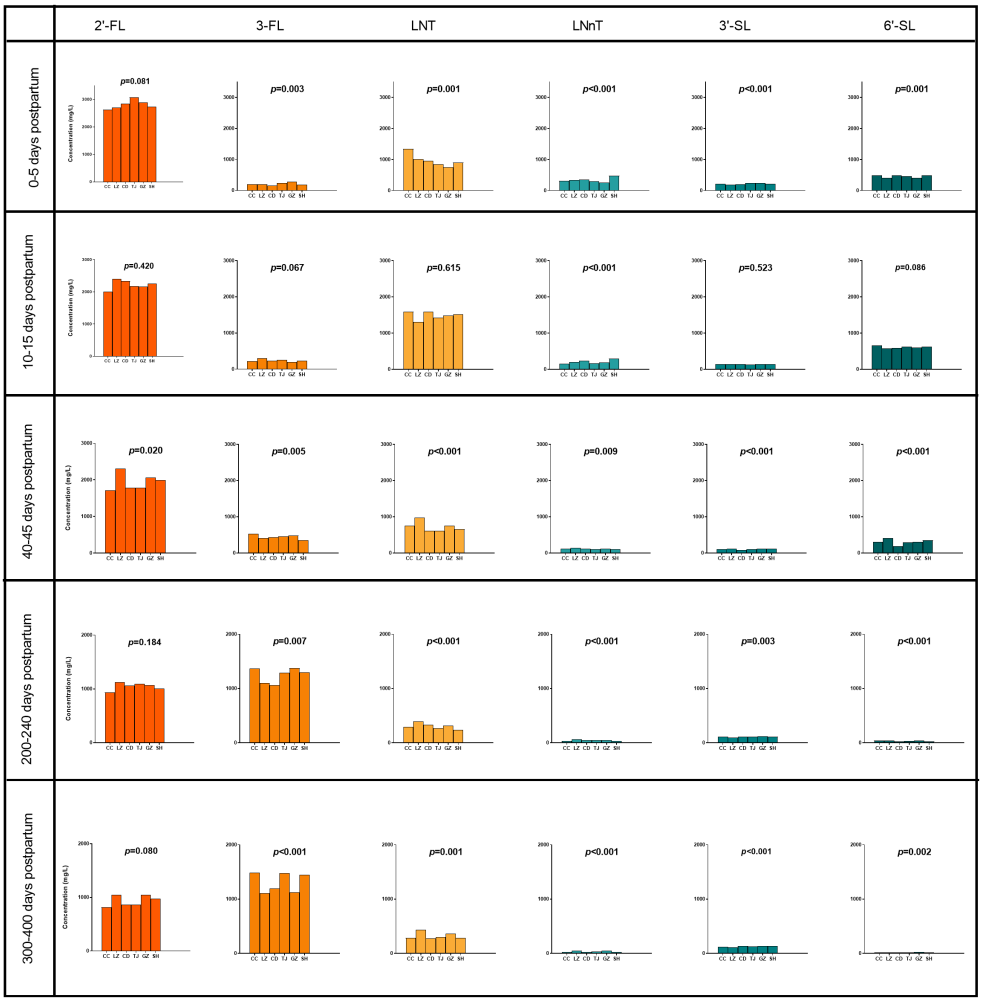

FIGURE 2
Concentration of six human milk oligosaccharides (HMOs) at six geographical sites at 0–13 months postpartum. 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; LNT, Lacto-N-tetraose; LNnT, Lacto-N-neotetraose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose. Total HMO concentration was calculated as the sum of 2′-FL, 3-FL, LNT, LNnT, 3′-SL, and 6′-SL. CC, Changchun; LZ, Lanzhou; CD, Chengdu; TJ, Tianjin; GZ, Guangzhou; SH, Shanghai. Nonparametric test-Kruskal Wallis one-way ANOVA (multiple comparisons: all pairwise, adj-*p*).

different locations, standardized sample processing techniques and HPAEC-PAD-based analytical methods were uniformly employed. This multicenter study displayed the dynamic change in the concentrations of individual HMOs in breast milk collected from multiple sites and covering the first 13 months of lactation. Our results provide a comparative representation of 6 HMO concentration in the breast milk of Chinese mothers. This data provides a scientific basis for the fortification of this 6 HMOs in infant formula and serves as a reference for comparing HMO content in China.

Our findings contribute additional data on HMO content in colostrum, supplement the available literature on HMO content in breast milk up to one year postpartum, and enhance the comprehensive characterization of HMO content in breast milk within China (Figure 5). Ren et al. (39) reported the concentration of 24 HMOs at three lactational stages (0–6 days, 7–14 days, and 15–340 days postpartum). When comparing the variation trend of individual HMO during lactation as observed by Ren et al., we noticed a difference in the variation trend of 3′-SL. Ren et al. reported a gradual decrease in the concentration of 3′-SL throughout the sampling period. However, our observations revealed a U-shaped pattern in the variation of 3′-SL content across five lactational stages. Specifically, the concentration of 3′-SL in breast milk at 300–400 days postpartum (127 mg/L) was significantly 15% higher than that in breast milk at 200–240 days postpartum (110 mg/L). The study conducted by Zhang et al. (40). reported the content of HMOs in 203 breast milk samples from mothers in 8 cities in China at 15–180 days postpartum. The average concentration of 2′-FL in breast milk at 6 months postpartum was 1,180 mg/L. In our study, we found that the average concentration of 2′-FL in breast milk was 1,032 mg/L at 6–8 months postpartum. And the average concentration of 2′-FL was 961 mg/L at 10–13 months postpartum. Compared with our previous study (37), the number of geographical locations where samples were collected was increased from one to six. The approach accounts for geographic variation and is therefore more representative of the concentrations of HMOs in the breast milk of Chinese mothers. In addition, the variation in 3-FL concentration across the period of lactation was clarified for the first time. This study and our previous

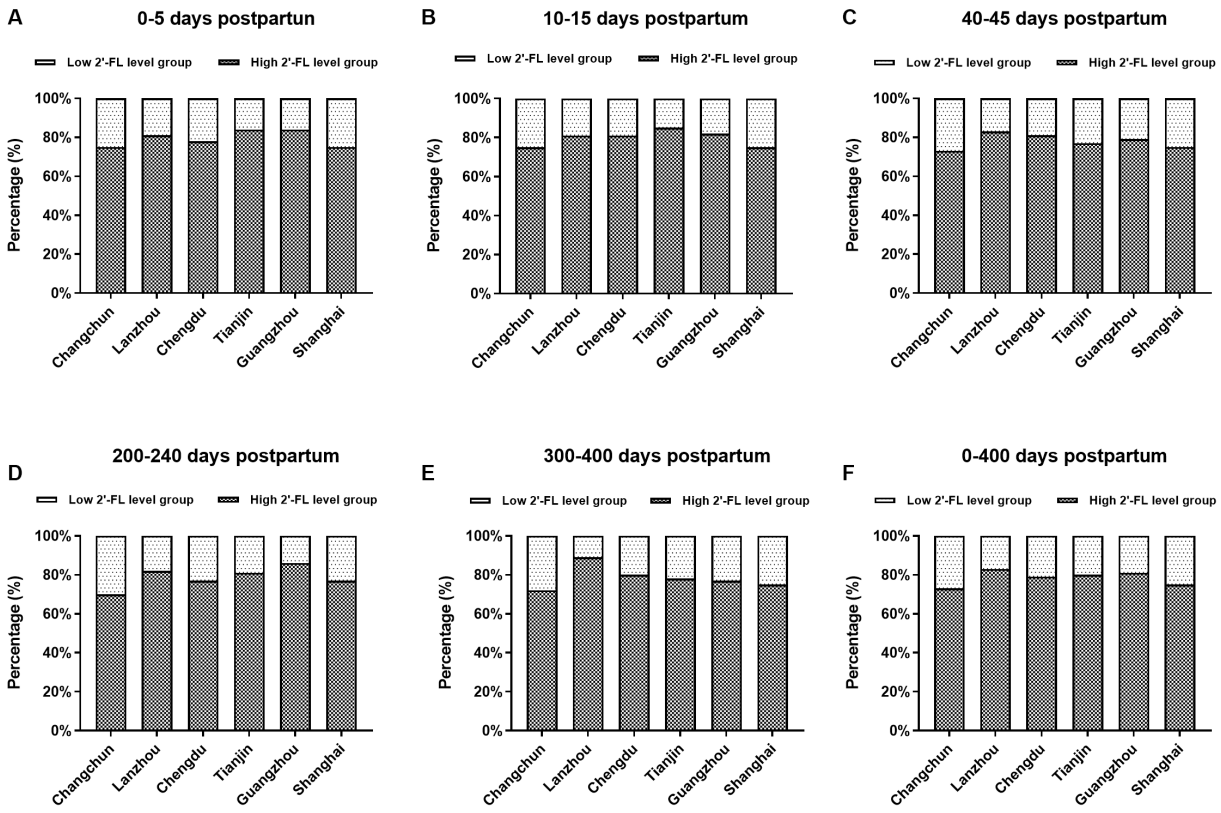

FIGURE 3
The proportion of high and low 2′-FL level groups among the six regions at 0–5 days postpartum **(A)**, 10–15 days postpartum **(B)**, 40–45 days postpartum **(C)**, 200–240 days postpartum **(D)**, 300–400 days postpartum **(E)**, and 0–400 days postpartum **(F)**.

study both report that 3-FL increased during lactation, although there was no significant difference between 200–240 days and 300–400 days postpartum. In previous studies, 3-FL was present at 1,421 mg/L and 1,128 mg/L in milk at 200–240 days and 300–400 days postpartum, respectively. However, in this study with an enlarged sample size, the concentrations of 3-FL were 1,230 mg/L and 1,325 mg/L at 200–240 days and 300–400 days postpartum, respectively. 2′-FL was more abundant and 3-FL was less abundant at 0–45 days postpartum compared to other Chinese studies (10, 26, 41). This may be due to the earlier sampling time in this study, as 2′-FL decreased while 3-FL increased during lactation. Other HMOs were within the range reported by multiple previous studies (10, 26, 42, 43).

In comparing the HMOs content of Chinese breast milk with studies conducted in other countries and regions, Chinese mothers have higher levels of 3-FL in their breast milk collected at 4 months after postpartum. However, the concentration of LNT and LNnT in Chinese breast milk appeared to be lower compared to that observed in other countries and regions (Supplementary Table S3) (26, 27, 44–46). At 4–8 months postpartum, the concentration of 3-FL in this study was 1,230 mg/L, compared to 1,074 mg/L in Europe (46), 1,194 mg/L in the UAE (27), and 1,146 mg/L in Malaysia (26). At 10–13 months postpartum, the concentration of 3-FL in this study was 1,325 mg/L, compared to 1,138 mg/L in Malaysia (26). Additionally, it is worth noting that 3-FL was detected in 99.9% of the samples in this study which means 3-FL was ubiquitously present in the breast milk of Chinese mothers. This indicates that 3-FL in Chinese breast milk might have an important function in the health outcomes of Chinese infants. 3-FL is one of the few HMOs known to increase significantly during the course of lactation (12, 47, 48). This suggests an important physiological role of 3-FL and warrants further investigation. The concentration of LNT in the breast milk was 701 mg/L at 40–45 days postpartum in this study, 876 mg/L at 30 ± 3 days postpartum in Europe (46) and 1,026 mg/L at 28–50 days postpartum in Brazil (44). The concentration of LNnT in this study was 215 mg/L at 10–15 days postpartum and 61 mg/L at 200–240 days postpartum, respectively. In another study conducted in the United Arab Emirates (27), LNnT was found to be 765 mg/L at 5–15 days postpartum and 250 mg/L at 180 days postpartum, respectively. Furthermore, LNnT was more abundant in milk from Brazilian mothers at 28–50 days postpartum (198 mg/L) (44) compared to the Chinese mothers at 40–45 days postpartum (113 mg/L) measured in this study.

The results of this study showed that 79% of Chinese mothers have the secretor phenotype and 21% express a non-secretor phenotype, although high and low 2′-FL level categories were used instead of the terms secretor and non-secretor in this study. Our study is not the first to use a cut off of 200 mg/L 2′-FL to determine secretor type. A study conducted by Wu et al. (43) in China (Guangzhou) reported that a 2′-FL concentration cut off of 200 mg/L separated secretors and non-secretors with 100% accuracy compared to serological tests. Wu et al. (43) reported that 2′-FL concentration in the breast milk of secretor's mothers was 3,020 mg/L, 2,540 mg/L, 1,960 mg/L, and 1,280 mg/L at 3, 7, 42, and 168 days postpartum, respectively. In our

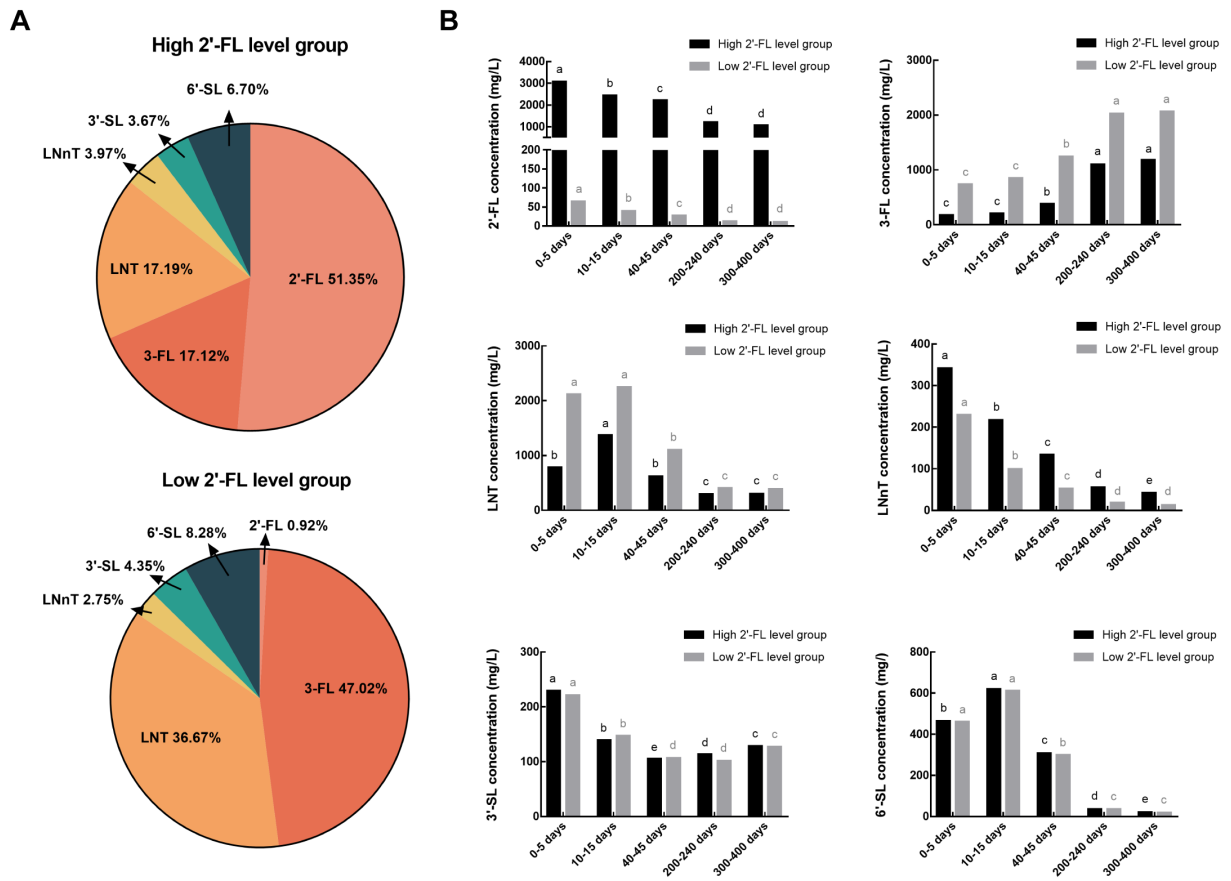

FIGURE 4
The proportion of single HMOs **(A)** and the variations of six HMOs from 0–13 months postpartum **(B)** in the high and low 2′-FL level groups. 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; LNT, Lacto-N-tetraose; LNnT, Lacto-N-neotetraose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose. Total HMO concentration was calculated as the sum of 2′-FL, 3-FL, LNT, LNnT, 3′-SL, and 6′-SL.

study, 2′-FL concentration in secretor's breast milks was 3,442 mg/L, 2,516 mg/L, 2,261 mg/L, 1,306 mg/L, and 1,228 mg/L at 0–5, 10–15, 40–45, 200–240, and 300–400 days postpartum, respectively. Our result from six geographical sites is similar to that of other studies conducted in China which reported 77% secretor phenotype in Guangzhou (43), 80% secretor phenotype in Hohhot (28) and 81% secretor phenotype in (Beijing, Xuchang and Suzhou) (38). However, the prevalence of secretor phenotypes among Chinese mothers differs from the prevalence worldwide (7, 12, 27–30, 44, 45, 49–51). For example, secretor phenotype prevalence is 87% in Finland (45), 89% in Brazil (44), 72% in Kenya (49), 74% in UAE (27), 66% in Spain (7) and 64% in Gambia (30). A recent study also reported that The proportion of mothers who are secretors in South Africa is higher compared to other cohorts (32). Our findings provide data on the prevalence of the secretor phenotype among Chinese mothers, and add to the total literature describing the frequency of the secretor phenotype worldwide.

HMO composition may have a significant effect on the function of breast milk. Studies over the past decade have revealed that the function of HMOs is to some extent related to their structure (18, 20, 22, 52, 53), and secretor and non-secretor milk may function differently in some ways. An observational study showed that infants who received secretor milk had a higher relative abundance of *Lactobacillus* in the gut at 6 months of age compared to non-secretor, while infants who received non-secretor's milk exhibited higher relative abundances of *Bacteroides* than secretor (54). An *in vitro* study showed that secretor milk inhibits the growth of *Streptococcus agalactiae* by reducing the production of the *Streptococcus agalactiae* envelope, while non-secretor milk inhibits the growth of *Streptococcus agalactiae* by changing the morphology of the biofilm (55). Two observational studies indicated that specific milk oligosaccharide structures may associate with the fecal microbiome alteration of either term or preterm infants (56, 57). For term infants, those fed by secretor mother's milk were reported to have quicker establishment of bifidobacterial-laden microbiota than non-secretor mother's milk. For premature infants, those fed by non-secretor mother's milk had higher levels of *Proteobacteria* and lower levels of *Firmicutes* than non-secretor mothers. In this study, the concentration of six HMOs was stratified according to high and low 2′-FL level categories within a cohort of Chinese mothers. The individual HMO concentrations measured in this study were comparable to a prior study conducted in China which also reported on HMO concentrations in milk at 3–168 days postpartum in secretor and non-secretor mothers (43). In addition, the proportions of the six HMOs in the high 2′-FL level group were as follows: 51% 2′-FL, 17% 3-FL, 17% LNT, 4% LNnT, 4% 3′-SL and 7% 6′-SL. The proportions of the six HMOs in the low 2′-FL level group were as follows: 1% 2′-FL, 47% 3-FL, 37% LNT, 2% LNnT, 4% 3′-SL and 8% 6′-SL. Thus, milk from the high and low 2′-FL level groups displayed distinct profiles of HMOs.

TABLE 2 The concentration of human milk oligosaccharides (HMOs) at six geographical sites over 0–400 days postpartum in high 2′-FL level groups [Median (p25, p75)] (mg/L).

HMOs	Lactational stages	Geographical sites						*p* value
		Changchun	Lanzhou	Chengdu	Tianjin	Guangzhou	Shanghai	
2′-FL	0–5 days	2,895 (2,471, 3,447) [c]	2,831 (2,162, 3,528) [c]	3,296 (2,552, 3,900) [a,b]	3,571 (3,017, 4,572) [a]	3,263 (2,551, 5,222) [a,b]	2,838 (2,301, 3,765) [b]	<0.001
	10–15 days	2,271 (1,833, 2,776)	2,564 (2,167, 2,985)	2,472 (2 065, 2,875)	2,504 (2,080, 2,900)	2,446 (1,934, 2,922)	2,432 (1,965, 3,222)	0.661
	40–45 days	2,201 (1,678, 2,683) [a,b]	2,490 (2,162, 2,926) [a]	1,958 (1,579, 2,326) [b]	2,148 (1,644, 2,614) [b]	2,313 (1,803, 2,941) [a,b]	2,159 (1,627, 2,764) [a,b]	<0.001
	200–240 days	1,153 (905, 1,633)	1,421 (923, 1,833)	1,272 (896, 1,742)	1,228 (875, 1,556)	1,238 (935, 1,671)	1,071 (802, 1,424)	0.081
	300–400 days	1,019 (775, 1,507) [a,b]	1,243 (916, 1,673) [a]	945 (695, 1,486) [b]	1,030 (686, 1,364) [a,b]	1,183 (896, 1,607) [a,b]	1,073 (843, 1,385) [a,b]	0.008
3-FL	0–5 days	150 (98, 220) [a,b]	169 (115, 263) [a,b]	133 (100, 209) [b]	176 (117, 294) [a,b]	198 (132, 326) [a]	148 (93, 225) [b]	0.004
	10–15 days	192 (125, 281) [b]	266 (188, 411) [a]	209 (146, 284) [a,b]	206 (133, 295) [b]	170 (121, 254) [b]	209 (93, 290) [b]	0.002
	40–45 days	433 (320, 581) [a]	338 (200, 482) [b]	388 (311, 560) [a,b]	375 (230, 501) [a,b]	397 (289, 597) [a,b]	298 (193, 505) [b]	<0.001
	200–240 days	1,047 (757, 1,516) [a,b]	1,051 (707, 1,312) [a,b]	991 (607, 1,277) [b]	1,180 (891, 1,469) [a,b]	1,148 (811, 1,553) [a,b]	1,247 (908, 1,477) [a]	0.007
	300–400 days	1,355 (964, 1,611) [a]	1,018 (592, 1,374) [b]	1,132 (808, 1,552) [a,b]	1,373 (976, 1,616) [a]	1,011 (712, 1,295) [b]	1,233 (878, 1,611) [a,b]	<0.001
LNT	0–5 days	1,150 (757, 1,487) [a]	829 (464, 1,304) [a,b]	918 (667, 1,218) [a,b]	627 (438, 1,089) [b]	538 (256, 1,061) [b]	810 (425, 1,355) [a,b]	<0.001
	10–15 days	1,495 (1,166, 2,018)	1,230 (818, 1,691)	1,463 (990, 1,670)	1,299 (967, 1,630)	1,393 (962, 1,770)	1,365 (1,074, 1,865)	0.450
	40–45 days	682 (471, 943) [a,b]	851 (580, 1,287) [a]	581 (429, 798) [b]	530 (346, 781) [b]	617 (449, 904) [b]	583 (392, 782) [b]	<0.001
	200–240 days	260 (182, 423) [b]	389 (272, 530) [a]	330 (214, 500) [a,b]	255 (199, 379) [b]	287 (193, 395) [b]	238 (172, 350) [b]	<0.001
	300–400 days	245 (151, 418) [b]	419 (228, 537) [a]	272 (174, 401) [b]	297 (179, 466) [a,b]	329 (226, 456) [a,b]	277 (171, 425) [b]	0.002
LNnT	0–5 days	319 (262, 421) [b]	350 (271, 427) [b]	400 (294, 533) [a,b]	295 (244, 416) [b]	272 (194, 407) [b]	499 (364, 615) [a]	<0.001
	10–15 days	186 (133, 238) [b]	216 (146, 276) [b]	250 (171, 364) [a,b]	191 (137, 282) [b]	209 (140, 269) [b]	305 (242, 392) [a]	<0.001
	40–45 days	133 (96, 194) [a,b]	155 (115, 212) [a]	130 (86, 175) [b]	116 (73, 165) [b]	142 (85, 198) [a,b]	119 (77, 165) [b]	0.002
	200–240 days	50 (28, 74) [b]	85 (48, 126) [a]	52 (28, 93) [b]	59 (37, 102) [a,b]	53 (26, 103) [b]	39 (25, 68) [b]	<0.001
	300–400 days	36 (13, 66) [b]	62 (34, 99) [a]	32 (13, 72) [b]	41 (20, 82) [a,b]	57 (25, 90) [a,b]	30 (17, 60) [b]	<0.001
3′-SL	0–5 days	216 (175, 252) [a,b]	195 (137, 260) [b]	204 (167, 244) [b]	249 (201, 316) [a]	246 (207, 324) [a]	223 (168, 282) [a,b]	<0.001
	10–15 days	144 (133, 166)	132 (101, 158)	138 (124, 164)	132 (115, 160)	140 (123, 162)	135 (119, 154)	0.368
	40–45 days	104 (86, 124) [a,b]	109 (97, 131) [a,b]	83 (72, 99) [c]	104 (84, 119) [b]	112 (95, 132) [a,b]	115 (100, 136) [a]	<0.001
	200–240 days	117 (101, 142) [a]	100 (83, 125) [b]	109 (88, 135) [a,b]	113 (91, 142) [a,b]	119 (98, 139) [a]	110 (98, 132) [a,b]	0.013
	300–400 days	124 (101, 150)	116 (97, 150)	134 (108, 177)	135 (100, 165)	131 (110, 160)	133 (110, 170)	0.099

(Continued)

TABLE 2 (Continued)

HMOs	Lactational stages	Geographical sites						*p* value
		Changchun	Lanzhou	Chengdu	Tianjin	Guangzhou	Shanghai	
6′-SL	0–5 days	490 (416, 584) [a]	408 (349, 543) [a,b]	475 (420, 548) [a,b]	465 (389, 556) [a,b]	404 (299, 501) [b]	490 (420, 560) [a,b]	0.004
	10–15 days	660 (555, 795) [a]	568 (454, 699) [a,b]	584 (442, 657) [b]	640 (497, 763) [a,b]	611 (519, 783) [a,b]	630 (540, 758) [a,b]	0.034
	40–45 days	290 (210, 406) [b]	402 (320, 491) [a]	183 (139, 269) [c]	294 (181, 406) [b]	300 (216, 374) [b]	363 (277, 484) [a]	<0.001
	200–240 days	41 (20, 59) [a,b]	44 (28, 69) [a]	28 (20, 47) [b]	33 (21, 52) [a,b]	36 (22, 54) [a,b]	25 (16, 53) [b]	0.001
	300–400 days	22 (11, 34) [a,b]	18 (11, 28) [b]	15 (10, 23) [b]	21 (13, 38) [a,b]	26 (15, 42) [a]	20 (13, 40) [a,b]	0.001
Total six HMOs	0–5 days	5,445 (5,032, 5,923) [a,b]	4,936 (4,681, 5,566) [b]	5,497 (4,978, 6,096) [a,b]	5,802 (5,190, 6,533) [a]	5,439 (4,871, 6,794) [a]	5,389 (4,905, 5,919) [a,b]	<0.001
	10–15 days	5,063 (4,705, 5,381)	5,182 (4,742, 5,729)	5,205 (4,891, 5,460)	5,173 (4,498, 5,612)	5,128 (4,691, 5,583)	5,314 (4,906, 5,950)	0.285
	40–45 days	3,909 (3,600, 4,308) [c]	4,492 (4,146, 5,025) [b]	3,528 (3,166, 3,736) [d]	3,756 (3,119, 4,191) [c,d]	4,179 (3,704, 4,545) [a]	3,854 (3,338, 4,491) [c]	<0.001
	200–240 days	2,918 (2,672, 3,342) [a,b]	3,156 (2,820, 3,422) [a]	2,915 (2,617, 3,202) [b]	2,931 (2,752, 3,176) [a,b]	3,095 (2,748, 3,358) [a,b]	2,803 (2,622, 3,075) [b]	<0.001
	300–400 days	2,966 (2,718, 3,312)	3,016 (2,739, 3,294)	2,813 (2,506, 3,133)	2,934 (2,639, 3,245)	2,908 (2,689, 3,141)	2,919 (2,745, 3,200)	0.081

[a–d]Values within a row in individual cities with different superscript letters were significantly different (adjusted $p < 0.05$ by the Bonferroni correction for multiple tests) according to an independent nonparametric test (Kruskal–Wallis one-way ANOVA, all pairwise). 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; LNT, Lacto-N-tetraose; LNnT, Lacto-N-neotetraose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose. Total HMO concentration was calculated as the sum of 2′-FL, 3-FL, LNT, LNnT, 3′-SL, and 6′-SL.

By using the same sample collection and HMO measurement methods, our data from six geographical sites demonstrated that geographical location has an effect on the concentration of HMOs in the Chinese milks. The effect of geographical location on HMO concentration in China has been previously investigated by Ren et al. (39). Their study reported no significant difference in the total content of 24 HMOs in breast milk from mothers in coastal and inland areas of China. However, in our current study, we analyzed the variations in individual HMO content among six regions at different lactation stages. We observed that breast milk from coastal cities (Guangzhou and Shanghai) exhibited significantly higher concentrations of 3′-SL compared to inland cities (Lanzhou) at 200–400 days postpartum. Global research has documented geographical factors affecting HMO concentration (13, 32). A study conducted by McGuire et al. (13) in 11 global cohorts with 410 breast milk samples confirmed that HMO concentrations and profiles vary geographically. 2′-FL concentration was 3.9 times higher in milk collected in the US (2,740 mg/L) than in milk collected in Ghana (700 mg/L) at 2 weeks-5 months postpartum. Ma et al. (26) analyzed a total of 243 breast milk samples from China (Guangzhou) and Malaysia and reported that the 2′-FL mean (SD) concentration in breast milk of Chinese (Guangzhou) and Malaysian mothers is 704.1 (752.4) mg/L and 1,002.8 (803.4) mg/L, respectively, at 180 days postpartum. 3-FL mean (SD) concentration in the breast milk of Chinese (Guangzhou) and Malaysian mothers is 1,475.8 (789.9) mg/L and 1,146.1 (868.5) mg/L, respectively at 180 days postpartum. In our study, the concentration ranges of 2′-FL and 3-FL were 935–1,123 mg/L and 1,061–1,375 mg/L, respectively, at 200–240 days postpartum. Compared with other studies, variation among different geographical sites within China is smaller than the variation observed between different countries.

We also observed that geographical variations in concentration were not consistent across the 5 sampling times. For example, the breast milk of mothers in Chengdu had higher levels of 6′-SL than Guangzhou at 0–5 days postpartum, however, the breast milk of mothers in Chengdu had lower levels of 6′-SL than Guangzhou at 300–400 days postpartum. The influence of geographic factors is complex as geographic location may include multiple aspects, such as ethnicity, maternal nutrition, and climate. Although China is a large country, the majority of the population in the six geographical sites involved in this study are of Han ethnicity (>90% of recruited subjects). In this case, differences occurred despite similar genetic backgrounds, suggesting that environmental factors may be important. A study conducted by Seferovic et al. (58) observed that compared to a high carbohydrate diet, a high-fat diet decreased the concentrations of sialylated HMOs. Seppo et al. (59) demonstrated that the concentrations of 3-FL and 3′-SL were higher in colostrum from mothers in the probiotic supplementation group, whereas difucosyllacto-N-hexaose, LNT, lacto-N-fucopentaose I, and 6′-SL were lower in mothers who received probiotic supplementation relative to a control group. A published study from our team showed that dietary vitamins, vegetables and metal elements are positively associated with HMO concentration using a mixed effect model with lactational stage and secretor status as the random effect (60). In addition, investigations by Davis et al. (50) revealed that mothers nursing in the dry season produced significantly more total HMOs compared to mothers nursing in the wet season. Therefore, maternal nutrition, and climate need to be further examined in order to explain the effect of geographical location on HMO concentration.

HMOs are a research hotspot of infant nutrition (61–66). Several studies have investigated the safety and tolerability of infant formula

TABLE 3 Concentration of human milk oligosaccharides (HMOs) at six geographical sites over 0–400 days postpartum in low 2′-FL level groups [Median (p25, p75)] (mg/L).

HMOs	Lactational stages	Geographical sites						*p* value
		Changchun	Lanzhou	Chengdu	Tianjin	Guangzhou	Shanghai	
2′-FL	0–5 days	64 (45, 79)	51 (46, 75)	59 (45, 74)	69 (54, 94)	64 (47, 86)	38 (21, 90)	0.467
	10–15 days	37 (32, 43)	40 (30, 42)	40 (35, 46)	38 (28, 50)	39 (36, 47)	40 (30, 53)	0.962
	40–45 days	25 (21, 32) [b]	37 (33, 43) [a]	29 (22, 35) [a,b]	30 (24, 41) [a,b]	14 (5, 20) [c]	23 (20, 29) [b,c]	<0.001
	200–240 days	14 (9, 17) [a,b]	14 (10, 18) [a,b]	11 (9, 16) [a,b]	16 (12, 22) [a]	15 (2, 20) [a,b]	8 (6, 12) [b]	0.012
	300–400 days	8 (5, 10) [b]	12 (8, 20) [a,b]	14 (10, 26) [a,b]	16 (11, 22) [a]	12 (7, 13) [a,b]	7 (4, 10) [b]	0.001
3-FL	0–5 days	756 (480, 947)	518 (385, 818)	728 (394, 1,114)	714 (577, 962)	944 (674, 1,302)	721 (421, 1,138)	0.190
	10–15 days	933 (647, 1,187)	1,013 (819, 1,408)	944 (547, 1,605)	813 (649, 1,073)	758 (661, 998)	1,064 (561, 1,115)	0.630
	40–45 days	1,299 (826, 1,692) [a,b]	1,305 (841, 1,618) [a,b]	1,170 (774, 1,692) [a,b]	1,090 (854, 1,460) [a,b]	1,641 (1,192, 1,907) [a]	1,075 (782, 1,462) [b]	0.035
	200–240 days	1,945 (1,514, 2,217)	2,195 (1,468, 2,454)	1,991 (1,264, 2,165)	2,163 (1,387, 2,474)	2,336 (1,814, 2,743)	1,930 (1,627, 2,558)	0.181
	300–400 days	2,131 (1,590, 2,567)	2,041 (1,540, 2,628)	1,996 (1,483, 2,405)	2,050 (1,687, 2,361)	1,896 (1,270, 2,403)	2,367 (1,892, 2,757)	0.450
LNT	0–5 days	2,126 (1,670, 2,445)	2,115 (1,415, 2,644)	2,488 (1,495, 3,150)	2,052 (1,443, 2,557)	2,021 (1,067, 2,434)	2,065 (1,764, 2,332)	0.790
	10–15 days	2,030 (1,115, 2,331)	2,037 (1,947, 2,700)	2,298 (1,499, 2,787)	2,241 (1,808, 2,622)	2,299 (1,712, 2,759)	3,121 (2,124, 3,852)	0.072
	40–45 days	1,005 (837, 1,294)	1,446 (957, 1,769)	803 (583, 1,216)	977 (800, 1,366)	1,104 (805, 1,314)	1,184 (883, 1,962)	0.064
	200–240 days	318 (222, 536)	508 (354, 806)	417 (246, 543)	383 (176, 606)	443 (280, 648)	345 (154, 842)	0.177
	300–400 days	340 (271, 541)	489 (285, 785)	344 (242, 617)	336 (253, 599)	671 (353, 785)	363 (185, 495)	0.055
LNnT	0–5 days	234 (159, 458)	208 (134, 400)	219 (120, 373)	273 (205, 372)	211 (153, 269)	236 (164, 396)	0.556
	10–15 days	76 (56, 129)	75 (51, 142)	73 (41, 238)	101 (60, 152)	115 (70, 164)	88 (62, 261)	0.733
	40–45 days	41 (28, 81)	56 (36, 115)	44 (28, 67)	62 (39, 100)	49 (29, 88)	56 (34, 82)	0.407
	200–240 days	14 (6, 23)	23 (17, 41)	18 (11, 38)	20 (13, 35)	19 (12, 40)	10 (4, 18)	0.083
	300–400 days	7 (4, 15)	15 (6, 27)	7 (6, 22)	14 (7, 27)	20 (10, 34)	11 (5, 18)	0.051
3′-SL	0–5 days #	169 (147, 242)	167 (108, 250)	218 (172, 251)	240 (220, 301)	216 (197, 295)	215 (203, 222)	0.029
	10–15 days	142 (121, 167)	156 (130, 198)	159 (147, 172)	142 (116, 169)	141 (124, 175)	145 (134, 163)	0.462
	40–45 days	93 (78, 115) [b]	118 (99, 146) [a]	95 (72, 104) [b]	99 (76, 125) [a,b]	111 (91, 135) [a,b]	113 (104, 128) [a,b]	0.001
	200–240 days	98 (89, 122) [a,b]	90 (74, 108) [b]	99 (88, 145) [a,b]	100 (84, 116) [a,b]	103 (90, 126) [a,b]	117 (101, 150) [a]	0.043
	300–400 days	113 (93, 138) [b]	104 (87, 124) [b]	137 (107, 145) [a,b]	126 (90, 143) [a,b]	144 (127, 170) [a]	140 (114, 179) [a,b]	0.001
6′-SL	0–5 days	493 (398, 552)	427 (285, 576)	588 (404, 721)	452 (397, 507)	431 (312, 561)	530 (376, 607)	0.454
	10–15 days	654 (558, 763)	630 (537, 765)	666 (544, 738)	602 (474, 742)	568 (512, 734)	566 (509, 772)	0.869
	40–45 days	305 (251, 428) [a,b]	425 (296, 551) [a]	228 (150, 274) [b]	267 (163, 382) [b]	299 (219, 365) [a,b]	325 (212, 451) [a,b]	0.001
	200–240 days #	40 (20, 73)	43 (33, 86)	29 (20, 37)	29 (10, 45)	44 (34, 64)	28 (14, 40)	0.015
	300–400 days	16 (9, 33)	29 (18, 58)	19 (13, 29)	17 (7, 27)	21 (15, 30)	19 (12, 34)	0.241
Total six HMOs	0–5 days	3,953 (3,211, 4,852)	3,534 (3,128, 4,334)	4,261 (3,676, 4,846)	3,882 (3,579, 4,191)	3,678 (3,416, 4,264)	3,960 (3,682, 4,052)	0.535
	10–15 days	3,723 (3,140, 4,255) [b]	4,269 (3,693, 4,858) [a,b]	4,125 (4,002, 4,846) [a,b]	4,095 (3,570, 4,568) [a,b]	3,912 (3,598, 4,428) [a,b]	4,643 (4,108, 5,880) [a]	0.039
	40–45 days	2,979 (2,466, 3,175) [a,b]	3,444 (2,773, 3,832) [a]	2,506 (2,143, 2,990) [b]	2,639 (2,412, 3,084) [b]	3,271 (2,968, 3,480) [a]	2,846 (2,503, 3,295) [a,b]	<0.001
	200–240 days	2,388 (2,114, 2,909) [b]	2,936 (2,403, 3,234) [a,b]	2,670 (2,057, 2,786) [b]	2,807 (2,317, 3,097) [a,b]	2,938 (2,733, 3,418) [a]	2,738 (2,180, 3,062) [a,b]	0.005
	300–400 days	2,591 (2,193, 3,068)	2,860 (2,629, 3,190)	2,542 (2,152, 3,136)	2,687 (2,451, 3,072)	2,789 (2,236, 3,216)	2,884 (2,527, 3,179)	0.540

[a–c]Values within a row in individual cities with different superscript letters were significantly different (adjusted $p < 0.05$ by the Bonferroni correction for multiple tests) according to an independent nonparametric test (Kruskal–Wallis one-way ANOVA, all pairwise). 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; LNT, Lacto-N-tetraose; LNnT, Lacto-N-neotetraose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose. Total HMO concentration was calculated as the sum of 2′-FL, 3-FL, LNT, LNnT, 3′-SL, and 6′-SL.

with added HMOs, including containing single (61, 64), dual (67, 68), or five types of HMOs (69, 70). Research to date, showed 3-FL increased through lactation, especially in Chinese populations, in which 3-FL is reported to be higher than in other counties. LNT, the most abundant acetylated HMO is globally reported to be at a higher level than LNnT in mature milk (26, 27, 44, 45, 71). The six types of HMOs analyzed in this study are among the first to potentially be added to infant formula in China. The data from Chinese breast milk presented in this study can serve as a reference for adding these HMOs into formula milk in China.

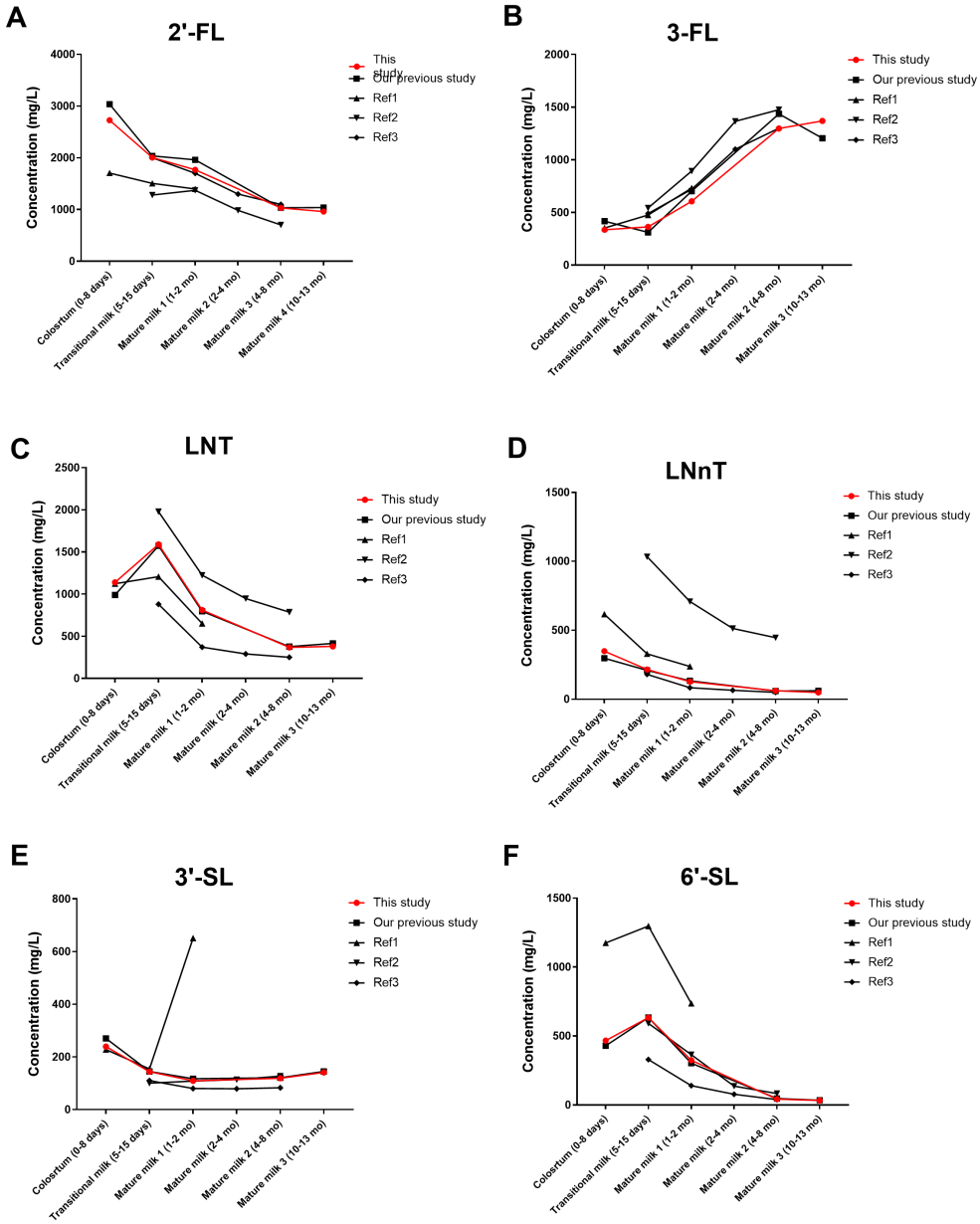

FIGURE 5
Comparison of HMO concentrations in this study and other studies in Chinese populations **(A–F)**. Our previous study (37); Ref 1, reference 1 (41); Ref 2, reference 2 (26); Ref 3, reference 3 (10). 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; LNT, Lacto-N-tetraose; LNnT, Lacto-N-neotetraose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose. Total HMOs concentration was calculated as the sum of 2′-FL, 3-FL, LNT, LNnT, 3′-SL, and 6′-SL. The data points represent the average value.

This study has some limitations. First, we were unable to report on the maternal Lewis status of Chinese mothers due to the lack of data on α1-4 linked HMOs. Several studies have classified mothers as Lewis-positive and Lewis-negative according to the amount of Lacto-N-fucopentaose II (LNFP II) in breast milk because LNFP II contains the α1-4 linkage (38, 72, 73). Second, we did not investigate the effect of maternal nutrition on HMO concentration in lactating mothers. Future studies are needed to reveal the relationship between HMO concentration and maternal nutrition.

In conclusion, HMOs are an abundant class of carbohydrates found in human milk and have been linked to developmental outcomes in infants, specifically cognition, the developing immune system, and the gut microbiome. We systematically analyzed the dynamic changes in six abundant HMOs in 2,616 breast milk samples from 1,758 healthy mothers at 0–13 months postpartum collected across six geographical sites in China. The concentrations and proportions of these six HMOs varied dynamically during lactation, but this variation was consistent across multiple geographical sites. We determined that 79% of breast milk was classified as containing high 2′-FL levels and 21% of breastmilk contained low 2′-FL levels in this large Chinese cohort. This analysis of the HMO composition of human milk samples collected from six sites in China provided the most extensive data set reported to date, which is helpful for comparison with HMO data worldwide and will provide a strong foundation of scientific principles to further guide infant formula fortification strategies. Future studies may determine the relationship

between changes in HMO composition and maternal and infant health.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The protocol was approved by the Ethics Committee of Tianjin hospital of ITCWM Nankai hospital. This study was registered in the China Clinical Trial Center (ChiCTR1800015387). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SL: Formal analysis, Software, Visualization, Writing – original draft. YM: Conceptualization, Investigation, Validation, Writing – original draft. JW: Data curation, Methodology, Validation, Writing – review & editing. FT: Methodology, Software, Visualization, Writing – review & editing. DH: Writing – original draft. XX: Investigation, Validation, Writing – review & editing. XL: Data curation, Supervision, Writing – review & editing. YZ: Resources, Supervision, Writing – review & editing. SW: Funding acquisition, Project administration, Writing – original draft.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1267287/full#supplementary-material

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Characteristics and factors influencing the volume of breastmilk donated by women to the first human milk bank in Vietnam

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Background: Donor human milk (DHM) is essential to the operation of human milk banks (HMB). This study examined characteristics and factors associated with higher volumes of DHM donation at the first HMB in Vietnam.
Method: Data from an online HMB monitoring system collected between February 2017 and July 2022 included demographic characteristics, child information, the timing of donation, and the volume of DHM. Higher volume is defined as equal to or greater than the median DHM volume per donor of 14.4 liters (L). Potential contributors to higher DHM volume were examined using the chi-square test in univariate and multivariable logistic regression analysis.
Results: During the 5.5-year operation, this HMB recruited 517 donors with an average age of 28.6 years. Approximately 60.9% of donors had a college or higher degree and 97.3% gave birth in Da Nang city. Of these donors, the prevalence of cesarean birth was 48.2%, preterm births was 40.2%, and 27.9% had babies with a birth weight of less than 1,500 g. There was a similar proportion of donors between the hospital (48.2%) and community (51.8%). On average, hospital donors started their donations 15 days after birth when their newborns were 33.9 weeks corrected age compared to 63 days and 47.7 weeks among community-based donors. The overall median volume of DHM per donor was 14.4 L over a period of 46 days. The amount and duration were higher in community-based donors (17.5 L in 72 days, 300 ml/day) than those in the hospital (8.4 L in 30 days, 258 ml/day). More than 37% of donors contacted the HMB themselves; the remainder were introduced by health professionals. Factors associated with higher volumes of DHM included higher education (OR: 1.77; 95% CI: 1.09, 2.87), having a full-term newborn (OR: 2.46; 95% CI: 1.46, 4.13), and community-based donors (OR: 2.15; 95% CI: 1.22, 3.78).
Conclusions: Mothers with higher education and from the community donate more breastmilk over a longer duration than those with lower education and from the hospital. Breastfeeding protection, promotion, and support should be offered to all mothers with specialized breastfeeding support for mothers of preterm and sick infants. This will ensure mothers have sufficient breastmilk for their newborns and potentially surplus breastmilk for donation.

KEYWORDS
breastfeeding, breastmilk, donor human milk, human milk bank, newborn

1. Introduction

Breastfeeding is the biological norm for feeding infants and is vital for the survival and development of sick and very preterm newborns. When mothers' own breastmilk is not available, the World Health Organization (WHO) recommends donor human milk (DHM) from a human milk bank (HMB) as the next option to the mother's own breastmilk (1, 2). An HMB recruits and screens donors, stores, processes, screens and provides safe DHM to infants in need (3). Since the first HMB opened in Austria in 1909, there are almost 800 HMBs in 70 countries (3, 4). Although HMBs are operated differently across countries, the women who donate their surplus breastmilk are always essential to the operation of HMBs (5).

The first HMB in Vietnam was established in Da Nang in 2017 (6) with donors recruited from both the community and the Da Nang Hospital for Women and Children (DNHWC) where the HMB is located. During the last five years of operation, the HMB received more than 10,036 liters (L) of DHM and provided pasteurized DHM to 23,016 infants whose own mother's milk was not available or insufficient. Although this HMB is non-profit and receives valuable DHM for free, resources are needed for its operation and maintenance. Based on the expenditure on consumables, electricity, water, DHM transport, microbiological testing, garbage collection, property maintenance, depreciation, salary, and research and learning, the HMB estimated the price of pasteurized DHM and got approval from the Da Nang Department of Health. The current subsidized price of about USD 60 per L does not account for down payments of substantial startup investments and the level of efforts by the majority of staff involved are covered by other hospital departments such as initial donor recruitment, storing and distributing DHM as well as managing DHM refrigerators in the neonatal unit and postnatal wards (6, 7). Because social health insurance has not covered the cost of pasteurized DHM, families of vulnerable newborns must pay for its use. In addition to ongoing advocacy for social health insurance to cover the use of pasteurized DHM for vulnerable newborns, efforts to reduce and cover associated costs are needed.

An approach to reduce the operational cost is to focus on increasing donors who have the potential to provide a higher volume of DHM over a longer period. All donors in Da Nang are screened for human immunodeficiency virus (HIV), hepatitis B and C, and syphilis. DHM also needs to pass microbiological tests pre- and post-pasteurization. Because DHM is only pooled from one mother for pasteurization, the HMB must store individual DHM until a sufficient amount is available for pasteurization (6). These processes require resources with associated costs including the testing and electricity used. An operational research question for the HMB is how to identify potential women who could safely donate a higher volume of DHM for a longer duration. To address this knowledge gap, this study aims to investigate the characteristics of the donors and factors associated with a high volume of DHM using monitoring data from more than five years of operation of the first HMB in Vietnam. The study will also contribute to global data on human milk banking (8) and inform more effective donor recruitment and support strategies.

2. Methods

2.1. Study setting

Da Nang is a class-1 municipality city in the central coastal area of Vietnam with a total area of 1,284.73 square kilometers. It is the economic, cultural, and educational center of Central Vietnam. Economic revenue for Da Nang is from service (68.4%), industry and construction (20.4%), tax (9.2%), and agriculture (2.0%) (9). Da Nang has a population size of 1,195,490 and a crude birth rate of 18.68 per thousand population or approximately 22,400 infants born each year. The infant mortality rate in Da Nang City in 2021 was 8.19 deaths per thousand live births, which is lower than the national rate of 13.65 deaths per thousand live births (10).

DNHWC is a tertiary hospital for obstetrics, gynecology, and pediatrics, which has 1,200 beds and serves mainly three provinces with a population of 4.4 million in 2019–2020 (11). This hospital supports more than 15,000 births annually (11) and receives high-risk pregnancies and sick children as a referral hospital for the central region of Vietnam (11). The neonatal unit admitted approximately 4,000 newborns with more than 30% being preterm. The hospital is recognized nationally and internationally as a center of excellence for the implementation of Ten Steps to Successful Breastfeeding, early essential newborn care, and kangaroo mother care (KMC) (12, 13). The inclusion criteria for human milk donors include passing a questionnaire for medical history screening, general health status, and lifestyle (6). The questionnaire includes screening for medications that are contraindicated for breastfeeding, having received a blood transfusion, or tattoos within the previous six months (6). Donors must also pass the serological screening tests for HIV, hepatitis B virus, hepatitis C virus, and syphilis (6). Following completion of the screening process, accepted donors attend face-to-face educational training by HMB staff on hand hygiene, equipment cleaning, and DHM storage with final approval from the HMB manager to begin donation (6).

2.2. Study data

Data was collected during the normal operation of the HMB from February 2017 to July 2022. The data were collected and updated daily to a web-based package that captures all aspects of the donors including donor recruitment, screening, donor information, and volume of DHM (11). For this study, we extracted data directly from the HMB software and removed identifiable information from all records before data analysis.

2.3. Variables

An outcome variable for this study was the total volume of DHM, which was the summation of the amount of DHM of each donor. The donation duration was calculated by subtracting

the donor approval date from the date of the last donation. The average volume of DHM per donor was calculated using the median volume of DHM per donor. For the regression analysis, we dichotomized the total volume using the median value: higher volume was defined as equal to or greater than the median volume of DHM per donor of 14.4 L.

The origin of the donor was defined at enrolment. A hospital donor begins donation while her newborn was cared for in the neonatal unit, while a community donor begins donation while her infant was at home. Thirty-seven out of 249 hospital donors continued donating breastmilk after their infants' discharge. We grouped them as hospital donors during the data analysis (intention-to-treat).

Maternal demographic characteristics including age, education, profession, and residency were recorded. Experience relating to the most recent birth was recorded including the number of children, place of birth, mode of birth, child sex, gestational age at birth, and birthweight.

In addition, we also extracted data on the sources of information relating to HMB and donation of breastmilk that the donors received and if the donor proactively approached HMB staff for donation.

2.4. Data analysis

We firstly performed descriptive analysis with stratification by donor origin for (1) demo- socio-economic characteristics and their birth experience, (2) sources of information relating to HMB and donation of breastmilk, and (3) volume and duration of donations. We presented descriptive statistics as the mean and standard deviation for normally distributed continuous variables or the median and interquartile range for skewed continuous variables. Counts and percentages were used for binary and categorical variables.

Secondly, we performed crude, binary analysis to examine the association between donors who contacted the HMB to donate, donor origin, donor characteristics, and birth experience for the prediction of a higher volume of DHM (≥14.4 L) using the chi-square test.

Thirdly, we performed adjusted logistic regression models with all the above covariates using Wald statistics.

The results were presented as crude and adjusted odds ratios with 95% confidence intervals. A two-tailed p-value of <0.05 defined statistical significance. We used Stata 15.1 (Stata Inc., TX, USA) to analyze the data.

3. Results

3.1. Donor characteristics

During five and a half years of operation, the HMB had 517 donors: 48.2% were recruited during inpatient stays, and the remaining 51.8% from the community (Table 1). The average age of the donors from both the hospital and the community was 28.6 years. Overall, 56.9% of the donors had a college higher degree of education and 60.9% had a white-collar job. Donors from the community had a higher education and job profile than those from the hospital (Table 1). Donors from the community were mostly living in Da Nang city (93.7%), which was higher than donors recruited from the hospital (45.8%) (Table 1).

3.2. Experiences relating to the most recent childbirth

Around 70.6% of the donors came from Da Nang City and 78.9% gave birth at the DNHWC where the HMB is located. The proportion of cesarean and vaginal births was similar among the donors (48.4% and 51.6%); 60.3% donated following the birth of their first child with 40.2% of donors having preterm infants, and 27.9% had babies with a birth weight of less than 1,500 g this time (Table 1).

TABLE 1 Characteristics of donors at the human milk bank from 2017 to 2022[a].

	Hospital (n = 249)	Community (n = 268)	Total (n = 517)
Demo- socio-economic characteristics			
Age, y mean (SD)	28.2 ± 4.6	28.9 ± 3.7	28.6 ± 4.2
Education			
High school	100 (40.2)	33 (12.3)	133 (25.7)
Diploma	55 (22.1)	35 (13.1)	90 (17.4)
College, university and higher	94 (37.8)	200 (74.6)	294 (56.9)
Profession			
Housewives	63 (25.3)	34 (12.7)	97 (18.8)
White-collar jobs	115 (46.2)	200 (74.6)	315 (60.9)
Blue color jobs	41 (16.5)	17 (6.3)	58 (11.2)
Trader	30 (12.0)	17 (6.3)	47 (9.1)
Residence place			
Da Nang	114 (45.8)	251 (93.7)	365 (70.6)
Other provinces	135 (54.2)	17 (6.3)	152 (29.4)
Number of children			
1	140 (56.2)	172 (64.2)	312 (60.3)
2	93 (37.3)	87 (32.5)	180 (34.8)
3+	16 (6.4)	9 (3.4)	25 (4.8)
The most recent birth experience			
Sex			
Male	126 (50.6)	145 (54.1)	271 (52.4)
Female	123 (49.4)	123 (45.9)	246 (47.6)
Gestational age			
Mean (SD)	31.4 ± 3.9	37.7 ± 3.5	34.7 ± 4.9
<37 weeks	190 (76.3)	18 (6.7)	208 (40.2)
≥ 37 weeks	59 (23.7)	250 (93.3)	309 (59.8)
Birth weight, g mean (SD)	1,639 ± 736	3,022 ± 743	2,356 ± 1,012
Mode of births			
Vaginal	150 (60.2)	117 (43.7)	267 (51.6)
Cesarean	99 (39.8)	151 (56.3)	250 (48.4)
Place of birth			
DNHWC	233 (93.6)	175 (65.3)	408 (78.9)
Other hospitals in Da Nang	8 (3.2)	87 (32.5)	95 (18.4)
Hospitals in other provinces	8 (3.2)	6 (2.2)	14 (2.7)

DNHWC, Da Nang Hospital for Women and Children.
[a]Data were presented as number (%) or mean ± standard deviation (SD) when specified.

TABLE 2 Sources of information relating to the human milk bank (HMB) and donation of breastmilk[a].

	Hospital ($n = 249$)	Community ($n = 268$)	Total ($n = 517$)
Proactiveness in donation			
Health professionals contacted donors	217 (87.1)	108 (40.3)	325 (62.9)
Donors contacted HMB	32 (12.9)	160 (59.7)	192 (37.1)
People who talked with donors about donating human milk			
Neonatal unit staff	198 (79.5)	54 (20.1)	252 (48.7)
HMB staff	15 (6.0)	34 (12.7)	49 (9.5)
Postnatal staff	3 (1.2)	13 (4.9)	16 (3.1)
Antenatal clinic staff	6 (2.4)	7 (2.6)	13 (2.5)
Previous donors	0 (0.0)	8 (3.0)	8 (1.5)
Others	31 (12.4)	99 (36.9)	130 (25.1)
Other sources of information about donating human milk			
Facebook Fan page	20 (8.0)	142 (53.0)	162 (31.3)
Poster	20 (8.0)	22 (8.2)	42 (8.1)
Websites	11 (4.4)	19 (7.1)	30 (5.8)
Events	1 (0.4)	4 (1.5)	5 (1.0)
Newspaper, magazines	3 (1.2)	1 (0.4)	4 (0.8)
Brochures	1 (0.4)	0 (0.0)	1 (0.2)
Others	7 (2.8)	12 (4.5)	19 (3.7)

[a]Data were presented as number (%) HMB, Human milk bank.

3.3. Donor recruitment

The proportion of donors who proactively contacted the HMB was 37.1%, and this was higher in donors from the community (59.7%) than those from the hospital (12.9%) (Table 2).

For donors from the hospital, the recruitment was done mostly by staff at the neonatal unit (79.5%), followed by HMB (6.0%), antenatal clinic (2.4%), and postnatal care (1.2%) (Table 2). Alternatively, the main sources of information for community donors were Facebook (53.0%), neonatal unit staff (20.1%), HMB staff (12.7%), posters (8.2%), and websites (7.1%). However, traditional mass media such as newspapers, magazines, and brochures were not a common source of information (Table 2).

3.4. Human milk donations

Throughout the duration of five and a half years, 517 women donated 10,104 L of DHM, of which 67.1% were from community donors (Table 3). On average, donors from the hospital started their donation 15 days after birth and when their newborns were 33.9 weeks of corrected age while donors from the community started 63 days after birth when their children were about 47.7 weeks. The average volume of DHM for each donor was 14.4 L over 46 days: the amount and duration were more than double in donors from the community than those in the hospital (17.5 vs. 8.4 L, 72 days vs. 30 days). The median daily volume of DHM was also higher among community donors as compared to hospital donors (300 ml vs. 258 ml) (Table 3).

3.5. Associated factors for providing a high volume of DHM

In crude models (Table 4), a higher volume of DHM was positively associated with mothers' proactively approaching the HMB to become donors (OR: 2.15; 95% CI:1.50, 3.10), mothers with college, university, or higher education (OR: 2.28; 95% CI:1.60, 3.25), a white-collar job (OR: 1.49; 95% CI: 1.04, 2.12), full-term birth (OR: 4.05; 95% CI:2.81, 5.84), residency in Da Nang (OR: 2.33; 95% CI:1.57, 3.44), and being a community donor (OR: 4.10; 95% CI: 2.84, 5.92).

In the adjusted logistic regression model (Table 4), factors associated with a higher volume of DHM included higher education (OR: 1.77; 95% CI: 1.09, 2.87, $p = 0.02$), having a full-term infant (OR: 2.46; 95% CI: 1.46, 4.13, $p = 0.001$), and community donors (OR: 2.15; 95% CI: 1.22, 3.78, $p = 0.008$).

4. Discussions

4.1. Hospital and community-based donors were mostly under 30 years of age

The demographic characteristics of women providing DHM to the first HMB in Vietnam at the DNHWC showed both similarities and differences when compared to other settings. The average donor age in this study was 28.6 years which is higher than the early- to mid-twenties found in Brazil and Indian studies (8) but similar to Chinese studies (14, 15) and younger than donors in Spain, Korea, Taiwan, or the US (8, 16, 17). Donors to the first HMB in Vietnam

TABLE 3 Volume and duration of donations to the human milk bank from 2017 to 2022.

	Hospital ($n = 249$)	Community ($n = 268$)	Total ($n = 517$)
Total DHM volume, L	3,322	6,782	10,104
Average donation duration, median (IQR) days	30.0 (20, 58.5)	72.0 (31.3, 118)	46.0 (24, 100)
Average volume of DHM, median (IQR) L	8.4 (5.4, 16.9)	17.5 (8.7, 26.3)	14.4 (7.6, 25.2)
Average volume of DHM per day, median (IQR) ml/day	258 (192–346)	300 (193–380)	279 (193–363)
Donation starting time after birth, median (IQR) days	15.0 (7.0, 34.0)	63.0 (26.0, 105.0)	31.0 (10.0, 75.5)
Child gestational age at starting of donation, mean (SD) weeks	35.1 ± 6.4	48.2 ± 9.8	41.9 ± 10.6
Child gestational age at finishing of donation, mean (SD) weeks	42.1 ± 8.8	60.8 ± 13.6	51.8 ± 14.9
Average donation duration, median (IQR) child days old	61.0 (37.0, 95.5)	149.0 (106.3, 202.5)	104.0 (54.5, 167)
Duration of donation of ≥46 days, *n* (%)	83 (33.3)	178 (66.4)	261 (50.5)
Volume of DHM ≥ 14.4 L, *n* (%)	81 (30.2)	178 (66.4)	259 (50.1)

DHM, Donor human milk; IQR, Interquartile range; SD, Standard deviation.

TABLE 4 Factors associated with a higher volume of pasteurized human donor milk.

	Donated at least 14.4 L		Crude model		Adjusted model	
	No ($n = 258$)	Yes ($n = 259$)	OR (95% CI)	*p*-value	aOR (95% CI)	*p*-value
Donors contacted HMB for donation, *n* (%)	73 (28.3)	119 (45.9)	2.15 (1.50, 3.10)	**<0.001**	0.82 (0.51, 1.32)	0.41
Community donor, *n* (%)	**90 (34.9)**	**178 (68.7)**	**4.10 (2.84, 5.92)**	**<0.001**	**2.15 (1.22, 3.78)**	**0.008**
Mother age (SD)	28.3 ± 4.5	28.8 ± 3.9	1.31 (0.92, 1.86)	0.14	1.45 (0.93, 2.25)	0.10
College, university, or higher, *n* (%)	**121 (46.9)**	**173 (66.8)**	**2.28 (1.60, 3.25)**	**<0.001**	**1.77 (1.09, 2.87)**	**0.02**
Obtained white-collar jobs, *n* (%)	145 (56.2)	170 (65.6)	1.49 (1.04, 2.12)	0.03	0.71 (0.44, 1.15)	0.71
Resided in Da Nang city, *n* (%)	160 (62)	205 (79.2)	2.33 (1.57, 3.44)	**<0.001**	0.95 (0.58, 1.55)	0.82
Had the first child, *n* (%)	155 (60.1)	158 (61.0)	1.04 (0.73, 1.48)	0.83	1.09 (0.70, 1.70)	0.72
Male, *n* (%)	133 (51.6)	137 (52.9)	1.06 (0.75, 1.49)	0.76	1.02 (0.70, 1.48)	0.94
Full-term births, *n* (%)	**94 (36.4)**	**181 (69.9)**	**4.05 (2.81, 5.84)**	**<0.001**	**2.46 (1.46, 4.13)**	**0.001**
Cesarean births, *n* (%)	120 (46.5)	130 (50.2)	1.16 (0.82, 1.64)	0.40	1.27 (0.86, 1.88)	0.23
Giving birth at DNHWC	224 (86.8)	184 (71.0)	0.37 (0.24, 0.58)	**<0.001**	0.66 (0.39, 1.12)	0.13

DNHWC, Da Nang Hospital for Women and Children. Adjusted odds ratios (aOR) and 95% CI from multiple logistic regression models, controlled for covariates in this table. The bold highlighted values corresponding to variables with statistically significant ($p < 0.05$).

had higher education levels compared to donors from Brazil and similar to that from China, Norway, and Spain (8). Donors in this study had higher cesarean birth rates (48.4%) than those in China (32.8%) (15) or India (44.0%) (18). A potential explanation could be that this hospital is a referral facility that receives clients with high-risk pregnancies within the province and from other provinces in the central part of Vietnam (19). Cesarean births and vaginal births with episiotomy are barriers to breastfeeding (20–22). A study from eight countries in the Western Pacific region showed that where mothers received early and uninterrupted skin-to-skin contact in a supportive environment such as rooming-in practice and prohibiting formula use as policy, the breastfeeding rate was still high (23). Therefore, regardless of the mode of birth, early support and skin-to-skin contact could improve the likelihood of women establishing a good breastmilk supply and having surplus breastmilk for their infants and donations.

4.2. Mothers with higher education, from the community, and having full-term newborns donated more breastmilk

In this study, we found that donors with higher education, from the community, and having a full-term newborn were more likely to donate a higher volume to the HMB. Higher education was found to be associated with a higher volume of DHM. Previous studies have proved maternal higher education to be an important enabler for successful breastfeeding (24, 25). Mothers with higher education have more opportunities to seek information and are more confident in contacting HMBs directly for information or to donate. As found in a study from Brazil, higher education was a benefit for HMBs because more donors in this group donated more than one time (i.e., repeat donors) compared to the lower education group (26). Future support should focus on women with lower education by increasing their breastfeeding knowledge and skills to enable them to exclusively breastfeed their infants and, where appropriate, donate any surplus milk.

Mothers of full-term infants provided a higher volume of DHM compared with mothers of pre-term infants. There may be several reasons to explain this association. The mothers with preterm infants tended to donate human milk during their hospital stay and finishing after the infant was discharged. This can be seen by the infant age at the start of donation, 35.1 weeks, and the age at completion of the donation of 42.1 weeks. In contrast, mothers of healthy infants at home have greater comfort and the potential for a surplus of their own milk. In addition, support from family members was important to enhance breastfeeding (27, 28) and subsequently support human milk donation. These mothers expressed and stored milk for their infants for future use (e.g., after going back to work) and decided to donate some to the HMB. Studies from India showed that on average, hospital donors provided only 268 ± 386 ml (18). In comparison, a community donor from Taiwan could provide 17 L, and in Norway 28l (17, 29).

4.3. Although it is more challenging for donors from the hospital, their DHM is important

Staying in the hospital and caring for a preterm infant, can be challenging and a source of anxiety which may influence the mother's lactation and milk volume. In addition, the hospital's KMC rooms always have a high occupancy of mothers and newborns, sharing space and this would not always be optimal and comfortable for mothers to express breastmilk. However, KMC is an important intervention proven to prevent infant mortality and morbidity as strongly recommended by World Health Organization (1). Furthermore, mothers of preterm infants in our hospital roomed in with their infants 24 h a day for KMC which helped improve breastmilk supply thus supporting the donation of their surplus breastmilk. As donor milk from preterm mothers may have a higher protein concentration than that from full-term mothers, this provides more optimal nutrition for recipients who are preterm and sick (30) and so is a critical source of DHM for other preterm newborns. A systematic review of 28 studies of donor characteristics showed that preterm mothers accounted for a

minority percentage, with only two studies from India and Brazil showing around half of the donors having preterm births (8). The large proportion of preterm donors in our setting is an important advantage for the HMB operation and reflects good practice for preterm mothers in neonatal units by maintaining exclusive human milk feeding when their infants are still premature and not able to have full direct breastfeeding.

There are fewer donors from hospitals with less volume of DHM in many HMBs worldwide (8). Conversely, hospital donors who often have preterm infants staying in the neonatal unit, are accessible for conversations about donation as well as transporting DHM within the hospital. Therefore, improving recruitment within this group is beneficial to the HMB. To support these women and to promote their donation of breastmilk whilst resident in the hospital, reasonable measures should be made to improve facilities including additional comfortable spaces and bathrooms, and support mothers with preterm infants to begin early breastmilk expression even when their newborns are in the neonatal intensive care unit. It is important to provide coaching on the hand-expression of breastmilk, knowledge on handling of expressed breastmilk, and suitable facilities for breastmilk storage for their infants. Throughout, the act of donation of breastmilk to an HMB should never be a disadvantage to the donor or her infant.

Most hospital donors started donating DHM when their infant was around one month. The age of the infants of donors from the hospital was much lower than community-based donors (15 vs. 63 days). Overall, this was still lower than those from Poland where the donors started their donation around 14 weeks after birth (31), and Norway at 7 weeks. Similarly, a study from Taiwan showed less than 16% of the first donation happened in the first 2 months. The systematic review of donor characteristics also showed most donors provided DHM after their infant was one month old (8). The earlier donation seen in our HMB may have resulted from the fact that mothers were encouraged to practice early, continuing KMC, and received specialized breastfeeding support in the neonatal unit. Globally, community donors are usually encouraged to wait until their breastfeeding is fully established before starting to express breastmilk for donation. This is to help prevent the overproduction of breastmilk to the detriment of the donor.

Although the time to start the donation for hospital donors was earlier than for community donors the duration was shorter. More than 54% of hospital donors came from other provinces so their donation period would end once the infants were discharged. The duration of the donation was shorter in this first HMB in Vietnam as compared to HMBs in Norway or Korea (about one month vs. more than two months) (16, 29). Future interventions should focus on improving the duration of the donations, if appropriate and not likely to disadvantage the mother or her child. Enabling more optimal conditions for lactation and breastfeeding could encourage earlier donations for both hospitals and, where appropriate, community donors. In Vietnam, maternity leave is six months for women working in formal sectors. If the donation period started within two months following births, as in the community donors in this study, then the donation time potentially could be maximized for up to four months. The HMB should consider investing more in encouraging mothers in the community to donate to maximize the potential of a longer duration of donation if ethically and practically appropriate.

4.4. The average volume of DHM and comparison with other HMBs in the world

The median volume of DHM from donors in this study was much higher than that from India (18) and relatively higher than a report from the North American HMB with a median of 11.4 L (32). However, the volume of donors in Da Nang was much lower than that from Taiwan or Norway with a median of 17 L and more than 28 L respectively (17, 29). A shorter donation period and lower volume compared to several HMBs may be influenced by the hospital donors where their duration and volume were much lower than that of the community. In addition, the median daily volume from hospital donors was also lower than that from community donors (258 ml vs. 300 ml). In DNHWC, breastfeeding support policies were implemented from antenatal consultations, then early and continuing skin-to-skin contact was provided to all preterm newborns who did not require resuscitation. In the neonatal unit, all preterm newborns were provided continuing KMC by the mother and family members. Neonatal staff promoted and supported early breastmilk expression. Commercial milk formula (CMF), feeding bottles, and pacifiers were prohibited. Pasteurized DHM was provided if the mother's own milk was not sufficient. Each KMC room had a refrigerator with a freezer compartment for mothers to store breastmilk for their infants. For infants who were not able to breastfeed themselves and required alternative methods of the gastric tube or cup feeding, mothers express breastmilk at least eight times a day. While very small newborns in the neonatal unit only required small amounts of breastmilk, these mothers would have a surplus to donate to the HMB after reserving what was needed for their infants. Supporting donors with parent facilities, a comfortable space, individual breast pumps, and sterilizers or coaching on hand expression based on informed decisions would promote further human milk feeding and donation in the neonatal unit (33).

4.5. The importance of health staff and social media in donor recruitment strategies

Finally, to encourage donor recruitment, we have used various measures to approach potential donors including health professional consultation, fan pages, posters, and other written materials. About one-third of donors contacted our HMB by themselves while most donors were referred by health staff. These were often hospital donors whom health professionals recognized their potential and HMB staff visited them to encourage recruitment.

Health professionals were a major source of communication and information sharing for potential donors in Da Nang. This was also reported in a Brazilian study (26). On the contrary,

online sources played important roles in China and Korea (8). As around half of our donors came from the hospital, the role of health professionals is important, especially in the Neonatal unit where mothers of newborns in KMC remained with their infants. This supports the integration of breastfeeding support and donor recruitment by neonatal staff.

Facebook is the second most common measure in providing information on donations to the HMB. In Vietnam, as well as other countries, the internet is widely used, especially by women between the age of 20–30 s who grew up with access to the internet. Social media is now commonly used by mothers (34), for information on breastfeeding, HMB, and human milk donation. There are more articles written and available on public media and social network sites rather than on traditional media such as newspapers or magazines. A Chinese study in 2013–2016 on 2,680 donors from 14 HMBs showed that most of the information came from the internet (32.5%), then health professionals (29.4%), television (14.9%), newspapers, and magazines (10.1%) (15). In our study, apart from Facebook, other methods were not popular. Advertising HMB on television and in newspapers often requires a fee although free advertising was given on a few occasions. Therefore, free social networks would be preferable. The HMB should invest in increasing information and confidence in breastfeeding and raising awareness and encouraging donations via social media. Other measures should be enhanced including education on donation and donor recruitment for health professionals from the public health system where infant immunization occurs and encouraging television and newspapers to participate in breastfeeding promotion and donation whenever appropriate.

4.6. Strengths and limitations

To our knowledge, this study is among the few studies that have examined characteristics of donors and the volume of DHM donated to an HMB in lower-middle-income countries. The use of the online system with structured forms and pre-coded options as well as built-in verification functions helps to reduce the workload, cost, and reduced recall bias while ensuring a large sample size and the quality of the data. In addition, because the data from this monitoring system are regularly used to optimize the functionality of the HMB, it ensures compliance with standardized protocols, facilitate networking and information sharing among HMBs, and that the quality of the data is verified and improved.

Our study also has limitations. First, the intensity of recruitment of donors might vary depending on the demand of DHM. Secondly, the characteristics of donors might have been different during the COVID-19 pandemic in Da Nang (from March 2020 to July 2022), when the number of recipients decreased because there was a decrease in referrals of high-risk pregnant women and newborns from surrounding provinces but also fewer donors and DHM due to the lockdown (11, 35). However, the coverage of five and a half years would still capture key characteristics of donors. It is beyond the scope of this study, but this study could have benefitted from some qualitative data, which would explain how potential donors are reached as well as barriers and motivators for donation.

5. Conclusions

Women enabled to breastfeed their infants according to the WHO recommendations and willing to donate their surplus breastmilk is essential for the sustainable operations of HMBs. Mothers with higher education levels from the community donate more breastmilk over a longer duration compared with those with lower education levels and from the hospital.

We recommend that HMBs focus on community-based donors with higher education levels by providing information about HMB and breastmilk donation. This subsequently can stabilize operational expenses at the HMB, which is of paramount importance when the costs for the use of HDM are ultimately recovered by charging these vulnerable families and with the limited public financing mechanisms currently available. Donor engagement and support strategies for the HMB should continue focusing on universal breastfeeding protection, promotion, and support to enable and sustain donations of surplus breastmilk. Health workers are to provide targeted and specialized support based on care needs and risk assessments of more vulnerable mother-and-infant dyads when these mothers show a willingness to provide their surplus breastmilk while at the hospital. Also, health workers would need to provide tailored support to donors from the hospital so that they continue donation after the hospital discharge.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of Da Nang Hospital for Women and Children and Da Nang Health Department (protocol code 52.18; Decision number 2331/SYT-NVY dated 2/6/2021). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization HT and TN; methodology, HT and TN; formal analysis, HT, TN, and ON; validation, HT and TN; investigation, HT and ON; resources: HT and RM; data curation, HT, TN, and ON; First draft of the manuscript HT and TN; Review and editing, HT, TN, DB, GW, and RM; visualization, HT, TN, and ON; supervision, HT; project administration

HT and ON; funding acquisition, HT and RM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Human milk oligosaccharide composition and associations with growth: results from an observational study in the US

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Background: Breast milk is the recommended source of nutrients for newborns and infants. Human milk oligosaccharides (HMO) are the third most abundant solid component in human milk and their composition varies during lactation.

Objectives: Our objective was to investigate longitudinal and cross-sectional changes in HMO composition and whether these changes were associated with infant growth up to 24 months of age. Associations with maternal characteristics were also investigated.

Methods: 24 HMOs were quantified in samples taken at 2 weeks ($n = 107$), 6 weeks ($n = 97$) and 3 months ($n = 76$), using high performance liquid chromatography. Body length, weight, and head circumference were measured at 8 timepoints, until 24 months. Clusters of breast milk samples, reflecting different HMO profiles, were found through a data-driven approach. Longitudinal associations were investigated using functional principal component analysis (FPCA) and used to characterize patterns in the growth trajectories.

Results: Four clusters of samples with similar HMO composition were derived. Two patterns of growth were identified for length, body weight and head circumference via the FPCA approach, explaining more than 90% of the variance. The first pattern measured general growth while the second corresponded to an initial reduced velocity followed by an increased velocity ("higher velocity"). Higher velocity for weight and height was significantly associated with negative Lewis status. Concentrations of 3'GL, 3FL, 6'GL, DSNLT, LNFP-II, LNFP-III, LNT, LSTb were negatively associated with higher velocity for length.

Conclusion: We introduced novel statistical approaches to establish longitudinal associations between HMOs evolution and growth. Based on our approach we propose that HMOs may act synergistically on children growth. A possible causal relationship should be further tested in pre-clinical and clinical setting.

KEYWORDS

human milk oligosaccharides, child development, longitudinal modeling, cluster analyses, statistical methodologies, infant nutrition (including breastfeeding)

Introduction

Human milk oligosaccharides (HMO) are the most abundant solid component of human milk, after fat and lactose, with an estimated concentration ranging between 5 and 15 g/L (1). Although largely not digestible by the infant's gut, HMOs are known to be associated with a range of biological functions, such as gastrointestinal development (2–8), protection against infection (9–11) and inflammation (12) and more recently in neurodevelopment (13). Breastmilk composition, and in particular the concentration of HMOs, is known to evolve during the lactation period (1, 14, 15); the total concentration of HMOs has been observed to decrease, as well as the concentration of most of the individual HMOs, with the notable exception of 3FL which increases over time (1, 15, 16). Berger et al. (17) have speculated that these dynamic changes in HMO concentrations may reflect their involvement in biological functions beyond the first few months.

The concentration of HMOs in breast milk varies among individuals and particularly depends on the expression of α1-2-fucosyltransferase (FUT2) and α1-3,4-fucosyltransferase (FUT3) genes which determine the secretor (Se) and Lewis (Le) status, respectively (18). 2′-fucosyllactose (2'FL) is absent in the milk of non-secretor women, while it is the most abundant HMO in the milk of secretors. While maternal genetic polymorphisms for the Se and Le genes explain, to a large extent, the HMO variability between mothers (18), additional maternal or infant factors like pre-pregnancy BMI, age, mode of delivery and gestational age may contribute as well to the interindividual variability (15).

The first objective of this exploratory work was to characterize the temporal and inter-individual variability of the HMO concentrations in a US cohort over the first 3 months of lactation. 24 HMOs were measured at three time points; although approximately 150 HMOs have been identified in the literature, it is known that the HMOs used in our study account for roughly 90% of the total HMO (1). We developed a novel data-driven approach to cluster breast milk samples based on the concentrations of HMOs, and tested if these clusters were associated to maternal factors, such as mode of delivery or pre-pregnancy BMI. The data-driven clustering was based on the abundances of all the measured HMOs, assigning samples to milk types sharing similar HMO profiles, and we compared the resulting clusters to the milk types defined by the presence/absence of specific HMOs (e.g., 2'FL and LNFP-I) as proxies for the Se, Le status (18). Van Leuween (19) observed that it may not always be correct to determine the milk type based on HMOs composition at one single time-point, and in our analysis we also considered the intra-individual variability, by describing trajectories of individual HMOs within each cluster.

As a second objective, we investigated the effect of HMO concentrations on growth parameters (head circumference, body weight and body length, measured at 8 time points up to 24 months of age). HMOs are hypothesized to affect growth through several mechanisms, including a possible role in functional gut maturation, therefore improving nutrient absorption (20). Moreover, in some studies, multiple HMOs (eg 2'FL, and 3FL) have been detected in the urine and plasma of breastfed infants, but not in formula-fed infants (21), suggesting the possible existence of systemic effects, yet to be identified (14, 16, 22–27).

The biological functions of many, if not most, HMOs remain unknown. Selected HMOs are increasingly added to infant formulas for their beneficial health benefits (6). It is therefore important to understand better these benefits, whether they might be supported by synergistic effects and what are the natural variations of the individual HMO concentrations over the course of lactation. This knowledge might help to target the HMOs that are the most relevant at critical windows of infant development. In turn, such investigations pose methodological challenges for the data analysis, that the present study intended to address.

Patterns of development are often non-linear, therefore linear mixed effect models that are often used in modelling growth trajectories may not describe the data accurately. Non-linear parametric approaches are an alternative to linear mixed models, however, they rely on parametric assumptions that might not be realistic. In addition, the sparsity often found in real data is a challenge for fitting the data into those models (23). Functional data analysis offers a non-parametric, non-linear approach to model sparse, non-linear longitudinal data. The FPCA approach allows extracting the patterns of growth from a set of trajectories and has been successfully applied in many domains, including neurodevelopment (28) and gene expression (29, 15).

Materials and methods

Endpoints

Table 1 summarizes the timepoints available for the milk samples and the body measures.

Description of study population

Children for this study were selected from the observational breastfeeding arm of a prospective longitudinal randomized control trial. Results for the randomized arm were analyzed in a previous publication (30). Infants for the trial were recruited and followed over 24 months at two study sites in the United States (Rhode Island Hospital in Providence, RI, and Pennington Biomedical Research Center, in Baton Rouge, LA). Maternal screenings were performed during the third trimester of pregnancy up to and including post-delivery. Following written informed consent (screening visit), sociodemographic information, medical and family histories were collected, as well as a physical and neurological examination of the infant. Withdrawal from the study was possible at any point and with no further evaluations and any additional data collection. The research ethic boards at both clinical sites approved the protocol.

In total, 107 breast milk samples were available for analysis. The demographic characteristics of the mothers are summarized in Table 2. 61 (57%) children were female, 46 (43%) were male. Mean age of the mothers at recruitment ranged from 19 to 43 years, with an average of 32 years. Most mothers were white (70%) BMI before pregnancy ranged from 19.1 to 39.9, with an average of 27.2 and a median of 26. 72% of children were born through vaginal delivery.

Supplementary Table 1 summarizes the anthropometric characteristics of the breastfed children involved in the study.

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TABLE 1 Overview of time points.

	V0 (2–5 week)	V1 (6 ± 1 week)	V2 (3 month ± 2 week)	V3 (6 month ± 2 week)	V4 (9 month ± 2 week)	V5 (12 month ± 2 week)	V6 (18 months ± 3 week)	V7 (24 month ± 4 week)
Breast milk samples	X	X	X					
Body measures	X	X	X	X	X	X	X	X

Body measures include head circumference body length and body weight.

Exclusion criteria were: (i) Birth>41 weeks +6 days gestation as reported in medical record when available; (ii) Birth Weight < 2000 g or small for gestation age (birth weight less than the 10th percentile for the gestational age) or large for gestational age (weight, length, or head circumference above the 90th percentile); (iii) Any unsafe psychopharmacological treatment of mother using prohibited medications during pregnancy or lactation as assessed by medical interview. This included anticonvulsants, antidepressants, benzodiazepines, cytotoxic drugs, dopamine agonists, opioids.

Milk samples

Breast milk was sampled from mothers longitudinally at defined study visits, each time between 10 AM–12 PM from the right breast using a hospital grade electric breast pump. Mothers were asked to empty the right breast approximately 2 h prior to milk sampling and the time of milk sampling the complete breast was emptied using a pump (single full breast milk sampling methodology). Only a fraction of the collected milk was aliquoted for research and the rest was returned to mother to feed the baby at a later time.

Data on feeding mode and introduction of solid food were collected via questionnaires at baseline, 6 weeks, 3, 6, 9, and 12 months. Up to at least 4 months of age children received breastmilk for more than 90% of nutritional intake and no infant formula or solids for more than 10% of the nutritional intake (approximately 6 feeds per week).

HMO data (Table 3) were available for $N = 107, 97, 76$ individuals at 2–5 weeks, 6 weeks, 3 months (visits V0–V2), respectively. 76 mothers had complete HMO data for all three visits.

HMOs were analyzed by ultra-high performance liquid chromatography with fluorescence detection (UHPLC-FLD), according to the method of Austin and Benet (31). We refer to Table 3 for the full list of measured HMOs and their respective abbreviations. 2'FL, 3FL, 3'SL, 6'SL, LNT, LNnT and LNFP-I were quantified against standards of analytical quality all other HMOs were quantified against maltotriose assuming equimolar response factors. Mother's genotypes to define Secretor and Lewis status were not available.

Statistical analysis

We first calculated the sum of all HMO concentrations at each time point in each sample and tested for significant differences between visits V0 and V1, and between V1 and V2. We compared the Shannon diversity of the HMOs between timepoints. The Shannon diversity index is low when all HMOs are present in similar concentrations. The more unequal the abundance of HMOs, the larger the corresponding Shannon entropy.

In this work, we analyzed both the proportion of each HMO, expressed as % of the sum of the 24 measured HMOs, and their concentration expressed in mg/L. Since the volume of milk per feeding was not measured, total amounts of HMOs or other milk components are not known, and only relative information was available for analysis.

We applied a data-driven method to assign mothers to clusters of HMO composition. The number of clusters was data-driven as well. Basically, we clustered together samples sharing a similar HMO profile. Our approach was based on network theory (32), where each

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TABLE 2 Maternal characteristics.

Ethnicity	*n*	%
White	75	70
African American, black	8	7
Mixed race	8	7
Hispanic	5	5
Asian	3	3
Latino	2	2
Other	6	5
Income	*n*	*%*
I prefer not to answer	23	21
200,000 USD or more	7	6
150,000–199,999 USD	10	9
110,000–149,999 USD	17	16
90,000–109,999 USD	9	8
70,000–89,999 USD	10	9
50,000–69,999 USD	10	9
30,000–49,999 USD	10	9
10,000–29,999 USD	9	8
Missing	2	2
Number of siblings	*n*	*%*
0	39	36
1	44	41
2	14	13
3	7	7
4	1	1
5	2	2
Body measures	*Mean (SD)*	*[Min, Max]*
BMI before pregnancy	27.3 (5.8)	[19.1, 39.9]
Gestational age	*Mean (SD)*	*[Min, Max]*
Weeks	39.3 (1.11)	[37.0, 41.0]
Age at recruitment	*Mean (SD)*	*[Min, Max]*
Years	32 (5)	[19, 43]
Mode of delivery	*n*	*%*
Vaginal	77	72
C-section	30	28
Maternal education	*n*	*%*
High school not graduated (10th or 11th grade)	2	2
High school graduate	8	7
Partial college/university, not graduated	24	22
Profession training/graduate degree/master/doctorate/MBA	30	28
Other	43	40

node of the network corresponds to a milk sample and two nodes are connected based on the similarity between their HMO profiles. We first calculated the Aitchison (33) distances between all the pairs of samples, all subjects and all timepoints. The resulting matrix of distances allows us to identify the closest neighbor to a given sample. The edges in the network were weighted, with weights inversely proportional to the distance between the samples. Therefore, two samples with similar HMO composition have a short distance and therefore will be connected with a high weight in the network. We applied the leading eigenvalues method (34) to identify clusters in the network. These clusters correspond to groups of samples sharing a similar HMO composition. We applied a Kruskal-Wallis test to each HMO to test whether its concentration was significantly different between the clusters. We also made qualitative comparisons with clusters of HMOs that can be derived from presence or absence of 2'FL and LNFP-II (3, 6).

We tested for differences between HMO clusters for several covariates: mother's BMI before pregnancy, BMI status, mode of delivery, ethnicity, and gestational age. BMI status was defined as 'overweight' (OW) for a BMI $\geq$25 kg/m (2), 'obese' (OB) if BMI $\geq$30 kg/m^2, 'normal weight' (NW) otherwise. Fisher's exact tests were used to test the independency between two categorical variables. Permutational analysis of variance (PERMANOVA) was used to test the equality of the overall HMO composition between groups (e.g., clusters). Kruskal-Wallis test was used to check the independence between a continuous and a categorical variable, followed by a Dunn *post-hoc* test.

We calculated all pairwise correlations between the individual HMOs, at each timepoint, and visualized them as a heatmap, to summarize and display patterns in the data. In particular, the existence of strong correlations between pairs of HMOs is an important background information when interpreting associations arising from the application of univariate models, and suggests a possible synergistic effect of several HMOs acting in combination.

All correlations reported in this study were Spearman correlations, and a Benjamini-Hochsberg correction for multiple testing was applied whenever applicable, using a false discovery rate of 5%.

Previous work (14, 16) has explored the influence of individual HMOs on growth, using linear mixed models with growth parameters as response variable and the HMO level as predictor. We propose an alternative approach derived from functional data analysis (22–27) to describe the growth trajectories and their main modes of variation; this approach allowed us to identify distinct growth patterns in the data, that could not be properly detected using linear models. These patterns were then investigated in association with the clusters derived in the first part of the analysis.

We investigated the associations between the HMO composition and the child's growth over time.

We plotted the growth data separately for boys and girls, using the WHO reference values.[1] Growth trajectories were analyzed within the methodological framework of functional data analysis (23). More specifically, we applied Functional Principal Component Analysis (FPCA) to describe trajectories using a limited number of numerical parameters, the FPCA scores (11). FPCA models longitudinal data as samples from smooth curves, so that the time-varying trait of the i-th subject admits a Karhunen-Loeve expansion

$$X_i(t) = \mu(t) + \sum_{k \geq 1} \xi_{i,k} \phi_k(t)$$

1 https://www.who.int/tools/child-growth-standards/standards

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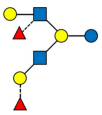
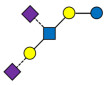
TABLE 3 Overview of all HMOs measured, with chemical structure and abbreviation.

HMO		Abbreviation
2'-FUCOSYLLACTOSE		2'FL
3'-GALACTOSYLLACTOSE		3'GL
3'-SIALYLLACTOSE		3'SL
3,2'-DIFUCOSYLLACTOSE		DFL
3-FUCOSYLLACTOSE		3FL
6'-GALACTOSYLLACTOSE		6'GL
6'-SIALYLLACTOSE		6'SL
A-TETRASACCHARIDE		A-TETRA
DIFUCOSYLLACTO-N-HEXAOSE-a		DFLNHa
DISIALYLLACTO-N-TETRAOSE		DSLNT
Unidentified hexasaccharide of composition Hex4 HexNAc2	unidentified	Hex4 HexNAc2
LACTO-N-DIFUCOHEXAOSE-I		LNDFH-I
LACTO-N-FUCOPENTAOSE-I		LNFP-I
LACTO-N-FUCOPENTAOSE-II		LNFP-II
LACTO-N-FUCOPENTAOSE-III		LNFP-III
LACTO-N-FUCOPENTAOSE-V		LNFP-V
LACTO-N-HEXAOSE		LNH

(Continued)

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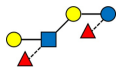
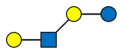
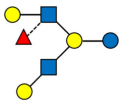
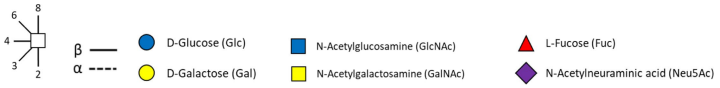
TABLE 3 (Continued)

HMO		Abbreviation
LACTO-N-NEODIFUCOHEXAOSE		LNnDFH
LACTO-N-NEOFUCOPENTAOSE-V		LNnFP-V
LACTO-N-NEOTETRAOSE		LNnT
LACTO-N-TETRAOSE		LNT
MONOFUCOSYLLACTO-N-HEXAOSE-III		MFLNH-III
SIALYLLACTO-N-TETRAOSE-B		LSTb
SIALYLLACTO-N-TETRAOSE-C		LSTc
		

where $\mu(t)$ represents the average trajectory and the $\xi_{i,k}$ are the FPCA scores. The sum in the above formula can be truncated to a fixed number of terms, and the fraction of variance explained will depend on how many terms are kept in the sum. We applied FPCA separately to the length, weight, and head circumference data.

For our applications, based on the percentage of variation explained, we selected the first 2 scores as descriptors for the trajectories, FPCA1 and FPCA2. We therefore effectively achieve a dimensionality reduction, where a trajectory, *a priori* defined by 8 time points, can be effectively described by just 2 parameters.

We then tested whether the FPCA scores were associated with the HMO clusters, with the single HMO concentrations or with the maternal baseline characteristics.

We then further investigated which of the HMOs concentrations were associated with higher velocity, by running linear models adjusted for clustering and with an interaction term between cluster and HMO concentration:

$$FPCA2 = Intercept + HMO + cluster + cluster * HMO \quad (1)$$

where HMO stands for the HMO concentration in mg/L, cluster is a categorical variable 1–4 and the last term accounts for the interaction.

It is expected that the body length, weight, and head circumference are correlated, therefore we report the correlations between the FPCA scores associated to the various growth measures (length, weight, head circumference).

All analyses were performed with R, version 4.0.2. Network analysis was performed with the package *igraph (v 1.3.0)* and functional PCA was performed using the package *fdapace (v 0.5.8)*.

Results

Description of HMO composition over the lactation period/time

The total measured HMO concentration decreased over the first 3 months of lactation from 9182 (2013) mg/L, at V0, to 7,887 mg/L (1813) at V1 and 6,248 mg/L (1322) (mean (SD)) at V2.

Consistently with previous findings (15) we observed a decrease of almost all HMOs with time of lactation (Supplementary Table 2). A noticeable exception to this decreasing pattern was 3FL, which increased from 704 mg/L (538 mg/L) at V0 to 1,118 mg/L (698 mg/L) at V2.

Mothers can be deemed as secretors/non-secretors based on the presence/absence of α-1-2-linked fucosylated HMOs (2'FL and LNFPI). In secretor mothers, 2'FL was the most abundant HMO, representing up to 58% of the total measured HMO (Supplementary Table 2).

The Shannon diversity at V2 was lower than at V1 (Wilcoxon test, $p < 0.01$), and lower than at V0 (Wilcoxon test, $p < 0.01$). This is reflected in the fact that the cumulative proportion of the two most

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abundant HMOs, 2'FL and 3FL, increased from 29% at V0 to 34% at V1 and increased to 41% at V2. There was no significant difference in diversity between V0 and V1.

Pairwise correlations between the concentrations of individual HMOs are reported in the supplementary materials (Supplementary Figures 1–3): significant correlations ranged between −0.9 (2'FL and LNFP-V at V0) and 0.9 (3FL and LNFP-II at V1). In general, we observed very strong positive correlations between 2'FL, DFNLHa, LNFP-I, between 3FL and LNFP-II, and between DSLNT, LSTb, LNFP-V, LNT at all timepoints. Also, Hex4 HexNAc2 was positively correlated (Spearman $\rho = 0.7$ at V0) with LNnT, and 6'SL with LSTc ($\rho = 0.7$ at V0, V1, $\rho = 0.8$ at V2). 2'FL and LNFP-II were not significantly correlated with LNH, 3'GL, 6'GL, 6'SL, LSTc. LNFP-I was significantly and negatively correlated with 3FL ($\rho = -0.8$ at V0), LNFP-V ($\rho = -0.5$ at V0), LNFP-II ($\rho = -0.5$ at V0), LNnFP-V ($\rho = -0.4$ at V0), LNFP-III ($\rho = -0.3$ at V0). In most cases, these correlations and their statistical significance were similar across visits. Other HMOs, like A-Tetrasaccharide or 6'GL, were weakly correlated, or not significantly correlated with the other HMOs.

We calculated the pairwise Aitchison distances between all samples, resulting in a 276 × 276 matrix. Figure 1 compares the distances between samples from the same donors and samples from different donors. As expected, the intra-individual distances between samples were significantly smaller than the inter-individual distances.

We then defined a weighted network with the samples as nodes and edges weighted by the inverse of their distance. Each node corresponds to a subject at a given timepoint. The resulting weighted network was partitioned, producing four clusters of sizes 97(35%), 89 (32%), 62 (22%), 28 (10%), consisting of samples sharing a similar HMO composition.

The average value of each HMO at each timepoint and in each cluster was plotted, as concentrations in mg/L (Figure 2). Overall, it appeared that several HMOs (e.g., 3FL, 2'FL, LNFP-I, LNFP-II) had well separated, non-overlapping, trajectories in the different clusters, while for other HMOs (e.g., 6'SL, LSTc, LSTb) the trajectories were overlapping or even crossing. The concentrations of 6'SL decreased in all clusters, from an average of 468 mg/L at V0 to 119 mg/L at V2 in cluster 1, from 489 mg/L to 147 mg/L in cluster 2, from 535 mg/L to 155 mg/L in cluster 3 and from 588 mg/L to 203 mg/L in cluster 4. In Figure 3, we looked at the temporal consistency of clustering: whether the milk samples remained in the same cluster across visits. All subjects belonging to cluster, 3 or 4 at the first visit, remain in the same cluster afterwards. Some of the subjects starting in cluster 2 switched to cluster 1 afterwards.

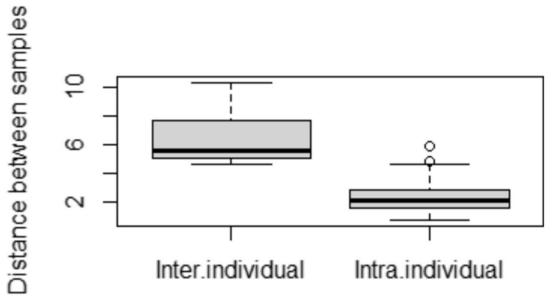

FIGURE 1
Comparison of distances between samples. Inter-individual distances were calculated between different mothers, and intra-individual distances between samples from the same mother but at different time points.

Cluster 1 was characterized as having the highest concentrations of A-TETRA, DFL, LNDFH-I and LNnFP-V and second highest proportions of 3FL, LNFP-II (Figure 2). It also had the lowest levels of MFLNH-III. Cluster 2 was characterized by the highest concentrations of LNnT and LNH, and second highest concentrations of 2'FL, A-TETRA and LNDFH-I. Also, the concentration of 3FL in this cluster was roughly stable across visits. Cluster 3 was characterized by highest concentrations of 3FL, LNFP-II, LNFP-V, LNT, MFLN-III, and lowest concentrations of 2'FL, LNDFH-I, LNFP-I, LNnT. 2'FL was actually absent in this group, which then corresponds to the non-secretor group. LNFP-I was also absent in this cluster. Cluster 4 is characterized by highest concentrations of 2'FL, DFLNHa, LNFP-I, and lowest concentrations of 3FL, LNDF-I, LNFP-II, LNFP-V. Moreover, LNDFH-I and LNFP-II were absent in this cluster. Clusters 3 and 4 had significantly smaller Shannon diversity at all time points compared to clusters 1 and 2, cluster 4 had a significantly lower diversity than cluster 2 at visits V0 and V1, and cluster 2 had lower diversity than cluster 1 at visit V1.

A majority of HMOs had significantly different concentrations between the clusters, at least one timepoint, with the exception of 3'GL, 6'GL, 6'SL, LNnDFH, LSTc (Figure 4).

The total measured HMO concentration varied between the clusters and was consistently the lowest in cluster 3 (Figure 5).

Association with maternal and baseline characteristics

HMO clustering was not associated with mother's age at recruitment, pre-pregnancy body mass index (ppBMI) or gestational age.

We further tested the impact of BMI status on HMO concentrations, First, total HMO concentration and Shannon diversity index were not different between overweight (OW), obese (OB) and normal weight (NW) groups, at any visit. We then compared the average trajectories for each of the HMOs between the BMI groups regardless of clusters, using functional ANOVA (23): tests were significant only for 6'GL ($p = 0.04$), LNH ($p = 0.04$), LSTc ($p = 0.04$) (Supplementary Figure 4). We also tested if HMO concentrations were different between vaginal delivery and C-section. Specifically, the concentration of LNnT and Hex4 HexNAc2 (2) at 3 months was affected by birth mode and lower in the group of mothers who delivered through C-section (Table 4).

Association with growth

Children in this study showed a normal development for length and weight (see Supplementary Figures 5–8).

Although the differences did not reach statistical significance, children from mothers in cluster 3 (non-secretors) were on average 1 cm shorter than the rest.

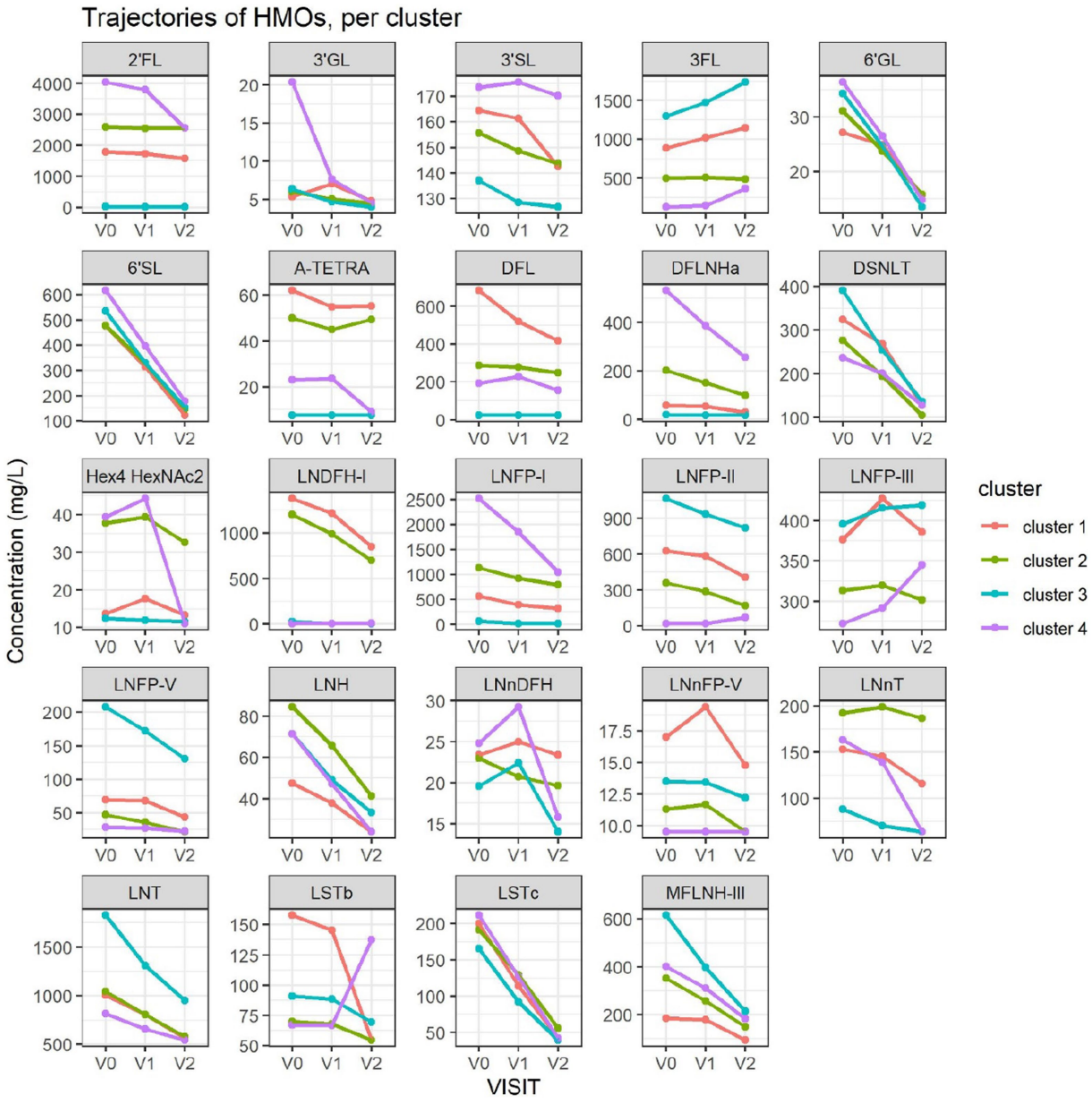

FIGURE 2
Each dot represents the average concentration of each HMO, in each cluster and for each time point. Several clustering variables show consistent temporal patterns: for example, LNFP-V is consistently highest in cluster 4, while 3FL is consistently highest in cluster 3 (non-secretors). V0 = 2–5 weeks, V1 = 6 weeks, V2 = 3 months. For other HMOs, like 6'GL, LSTc and DSLNT, the concentration is similar between the clusters. Several HMO concentrations exhibit a non-linear trend, with an increase between the first and second visit and a decrease between the second and third visit.

Length

When applied to the trajectories of body length, FPCA resulted in 97% of the variance explained by the first two scores, the first score FPCA1 explained 93% of the variance, and was positively correlated with the length z-scores at all time points (Figure 6). The second score (FPCA2) explained 4% of the variance; it was negatively correlated with length at early timepoints, and positively correlated at V7, so positive values of FPCA2 correspond to trajectories having a slow growth before V3 months and an increase at V6 and V7 (Figure 7). We will refer to FPCA1 as 'general growth' and to FPCA2 as 'higher velocity'. See Figure 8 for an illustration of these patterns.

General growth was higher for boys (T test, $p < 0.01$), indicating that in girls most trajectories of length were below the trajectories for boys. Higher velocity rates were not significantly different between males and females.

Clusters of HMOs at V2 were significantly associated with higher velocity (FPCA2 score), but not with general growth (FPCA1 score), with cluster 4 having significantly higher values than cluster 1 (Figure 9). The same trend was observed at V0 and V1, albeit not significant.

Concentrations of 3'GL, 3FL, 6'GL, DSNLT, LNFP-II, LNFP-III, LNT, LSTb were negatively associated with higher velocity

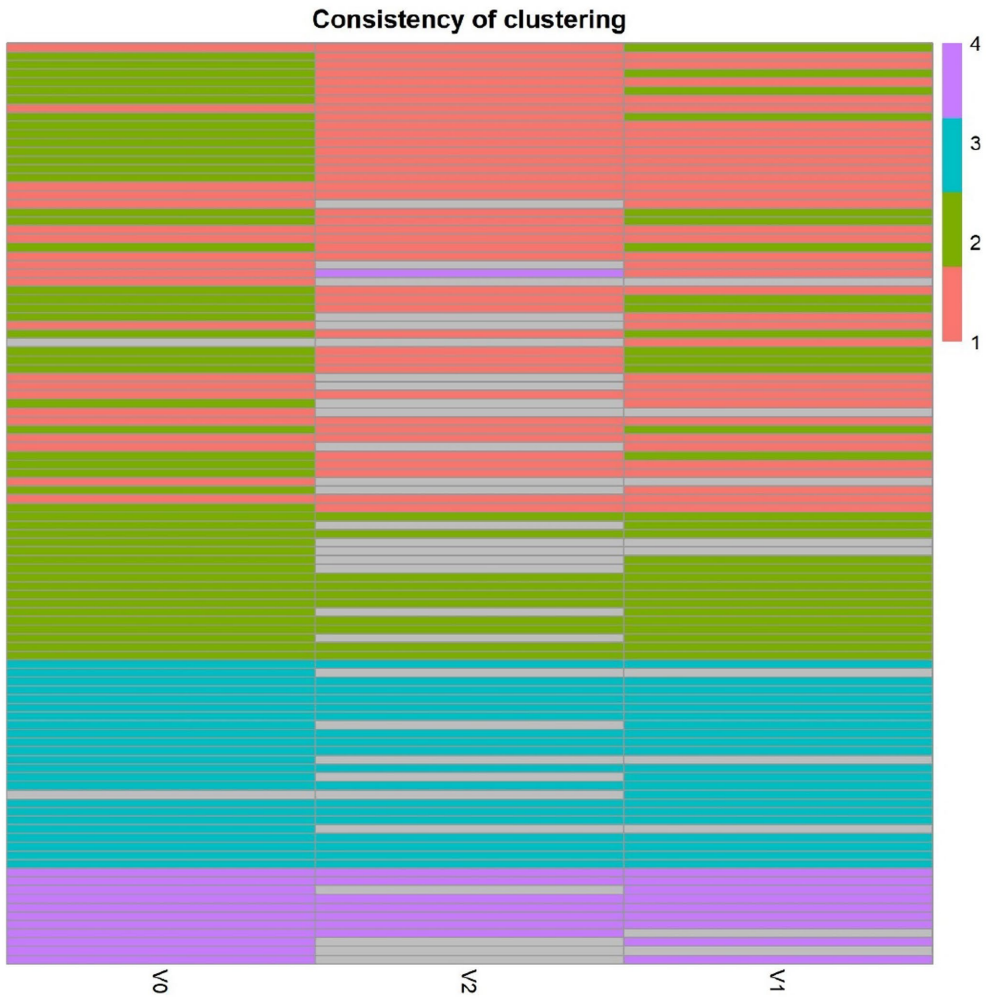

FIGURE 3
Temporal consistency of clustering: each row corresponds to a mother, the heatmap describes whether the milk samples remained in the same cluster between visits. Grey cells are missing values. All subjects belonging to cluster, 3 or 4 at the first visit, remain in the same cluster afterwards. Some of the subjects starting in cluster 2 (green) switch to cluster 1 (orange) afterwards.

(Supplementary Table 3). For 3FL, this negative association was consistent across the time of lactation (V0, V1, V2).

Weight

For the weight, the first score FPCA1 explained 91% of the variance, and was highly correlated with the weight z-scores ($\rho = 0.58$ at V0, 0.72 at V1, 078 at V2, 0.84 at V3, 0.9 at V4, 0.9 at V5, 0.93 at V6, 0.9 at V7, all significant). The second score FPCA2 explained 7% of the variance, and Spearman correlation coefficients with z-scores were −0.5 at V0, −0.57 at V1, −0.59 at V2, −0.53 at V3, −0.37 at V4, all significant ($p < 0.01$); correlations at V5-V6 were not significant. At V7, there was a significant positive correlation between FPCA2 and the z-score ($\rho = 0.21$); higher values of FPCA2 correspond to weight trajectories that are below the average before 9 months and above the average afterwards. FPCA1 was positively and significantly correlated with growth velocity at V2, 3 months ($\rho = 0.48$); at later time points FPCA1 and velocity were not significantly correlated. FPCA2 was significantly positively correlated with velocity at V4, 9 months ($\rho = 0.49$) and V5, 12 months ($\rho = 0.57$).

As in the case of length, clusters of HMOs at V2 were significantly associated to higher velocity for weight, as measured by FPCA2 (Kruskal-Wallis, $p = 0.02$, average values were −0.7 for clusters 1,2, −0.2 for cluster 3, 2.7 for cluster 4). A post-hoc Dunn comparison test resulted in significantly higher values in cluster 4 compared to the other clusters.

General growth (FPCA1) was higher for boys (T test, $p = 0.02$) with a mean value of 2.02 kg, versus a mean value of −1.73 for girls. FPCA2 was higher for girls (T test, $p = 0.04$), with a mean value of 0.33 kg, versus a mean value of −0.57 for boys.

Head circumference

For the head circumference, the first score FPCA1 explained 93% of the variance, and the second score FPCA2 explained 4% of the variance. Again, we can interpret FPCA1 as a measure of general growth and FPCA2 as a measure of higher velocity. FPCA scores for head circumference trajectories were not significantly associated with the HMO clustering.

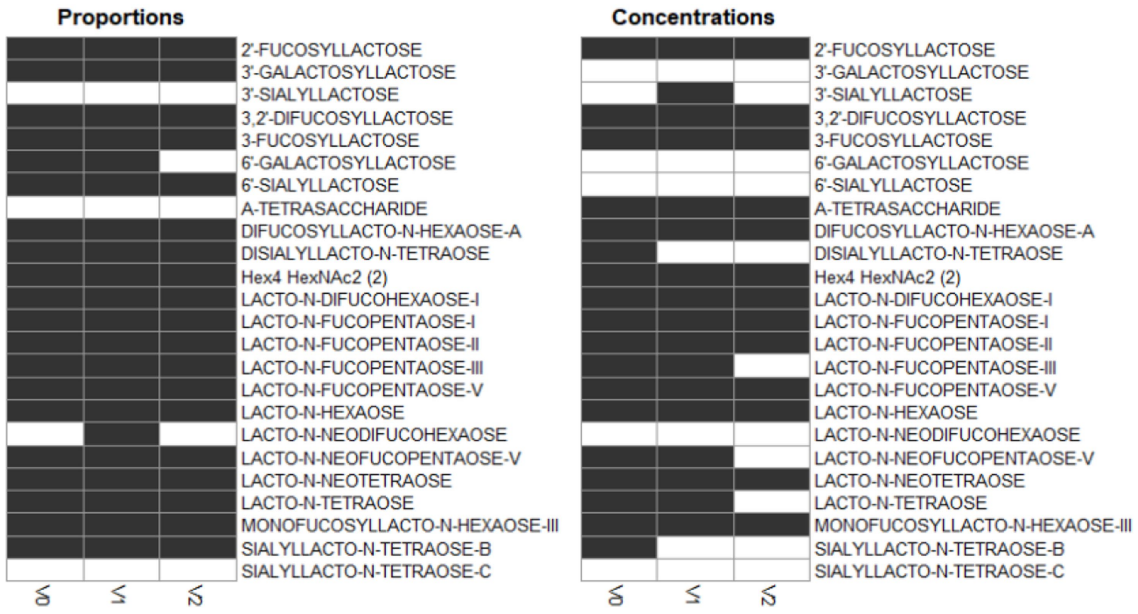

FIGURE 4
Dark cells correspond to significant difference between the clusters (kruskal test). On the left, HMOs are expressed in relative concentrations (as % of total HMO), on the right, they are expressed in mg/L.

Discussion

Our analysis aimed to explore the temporal and interindividual variability of the HMOs during the first 3 months of lactation, and to investigate the potential effect on growth. Since several groups of HMOs are highly correlated, multivariate methods may help to understand their interactions, and their cumulative effects. In this exploratory analysis, we proposed a modelling methodology to overcome some of the difficulties that occur when analyzing such data, in particular to address at the same time the non-linearity of growth patterns and their potential association with multi-dimensional, highly correlated HMO concentrations.

We applied a data-driven algorithm to assign mothers to clusters sharing similar HMO profiles. The significance of this clustering is that it is based on the concentrations of a panel of 24 HMOs, and takes into account their mutual correlations. The clustering algorithm produced 4 clusters, distinct from the 4 milk types that are usually defined based on fucosyltransferase (*FUT2* and *FUT3*) polymorphisms. However, the two partitions overlap: for example, our clusters 1 and 2 were composed of samples from mothers belonging to the ($FUT2^+$, $FUT3^+$) group and they differed in their respective concentrations of several fucosylated HMOs, including 3FL (higher in cluster 1), LNnT (higher in cluster 2), DFL (higher in cluster 1). Therefore, clusters 1 and 2 split the ($FUT2^+$, $FUT3^+$) group in two subgroups, with distinct levels of several of the most abundant HMOs; this might reflect differences in FUT enzymatic activity with FUT3 having potentially a stronger activity in cluster 1 compared to cluster 2. Cluster 3 corresponded to the ($FUT2^-$, $FUT3^+$) type. Cluster 4 was analog to the milk group 3 ($FUT2^+$, $FUT3^-$) with the highest concentrations of 2'FL, DFLNHa, LNFP-I, and lowest concentrations of 3FL, LNDF-I, LNFP-II, LNFP-V.

Previous studies (15) reported that 3FL, LNFP-II and LNnFP-V had their highest concentration in the ($FUT2^-$, $FUT3^+$) group, similarly to what we observed in our cluster 3. Likewise, 2'FL, LNFP-I and DFLNHa were highest in the ($FUT2^+$, $FUT3^-$) group, corresponding to our cluster 4. These dynamics can be explained by the substrate and enzyme availability as well as the competition between FUT2 and FUT3 enzymes. Cluster 1 had a lower FUT2 activity compared to FUT3. The opposite is true for Cluster 2. When both enzymes are necessary to synthesize an HMO like in the case of LNDFH-I, more is synthesized in the cluster with a presumably stronger FUT2.

In general, the intra-individual variability was significantly lower compared to inter-individual variability. While subjects were consistently clustered in clusters 3 and 4, independently of the time of lactation, some subjects switched from cluster 2 to 1. This might be explained by a difference in gene regulation and enzyme activity over time, in clusters 1 and 2 with the same Se and Le status, which may follow different patterns of change for each HMO. This means that the overall breastmilk composition, while remaining in the ($FUT2^+$, $FUT3^+$) group, can vary over the lactation period, with some HMOs decreasing more rapidly than others, changing the ratios between HMOs. We therefore suggest that it may be important to distinguish these subgroups in a statistical analysis, as these different rates of change might have a biological relevance.

When HMOs were expressed as % of total measured HMO, comparing Figure 2 with Supplementary Figure 9, patterns look very similar for several HMOs, including 3FL and LNFP-I, but differences appear for 3'SL and other HMOs. This highlights the fact that the concentration of a specific HMO can increase over time while decreasing as % of total measured HMO.

Only a few studies to date have investigated variations in the HMO composition in relation to maternal age (35), but the results are in general not conclusive. Our clusters of HMO composition were not associated with maternal characteristics such as age and maternal ethnicity. The same lack of association was observed for the levels of

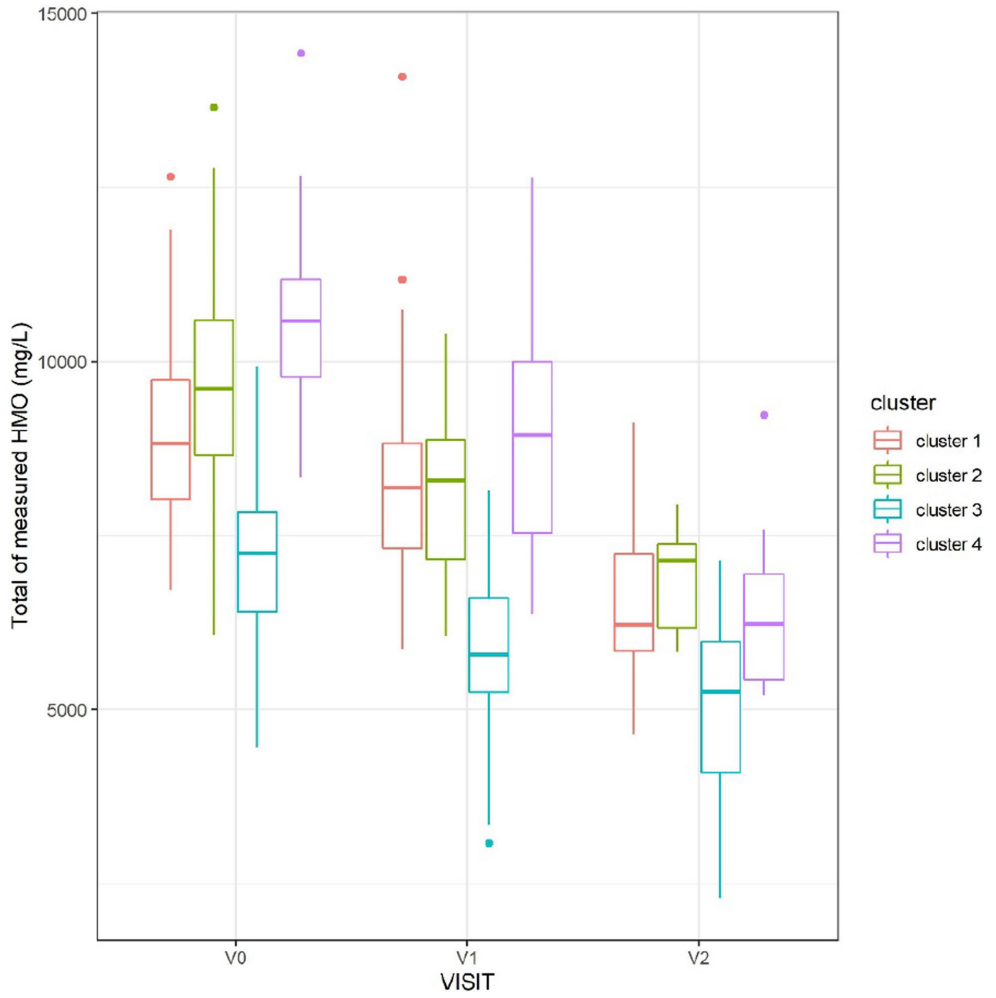

FIGURE 5
Sum of measured HMO concentrations, at each visit, split by cluster. Cluster 3, corresponding to non-secretors, had the lowest total concentration of HMOs.

TABLE 4 Concentrations of Hex4 HexNAc2 and LNnT at 3 months are lower in the C-section group.

HMO	C-section Mean concentration (mg/L)	Vaginal delivery Mean concentration (mg/L)	Adjusted *p* value
Hex4 HexNAc2	11.2	16.7	0.048
LNnT	85.6	116.0	0.039

2'FL and LNFP-II and, a fortiori, for the clustering based on the FUT2, FUT3 levels.

A negative association of 3'SL with ppBMI had been reported by Saben et al. (36), who suggested that HMO sialylation may be negatively associated with maternal adiposity. According to Samuel et al. overweight women from a European cohort had significantly higher concentrations of 3′SL, 6′GL and DSLNT (at day 2), 6′SL (at day 17) and LNFP-V (at 3 and 4 months), while lower concentrations of LNnT (at day 2), LNT (at 1 and 3 months) and LNFP-V (at 2 months) compared to normal weight women ($p < 0.05$ for all). Other studies reported non-significant associations between HMO concentrations and ppBMI (37). We observed in our data that 6'GL, LNH and LSTc were consistently higher in the overweight group. To the best of our knowledge, associations of LNH and LSTc concentrations with ppBMI have not been reported before.

Samuel et al. reported lower concentrations of 2'FL, 3'SL and 6'GL at 2 days, among women delivering through C-section (15). Although we observed the same trend for these HMOs all time points, it was not significant, possibly because this association might be stronger at a very early stage of lactation.

It was proposed that HMO composition might affect child growth by altering the composition of the gut microbiome (38). Our results suggest a possible association of the Lewis negative status with a specific longitudinal growth pattern, as discussed below.

Higher HMO diversity and evenness at 1 month have been associated to lower total and percentage fat mass at 1mo (39). In Lagström et al. (40), the concentration of LNnT (at 3 months) was inversely associated and that of 2'FL (3 months) was directly

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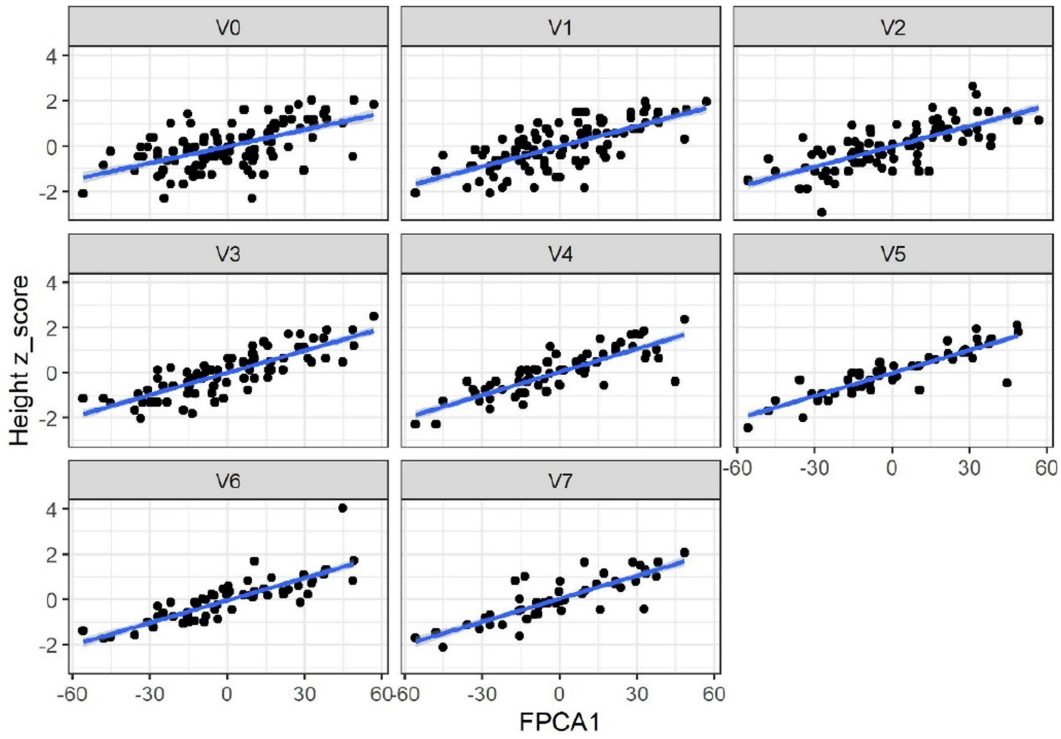

FIGURE 6
FPCA1 score is positively correlated with the z-scores for length (cm). Correlations: 0.54 (V0 = 2–5 wk), 0.70 (V1 = 6 wk), 0.73 (V2 = 3 mo), 0.82 (V3 = 6 mo), 0.81 (V4 = 9 mo), 0.88 (V5 = 12 mo), 0.88 (V6 = 18 mo), 0.78 (V7 = 24 mo). All *p*-values <0.01.

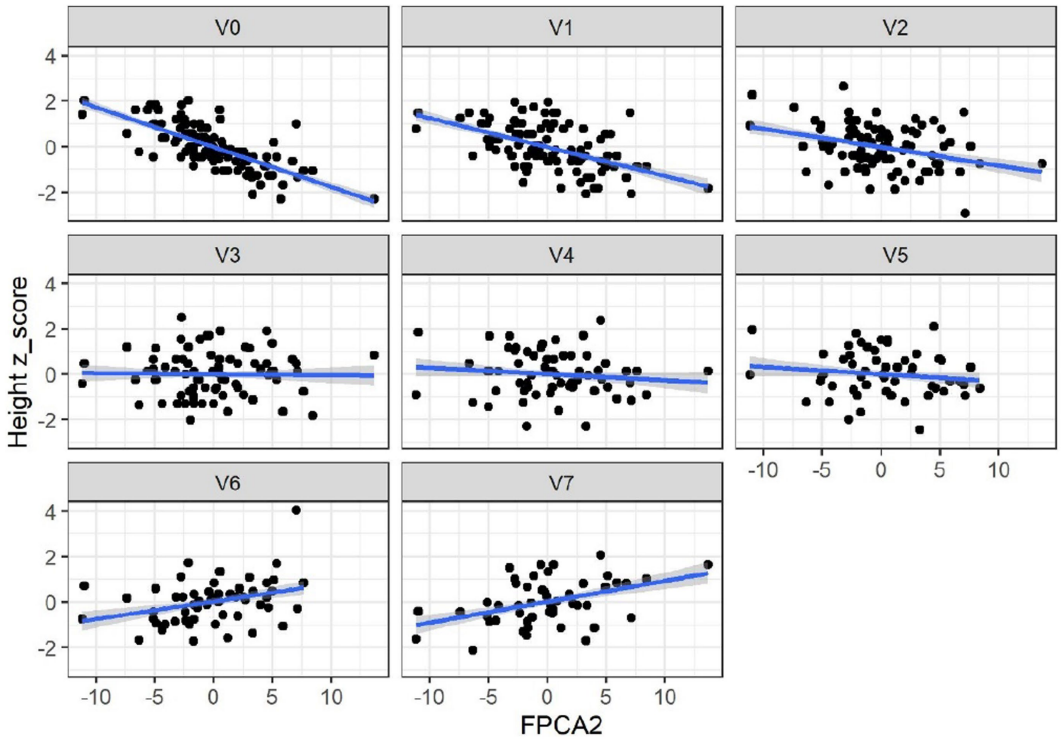

FIGURE 7
Correlations between FPCA2 scores and length (cm). Correlations: −0.79 (V0), −0.56 (V1), −0,37(V2), −0.05 (V3), −0.13 (V4), −0.12 (V5), 0.3 (V6), 0.35 (V7). Correlations were significant only at V0-V2 and V6-V7.

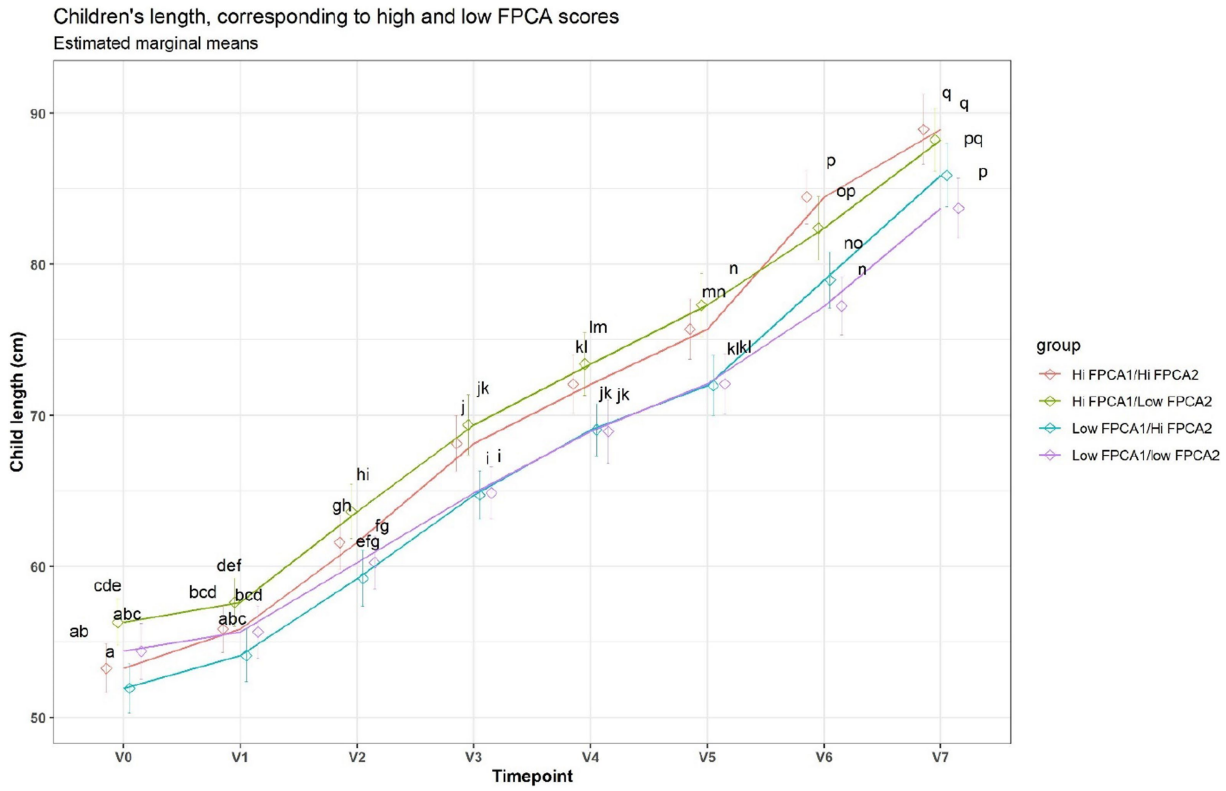

FIGURE 8
Average trajectories for length. Trajectories were split in 4 groups, based on median FPCA scores. A shared letter indicates a non-significant difference at the corresponding time point. For example, the 'Low FPCA1l/high FPCA2' is significantly lower than the 'Hi FPCA1/low FPCA2' at V0, V1, V2, V3, but not at V7.

associated with child length and weight z-scores in a model adjusted for maternal pre-pregnancy BMI, mode of delivery, birthweight z-score, sex and time. In an exploratory study, Sprenger et al. (16) found that FUT2 related alterations of breast milk HMOs composition as assessed through 2'FL concentrations, did not impact growth of breastfed infants during the first 4 months of life. However, they reported a non-statistically significant trend, with males from mothers with low 2'FL milk appearing to have a slightly higher BMI at 1 month, which was not seen any more at 4 months of age when they rather had a smaller BMI and body weight gain. Our approach suggests a complex, dynamic association between growth and HMO composition, illustrated by diverging trajectories starting from 3 months. However, these associations did not address the question of how changes in growth trajectories could be associated with early exposure to higher concentrations of HMO. Therefore, we analyzed the trajectories of length, weight, and head circumference over a period ranging from 2–5 weeks to 24 months, applying a data-driven approach derived from functional data analysis (23, 28, 29). In our study, each trajectory could be accurately described by two numbers (FPCA1, FPCA2), the first describing general growth (higher scores correspond to trajectories above the average at all time points), and the second describing a pattern of increased velocity at 12–18 months. These two scores jointly describe accurately the shape of the individual trajectories and can be therefore taken as a space of coordinates describing the growth trajectories.

Children fed with breast milk of cluster 4 (high in 2'FL, DFNHA, LNFP-I, LNDFH-I, low in 3FL) at 3 months, experienced a slight growth spurt after 12 months, compared to children fed with breast milk of cluster 1 (high in DFSL, LNDF-I). Since cluster 1 belongs to the ($FUT2^+$, $FUT3^+$) group and cluster 4 belongs to the ($FUT2^+$, $FUT3^-$) group, this can be interpreted as a possible association of the Lewis negative status with this particular longitudinal growth pattern. This supports the possibility that HMO concentrations should be looked at in combination when assessing associations with development, and that the non-linear patterns in the developmental trajectories must be appropriately modelled.

To the best of our knowledge, this is the first study to investigate the longitudinal association between clusters of HMO composition and child's physical development. Among the limitations, we acknowledge the modest sample size and the limited age range 0–24 months, limiting the interpretation of the growth patterns to early growth.

Using longitudinal data, we introduced novel methodological approaches, like network analysis of compositional data and functional PCA, to address the multivariate nature of the data. This approach may help to reveal complex longitudinal patterns, and can help future studies in the field.

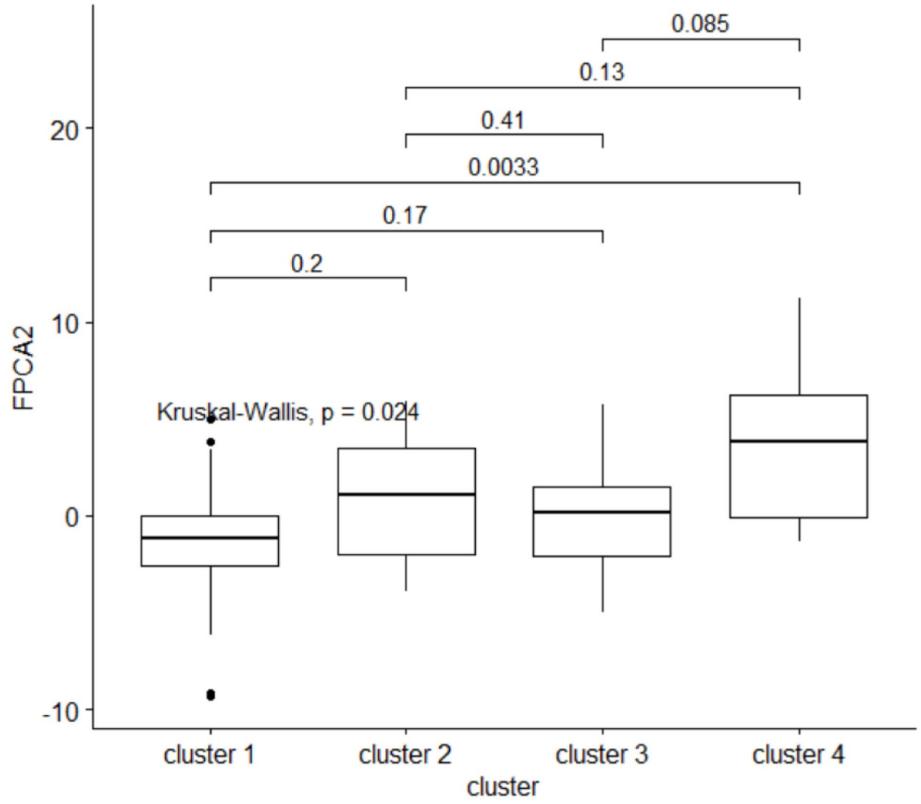

FIGURE 9
The second FPCA score for the child's length is associated with the HMO clustering. Above the boxplots, are shown the *p*-values from a *post-hoc* Dunn pairwise comparison test, with multiplicity correction.

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 humans were approved by Ethics boards at Rhode Island Hospital in Providence, RI, and Pennington Biomedical Research Center, in Baton Rouge, LA. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

FM conducted the data analyses and wrote the manuscript draft. AB, PR, SA, SD, and NS reviewed the manuscript. SA performed the quantification of the HMOs. NS and SD designed the study. All authors contributed to the article and approved the submitted version.

Conflict of interest

FM, PR, AB, SA, and NS were employed by Nestlé Research.

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

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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.2023.1239349/full#supplementary-material

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Infant feeding practices in three Latin American countries in three decades: what demographic, health, and economic factors are relevant?

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Introduction: Studies in Latin America have focused either on analyzing factors associated with exclusive breastfeeding (EBF) or infant formula (IF).

Purpose: Analyze the association between economic, sociodemographic, and health factors with EBF, mixed milk feeding (MixMF), and exclusive use of IF in three Latin American and Caribbean countries in the 1990s, 2000s, and 2010s.

Methods: Cross-sectional time-series study using data from Demographic and Health Surveys between the 1990s and 2010s in Colombia (1995–2010), Haiti (1994–2017), and Peru (1996–2012) accounting for a sample of 12,775 infants under 6 months. Hierarchical logistic multilevel regression models were used to estimate the adjusted association between infant feeding outcomes (EBF, MixMF, exclusive use of IF) and contextual level DHS survey decade (1990s, 2000s and 2010s) and economic factors (Gross Domestic Product by purchasing power parity, female wage and salaried workers, labor force participation rate female) as well as individual level sociodemographic (maternal age, maternal education, number of children in the household, wealth index, mother living with a partner, area of residence, mother working outside of home), and health factors (breastfed in the first hour, C-section).

Results: Factors associated with EBF cessation were c-section (OR: 0.76; 95%CI: 0.64, 0.92), mothers working outside of the home (OR: 0.79; 95%CI: 0.69, 0.90), families in the highest income quintile (OR: 0.64; 95%CI: 0.49, 0.84), and female wage and salaried workers (OR: 0.92; 95%CI: 0.91, 0.94). MixMF was associated with women with higher education (OR: 1.54; 95%CI: 1.21, 1.97), mother working outside of the home (OR: 1.26; 95%CI: 1.10, 1.43), c-section (OR: 1.37; 95%CI: 1.15, 1.62), families in the highest income quintiles (OR: 2.77; 2.10, 3.65). and female wage and salaried workers (OR: 1.08;95% CI: 1.05, 1.09). Exclusive use of IF was associated with a mother working outside of the home (OR: 2.09; 95%CI: 1.41, 3.08), c-section (OR: 1.65; 95%CI: 1.09, 2.51), families in the highest income quintiles (OR: 12.08; 95% CI: 4.26, 34.28), the 2010s (OR: 3.81; 95%CI: 1.86, 7.79), and female wage and salaried workers (OR: 1.12; 95%CI: 1.07, 1.16).

Discussion/Conclusion: Factors related to women empowerment and gender equality jeopardized EBF and favored the exclusive use of IF in Latin America.

Therefore, workplace interventions to promote, protect, and support breastfeeding practices are key to reducing exclusive use of IF.

KEYWORDS

child nutrition, breastfeeding, demographic factors, economic factors, Latin America, health

Introduction

The global prevalence of exclusive breastfeeding (EBF) among infants under 6 months, as recommended by the World Health Organization, is 48%, which means significant progress is required to meet the Global Nutrition Goal of 70% by 2030 (1). However, the increased use of infant formula (IF) in low-and middle-income countries is concerning and has become a threat to achieving breastfeeding goals (2). A recent analysis indicated that countries could meet this goal by 2030, if national efforts to support breastfeeding practices and reduce the exclusive use of IF were scaled up (1). Therefore, understanding the modifiable factors associated with infant feeding practices can help countries protect, promote, and support breastfeeding (3).

Cessation of EBF before the 6 months of life, as recommended by the WHO, can occur due to sociodemographic, economic, and health factors (3). Teenage motherhood (4), primiparity (4), the lack of a skilled attendant at birth (5), and the absence of a partner (6) are associated with the early interruption of EBF (4–6). While breastfeeding in the first hour of life (BF1h) is a strong predictor for EBF (3, 7), cesarean delivery is associated with introducing milk-based prelacteal in hospitals (3, 8). Some studies have observed lower prevalence of EBF in urban areas compared to rural areas (9, 10), and one study found an increased prevalence of IF in urban areas (10). A meta-analysis of Brazilian studies noted that low education and low family income are also among the factors associated with EBF discontinuation before 6 months of age (4). In contrast, another study found that women with higher education contributed concomitantly to a significant increase in the prevalence of EBF and to the use of IF (2). Returning to work (11) and full-time work schedules are considered factors that contribute to the interruption of EBF (3, 12). Additionally, more than half of mothers who work outside the home offer food before 6 months of age (13). One study showed a negative correlation between Gross Domestic Product (GDP) and EBF and a positive correlation between GDP and breast-milk substitutes such as IF, meaning that IF use may become widespread as a country develops (14).

In Latin America and the Caribbean, the rate of EBF has increased slightly from 35% in 2005 to 38% in 2018, but at this annual growth rate, it would take more than 40 years to reach the 70% goal (15). A study of six Latin American and Caribbean countries that analyzed national surveys between the 1990s and the 2010s observed an increase in IF in infants under 6 months, especially among those living in urban areas (10). Likewise, a progressive increase in mixed milk feeding (MixMF, i.e., the provision of formula and/or animal milk along with breast milk) across all children's age groups was observed (10). On the other hand, a progressive increase in EBF was observed, especially between the 1990s and 2000s and in the rural area. Findings from this study concluded that the increased use of IF has been a major barrier to achieving the 2030 Global Nutrition Goal for Exclusive Breastfeeding (10). However, prior studies in Latin America and the Caribbean have analyzed separately the factors associated with EBF or IF (4, 5, 16). In the present study, we aim to verify association between sociodemographic, economic, and health factors and EBF, MixMF, and the exclusive use of IF in three Latin American and Caribbean countries in the 1990s, 2000s, and 2010s.

Methods

Study design and data source

This cross-sectional time-series study used data from the Demographic and Health Surveys (DHS) Program conducted in the 1990s, 2000s, and 2010s. The DHS surveys are nationally representative household-based surveys that are comparable across and within countries at different time intervals. All DHS surveys used in this study are available on the DHS Program website[1] (17) and were approved by each country's ethics committee. The informed consent form was presented and signed by the respondents prior to the interview[2] (18). In addition, we gathered data from the World Bank website, which is a publicly available website (19).

Countries selection

The inclusion criteria used to choose countries in Latin America and the Caribbean were (1) the availability of at least one DHS survey for each decade (1990, 2000, and 2010), (2) the availability of variables on infant feeding on the day before the survey, and (3) the availability of variables on sociodemographic and health factors in the DHS survey databases. The Dominican Republic was not the only country considered eligible to this study because of sharp variation in the estimative of IF between datasets from the 2000s and 2010s, as our research group analyzed before (20).

The DHS Program website has available datasets for 15 countries in Latin America and the Caribbean. However, 11 countries did not have surveys for all decades (Brazil, Bolivia, Ecuador, El Salvador, Guatemala, Guyana, Honduras, Mexico, Nicaragua, Paraguay, and Trinidad and Tobago). The three countries with surveys in the three

1 https://dhsprogram.com/

2 https://dhsprogram.com/Methodology/Protecting-the-Privacy-of-DHS-Survey-Respondents.cfm

decades were Colombia, Haiti, and Peru. A total of 13 DHS surveys were included in this study: Colombia (4 databases: DHS III—1995, DHS IV—2000; DHS V—2005; DHS VI—2010), Haiti (5 databases: DHS III—1994/1995; DHS IV—2000; DHS V—2005/2006; DHS VI—2012; DHS VII—2016/2017), and Peru (4 databases: DHS III—1996; DHS IV—2000; DHS V—2007/2008; DHS VI—2012).

DHS sample, target population, and analytical sampling

All DHS surveys feature complex two-stage probability sampling. In the first stage, the census sectors (conglomerates) were selected, and in the second stage, the households to survey were selected (21). In most DHS surveys, people eligible for the individual interview include women of reproductive age (15–49) with an infant under the age of three or five (22). The target population of this study consisted of infants under 6 months of age who were alive at the time of the interview and who lived with the respondent. Thus, the pooled analytical sample from the 13 DHS surveys included in this study consisted of 12,775 infants under 6 months of age. The percentage of infants excluded was 1.3% in Colombia and 4.6% in Haiti due to the exclusion of children older than 6 months, dead children, and those who did not live with the respondent, as recommended by the WHO (23, 24).

Outcomes

This study selected three infant feeding outcomes defined by a recent WHO recommendation (22):

- *Exclusive breastfeeding* (EBF) is defined as the provision of only breast milk (numerator: infants under 6 months of age who were on EBF in the previous 24 h/denominator: infants 0–5 months old). Prescribed medicines, oral rehydration solution, vitamins, and minerals are not counted as fluids or foods (24).
- *Mixed milk feeding* (MixMF) is defined as the provision of formula and/or animal milk in addition to breast milk (numerator: infants 0–5 months of age who received breast milk, milk, and IF in the previous 24 h/denominator: infants 0–5 months old) (24).
- *Exclusive use of Infant formula* (exclusive use of IF) is defined as the exclusive use of IF to infants under 6 months old in the previous 24 h (numerator: infants younger than 6 months who exclusively received IF in the last 24 h/denominator: infants 0–5 months).

The outcomes were configured as dichotomous variables (no/yes). Missing data and the category "do not know" were less than 1% and were considered as "not consumed," as recommended by the WHO (23, 24).

Predictors

Sociodemographic, health, and economic predictors selection was guided by the hierarchical framework (25) developed based on models presented by previously studies supporting the associations with infant feeding (3, 26, 27) and according to the availability of information in the selected DHS surveys. These determinations informed the hierarchical framework illustrating potential associations of infant feeding used to guide our modeling approach (Figure 1).

Individual-level factors included sociodemographic and health obtained from the DHS surveys. The *sociodemographic factors* were maternal age (<20, 20–24 years, 25–29 years, ≥30 years), maternal education (no education, high school, and college education), number of children in the household (1, 2–3, and ≥4), wealth index (first quintile/s quintile/third quintile/fourth quintile/fifth quintile), mother living with a partner (no/yes), area of residence (urban/rural), mother working outside of the home (formal/informal) (no/yes). The wealth index refers to detailed information collected on the availability of consumer assets and housing characteristics that are directly related to the socioeconomic status (17). Each family received a score generated through a principal component data analysis to create the WI quintiles (17). The *health factors* were breastfeeding in the infant's first hour of life (BF1h) (no/yes) and c-section (no/yes) (Figure 1).

Contextual level factors included economic variables obtained from the World Bank website and the DHS survey decade. The *economic factors* were gross domestic product by purchasing power parity (GDP PPP) (continuous variable), female wage and salaried workers (salaried workers/self-employed workers) (% of female employment), and female labor force participation rate (% of female population ages 15–64). The GDP PPP refers to the sum *per capita* in current international dollars of all final goods and services produced in a country in a given period. The female wage and salaried workers variable refers to the employment status of women that is distinguished into two categories of total employees: (a) salaried workers (also known as employees) and (b) self-employed workers. The female labor force participation rate variable refers to the proportion of the female population age 15 and over who provide labor for the production of goods and services and includes both employed and unemployed women. Both variables related to working women are estimates modeled by the International Labor Organization (ILO) (19). The *DHS survey decades* were grouped as follows: DHS surveys from 1990 to 1999 were grouped into the 1990s, DHS surveys from 2000 to 2009 into the 2000s, and DHS surveys from 2010 to 2019 into the 2010s.

Data analysis

First, descriptive analysis of the outcome and predictors for each decade and each country was conducted individually (Supplementary Tables S1–S3). For these analyses, the complex sampling structure (stratification and conglomeration) was considered by weighting the importance and domain of the sample and the primary sampling unit using the *surveyset* command available in Stata.

Subsequently, the databases for each country and each decade were integrated (i.e., pooled database of countries). Descriptive analysis of the sociodemographic and health variables was conducted, including prevalence and respective confidence intervals of 95% by decades. Similarly, the average of the economic variables was calculated by decades for the pooled database of countries.

To verify the associations between the predictors and each outcome (EBF, MixMF, and exclusive use of IF), multilevel logistic regression models were conducted to estimate the odds ratios (OR)

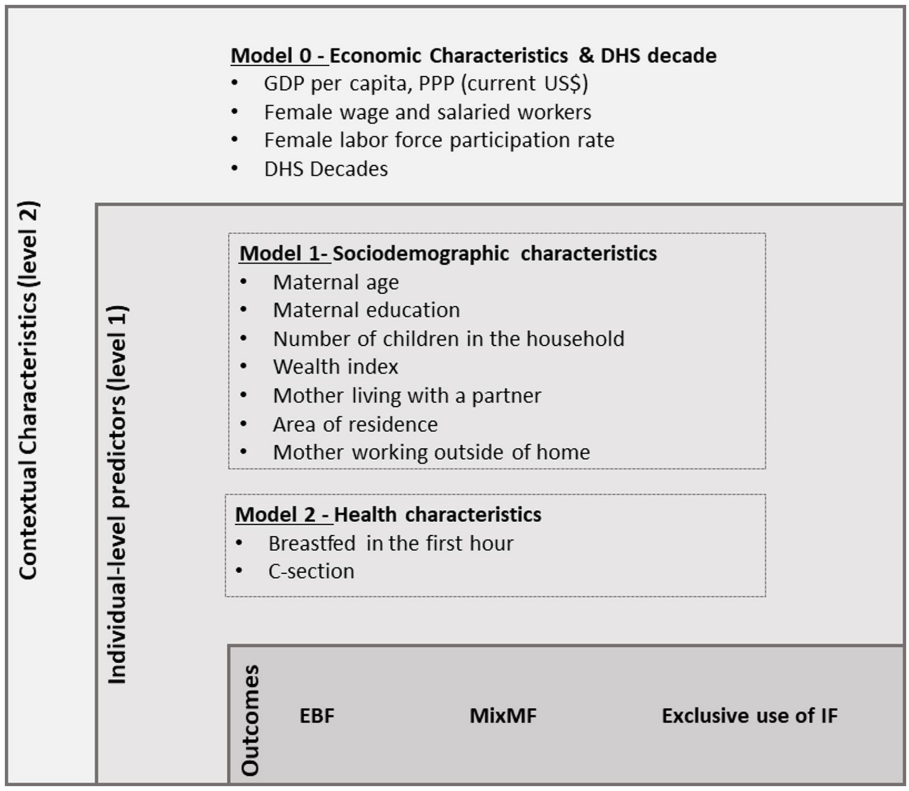

FIGURE 1
Analytical model of sociodemographic, health, and economic variables associated with exclusive breastfeeding (EBF), mixed milk feeding (MixMF), and exclusive use of infant formula (IF).

and 95% confidence intervals. For the multilevel logistic regression of the pooled database of countries, the weighting of strata, conglomerates, and sample weight was recoded to take into account the contextual level. For recoding the stratum, the cluster number, survey year, country code, and survey phase were considered. For recoding the conglomerate, the sample domain, country code, survey phase, and survey year were considered. For recoding the sample weight, the country code and survey phase were considered. The complex structure of the strata, conglomerates, and sample were incorporated into the multilevel analyses.

At this stage, economic and DHS survey decade factors comprised level 2 (contextual level), and sociodemographic and health factors comprised level 1 (individual level). A hierarchical approach was used to estimate the individual effect of the factors on the outcome (25), following a theoretical model established *a priori* (Figure 1). The hierarchical modeling data analysis approach (25) has been extensively used to study determinants of infant feeding practices (16, 27), as in the case of our analysis. First, the economic and DHS survey decade factors were added to the initial model (model 0), organizing individual data according to the clusters used in the multilevel analysis. Subsequently, in model 1, the sociodemographic factors were included; these factors were adjusted for in subsequent models. Likewise, factors related to health characteristics were added in model 2 to model 1 variables. At each level model, a value of $p < 0.05$ was used as a statistical significance criterion to assess the correlation between factors and outcome. This hierarchical modeling process was repeated for each outcome individually. At the end of each model, the Intraclass Correlation Coefficient was calculated. All analyses were performed using STATA SE® version 15.1.

Results

The prevalence of EBF increased from 35.6% (95% CI = 33.1, 38.3) in 1990 to 44.8% (95% CI = 42.7, 46.9) in 2010. The prevalence of MixMF reduced from 37.9% (95% CI = 35.5, 40.4) in 1990 to 31.9% (95% CI = 30.1, 33.9) in 2010. The prevalence of exclusive use of IF increased from 1.9% (95% CI = 1.4, 2.8) in 1990 to 3.7% (95% CI = 2.9, 4.7) in 2010 (Figure 2).

In the pooled database of countries, no differences in the prevalence of outcomes were observed across area of residence and mothers' age group across the decades. Between 1990 and 2010, the proportions of women with high school and college education increased in all three countries. The prevalence of women who reported working outside of the home, as well as those who reported living with a partner, reduced between 1990 and 2010. However, the prevalence of breastfeeding in the first hour and c-section increased across the decades. For the contextual level variables, the average number of female wage and salaried workers increased slightly from 30.3% in 1990 to 32.2% in 2010. The average female labor force participation increased from 55.6% in 1990 to 65.4% in 2010. The GDP PPP of the countries grew by US$2,610 between 1990 and 2010 (Table 1). The individual country surveys between 1990 and 2010 found an increase in

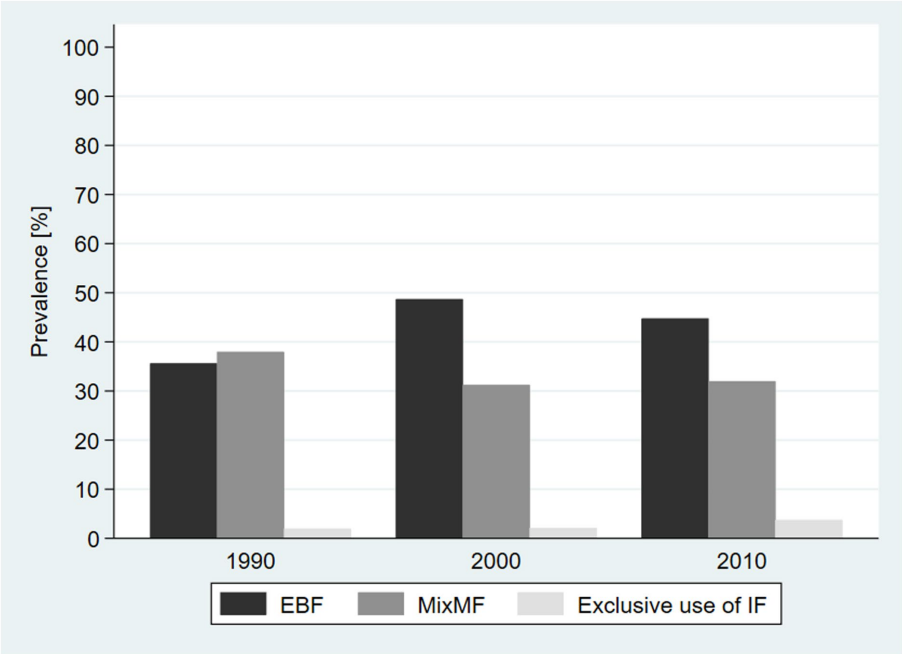

FIGURE 2
Prevalence of exclusive breastfeeding (EBF), mixed milk feeding (MixMF), and exclusive use of infant formula for the pool of countries by decade. DHS, 1990–2010.

maternal education, BF1h, c-section, the average female participation in the labor force, and the country GDP (Supplementary Tables S1–S3).

EBF interruption was associated with mother working outside of the home (OR: 0.79; 95% CI: 0.69, 0.90), families in the fifth income quintile (OR: 0.64; 95% CI: 0.49, 0.84), c-section (OR: 0.76; 95% CI: 0.64, 0.92), and female wage and salaried workers (OR: 0.92; 95% CI: 0.91, 0.94). The continuation of EBF was associated with women living with a partner (OR: 1.38; 95% CI: 1.17, 1.65), and the prevalence was higher in the 2000s (OR: 1.66; 95% CI: 1.39, 1.98) (Table 2).

MixMF was associated with women with a college education (OR: 1.54; 95% CI: 1.21, 1.97), families in the highest income quintiles (OR: 2.77; 95% CI: 2.10, 3.65), working outside of the home (OR: 1.26; 95% CI: 1.10, 1.43), c-section (OR: 1.37; 95% CI: 1.15, 1.62), and wage and salaried workers (OR: 1.08; 95% CI: 1.05, 1.09). Living with a partner (OR: 0.65; 95% CI: 0.54, 0.79), residing in rural areas (OR: 0.82; 95% CI: 0.68, 0.98), breastfeeding in the first hour (OR: 1.37; 95% CI: 1.15, 1.62), and the 2000s (OR: 0.65; 95% CI: 0.54, 0.77) reduced the odds of MixMF (Table 3).

Exclusive use of IF was associated with families in the highest income quintiles (OR: 12.08; 95% CI: 4.26, 34.28), working outside of the home (OR: 2.09; 95% CI: 1.41, 3.08), c-section (OR: 1.65; 95% CI: 1.09, 2.51), wage and salaried workers (OR: 1.12; 95% CI: 1.07, 1.16), and the 2010s (OR: 3.81; 95% CI: 1.86, 7.79). Having two or more children (OR: 0.57; 95% CI: 0.37, 0.88) and breastfeeding in the first hour (OR: 0.66; 95% CI: 0.46, 0.97) reduced the odds of exclusive IF use (Table 4).

Through the Intraclass Correlation Coefficient, we identified that 29% of the increase in EBF, 21% of the increase in MixMF, and 45% of the increase in exclusive IF use in this period were attributed to contextual factors (Tables 2–4).

Discussion

Our study is the first, as far as we know, to explore the individual and contextual factors associated with EBF, MixMF, and exclusive use of IF in three countries in Latin America and the Caribbean. The prevalence of EBF improved, with the largest increase seen between 1990 and 2000, followed by a stabilization of the rate between 2000 and 2010. These data corroborate information compiled in the Global Breastfeeding Scorecard, which indicated a worldwide increase of 10 % in EBF between 2010 and 2022, reaching 48% (1). However, such progress is uneven and insufficient (1). To achieve the 70% breastfeeding target set as a 2030 global goal, modifiable factors must be identified, disparities reduced, and equity promoted for women who need support to breastfeed.

Our study explored the association of sociodemographic, economic, and health factors with the indicators EBF, MixMF, and exclusive use of IF between the 1990s and 2010s. In the 1990s and 2000s, an increase in the prevalence of EBF was observed, while MixMF decreased and exclusive use of IF increased between 1990 and 2010. For women who were employed, who delivered by cesarean section, and who were classified in the highest income quintile, EBF decreased; however, for women who had a partner and in the 2000s, EBF increased. For women with a partner, who resided in rural areas and in the 2000s, MixMF reduced; while for women with a college education, this indicator increased. For women with greater numbers of children, who practiced breastfeeding in the first hour, and in the 2010s, exclusive use of IF consumption reduced. For women who were employed, who delivered by c-section, and who were in higher income quintiles, MixMF and exclusive use of IF consumption increased.

We found in our study that the likelihood of EBF increased when women living with a partner. These findings corroborate a Brazilian

TABLE 1 Characterization of sociodemographic, health, and economic factors in the set of Latin American countries in the 1990s, 2000s, and 2010s.

Factors	1990 OR (95% CI)	2000 OR (95% CI)	2010 OR (95% CI)
GDP per capita, PPP	4313.1	6215.6	6923.1
Female wage and salaried workers	30.3	33.8	32.2
Female labor force participation rate	55.6	63.3	65.4
Maternal age			
<20	15.4 (13.8, 17.1)	16.6 (15.4, 17.8)	17.2 (15.7, 18.7)
20–24	28.6 (26.5, 30.8)	27.1 (25.6, 28.6)	27.7 (25.9, 29.5)
25–29	23.1 (21.2, 25.1)	22.6 (21.2, 24.1)	23.2 (21.6, 24.9)
≥30	32.9 (30.7, 35.3)	33.8 (32.1, 35.4)	31.9 (30.1, 33.9)
Maternal education			
No schooling	13.8 (12.3, 15.5)	9.7 (8.7, 15.5)	6.6 (5.7, 7.6)
Primary school	38.6 (36.2, 41.1)	35.5 (33.9, 37.1)	30.8 (29.1, 32.7)
High school	36.9 (34.5, 39.4)	41.4 (39.7, 43.2)	48.3 (46.3, 50.4)
College	10.7 (9.2, 12.4)	13.3 (12.1, 14.6)	14.2 (12.8, 15.8)
Number of children in the household			
1	32.3 (30.1, 34.5)	35.3 (33.7, 36.9)	37.3 (35.3, 39.2)
2–3	39.6 (37.4, 41.9)	41.1 (39.5, 42.8)	44.4 (42.4, 46.5)
≥4	28.1 (25.9, 30.3)	23.6 (22.2, 25.1)	18.3 (16.9, 19.8)
Mother living with a partner			
Yes	88.9 (87.4, 90.3)	82.3 (81.0, 83.6)	81.9 (80.3, 83.3)
No	11.1 (9.7, 12.5)	17.7 (16.4, 18.9)	18.1 (16.7, 19.7)
Residence area			
Urban	55.4 (52.7, 58.2)	55.9 (53.8, 57.9)	56.8 (54.1, 58.9)
Rural	44.6 (41.8, 47.3)	44.1 (42.1, 46.2)	43.2 (41.1, 45.2)
Mother working outside of home			
No	66.5 (64.1, 68.8)	64.7 (62.9, 66.4)	71.6 (69.7, 73.5)
Yes	33.5 (31.2, 35.9)	35.3 (33.6, 37.1)	28.4 (26.5, 30.3)
Breastfed in the first hour			
Yes	42.6 (40.1, 45.2)	53.2 (51.4, 54.9)	53.5 (51.5, 55.6)
No	57.4 (54.8, 59.9)	46.8 (45.1, 48.6)	46.5 (44.5, 48.5)
C-section			
No	89.3 (87.6, 90.8)	82.3 (80.8, 83.7)	76.2 (74.5, 77.9)
Yes	10.7 (9.2, 12.4)	17.7 (16.3, 19.2)	23.8 (22.1, 25.5)

study that observed that not living with a partner was negatively associated with the prevalence of EBF (6). On the one hand, we found that EBF reduces with cesarean birth, indicating agreement with some studies that showed that c-section could increase the risk of pre-lacteal supplementation (8), and distribution of free samples of breast-milk substitutes in the hospital environment (3) hinders maintenance of EBF. On the other hand, vaginal delivery contributes to timely initiation of breastfeeding and facilitates the maintenance of EBF (3, 8). Some variables contributed to the negative outcome of breastfeeding, thus highlighting the importance of the family and community support network, breastfeeding counseling, and training health professionals to have adequate skills to provide training, practical knowledge, and confidence to women during prenatal and postpartum care.

The variables maternal work and formal or informal employment also showed negative outcomes in relation to EBF, corroborating two studies that showed that employed mothers may interrupt EBF early (13). The employment situation of mothers should be analyzed, including mothers who do not work outside the home and mothers who work outside the home but may or may not take maternity leave because the absence of maternity leave is also associated with the interruption of EBF (28). The maternal employment variable should be investigated considering whether or not the mother is on maternity leave because employed mothers on maternity leave probably have better conditions for maintaining EBF during the maternity leave period. Six-month maternity leave, a supportive work environment, and flexible work schedules are contextual factors that contribute to EBF up to 6 months of age (3, 12). Countries also need to be concerned with providing equity and reducing disparities in relation to the labor market for breastfeeding women because not all countries have laws that provide six-month paid maternity leave after the birth of the child and legislate the mandatory provision of paid breaks and support facilities for breastfeeding after returning to work. Protecting maternity in the workplace and promoting family and workplace leave policies are important indicators for successful breastfeeding (29).

Contextual factors, including income classification, are part of the determinants of breastfeeding (3). In this study, the prevalence of EBF also decreased when women were classified in the highest income quintile of the wealth index. Our study collaborates with other studies, which reported that mothers with higher income might breastfeed for less time (3, 30). This trend could be due to the misperception that breastfeeding is for the poor and unsophisticated and that breast-milk substitutes are modern and prestigious (3). In addition to national efforts to achieve breastfeeding goals, it would also be interesting to provide equity and quality counseling on the benefits of breastfeeding to women who are more likely to discontinue breastfeeding.

Although the prevalence of exclusive use of IF between the decades has increased, the prevalence is still low. Feeding a child exclusively with IF can be very expensive and almost unfeasible for infants older than 3 months when caregivers normally introduce other types of milk or foods for the infant. Perhaps this is one of the reasons why we found such a low prevalence of exclusive use of IF in this population. In previous studies (2, 14), the prevalence of IF was analyzed together with breastfeeding (MixMF), but the exclusive consumption of IF was not analyzed. Therefore, this study advances the understanding of factors associated with exclusive use of IF. The increase in economic development in low-and middle-income countries, the enrichment of the population, and the continuation of social changes unfavorable to breastfeeding could lead to increased commercialization of IF, as evidence shows that IF is a product that is resistant to market crises (3, 14). One possible factor currently contributing to the increase in IF consumption that was not examined in this study is the aggressive marketing of IF. Evidence is powerful of the connection between marketing of breast-milk substitutes and the decision of families with infants and toddlers about breastfeeding and infant health (31). To enhance efforts by countries to reduce IF consumption and increase breastfeeding rates, trends in breastfeeding and infant feeding indicators should be analyzed (31). Data from 2022

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TABLE 2 Association of sociodemographic, health, and economic factors with exclusive breastfeeding (EBF) in three selected Latin American countries in the 1990s, 2000s, and 2010s.

Predictors	Exclusive breastfeeding (EBF)		
	Model 0 OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Level 2—contextual predictors			
GDP per capita, PPP	1.00 (1.00, 1.00)		
Female wage and salaried workers	0.92 (0.91, 0.94)		
Female labor force participation rate	0.98 (0.97, 1.00)		
Decades			
1990	1.00 (ref)		
2000	1.66 (1.39, 1.98)		
2010	0.83 (0.68, 1.00)		
Level 1—individual-level predictors			
Model 1—sociodemographic characteristics			
Maternal age			
<20–24		1.00 (ref)	
25–29		1.07 (0.90, 1.26)	
≥30		0.99 (0.83, 1.19)	
Maternal education			
No schooling		1.00 (ref)	
High school		1.13 (0.98, 1.30)	
College		0.93 (0.72, 1.18)	
Number of children in the household			
1		1.00 (ref)	
2–3		1.12 (0.96, 1.30)	
≥4		1.10 (0.89, 1.38)	
Wealth index			
First quintile		1.00 (ref)	
Second quintile		1.02 (0.85, 1.23)	
Third quintile		0.89 (0.72, 1.10)	
Fourth quintile		0.79 (0.62, 1.00)	
Fifth quintile		0.64 (0.49, 0.84)	
Mother living with a partner			
No		1.00 (ref)	
Yes		1.38 (1.17, 1.65)	
Area of residence			
Urbana		1.00 (ref)	
Rural		1.09 (0.91, 1.32)	
Mother working outside of home			
No		1.00 (ref)	
Yes		0.79 (0.69, 0.90)	
Model 2—health characteristics			
Breastfed in the first hour			
No			1.00 (ref)
Yes			1.11 (0.98, 1.26)
C-section			

(Continued)

TABLE 2 (Continued)

Predictors	Exclusive breastfeeding (EBF)		
	Model 0 OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)
No			1.00 (ref)
Yes			0.76 (0.64, 0.92)
ICC	0.30	0.29	0.29

DHS, 1990–2010. Model 0: contextual predictors—level 2: Gross Domestic Product (GDP) per capita, purchasing power parity (PPP); female wage and salaried workers; female labor force participation rate; decades. Model 1: Individual-level predictors—Level 1: sociodemographic characteristics (maternal age, maternal education, number of children in the household, wealth index, mother living with a partner, area of residence, mother working outside of home) adjusted for all contextual variables. Model 2: Individual-level predictors—Level 1, sociodemographic characteristics and health characteristics (breastfed in the first hour, c-section) adjusted for all contextual variables. ICC, Intraclass Correlation Coefficient.

TABLE 3 Association of sociodemographic, health, and economic factors with breast milk supplemented with mixed milk feeding (MixMF) in the three selected Latin American countries in the 1990s, 2000s, and 2010s.

Predictors	Mixed milk feeding (MixMF)		
	Model 0 OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Level 2—contextual predictors			
GDP per capita, PPP	1.00 (1.0, 1.0)		
Female wage and salaried workers	1.08 (1.05, 1.09)		
Female labor force participation rate	1.01 (0.99, 1.02)		
Decades			
1990	1.00 (ref)		
2000	0.65 (0.54, 0.77)		
2010	0.97 (0.79, 1.18)		
Level 1—individual-level predictors			
Model 1 sociodemographic characteristics			
Maternal age			
<20–24		1.00 (ref)	
25–29		1.11 (0.94, 1.30)	
≥30		1.15 (0.95, 1.38)	
Maternal education			
No schooling		1.00 (ref)	
High school		1.09 (0.92, 1.29)	
College		1.54 (1.21, 1.97)	
Number of children in the household			
1		1.00 (ref)	
2–3		0.92 (0.79, 1.07)	
≥4		1.01 (0.81, 1.27)	
Wealth index			
First quintile		1.00 (ref)	
Second quintile		1.28 (1.03, 1.57)	
Third quintile		1.51 (1.21, 1.88)	
Fourth quintile		1.73 (1.35, 2.21)	
Fifth quintile		2.77 (2.10, 3.65)	
Mother living with a partner			
No		1.00 (ref)	
Yes		0.65 (0.54, 0.79)	
Area of residence			

(Continued)

TABLE 3 (Continued)

Predictors	Mixed milk feeding (MixMF)		
	Model 0 OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Urbana		1.00 (ref)	
Rural		0.82 (0.68, 0.98)	
Mother working outside of the home			
No		1.00 (ref)	
Yes		1.26 (1.10, 1.43)	
Model 2—health characteristics			
Breastfed in the first hour			
No			1.00 (ref)
Yes			0.87 (0.76, 0.98)
C-section			
No			1.00 (ref)
Yes			1.37 (1.15, 1.62)
ICC	0.27	0.21	0.21

DHS, 1990–2010. Model 0: contextual predictors—level 2: Gross Domestic Product (GDP) per capita, purchasing power parity (PPP); female wage and salaried workers; female labor force participation rate; decades. Model 1: Individual-level predictors—Level 1: sociodemographic characteristics (maternal age, maternal education, number of children in the household, wealth index, mother living with a partner, area of residence, mother working outside of home) adjusted for all contextual variables. Model 2: Individual-level predictors—Level 1: sociodemographic characteristics and health characteristics (breastfed in the first hour, c-section) adjusted for all contextual variables. ICC, Intraclass Correlation Coefficient.

TABLE 4 Association of sociodemographic, health, and economic factors with exclusive use of infant formula (IF) in the set of Latin American countries in the 1990s, 2000s, and 2010s.

Predictors	Exclusive use of infant formula (IF)		
	Model 0 OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Level 2—contextual predictors			
GDP per capita, PPP	0.99 (0.99, 0.99)		
Female wage and salaried workers	1.12 (1.07, 1.16)		
Female labor force participation rate	1.00 (0.94, 1.05)		
Decades			
1990	1.00 (ref)		
2000	1.02 (0.59, 1.74)		
2010	3.81 (1.86, 7.79)		
Level 1—individual-level predictors			
Model 1—sociodemographic characteristics			
Maternal age			
<20–24		1.00 (ref)	
25–29		1.19 (0.76, 1.87)	
≥30		1.37 (0.80, 2.32)	
Maternal education			
No schooling		1.00 (ref)	
High school		1.03 (0.64, 1.68)	
College		0.92 (0.50, 1.72)	
Number of children in the household			
1		1.00 (ref)	
2–3		0.57 (0.37, 0.88)	
≥ 4		0.41 (0.21, 0.81)	
Wealth index			

(Continued)

TABLE 4 (Continued)

Predictors	Exclusive use of infant formula (IF)		
	Model 0 OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)
First quintile		1.00 (ref)	
Second quintile		6.31 (2.40, 16.63)	
Third quintile		8.91 (3.45, 22.99)	
Fourth quintile		6.38 (2.31, 17.62)	
Fifth quintile		12.08 (4.26, 34.28)	
Mother living with a partner			
No		1.00 (ref)	
Yes		1.15 (0.74, 1.78)	
Area of residence			
Urbana		1.00 (ref)	
Rural		0.56 (0.32, 0.98)	
Mother working outside of the home			
No		1.00 (ref)	
Yes		2.09 (1.41, 3.08)	
Model 2—health characteristics			
Breastfed in the first hour			
No			1.00 (ref)
Yes			0.66 (0.46, 0.97)
C-section			
No			1.00 (ref)
Yes			1.65 (1.09, 2.51)
ICC	0.48	0.44	0.45

DHS, 1990–2010. Model 0: contextual predictors—level 2: Gross Domestic Product (GDP) per capita, purchasing power parity (PPP); female wage and salaried workers; female labor force participation rate; decades. Model 1: Individual-level predictors—Level 1: sociodemographic characteristics (maternal age, maternal education, number of children in the household, wealth index, mother living with a partner, area of residence, mother working outside of home) adjusted for all contextual variables. Model 2: Individual-level predictors—Level 1: sociodemographic characteristics and health characteristics (breastfed in the first hour, c-section) adjusted for all contextual variables. ICC, Intraclass Correlation Coefficient.

showed that the milk powder industry used systematic and unethical marketing strategies to influence families' infant feeding decisions (31), which may justify the progressive increase in IF consumption rates.

Regarding health-associated factors, one study noted that cesarean section was associated with a higher chance of introducing milk-based prelacteals in hospitals (8), and our study also noted that exclusive use of IF increased for women who had c-sections. The promotion and availability of IF through the marketing of breast-milk substitute industries influence the early introduction of foods in infants under 6 months of age (3). Factors associated with the organization of services and support network are also important, including the unpreparedness of the health professional team and the lack of organization of health services to assist the mother in breastfeeding (3, 12). Although these are important factors, they were not measured in the DHS surveys.

Exclusive use of IF also increased for women ranked in higher income quintiles. Furthermore, when adjusted for contextual variables, the 2010s appear to have contributed to the increase in exclusive use of IF, i.e., more than 44% of the increase in exclusive use of IF in this period was concentrated in the contextual factors in the countries studied. We emphasize that such results align with the findings that IF consumption in the first 8 months of life increased in Latin America and the Caribbean, the Middle East and North Africa, Eastern Europe, and Central Asia (14). Exclusive use of IF also increased for those women who were employed and is one of the main reasons for not breastfeeding or weaning early and facilitates the introduction of other foods for infants under 6 months (3).

Moreover, we observed that exclusive use of IF reduced for women with more children and when they did BF1h. Collaborating with our study, researchers have observed that primiparity influences the interruption of EBF (4) and that breastfeeding in the first hour facilitates EBF until the sixth month (3, 7). A recent analysis observed that more than half of parents and pregnant women were exposed to aggressive marketing for formula milk, according to the WHO and UNICEF (31). To meet the challenges of increasing EBF and reducing IF consumption, governments, healthcare professionals, and the baby food industry need to stop the abusive marketing of milk formula and must fully implement and comply with the requirements of the International Code of Marketing of Breast-milk Substitutes (31). This requires passing, monitoring, and enforcing laws to prevent the promotion of milk

formula in accordance with the International Code; investing in breastfeeding policies and programs; adequately funding paid parental leave; ensuring adequate quality support for breastfeeding; requiring the industry to publicly commit to full compliance with the Code and the 2023 World Health Assembly global decisions; and prohibiting health professionals from accepting sponsorship, scholarships, awards, grants, meetings, or events from companies that market foods for infants and young children (31).

Our study has some limitations to consider when interpreting the results. The first limitation is the differences in the number of food variables by survey year in the DHS surveys. In 2008, the WHO systematized the child nutrition indicators for Latin America and the Caribbean for the number of food variables for the surveys in these countries. Therefore, this increase could make the prevalence of EBF lower in the later years of the surveys compared to surveys in previous years, as it gives the mother more feeding options to remember. However, we found an increase in EBF from 1990 to 2010 and stabilization between 2000 and 2010, suggesting that potential misclassification was irrelevant. The lack of data in the DHS surveys regarding the age at which food was introduced is a limitation; therefore, we only estimated whether or not the infant consumed selected foods in the study. A limitation was the lack of information on maternity leave in the DHS surveys and the World Bank database since maternity leave facilitates the maintenance of EBF. Another limitation was the lack of more recent surveys for Colombia and Peru, as the most recent surveys for these countries were done more than 10 years ago. A DHS survey for Colombia in 2015 was available but does not have child-feeding data, and an ENDES survey for Peru in 2018 was available but did not contain sociodemographic and health data, so they were not included in our dataset. We checked the UNICEF website (32) and searched country websites, but no recent infant feeding data exists. Another limitation is the absence of infant feeding data along with sociodemographic and health data from Latin American countries, such as Brazil and Mexico with large populations of children under 2 years of age. These countries are important markets for the breast-milk substitutes and infant formula industries.

Despite these limitations, the strengths of our study are the use of nationally representative surveys, the analysis of three Latin American countries for three decades, and the association analysis of infant feeding indicators with sociodemographic, health, and economic factors to understand which factors impact EBF for infant up to 6 months of age as well as the supply of breast-milk substitutes and IF. Moreover, we used robust multilevel regression analysis for the pool of countries with recoding of the weighting aimed at reflecting the pool of countries and adjusting for the contextual level of the model. Thus, our analyses are important to support health professionals and especially health managers to understand the situation of infant feeding in the first 6 months of life in Latin America and the Caribbean, as national efforts are needed to increase rates of EBF until the sixth month and reduce rates of breast-milk substitutes and IF.

We concluded that the prevalence of EBF and exclusive use of IF both increased, while the prevalence of MixMF decreased in the studied decades. On the one hand, the cesarean delivery route and employed women with higher income exhibited a negative correlation with EBF. On the other hand, women living with a partner and the 2000s positively influenced EBF. Women who breastfed in the first hour, who had a partner, and who resided in rural areas, as well as the 2000s showed a negative correlation to MixMF. In contrast, women with a college education and wage and salaried workers influenced the increase of this indicator. Women who practiced breastfeeding in the first hour, who had 2–3 children, and ≥ 4 children were negatively associated with exclusive use of IF. Nevertheless, the 2010s and women with higher female labor force participation rate influenced the increase of this indicator. Both MixMF and exclusive use of IF increased among women who worked outside of the home, children born via c-section, and families ranked in the highest income quintiles. We also noted that the increases in EBF and MixMF were partly attributed to the 2000s, and the increase in exclusive IF use to the 2010s. We highlight the low prevalence of exclusive IF consumption for the pool of countries over the three decades because we do not frequently observe similar data in the literature.

The individual and contextual variables analyzed in this study captured the important influence of the political, economic, and social context on breastfeeding. This reinforces that breastfeeding does not have just one individual component but involves several components, including individual, contextual, and health. Hence, our analyses contribute to understanding factors that facilitate or impede the achievement of the Global Nutrition Goals for breastfeeding since it is not only about achieving breastfeeding prevalence rates, but also about equity for women who need support to breastfeed and reducing disparities in their environment.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found at: The DHS and MICS are free access and available at: https://dhsprogram.com/data/available-datasets.cfm and https://mics.unicef.org/surveys.

Author contributions

CM contributed to the conception and design of the study, data analysis, interpretation of results, writing of the manuscript, and approval of the final version. GB contributed to the data analysis, interpretation of results, writing of the manuscript, and approval of the final version. CA contributed to writing of the manuscript and approval of the final version. WC contributed to the conception and design of the study and approval of the final version. AR contributed to the conception and design of the study, data analysis, interpretation of results, writing of the manuscript, and approval of the final version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1239503/full#supplementary-material

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A nutritional supplement taken during preconception and pregnancy influences human milk macronutrients in women with overweight/obesity and gestational diabetes mellitus

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Rational: Maternal overweight/obesity and gestational diabetes mellitus (GDM) are associated with an increased risk of their offspring developing overweight/obesity or type 2 diabetes later in life. However, the impacts of maternal overweight/obesity and dysglycemia on human milk (HM) macronutrient composition are not well understood.

Objective: Through a double-blind randomised controlled trial, we investigated the effects of maternal supplementation from preconception throughout pregnancy until birth on HM macronutrient concentrations, in association with maternal and infant factors including maternal pre-pregnancy body mass index (BMI) and GDM status. In addition, we aimed to characterise longitudinal changes in HM macronutrients.

Methods: The control supplement contained calcium, iodine, iron, β-carotene, and folic acid. The intervention supplement additionally contained zinc, vitamins B_2, B_6, B_{12}, and D_3, probiotics, and myo-inositol. HM samples were collected across seven time points from 1week to 12months from Singapore and/or New Zealand. HM macronutrient concentrations were measured using a MIRIS Human Milk Analyser. Potential differences in HM macronutrient concentrations were assessed using linear mixed models with a repeated measures design.

Results: Overall, HM macronutrient concentrations were similar between control and intervention groups. Among the control group, overweight/obesity and GDM

were associated with higher HM fat and energy concentrations over the first 3 months. Such associations were not observed among the intervention group. Of note, mothers with GDM in the intervention group had lower HM fat by 10% ($p = 0.049$) and energy by 6% ($p = 0.029$) than mothers with GDM in the control group. Longitudinal changes in HM macronutrient concentrations over 12 months of lactation in New Zealand showed that HM fat and energy decreased in the first 6 months then increased until 12 months. HM lactose gradually decreased from 1 week to 12 months while crude protein decreased from 1 week to 6 months then remained relatively constant until 12 months of lactation.

Conclusion: Maternal overweight/obesity or GDM were associated with increased HM fat and energy levels. We speculate the intervention taken during preconception and pregnancy altered the impact of maternal BMI or GDM status on HM macronutrient composition. Further studies are required to identify the mechanisms underlying altered HM macronutrient concentration in the intervention group and to determine any long-term effects on offspring health.

Clinical trial registration: ClinicalTrials.gov, NCT02509988, Universal Trial Number U1111-1171-8056. Registered on 16 July 2015. This is an academic-led study by the EpiGen Global Research Consortium.

KEYWORDS

human milk, macronutrients, gestational diabetes mellitus, maternal BMI, maternal nutrition

1. Introduction

Human milk (HM) provides the essential nutrients and bioactive factors infants need for growth and development (1, 2). The World Health Organisation (WHO) recommends infants to be exclusively breastfed for at least 6 months (3), which has been associated with long-term infant outcomes including lower risks of obesity (4, 5) type 2 diabetes (6), infections (7), allergies, and asthma (8, 9), and improved neurodevelopmental outcomes (10). Some HM components have been associated with specific outcomes. For example, HM oligosaccharides have been associated with altered risks of allergies or infections (11, 12) and cognitive developmental scores in infants (13). Further, a recent pre-clinical study demonstrated that myo-inositol promotes neuronal connectivity, providing insights into the role of myo-inositol in infant brain development (14). HM macronutrient composition has been associated with infant body composition up to 12 months of age (15, 16). However, there is limited and inconsistent evidence in this area.

In addition to bioactive compounds such as immunological components and growth and metabolic hormones, HM provides nutrients and energy for infant growth and development. HM contains approximately 3.8% fat, 7% lactose, and 1% protein, each contributing about 50%, 40–45%, and 5–6% to total energy, respectively (17, 18). Infant formula contains proportions of macronutrients similar to HM, with fat providing approximately 45–50% of total energy, carbohydrate 40–45%, and protein 8–12% (18). While infant formula is a standardised solution over specific age ranges, HM is a dynamic compound, changing during a feed (19), throughout the day (20), and over the course of lactation (19, 21). Moreover, HM macronutrient composition may vary according to a range of maternal and infant factors, but these associations are not well understood. With the exception of fatty acids, maternal diet is reported to have no association with HM macronutrients (22). Positive associations have been reported between maternal body mass index (BMI) and HM fat and energy (23–26), and between maternal age and HM fat and carbohydrate (27–30). In addition, negative associations have been reported between infant gestational age and HM protein (27, 31), fat, and lactose (21, 30, 32). There have been inconsistent observations on the influence of maternal GDM status (27, 33, 34), infant sex (26, 35–38), parity (28, 39), and mode of delivery (24, 32, 36, 40) on HM macronutrient composition.

The Nutritional Intervention Preconception and During Pregnancy to Maintain Healthy Glucose Metabolism and Offspring Health (NiPPeR) study was a double-blind, randomised controlled trial investigating the effects of a nutritional supplement during preconception and pregnancy on maternal pregnancy outcomes and infant growth (41). Adequate maternal micronutrient status during pregnancy and lactation is essential for both mothers and infants (42). Micronutrient supplementation during pregnancy has been associated with lower risks of adverse pregnancy outcomes (43). For example, folic acid supplementation decreased occurrence of neural tube defect in the offspring (44); a combined supplementation of folic acid and iron reduced the risk of post-partum haemorrhage (45); calcium supplementation lowered the risk of pre-eclampsia (46); and vitamin D supplementation was associated with reduced risks of pre-eclampsia (47). In our recent publications, we showed that micronutrient supplementation during pre-conception and pregnancy increased HM concentrations of zinc (48) and vitamin D (49) in the first 3 months of lactation. However, there remains a paucity of data on the potential impacts of micronutrient supplementation during preconception and pregnancy on HM macronutrient composition during lactation. Therefore, the aim of this study was to describe HM macronutrient

composition following nutritional supplementation during preconception and pregnancy in association with maternal and infant factors including maternal pre-pregnancy BMI and GDM status.

2. Materials and methods

2.1. Study design

A detailed protocol for the NiPPeR study (ClinicalTrials.gov, identifier: NCT02509988, Universal Trial Number U1111-1171-8056; registered on July 16, 2015) has been published previously (41). Briefly, the NiPPeR study was a double-blind, randomised, controlled trial investigating the effects of a nutritional supplement taken from preconception and during pregnancy on maternal pregnancy and infant outcomes. The control supplement comprised of micronutrients that are present in commonly used pregnancy supplements: calcium, iron, iodine, folic acid, and vitamin A. The NiPPeR intervention supplement additionally contained zinc, vitamin B_2, vitamin B_6, vitamin B_{12}, vitamin D_3, myo-inositol, and probiotics (Table 1). The study supplements were packaged as a powder form in sachets and were taken twice daily, as a drink reconstituted with water. Adherence to the study protocol was assessed by sachet counting, with good adherence defined as at least 60% of the sachets consumed (53). The primary outcome of gestational glycaemia and the secondary outcome of GDM did not differ between control and intervention groups (53). The study was conducted in Southampton (United Kingdom), Singapore, and Auckland (New Zealand) and ethics approval was obtained at each site [Southampton—Health Research Authority National Research Ethics Service Committee South Central Research Ethics Committee (15/SC/0142); Singapore—the National Healthcare Group Domain Specific Review Board (2015/00205); and New Zealand—Northern A Health and Disability Ethics Committee (15/NTA/21)]. All participants provided written informed consent.

2.2. Study participants

Participants were recruited by self-referral after study information was distributed through local and social media advertisements. The key inclusion criteria were women aged 18–38 years who were planning to conceive within 6 months. The full inclusion, exclusion, and withdrawal criteria have been reported previously (41) and are provided in Supplementary Table 1. Eligible participants were randomised in a 1:1 ratio to either the control or the intervention group through the electronic study database (41), and stratified by site and ethnicity to ensure balanced allocation of participants.

2.3. Human milk sample collection

HM samples were collected in Singapore until 3 months (July 2016 to March 2019) and in New Zealand until 12 months of lactation (May 2017 to November 2019); practical constraints precluded collection in the United Kingdom (Figure 1). Samples were collected at four time points common to both sites: 1 week (±3 days), 3 weeks (±5 days), 6 weeks (±5 days), and 3 months (±10 days). In New Zealand, there were additional HM collections at 6 months (±14 days), 9 months (±14 days), and 12 months (±14 days); seven time points in total. In Singapore, samples were only collected until 3 months due to logistical constraints. Mothers were asked to refrain from breastfeeding for 2 hours prior to sample collection from the breast where samples would be collected. Under the supervision of trained staff, whole HM samples were collected in the morning from a single breast pumped for 15 minutes using an Ameda Lactaline breast pump (Ameda, Inc., Murarrie, Australia) or until fully emptied. Following collection, HM samples were homogenised, then stored at −80°C until analysis. HM samples were not collected if the mother had ceased breastfeeding, her milk supply was low, or there were complications with milk expression. Figure 1 shows the number of samples analysed at each time point.

TABLE 1 Detailed nutrient composition of the intervention and control drinks in the NiPPeR study.

Group	Nutrient	Intervention	Control	Daily dose	Recommended range[#]
Minerals	Calcium	✓	✓	150 mg	700–1,300 mg
	Iodine	✓	✓	150 µg	140–220 µg
	Iron	✓	✓	12 mg	14.8–27.0 mg
	Zinc	✓	✘	10 mg	7–15 mg
Vitamins	A (β-carotene)	✓	✓	720 µg	700–750 µg
	B_2 (Riboflavin)	✓	✘	1.8 mg	1.38–1.46 mg
	B_6 (Pyridoxine)	✓	✘	2.6 mg	1.2–1.9 mg
	B_9 (Folic acid)	✓	✓	400 µg	300–600 µg
	B_{12} (Cobalamin)	✓	✘	5.2 µg	1.5–2.6 µg
	D_3 (Cholecalciferol)	✓	✘	400 IU (10 µg)	5–10 µg
Other	Myo-inositol	✓	✘	4 g	n/a
	*Lactobacillus rhamnosus**	✓	✘	>1 × 10^9 CFU	n/a
	Bifidobacterium animalis ssp. *lactis*[†]	✓	✘	>1 × 10^9 CFU	n/a

[#]Recommended ranges for daily intake during pregnancy according to the reference nutrient intake for the United Kingdom (50), recommended dietary allowance for Singapore (51), and recommended daily intake for New Zealand (52). *NCC 4007 (CGMCC 1.3724). [†]NCC 2818 (CNCM I-3446). CFU, Colony-forming units; n/a, Not applicable. Table reproduced from Han et al. (48).

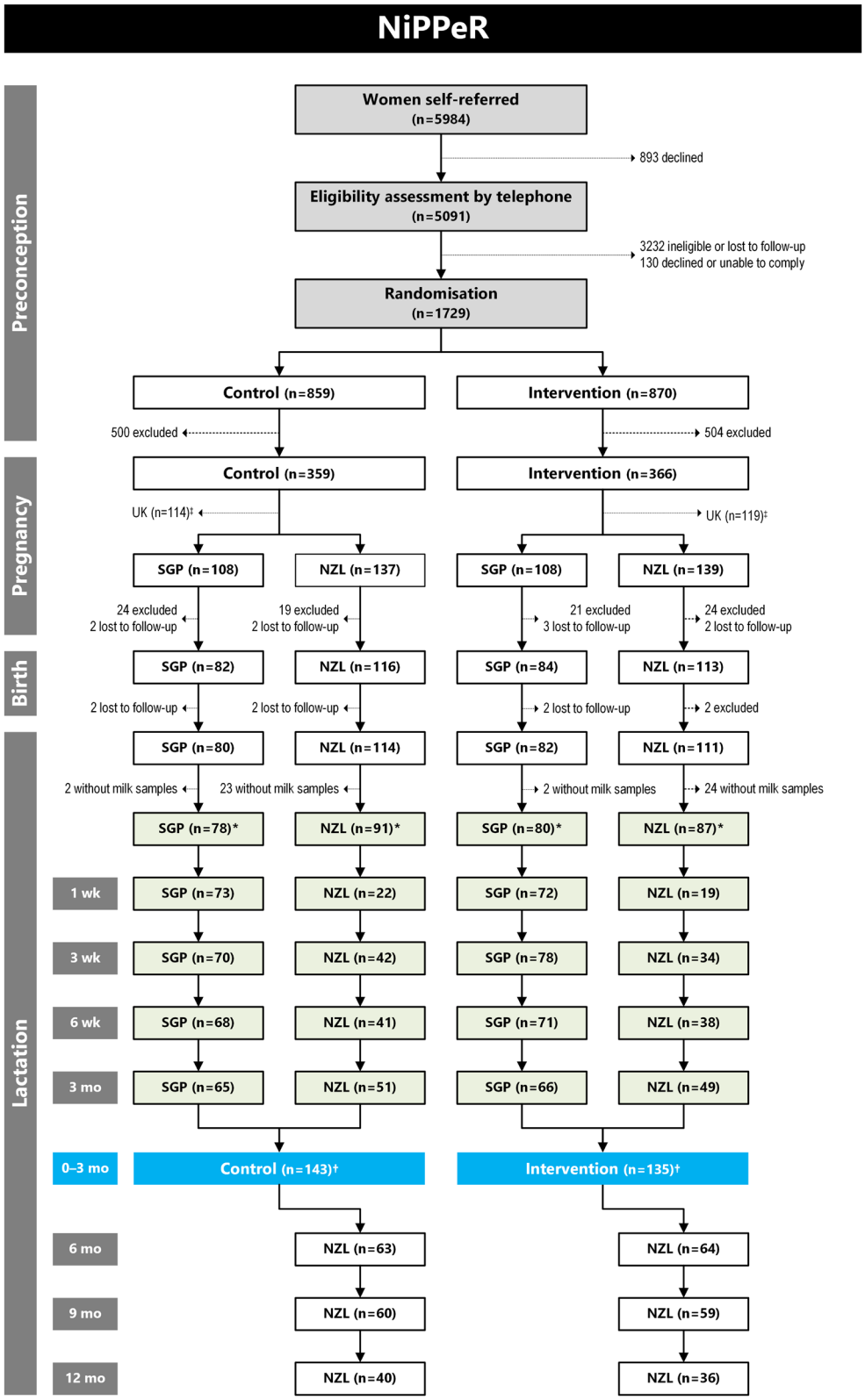

FIGURE 1
CONSORT diagram for the number of human milk (HM) samples analysed for macronutrients in the NiPPeR study. Reasons for exclusion during the preconception phase have been published previously (53), while reasons for exclusion during pregnancy and birth in Singapore (SGP) and New Zealand (NZL) are provided in Supplementary Table 2. ‡There were no HM samples collected in the United Kingdom (UK), so all participants from that site were excluded from this diagram. *Number of participants who provided at least one HM sample during the first 12months of lactation. †Number of participants who provided at least one HM sample during the first 3months of lactation. Diagram adapted from Han et al. (48).

2.4. Human milk macronutrient analyses

HM fat, energy, lactose, and crude protein were quantified via infrared transmission spectroscopy using a Miris Human Milk Analyser (HMA; Miris, Uppsala, Sweden) following the manufacturer's protocol. 1–3 mL of frozen HM samples were slowly thawed overnight at 4°C. Prior to analysis, they were warmed to 40°C in a water bath and homogenised using a Miris Ultrasonic Processor (Miris, Uppsala, Sweden) at a processing time of 1.5 s/mL. Quality control was performed using a HM sample with known macronutrient composition after every 20 samples and clean and check performances were done every 10 samples.

2.5. Statistical analyses

Descriptive statistics were calculated for maternal, infant, and birth-related characteristics. For intergroup comparisons, the independent samples *t*-test was used for continuous variables, and Chi-square tests were used to compare categorical variables between randomisation groups and between sites. For HM macronutrient measurements, samples with one or more macronutrient measurement value of '0' were excluded from analysis due to a possible risk of sample dilution. In addition, we adopted a conservative approach to removing outliers from analyses, excluding values outside the range of the mean ± 5 × standard deviations (SD; Supplementary Table 3).

Potential intervention effects on HM macronutrient concentrations were examined on the samples collected in the first 3 months of lactation only, collected in both Singapore and New Zealand. Key parameters included in linear mixed models were randomisation group, visit, their interaction term (group*visit), study site, maternal pre-pregnancy BMI, infant gestational age at birth, and adherence to the study supplements as a continuous variable. Participant study ID number was also included as a random factor to account for the repeated measurements. Subgroup analyses were performed to examine potential intervention effects over the first 3 months of lactation separately for Singapore and New Zealand. Temporal changes in HM macronutrients from 1 week to 12 months of lactation are described for the New Zealand site only as samples from the later time points were not available in Singapore.

As secondary analyses, potential interactions between the intervention and maternal metabolic status (pre-pregnancy BMI or GDM) and their associations with HM macronutrients were examined for the first 3 months of lactation in fully adjusted models. Pre-pregnancy BMI was defined using the WHO classification (54): underweight <18.5 kg/m^2, normal weight 18.5–24.9 kg/m^2, overweight 25.0–29.9 kg/m^2, and obesity ≥30 kg/m^2. Maternal GDM status was determined by an oral glucose tolerance test at 28 weeks gestation as defined by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria (55).

As exploratory analyses, potential associations between other maternal and infant factors with HM macronutrients were assessed. The mean HM macronutrient concentrations in the first 3 months were compared between binary categories of maternal ethnicity (Non-Asian vs. Asian), maternal age (< 35 vs. ≥ 35 years old), delivery mode (Vaginal vs. Caesarean-section), parity (Primiparous vs. Multiparous), infant sex (Male vs. Female), and infant gestational age (Term/Post-term ≥37 weeks vs. Preterm <37 weeks). Interactions between each of these maternal/infant factors and the intervention group were tested but none were statistically significant.

Study outcomes are reported as the back-transformed least-squares means (i.e., adjusted means) for each group or the adjusted mean difference (aMD) between groups and their respective 95% confidence intervals (CI). The aMD for back-transformed values represent proportional differences between the comparison groups. Statistical analyses was carried out using IBM SPSS Statistics for Windows, Version 26 (IBM Corp., Armonk, NY, United States) and SAS Version 9.4 (SAS Institute Inc., Cary, NC, United States). Graphs were created with GraphPad Prism version 9.3.1 (GraphPad Software, San Diego, California, United States). All statistical tests were two-sided with significance maintained at $p < 0.05$, without adjustments for multiple comparisons or imputation of missing values.

3. Results

3.1. Study population

In total, 336 of 387 participants (86.8%) from Singapore and New Zealand sites who continued to the postpartum stage of the study provided at least one HM sample in the first 12 months of lactation (Figure 1). Maternal pre-pregnancy BMI ($p = 0.047$) and passive smoking rates ($p = 0.035$) were lower in the intervention group compared with the control. Other baseline and perinatal characteristics, including GDM rates, were similar between control and intervention groups (Table 2). In Singapore, most participants were of Chinese ethnicity (78.5%), while in New Zealand, most were White Caucasian (75.8%; $p < 0.001$). Compared to the New Zealand cohort, the Singapore participants had lower pre-pregnancy BMI ($p < 0.001$), a higher GDM rate ($p < 0.001$), more vaginal deliveries ($p = 0.023$), lower infant birth weight ($p < 0.001$), and shorter gestation ($p < 0.001$), but the incidence of preterm delivery was not different between the two sites (Supplementary Table 4).

3.2. Impact of intervention on HM macronutrients

During the first 3 months of lactation, the mean HM macronutrient concentrations did not differ between the intervention and control groups with Singapore and New Zealand sites combined (Figure 2) or when each site was analysed separately (Supplementary Table 5).

3.3. Maternal pre-pregnancy BMI and GDM status on HM macronutrients

Among the control group, HM from mothers with overweight/obesity BMI had fat concentrations 11% higher [aMD (95% CI) = 1.11 (1.01, 1.21), $p = 0.023$] and energy content 5% higher [aMD (95% CI) = 1.05 (1.01, 1.10), $p = 0.017$] than mothers with underweight/normal weight BMI over the first 3 months of lactation (Figure 3). Such differences were not observed in the intervention group. Similarly, among the control group, HM fat concentrations were 11% higher [aMD (95% CI) = 1.11 (1.01, 1.22), $p = 0.030$] and energy content 6% higher [aMD (95% CI) = 1.06 (1.01, 1.11), $p = 0.011$] in mothers with GDM compared to those without GDM (Figure 4). Of note, mothers with GDM in the intervention group had HM fat concentrations 10% lower [aMD (95% CI) = 0.90 (0.81, 1.00), $p = 0.049$] and energy content 6% lower [aMD (95% CI) = 0.94 (0.90, 0.99), $p = 0.029$] than mothers with GDM in the control group.

TABLE 2 Baseline and perinatal characteristics of participants in the control and intervention groups in the NiPPeR study who provided at least one human milk sample during 12months of lactation.

	Overall (n = 336)		p-value
	Control	Intervention	
n	169 (50.3%)	167 (49.7%)	
Adherence (%)	89.2 (82.9–95.9)	90.4 (82.9–96.0)	n.s.
Duration of supplementation (days)	405.2 ± 105.0	393.4 ± 98.2	n.s.
Ethnicity			
Caucasian	69 (40.8%)	66 (39.5%)	n.s
Chinese	70 (41.4%)	69 (41.3%)	
South Asian	10 (5.9%)	10 (6.0%)	
Malay	10 (5.9%)	10 (6.0%)	
Other	10 (5.9%)	12 (7.2%)	
Maternal age			
Age at delivery (years)	31.9 ± 2.9	32.3 ± 3.2	n.s.
<35	145 (85.8%)	130 (77.8%)	n.s
≥35	24 (14.2%)	37 (22.2%)	
Maternal pre-pregnancy BMI status			
BMI (kg/m^2)	24.4 ± 5.2	23.3 ± 4.4	0.047
Underweight/ normal weight	115 (68.0%)	128 (76.6%)	n.s.
Overweight	31 (18.3%)	25 (15.0%)	
Obesity	23 (13.6%)	13 (7.8%)	
Missing	–	1 (0.6%)	
Highest level of education			
Bachelor's degree or higher	136 (80.5%)	135 (80.8%)	n.s.
Lesser qualification*	33 (19.5%)	32 (19.2%)	
Household income quintile			
5 (lowest)	4 (2.4%)	1 (0.6%)	n.s.
4	12 (7.1%)	16 (9.6%)	
3	43 (25.4%)	43 (25.7%)	
2	60 (35.5%)	55 (32.9%)	
1 (highest)	44 (26.0%)	42 (25.1%)	
Missing	6 (3.6%)	10 (6.0%)	
Smoking during pregnancy			
None	133 (78.7%)	148 (88.6%)	0.035
Passive	33 (19.5%)	16 (9.6%)	
Active	3 (1.8%)	3 (1.8%)	
GDM			
No GDM	123 (72.8%)	119 (71.3%)	n.s.
GDM	41 (23.4%)	42 (25.1%)	
Excluded	5 (3.0%)	6 (3.6%)	
Mode of delivery			
Vaginal delivery	124 (73.4%)	119 (71.3%)	n.s.
Caesarean section	45 (26.6%)	48 (28.7%)	
Infant birth weight			
Birth weight (kg)	3.24 ± 0.54	3.23 ± 0.53	n.s.
Appropriate for gestational age	143 (84.6%)	142 (85.0%)	n.s.
Large for gestational age	10 (5.9%)	6 (3.6%)	
Small for gestational age	16 (9.5%)	19 (11.4%)	
Infant gestational age			
Gestational age (weeks)	39.2 ± 1.6	39.2 ± 1.5	n.s.
Preterm	14 (8.3%)	11 (6.6%)	n.s.
Term or post-term	155 (91.7%)	156 (93.4%)	
Parity			
Primiparous	113 (66.9%)	95 (56.9%)	n.s
Multiparous	56 (33.1%)	72 (43.1%)	
Infant sex			
Male	75 (44.4%)	79 (47.3%)	n.s.
Female	94 (55.6%)	88 (52.7%)	

Data are n (%), mean ± SD, or median (Q1–Q3). p-values from independent samples t-test for continuous variables or Chi-square tests for categorical variables. Adherence to the study protocol was determined by sachet counting. Duration of supplementation calculated by counting the number of days from randomisation date to delivery date. Body mass index (BMI) status was defined as per World Health Organisation: Underweight /normal weight < 25.0 kg/m^2, overweight 25.0–29.99 kg/m^2, obesity ≥ 30.0 kg/m^2 (54). Gestational diabetes mellitus (GDM) was defined by International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria (55). Birth weight categories were determined using the Royal College of Paediatrics and Child Health 2009 U.K.-World Health Organisation growth charts (56). Gestational age was determined using a pre-specified algorithm as previously described (57) with preterm defined as birth < 37 weeks of gestation, and term or post-term as birth at ≥ 37 weeks of gestation. *Including incomplete and complete high school qualifications, and other tertiary level qualifications below bachelors (e.g., diploma or certificate). n.s., not statistically significant at p<0.05.

3.4. Changes in HM macronutrients over time in New Zealand (0–12 months)

Among the New Zealand cohort, longitudinal changes in HM macronutrient concentrations were observed in the first 12 months of lactation (Figure 5; Supplementary Table 6). HM fat and energy followed a similar decreasing pattern in the first 6 months, after which they increased until 12 months of lactation (Figures 5A,B). HM lactose gradually decreased from 1 week to 12 months of lactation but there was little intra-individual variation in absolute concentrations during this time (Figure 5C). HM crude protein continuously decreased from 1 week to 6 months, after which it remained relatively constant until 12 months of lactation (Figure 5D).

3.5. Other maternal and infant factors and HM macronutrients

HM macronutrient concentrations were associated with some maternal factors (Supplementary Table 7). Over the first 3 months of lactation, HM lactose concentrations were 2% lower in milk from younger

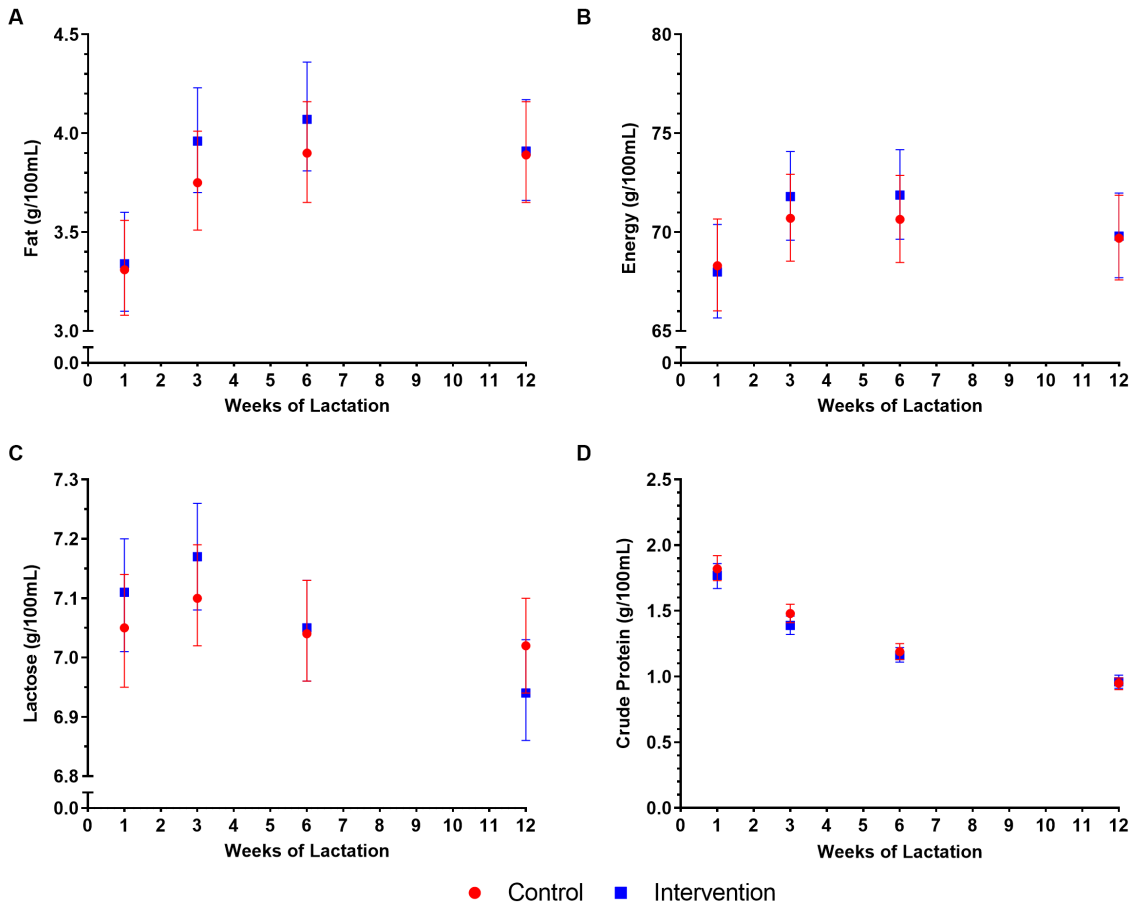

FIGURE 2
Macronutrient concentrations in human milk (HM) in control and intervention groups in the NiPPeR study, during the first 3 months of lactation: **(A)** fat, **(B)** energy, **(C)** lactose, and **(D)** crude protein. Data are the least-squares means (i.e., adjusted means) for each group, adjusted for randomisation group, visit, an interaction term (group*visit), study site, maternal pre-pregnancy body mass index, gestational age at birth, and adherence. Error bars represent the respective 95% confidence intervals.

mothers (< 35 vs. ≥ 35 years old, $p = 0.020$) and energy content was 3% lower after delivery by C-section ($p = 0.034$). Also, energy content ($p = 0.023$) and crude protein concentrations ($p = 0.022$) were 4% and 6% higher, respectively, following first childbirth compared to higher order of births (primiparous vs. multiparous). HM macronutrient composition did not differ between maternal ethnicity (Non-Asian vs. Asian), infant sex (Male vs. Female), or infant gestational age (Term/Post-term vs. Preterm).

4. Discussion

In our study, HM macronutrients were overall not influenced by the NiPPeR intervention supplement taken preconceptionally and during pregnancy. However, in the subpopulation of control group mothers with overweight/obesity or GDM, fat and energy levels were higher compared to the underweight/normal weight or non-GDM mothers, respectively, over the first 3 months of lactation. Such differences were not observed among the intervention group. Furthermore, among mothers with GDM, the intervention group had lower HM fat and energy levels than the control group. This suggests the impact of GDM status on HM macronutrients was altered by the NiPPeR intervention supplement.

Previous studies have reported that HM macronutrient composition is tightly regulated and is not strongly affected by maternal diet nutritional supplementation (2, 25, 58), with the exception of fatty acids (22). As such, the NiPPeR intervention supplement consumed in the pre-lactation period was not expected to strongly impact HM total fat, energy, lactose, and protein concentrations. Some studies have reported a positive association between maternal BMI and HM fat and energy (23–26), as observed in the present study. The impact of maternal GDM on HM macronutrients is less well understood. Among women with GDM, some have observed higher carbohydrate in colostrum (33), energy in colostrum, transitional, and mature milk (27), while others have reported lower fat and energy content in mature milk (34). Differences between study findings may be due to pre-analytical variations in HM collection protocols and processing. Fat is known to be the most dynamic component of HM but regulatory mechanisms underlying fat synthesis or transport in HM are not well understood. It has been speculated that metabolic dysregulation commonly reported in individuals with GDM, such as hyperglycemia, dyslipidemia, and insulin resistance, may contribute to increased HM fat in these mothers (59, 60). In the current study, using standardised collection methods, GDM status was associated with higher HM fat and energy levels in the control group but not in the intervention group. This suggests that the associations between GDM and

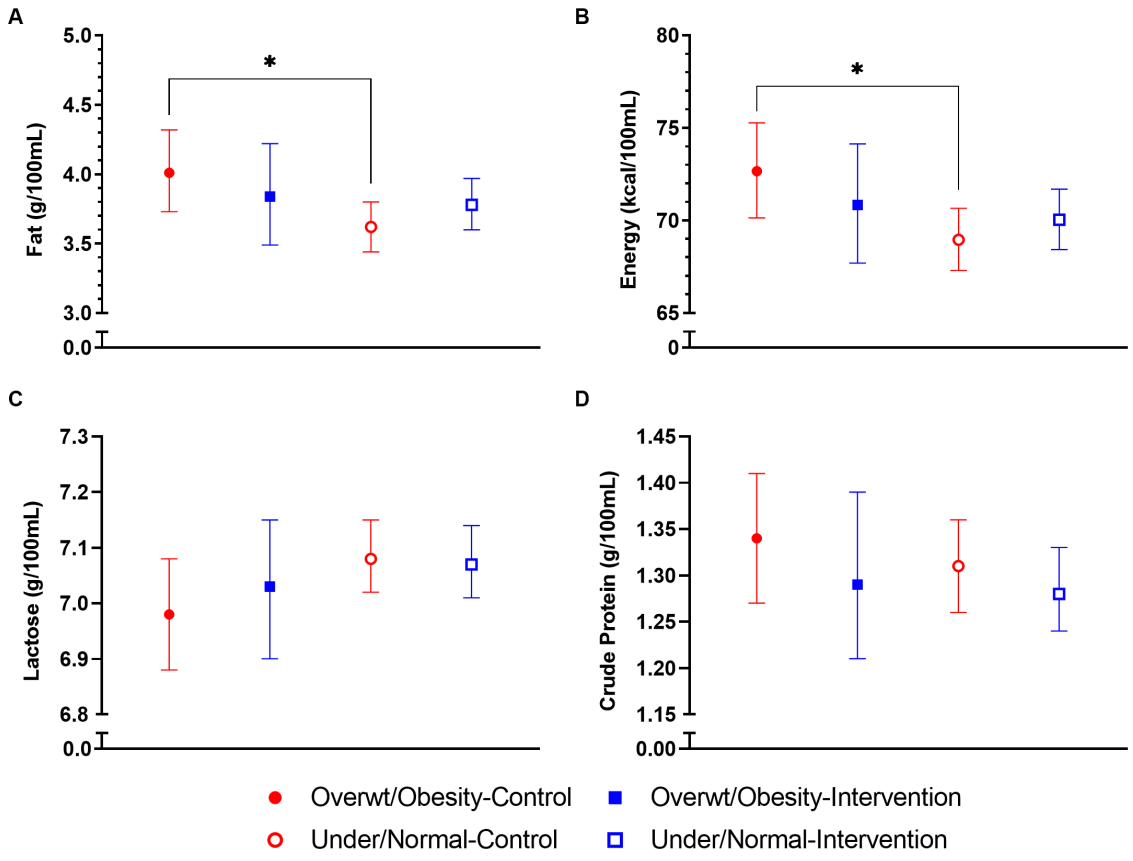

FIGURE 3
Average macronutrient concentrations in human milk (HM) in control and intervention groups by maternal pre-pregnancy body mass index (BMI) status in the NiPPeR study over the first 3 months of lactation: **(A)** fat, **(B)** energy, **(C)** lactose, and **(D)** crude protein. Data are the least-squares means (i.e., adjusted means) for each group, adjusted for randomisation group, BMI status, group*BMI interaction term, visit, study site, gestational age at birth, and adherence. Error bars represent the respective 95% confidence intervals. *$p<0.05$. Overwt/Obesity, Overweight/Obesity BMI; Under/Normal, Underweight/Normal weight BMI.

HM macronutrients could have been modified by some components in the NiPPeR intervention supplement. Previous studies have observed that supplementation of myo-inositol (61–63), probiotics (64–67), or zinc (68, 69) for 6–8 weeks in women already diagnosed with GDM at 24–28 weeks of gestation, improved glycemic control in these women, as reflected in lower maternal circulating insulin, glucose, triglycerides, total and LDL-cholesterol, and increased insulin sensitivity. In the current study, we speculate that myo-inositol, probiotics, and zinc components in the NiPPeR intervention supplement, postulated to act as insulin sensitizers, modified glucose or lipid metabolism in these mothers, leading to altered HM macronutrient composition. Further studies are required to assess the potential benefits of lower HM fat and energy for infant outcomes, particularly as these relate to growth during infancy and adiposity later in life.

The average HM fat, energy, lactose, and protein concentrations over 12 months observed in the current study are comparable to those reported previously: fat 3.0–4.0 g/100 mL, energy 61–65 kcal/100 mL, lactose 6.6–7.1 g/100 mL, and protein 0.9–1.4 g/100 mL (70–72). We also observed that HM macronutrients displayed various patterns of change over 12 months of lactation. As observed in previous studies, HM lactose remained relatively constant (19, 70, 73), and crude protein decreased (19, 32, 70, 74) until 6 months. Conversely, HM fat initially increased until 3 months, decreased from 3 to 6 months, then increased again from 6 to 12 months of lactation with HM energy content following a similar trajectory. Others have also observed a decrease in HM fat in early lactation followed by an increase in later stages of lactation (73, 75), reporting a positive correlation between HM fat and lactation stage (76). It has been suggested that such changes in HM fat are related to adaptation to changes in infant feeding and energy requirements during development. From about 6 months of age, infants start eating solid foods and breastfeeding becomes complementary. As a result, a reduction in milk volume could be counterbalanced by an increase in fat content to provide sufficient energy for the infant.

Previous studies have investigated the potential influences of maternal ethnicity (17), maternal age (27–30), infant gestational age (21, 27, 30–32), infant sex (26, 35–38), parity (28, 39), and mode of delivery (24, 32, 36, 40) on HM macronutrient composition. However, the results are conflicting, and the underlying mechanisms are not well understood. It has been suggested that anatomical changes of the mammary gland with maternal age (30, 77) and successive pregnancies (28), different hormonal releases associated with mode of delivery (78) and gestational age (79), and different energy demands according to infant sex (35) could contribute to altered HM macronutrient composition. In the current study, we did not observe differences in

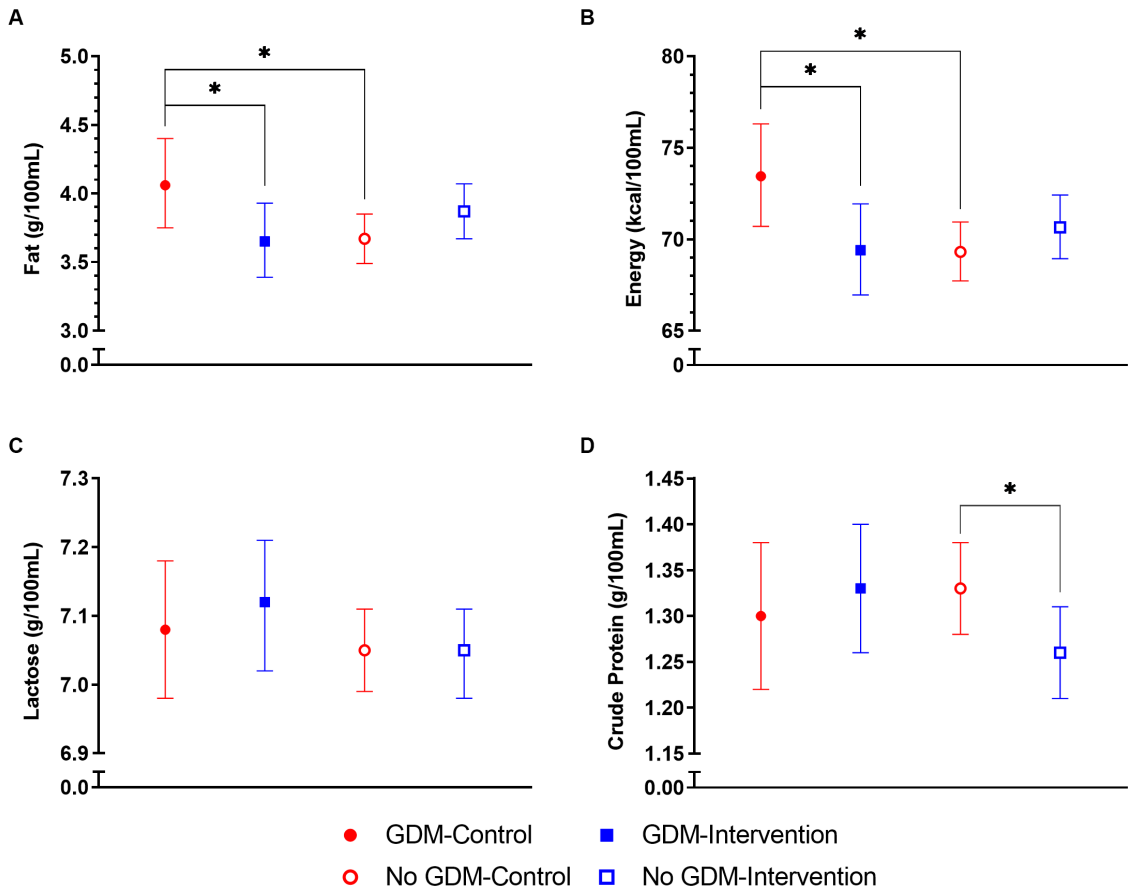

FIGURE 4
Average macronutrient concentrations in human milk (HM) in control and intervention groups by maternal gestational diabetes mellitus (GDM) status in the NiPPeR study over the first 3 months of lactation: **(A)** fat, **(B)** energy, **(C)** lactose, and **(D)** crude protein. Data are the least-squares means (i.e., adjusted means) for each group, adjusted for randomisation group, GDM status, group*GDM interaction term, visit, study site, gestational age at birth, and adherence. Error bars represent the respective 95% confidence intervals. *$p < 0.05$.

HM macronutrients according to maternal ethnicity, infant sex, or gestational age. Although we observed a relationship between maternal age and lactose, delivery mode and energy, and parity and energy and crude protein, the magnitude of differences ranged from 2% to 5% in the first 3 months of lactation. While these were statistically significant observations, further research is required to understand the physiological significance of such small absolute changes in HM macronutrient levels in relation to infant outcomes.

5. Strengths and limitations

This study has a few strengths to note: (i) HM macronutrient composition was examined from a large cohort of diverse ethnic groups, (ii) standardised HM sample collection, processing, and analytical methods were used, and (iii) the visit windows were tightly controlled, each time point being a distinctive stage of lactation. As longitudinal samples could not be collected from all participants, a repeated measures design was used for statistical analyses. There are some limitations to be acknowledged in the present study. The IADPSG criteria was used for determining GDM status and site-specific diagnostic criteria were not considered. Also, treatment for GDM was an independent decision by the local clinicians and specific for each site. While diet treatment was more common in Singapore, medication treatment with insulin or metformin was more common in New Zealand. Due to the imbalances in sample sizes for treatment types between control and intervention groups, and between sites, any potential impact of GDM treatment modality on HM macronutrients could not be assessed. Finally, infants of this cohort were born generally healthy, none under 28 weeks gestation (extremely preterm) and only 24 infants (7.1%) had low birth weight (< 2,500 g). This precluded investigation of potential influences of extreme infant characteristics on HM macronutrients.

6. Conclusion

In this study, we observed that maternal overweight/obesity and GDM were associated with increased HM fat and energy levels among controls but not in the intervention group. This suggests that the intervention supplement during preconception and pregnancy altered the impact of a high maternal BMI and

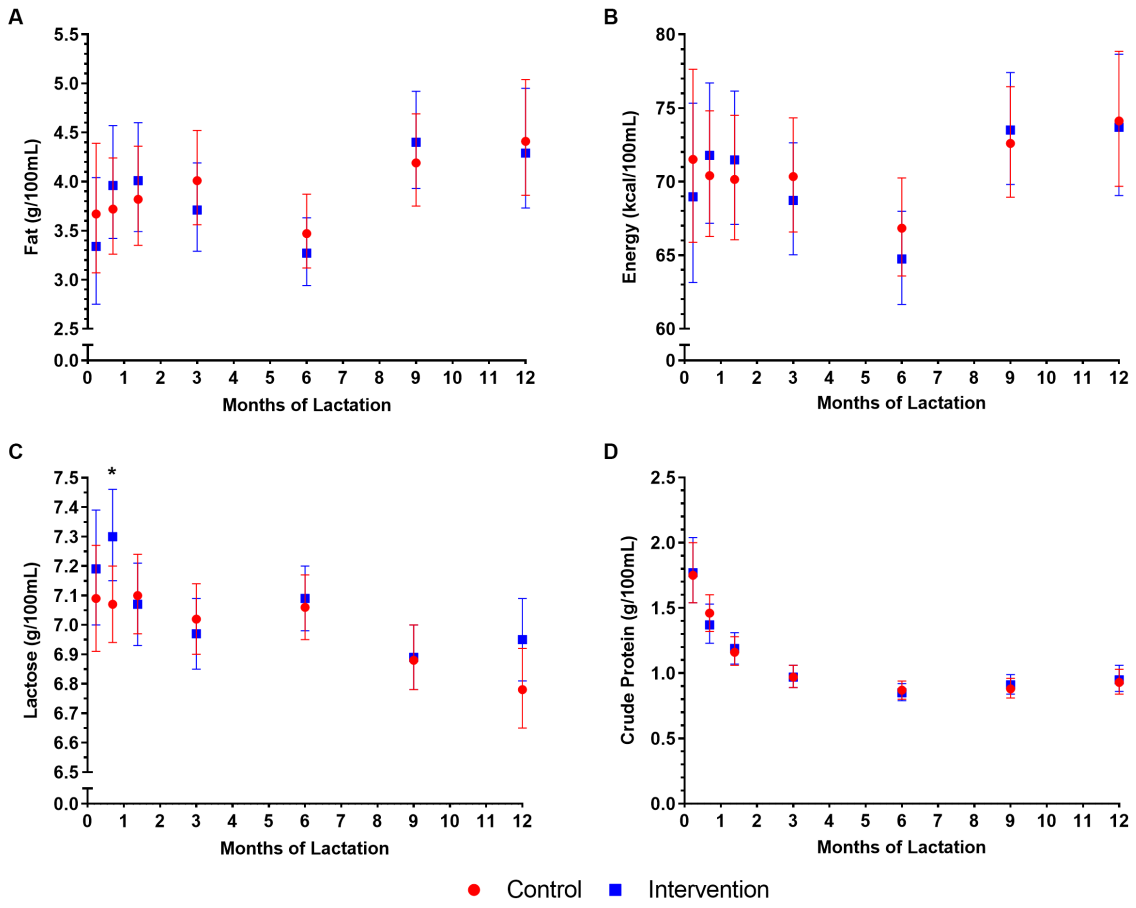

FIGURE 5
Macronutrient concentrations in human milk (HM) in control and intervention groups in New Zealand in the NiPPeR study during 12 months of lactation: **(A)** fat, **(B)** energy, **(C)** lactose, and **(D)** crude protein. Data are the least-squares means (i.e., adjusted means) for each group, adjusted for randomisation group, visit, an interaction term (group*visit), maternal pre-pregnancy body mass index, gestational age at birth, and adherence. Error bars represent the respective 95% confidence intervals. *$p < 0.05$ for a difference between intervention and control groups at a given time point.

GDM status on HM macronutrient composition. Further studies are required to identify the components in the intervention supplement associated with HM macronutrient composition, characterise the underlying mechanisms, and determine any long-term effects on offspring health.

Data availability statement

The datasets presented in this article are not readily available because public sharing of the data was not part of the original participant informed consent. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by ethics committees at each study site: Southampton - Health Research Authority National Research Ethics Service Committee South Central Research Ethics Committee (15/SC/0142), Singapore - the National Healthcare Group Domain Specific Review Board (2015/00205), and New Zealand - Northern A Health and Disability Ethics Committee (15/NTA/21).

Author contributions

SMH: Formal analyses, Investigation, Writing – original draft. JGBD: Formal analyses, Writing – review & editing. MHV: Writing – review & editing. SD: Writing – review & editing. FH: Writing – review & editing. WWP: Writing – review & editing. KMG: Funding acquisition, Writing – review & editing. S-YC: Funding acquisition, Writing – review & editing. SKT: Writing – review & editing, Supervision. WSC: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

KMG, S-YC, and WSC are part of an academic consortium that has received grants from Société des Produits Nestlé S.A. relating to the submitted work, and from Abbott Nutrition, Danone, and BenevolentAI Bio Ltd. outside the submitted work. SMH, JGBD, MHV, SD, FH, KMG, S-YC, SKT, and WSC are co-inventors on patent filings by Société des Produits Nestlé S.A. relating to the NiPPeR intervention or its components. FH, SD, and SKT are employees of Société des Produits Nestlé S.A.

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

The reviewer SP declared a past co-authorship with the authors JGBD, MHV, KMG, WSC, and S-YC to the handling editor.

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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.2023.1282376/full#supplementary-material

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Effects of human donor milk on gut barrier function and inflammation: *in vitro* study of the beneficial properties to the newborn

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Introduction: The gastrointestinal and immune systems of premature infants are not fully developed, rendering them more vulnerable to severe complications like necrotizing enterocolitis. Human milk offers a rich array of bioactive factors that collectively contribute to reducing the incidence of gut infections and inflammatory conditions. When a mother's milk is unavailable, preterm infants are often provided with donor human milk processed in Human Milk Banks. However, it remains uncertain whether pasteurized milk confers the same level of risk reduction as unprocessed milk. This uncertainty may stem from the well-documented adverse effects of heat treatment on milk composition. Yet, our understanding of the comprehensive impact on protective mechanisms is limited.

Methods: In this study, we conducted a comparative analysis of the effects of raw versus pasteurized milk and colostrum versus mature milk on cellular functions associated with the gut epithelial barrier and responses to inflammatory stimuli. We utilized THP-1 and HT-29 cell lines, representing monocyte/macrophages and gut epithelial cells, respectively.

Results: Our observations revealed that all milk types stimulated epithelial cell proliferation. However, only raw colostrum increased cell migration and interfered with the interaction between *E. coli* and epithelial cells. Furthermore, the response of epithelial and macrophage cells to lipopolysaccharide (LPS) was enhanced solely by raw colostrum, with a milder effect observed with mature milk. In contrast, both raw and pasteurized milk diminished the LPS induced response in monocytes. Lastly, we examined how milk affected the differentiation of monocytes into macrophages, finding that milk reduced the subsequent inflammatory response of macrophages to LPS.

Discussion: Our study sheds light on the impact of human milk on certain mechanisms that potentially account for its protective effects against necrotizing enterocolitis, highlighting the detrimental influence of pasteurization on some of these mechanisms. Our findings emphasize the urgency of developing alternative pasteurization methods to better preserve milk properties. Moreover, identifying the key components critically affected by these protective mechanisms could enable their inclusion in donor milk or formula, thereby enhancing immunological benefits for vulnerable newborns.

KEYWORDS

donor milk, human milk bank, gut inflammation, *in vitro* studies, Holder pasteurization

1 Introduction

The superior nutritional value of Human Milk (HM) in newborns (NB) is widely acknowledged. The World Health Organization advocates exclusive breastfeeding for the initial six months of an infant's life, followed by the gradual introduction of supplementary foods while continuing breastfeeding for at least two years (1). The provision of nutritional and bioactive elements in HM adjusts to the specific biological needs of NB. HM offers a well-suited and adaptable quantity of nutrients along with a multitude of bioactive constituents. Notably, a variety of these components contribute significantly to the defense against infections, development, and homeostasis of the immune system (2, 3). The aqueous fraction (AF) of HM contains the highest concentration and widest array of bioactive compounds. Within this fraction, prominent constituents such as lactoferrin, antibodies, α-lactalbumin, lysozyme, and Human Milk Oligosaccharides (HMO) exhibit the highest abundance (2–5). Furthermore, a diverse array of molecules are linked to milk fat globules, including lactadherin and mucins (6). Additionally, various components such as exosomes and microbiota are suspended in milk (7–9). Collectively, these components are believed to be responsible for the protective effects of human milk against infections and serious conditions associated with the underdeveloped gastrointestinal tract of premature infants (10–14). Necrotizing Enterocolitis (NEC) is one of the most devastating pathologies (15–17). The disruption of gut microbiota balance is believed to trigger this condition, initiating a key pathogenic mechanism involving the activation of Toll-like receptor 4 (TLR4) by lipopolysaccharides (LPS) (18). NEC has been correlated with certain attributes of the underdeveloped preterm gut, including: i) reduced production of mucus and antimicrobial molecules., ii) increased intestinal permeability associated with impaired epithelial regeneration, iii) elevated expression and activation of TLR4, and iv) compromised peristalsis (12, 19, 20). Numerous epidemiological studies have provided evidence for the protective impact of breastfeeding against NEC (17, 21, 22). However, only a limited number of studies have explored the potential mechanisms underlying the protective effects of human milk *in vitro*. Interestingly, it has been observed that colostrum can augment the response of epithelial cells to LPS, possibly due to its high concentration of sCD14 (23). Several *in vitro* and *in vivo* studies have examined the effects of the specific components present in human milk. For instance, IgA, lactoferrin, HMO, lactadherin, Trefoil Factor 3 (TFF3), exosomes, and lipids have been investigated for their potential effects on gut inflammation (24–30). Although these studies have provided valuable insights into the effects of specific components present in milk, it is important to note that human milk contains a complex mixture of hundreds of bioactive molecules present at varying concentrations. These molecules can interact synergistically, redundantly, or antagonistically, leading to intricate and multifaceted effects. As a result, the outcomes of these individual component studies may not fully capture the comprehensive impact of whole milk.

In situations where preterm NB are not able to receive their own mother's fresh milk, an alternative approach for their feeding is donated HM through Human Milk Banks (HMB) (31). Nonetheless, epidemiological data indicate that the protective effect of donated HM against NEC does not reach the same level as that of fresh milk (21, 22, 32, 33). This disparity can be attributed to the potentially detrimental effects of milk processing within the HMB, as well as the distinctive properties of milk associated with different lactation stages owing to the dynamic nature of HM composition (2, 3). Colostrum has the highest concentration of proteins and a plethora of bioactive components (2–4, 34). However, most donations received by HMB consist of mature milk. To ensure microbiological safety, donor milk undergoes pasteurization using the Holder method, which involves heating at 62.5°C for 30 min (31). This treatment has a negative impact on milk composition, leading to variable reductions in the concentration of most bioactive molecules (4, 35). Despite this potential reduction in bioactive molecules due to pasteurization, donated HM still contains a considerable concentration of many bioactive components that play a role in providing passive immunization to newborns (4). Nevertheless, only a limited number of *in vitro* studies have been conducted to evaluate the protective effects of donated human milk (4, 36, 37). Currently, knowledge regarding the mechanisms and specific bioactive

components responsible for the protective effects of donated human milk against intestinal inflammation is limited and incomplete.

The aim of this study was to enhance our understanding of the mechanisms underlying the beneficial effects of human milk in mitigating intestinal inflammation. Furthermore, we investigated how the Holder pasteurization method influences these properties using *in vitro* models. Our approach involved a comparative analysis of the effects of unprocessed and pasteurized colostrum and mature milk on the functionality of resident and recruited cells, which play pivotal roles in gut barrier integrity and inflammation control.

2 Materials and methods

2.1 Human donors and milk samples

Donated human milk (dHM) was collected from the Human Milk Bank (HMB) of Pereira Rossell´s Hospital in Montevideo (Uruguay) according to the Research Ethics Board of the Hospital guidelines. All donors had term delivery and met the HMB inclusion criteria (34); milk availability was an additional criterion for this study. Eleven individual samples of colostrum were obtained 3 days (median) after delivery (range:1–7), and 10 samples of mature milk were obtained at 6 months (median) of lactation (range:4–7). Aliquots of raw and Holder pasteurized milk (62.5°C for 30 min) were stored at −80°C until use. The aqueous fraction (AF) was obtained by two sequential centrifugation steps: 1,000 rpm for 10 min at 4°C to separate the cells and debris and 10,000 rpm for 30 min at 4°C to separate the fat (4). The endotoxin level was lower than 0.15 EU/mL in all AF samples, evaluated with the LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA, USA). To minimize inter-individual variation, the AF obtained from the pooled samples was used for all experiments. The AF of these pooled samples was stored in aliquots at −80°C until use. For each *in vitro* assay, AF was diluted and sterilized by 0.2 µm filtration (GVS Filter Technology, Sanford, USA). IL-1β, IL-6, and TNF-α were not detected in the pooled samples analyzed by ELISA (Duoset ELISA kits, R&D Systems, Minneapolis, MN, USA).

2.2 Cell lines and reagents

The HT-29 and THP-1 cell lines (ATCC, Manassas VA, USA) were cultured at 37°C in a controlled atmosphere with 5% CO_2 in complete medium: RPMI 1640 growth medium (Capricorn Scientific GmbH, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Capricorn Scientific), 100 µg/mL streptomycin and 100 U/mL penicillin (Sigma-Aldrich, St. Louis, USA). At 80%–90% confluence, HT-29 cultures were trypsinized, and the cells were counted and diluted according to each experimental design. Cultures of THP-1 at 1.0–1.2 × 10^6 cells/mL were centrifuged, and the cells were counted and diluted according to each experimental design. Both cell lines were cultured for no more than 15 passages. Chemical and biological reagents were purchased from Sigma-Aldrich, unless otherwise specified.

2.3 *E. coli* and LPS-specific antibodies in aqueous human milk

The levels of bacteria and LPS-specific antibodies (IgA, IgG and IgM) were assessed by ELISA according to Zeng et al. (2018) with a few modifications (38). One colony of *E. coli* (ER2738; Lucigen Corp. Middleton, USA) were grown in 5 mL of Luria-Bertani (LB) broth (Mast Group Ltd., Liverpool, UK) overnight (ON) at 37°C with agitation (250 rpm) and centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was washed twice with carbonate buffer (50 mM, pH 9.6) and heat inactivated at 65°C for 1 h. Then, $OD_{600\ nm}$ was adjusted to 0.5 and 100 µL/well was seeded in a high-binding 96-well ELISA plate (Thermo Fischer Scientific). For anti-LPS antibodies 100 µL/well of 2 µg/mL LPS (O26:B6) in carbonate buffer was seeded in the ELISA plate. After ON incubation at 4°C, the plates were washed with 0.05% Tween-20 in PBS and blocked with gelatin solution in PBS. Then, 100 µL/well of AF or commercial human colostrum IgA at 2-fold serial dilutions was incubated for 3 h at 37°C. The plates were washed and 100 µL/well of HRP-conjugated anti-human (IgA, IgG, or IgM) antibodies were incubated for 1 h at 37°C. Finally, the enzyme activity was developed as previously described (4) and the $OD_{450\ nm}$ was read (Labsystems Multiskan, Thermo Fisher Scientific).

2.4 Macrophage differentiation

THP-1 cells were differentiated into macrophage-like cells (dTHP-1) with phorbol 12-myristate 13-acetate (PMA), as described previously (39), with a few modifications. Briefly, cells were incubated with 50 ng/mL PMA in complete medium and after 3 days, the medium was removed, and the cells were rested for 24 h with fresh complete medium. To characterize the dTHP-1 macrophages obtained, the changes in morphology during PMA treatment were assessed by microscopy (Primovert Zeiss Microscopy GmbH, Germany), and FSC/SSC and mCD14 expression were assessed by flow cytometry (BD FACSCalibur™, New Jersey, USA). Cells were detached using Accutase (BioLegend, San Diego, CA, USA) and washed with PBS containing 0.1% BSA and 2 mM EDTA. The expression of surface CD14 was determined with a FITC-conjugated anti-human mCD14 antibody (BioLegend) using Propidium Iodide staining to exclude dead cells. Flowing 2.5.1. software (Turku Bioscience, Turku, Finland) was used for the data analysis. Detailed information about the cell density and volume of the growth medium for each experiment is described below.

2.5 Effect of LPS and milk aqueous fraction on cell viability

Viability was assessed using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) MTT assay (40). For HT-29 cells, 100,000 cells in 200 µL of complete medium were seeded in 96-wells culture plates (Greiner Bio-One, Frickenhausen, Germany) and after 24 h the cells were washed with PBS. For dTHP-1, 100,000 THP-1 cells in 200 µL were differentiated in 96-well culture plates, as described above. Then, the cells in 200 µL of incomplete medium (without FBS) containing 1 µg/mL LPS were incubated for 24 h with or without 0.3% AF. Cells were washed with PBS, and 200 µL/well of 0.5 mg/mL MTT in PBS was incubated for 4 h, and the formazan crystals were solubilized with 150 µL/well of DMSO; the $OD_{560\ nm}$ was recorded. For THP-1 monocytes, 100,000 cells in 200 µL of incomplete medium with 1 µg/mL LPS in the presence or absence of 0.1% AF were seeded in U-bottom 96-well culture plates (Greiner Bio-One). After 24 h, the cells were centrifuged to remove the supernatant, and 200 µL/well MTT was incubated for 4 h. After centrifugation, the supernatant was carefully removed and the formazan crystals were solubilized with 170 µL/well of DMSO. A total of 150 µL/well was transferred to an F-bottom 96-well ELISA plate and the $OD_{560\ nm}$ was recorded. The results were normalized to the conditions in the absence of LPS and AF.

2.6 Effect of milk aqueous fraction on epithelial cell proliferation

Proliferation was assessed using the Crystal Violet (CV) method (41). Briefly, 5,000 HT-29 cells in 200 µL complete medium were incubated in 96-well culture plates (Greiner Bio-One) for 24 h. The cells were washed with PBS and AF (0.3%–3%) in incomplete medium, and 10% FBS was used as a positive control. After 24 h, the cells were fixed with 50 µL/well 5% formaldehyde in PBS for 15 min. The cells were then stained with 50 µL/well 0.05% CV solution for 10 min. The cells were washed several times with PBS and air-dried for 2 h. CV was solubilized with 150 µL/well of methanol for 20 min, and the $OD_{560\ nm}$ was recorded. The data were normalized to conditions in the absence of AF.

2.7 Effect of milk aqueous fraction on wound healing

Migration was assessed using the scratch assay, as previously described (42). Briefly, 550,000 HT-29 cells in 1 mL complete medium were seeded in 12-well culture plates (Greiner Bio-One). When the cells reached 90% confluence, injury was induced by scratching the monolayer with a sterile pipette tip. The cells were washed with PBS and incubated with AF (0.1% to 1.0%) in incomplete medium supplemented with 1% FBS. Wound healing was assessed using optical microscopy, and images were taken before and after 24 h of incubation (Primovert Zeiss Microscopy GmbH). In some experiments, mitomycin-C was used as a proliferation inhibitor. Wound healing (%) was calculated as $100 - [(A_{24}/A_0) \times 100]$, where A_0 and A_{24} are the wound areas before and after incubation, respectively. Fiji/ImageJ (open source software, Bethesda, USA) was used for the analysis of wound areas.

2.8 Effect of milk aqueous fraction on bacterial adhesion to epithelial cell

The effect of AF on *E. coli* and HT-29 interaction was evaluated according to Letourneau et al. (43), with a few modifications. Briefly, 250,000 HT-29 cells in 500 µL complete medium without antibiotics were incubated for 48 h in 24-well culture plates. The medium was discarded, and the cells were washed with PBS. One colony of *E. coli* (ER2738, Lucigen Corp.) was grown in 2 mL LB broth for 3 h–4 h at 37°C with agitation. The bacterial concentration was estimated by $OD_{600\ nm}$ ($1\ OD_{600\ nm} \cong 8 \times 10^8$ bacteria/mL), and 500 µL of a bacteria; suspension in PBS was added to HT-29 (ratio 1:1) in the presence or absence of AF (1%–10%) or 100 µg/mL of commercial human colostrum IgA. After 3 h of incubation, the unattached bacteria were discarded and the cells were washed several times with PBS. Bacteria were detached with 100 µL/well of 1% Triton X-100 and 10-fold dilutions were incubated in LB agar plates at 37°C for 24 h to count the colonies. The data were normalized to the conditions in the absence of AF (maximum bacterial adhesion).

The effect of AF on bacterial proliferation has been investigated previously. Briefly, bacteria in PBS or LB broth with or without AF, were incubated for 4 h with agitation (250 rpm). The bacterial load was estimated at $OD_{600\ nm}$, as described above.

2.9 Effect of milk aqueous fraction on cell response to LPS

To evaluate the response of HT-29 cells to LPS, 200,000 cells in 500 µL complete medium were incubated for 48 h in a 24-well culture plate (Greiner Bio-One). For the dTHP-1 macrophages, 500,000 cells in 600 µL of complete medium were differentiated in a 24-well culture plate (Greiner Bio-One), as described in *Section 2.4*. Cells were washed with PBS, and 500 µL of incomplete medium containing 1 µg/mL LPS with or without AF (0.03%–0.3%) was added. After 24 h, the supernatant was collected and stored at −80°C until analysis. For THP-1 monocytes, 500,000 cells in 500 µL of incomplete medium containing 1 µg/mL LPS, with or without AF (0.01%–0.1%), were seeded in 24-well culture plates (Greiner Bio-One). After 24 h, the supernatant was obtained by centrifugation and stored at −80°C until analysis. The effects of sCD14 and human colostrum IgA (treated or not treated with Holder pasteurization) on LPS cell responses were assessed.

2.10 Cytokine determination

The concentrations of IL-1β, IL-6, IL-8, IL-10, TNF-α, and MIP-3α in the culture supernatants were measured in duplicate by

ELISA using the corresponding Human Duoset ELISA kit (R&D Systems) according to the manufacturer's instructions. Labsystems Multiskan MS (Thermo Fisher Scientific) plate reader was used to register $OD_{450\ nm}$. The lower concentration of the standard curve was 62.5 pg/mL for IL-1β, 9.4 pg/mL for IL-6, 31.2 pg/mL for IL-8, and IL-10, 15.6 pg/mL for TNF-α and MIP-3α.

2.11 Effect of milk aqueous fraction on macrophage differentiation

To study the impact of AF on macrophages (dTHP-1) differentiated from monocyte-like THP-1 cells, AF (0.03%–0.3%) was added during PMA treatment, and the differentiation process was studied as described above. The relative cell number after differentiation was assessed using the Crystal Violet method described in *Section 2.6*, using 20,000 THP-1 cells in 96-well culture plates. Viability was determined by MTT assay and the expression of mCD14 by flow cytometry, as described in Sections 2.5 and 2.4, respectively. The LPS (1 μg/mL) response of dTHP-1 was evaluated as described in Section 2.9.

2.12 Statistical analysis

In all studies, at least three analytical replicates were performed in three independent experiments. The results are expressed as the mean and Standard Error of the Mean (SEM) of independent experiments in the text, and results from one representative assay are shown in the figures. Pairwise comparisons performed made using Student's t-test and one-way ANOVA with Tukey's multiple comparisons *post-hoc* test for multiple comparisons. Paired comparisons of colostrum lactadherin content were performed using the Wilcoxon matched-pairs signed-rank test. GraphPad Prism (6.0 version) was used for statistical analysis. The statistical significance level was defined as 95% confidence and is indicated in the figures with asterisks: $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

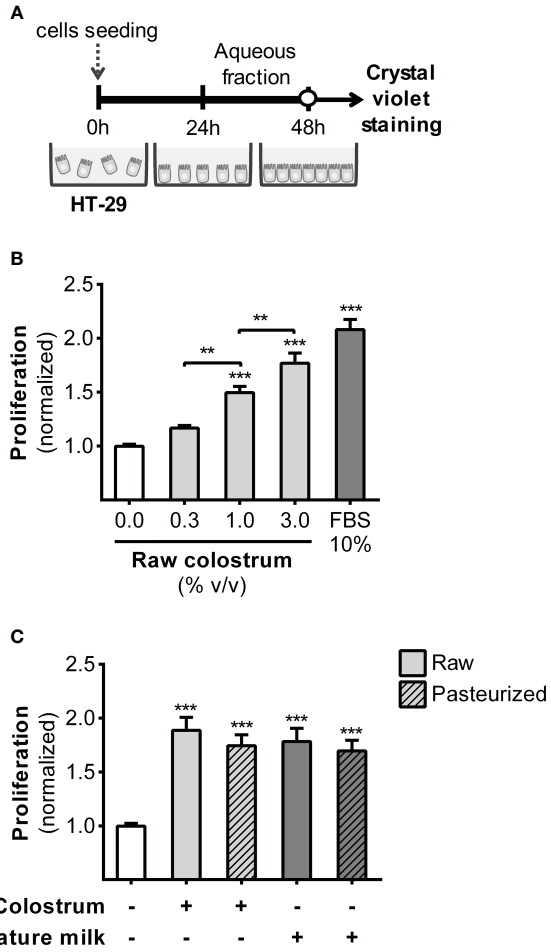

FIGURE 1
Effect of human milk AFs on gut epithelial cell proliferation. **(A)** Experimental design. **(B)** The effect of different concentrations of AF from raw colostrum on HT-29 cell proliferation. **(C)** Comparative effect of 3% AF derived from raw or pasteurized colostrum and mature milk on HT-29 cell proliferation. Data were normalized relative to the control condition (open bar). The bars represent the mean ± SEM of six analytical replicates, corresponding to one representative of three independent experiments. Asterisks on the bars indicate significant differences compared to the control condition, as determined by one-way ANOVA test (**p ≤0.01, ***p ≤0.001).

3 Results

3.1 Impact of donated milk on epithelial cell proliferation and migration

We first evaluated the effect of the AF of raw and pasteurized donated milk on strengthening of the epithelial barrier. This evaluation was performed by investigating the impact of milk AF on key cellular processes, such as proliferation and migration, using the HT-29 cell line. In this phase, our primary focus was to examine the effect of AF on cell proliferation (Figure 1A). As shown in Figure 1B, AF derived from raw colostrum exhibited a concentration-dependent effect on cell proliferation. Specifically, at a concentration of 3% AF, a significant fold-increase of 1.8 ± 0.2 (mean ± SEM) was observed compared to the basal condition. To ascertain whether this observed effect was unique to specific milk components, we conducted a comparative analysis by evaluating cell proliferation in the presence of Bovine Serum Albumin (BSA). BSA did not induce noticeable cell proliferation at any of the tested concentrations (Figure S1A). We compared the influence of the AF of raw and pasteurized colostrum and mature milk on cell proliferation. Interestingly, similar effects and magnitudes were observed for these distinct milk types (Figure 1C). A scratch assay was performed to assess the effect of milk on the migration of epithelial cells (Figure 2A). A concentration-dependent enhancement in wound healing was observed with the AF of raw colostrum (Figure 2B); a fold increase of 1.9 ± 0.1 (mean ± SEM) was noted with 1% v/v raw colostrum. To ascertain the specificity of this result for colostrum, we confirmed that BSA did not induce wound closure (Figure S1B). As wound healing relies on both cell migration and proliferation, we assessed the relative impact of raw and pasteurized colostrum and mature milk at a concentration of

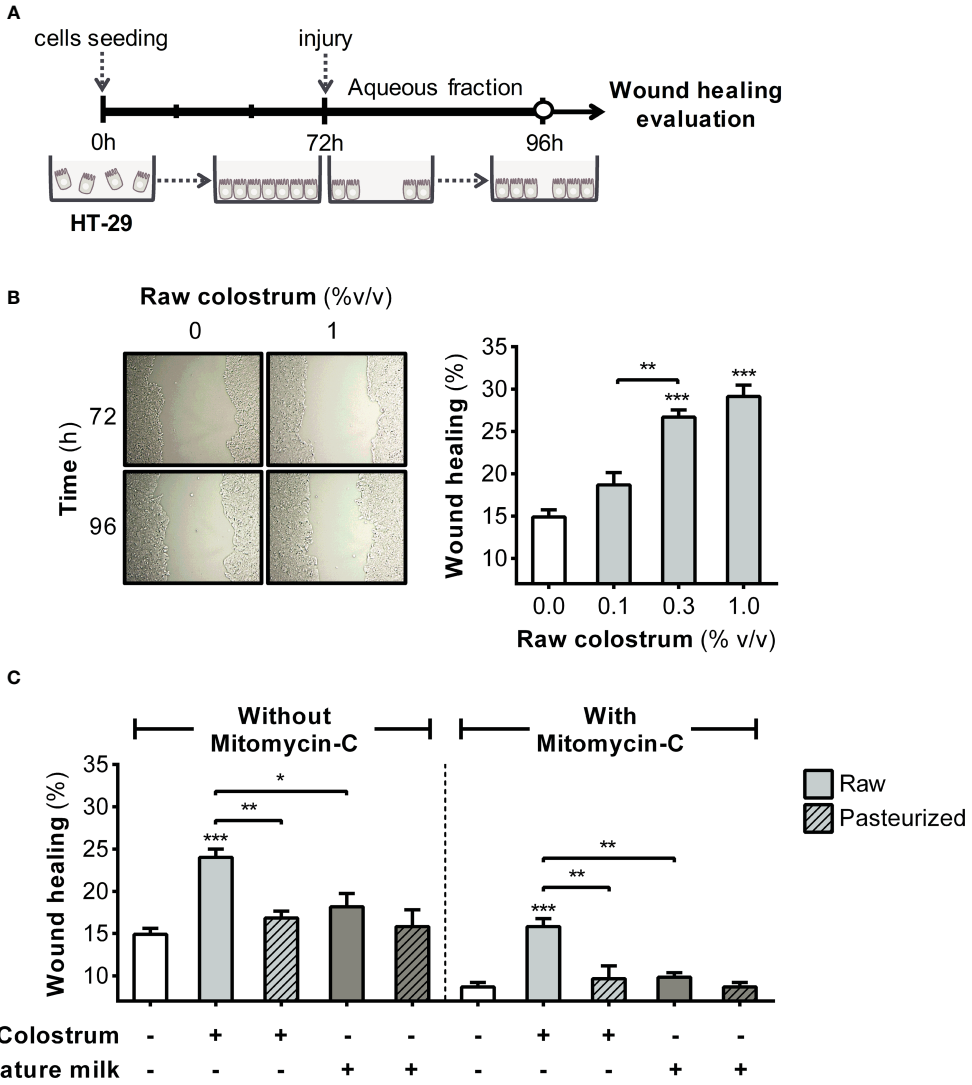

FIGURE 2
Effect of human milk AF on the wound healing of gut epithelial cells. **(A)** Experimental design. **(B)** Representative photograph (×10 magnification) illustrating the wound area before and after 24 h of incubation with raw colostrum. The graph depicts the dose-dependent effect of AF from raw colostrum on wound healing. **(C)** Comparative influence of 3% AF derived from raw or pasteurized colostrum and mature milk on wound healing, both in the absence and presence of 3 μg/mL mitomycin-C. Data were normalized relative to the control conditions (open bar). Bars represent the mean ± SEM of four analytical replicates, corresponding to one representative of three independent experiments. Asterisks on the bars denote significant differences in relation to the control condition, as determined by one-way ANOVA (*p ≤0.05, **p ≤0.01, ***p ≤0.001).

0.3% AF, which did not stimulate cell proliferation (Figure 1B). Our observations revealed that only the AF of raw colostrum stimulated cell migration (Figure 2C), which was verified by employing mitomycin-C as an inhibitor of cell proliferation (Figure 2C).

3.2 Effect of donated milk on the interaction between bacteria and epithelial cells

Because the interaction between bacteria and the gut epithelium marks their initial entry into the lamina propria, inducing immune system activation, we assessed whether HM influences the adhesion of *E. coli* to HT-29 cells (Figures 3A, B). A concentration-dependent inhibition of bacterial adhesion was observed when colostrum AF was used (Figure 3C). The normalized adhesion ratio decreased from 1 (in the absence of AF) to 0.42 ± 0.03 (mean ± SEM) with 3% AF of raw colostrum. However, this inhibitory effect was not observed in the AF of pasteurized colostrum or mature milk (Figure 3D).

Given the decreased bacterial adhesion, it is plausible that this effect may be due to the neutralizing influence of specific antibodies. Therefore, we quantified anti-*E. coli* and anti-LPS antibodies (IgA, IgG, and IgM) in HM using ELISA. Remarkably, colostrum exhibited elevated levels of specific IgA and IgM compared to

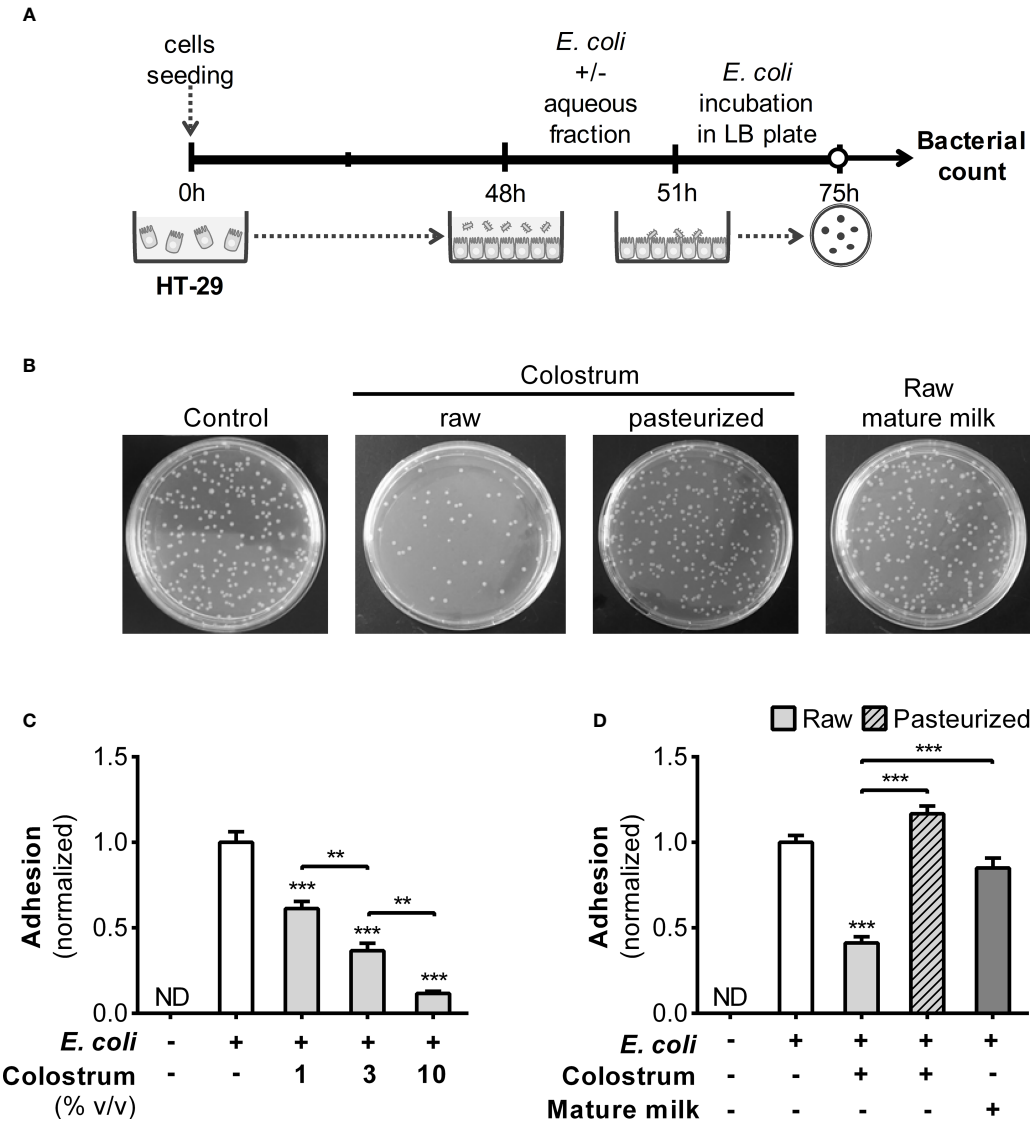

FIGURE 3
Effect of human milk AF on adhesion of *E. coli* to epithelial cells. **(A)** Experimental design. **(B)** Representative image of Luria–Bertani (LB) plates with or without the addition of 3% AF of colostrum or mature milk. **(C)** Dose-dependent influence of AF from raw colostrum on the interaction between *E. coli* and HT-29 cells (ratio 1:1). **(D)** Effect of 3% AF from raw and pasteurized colostrum as well as mature milk, on the interaction between *E. coli* and HT-29 cells. The data were standardized against the control conditions (open bar). Bars represent the mean ± SEM of four analytical replicates, corresponding to one representative of three independent experiments. The asterisks on the bars denote significant differences with respect to the control condition, as determined by one-way ANOVA (**p ≤0.01, ***p ≤0.001). ND, not detected.

those in mature milk, while IgG was predominantly detected in mature milk (Figure 4). The levels of antibodies across all isotypes were influenced by Holder pasteurization. These observations prompted us to investigate the effect of purified IgA from human colostrum on the adhesion of *E. coli* to epithelial cells. This commercial IgA displayed reactivity against *E. coli*, which was influenced by Holder pasteurization (Figure S2A). Consequently, when assessing their impact on bacterial adhesion, a reduction was observed in both untreated and treated IgA, although to a lesser extent following treatment (Figure S2B).

We controlled for the potential impact of AF on bacterial proliferation (Figure S3A); the presence of 30% AF did not exert any significant effect on bacterial growth (Figure S3B).

3.3 Effect of donated milk on the LPS-induced response of epithelial cells, macrophages, and monocytes

LPS is among the most potent bacterial signaling molecules capable of eliciting an exaggerated inflammatory response in the gut of preterm newborns, involving resident and recruited leukocytes, as well as epithelial cells. We first focused on the response of HT-29 cells to LPS, both in the presence and absence of AF, over a 24-hour period. We assessed the production of IL-8 and MIP-3α chemokines in the supernatant (Figure 5A), which augmented the production of both chemokines in a concentration-dependent manner (Figure 5B). Stimulation with LPS in combination with

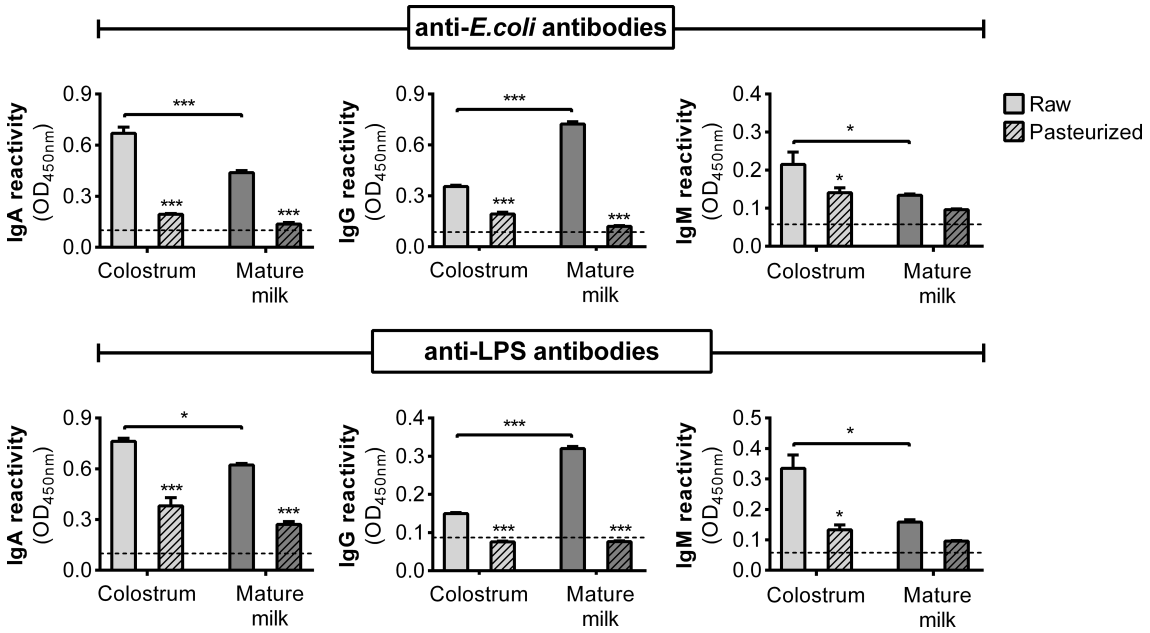

FIGURE 4
Specific antibodies against *E. coli* and LPS in the AF of human milk. Concentration of anti-*E. coli* and anti-LPS antibodies (IgA, IgG, and IgM) in raw and pasteurized colostrum and mature milk assessed by ELISA at non-saturating dilutions. The bars represent the mean ± SEM of the optical density at 450 nm based on data from four analytical replicates. Asterisks on the bars indicate significant differences between raw and pasteurized colostrum or mature milk, as determined by one-way ANOVA (*p ≤0.05, ***p ≤0.001).

0.3% of AF from raw colostrum resulted in a substantial fold-increase of IL-8 and MIP-3α levels, specifically 12.9 ± 3.3 and 15.0 ± 1.4 (mean ± SEM) respectively, compared to the basal condition. However, when HT-29 cells were exposed to raw mature milk, the increase in IL-8 production was significantly lower (fold-increase of 5.1 ± 1.8, mean ± SEM) compared to colostrum incubation. Interestingly, this effect was abolished when colostrum or mature milk was subjected to Holder pasteurization (Figure 5C).

Next, we examined the response of macrophages, a key resident cell type in the lamina propria responsible for orchestrating various aspects of the inflammatory process. To achieve this, we employed PMA-treated THP-1 cells (dTHP-1), a well-established *in vitro* model of macrophage-like behavior, to assess the influence of AF on cell response to LPS (Figure 6A). Initially, we confirmed that PMA incubation enhanced cell adhesion and increased the cell size, complexity, and expression of mCD14 (Figure S4). The addition of AF from raw colostrum increased the production of IL-6 and TNF-α in response to LPS. Specifically, a fold-increase of 9.0 ± 3.0 and 1.5 ± 0.1 (mean ± SEM) was observed for IL-6 and TNF-α levels respectively, when the cells were stimulated with LPS plus 0.3% AF. However, the IL-10 response to LPS remained unaltered (Figure 6B). The observed effect was significantly reduced when incubated with raw mature milk, resulting in a fold-increase of 4.9 ± 1.1 (mean ± SEM) for IL-6 levels. Notably, the effect observed with raw milk was abolished after Holder pasteurization (Figure 6C).

Monocytes, recruited effector cells, play a crucial role in acute inflammation and have the potential to differentiate into macrophages. Therefore, we studied the effect of AF on the response of THP-1 monocyte-like cells to LPS (Figure 7A). Incubation with LPS in the presence of 0.1% AF of raw colostrum led to decreased levels of IL-1β, IL-6, and TNF-α, with fold decrease of 11.6 ± 4.8, 3.0 ± 0.4, and 6.2 ± 0.9, respectively (mean ± SEM) (Figure 7B). These effects were less pronounced with mature milk, resulting in fold decreases of 1.8 ± 0.3, 2.0 ± 0.3, and 2.5 ± 0.6 (mean ± SEM) for IL-1β, IL-6, and TNF-α, respectively. The effect of milk on the response of THP-1 monocytes to LPS was not altered by Holder pasteurization (Figure 7C).

We assessed cell viability following LPS incubation in our *in vitro* model and found that the viability of HT-29 cells remained unaffected. A minor reduction in the viability of dTHP-1 and THP-1 cells upon LPS stimulation was observed; however, this effect was not influenced by the addition of AF (Figure S5).

3.4 Effect of donated milk on the differentiation of monocyte to macrophage

The functional phenotype of monocyte-derived macrophages depends on their microenvironments. Therefore, we examined whether inclusion of 0.3% AF during PMA treatment could alter the attributes of the resulting dTHP-1 cells (Figure 8A). Initially, we ensured that the cell count and viability remained unaffected by PMA treatment and AF under all experimental conditions after 24 h of resting (Figure 8B). Moreover, based on the cell FSC/SSC parameters, the morphology of dTHP-1 cells was similar across all conditions (Figure 8C). The expression of mCD14, a differentiation marker, was slightly decreased in dTHP-1 cells cultured with AF from all milk types compared to the basal condition (Figure 8D).

Next, we assessed the response of dTHP-1 cells obtained under various conditions (Figure 9A). dTHP-1 cells differentiated in the

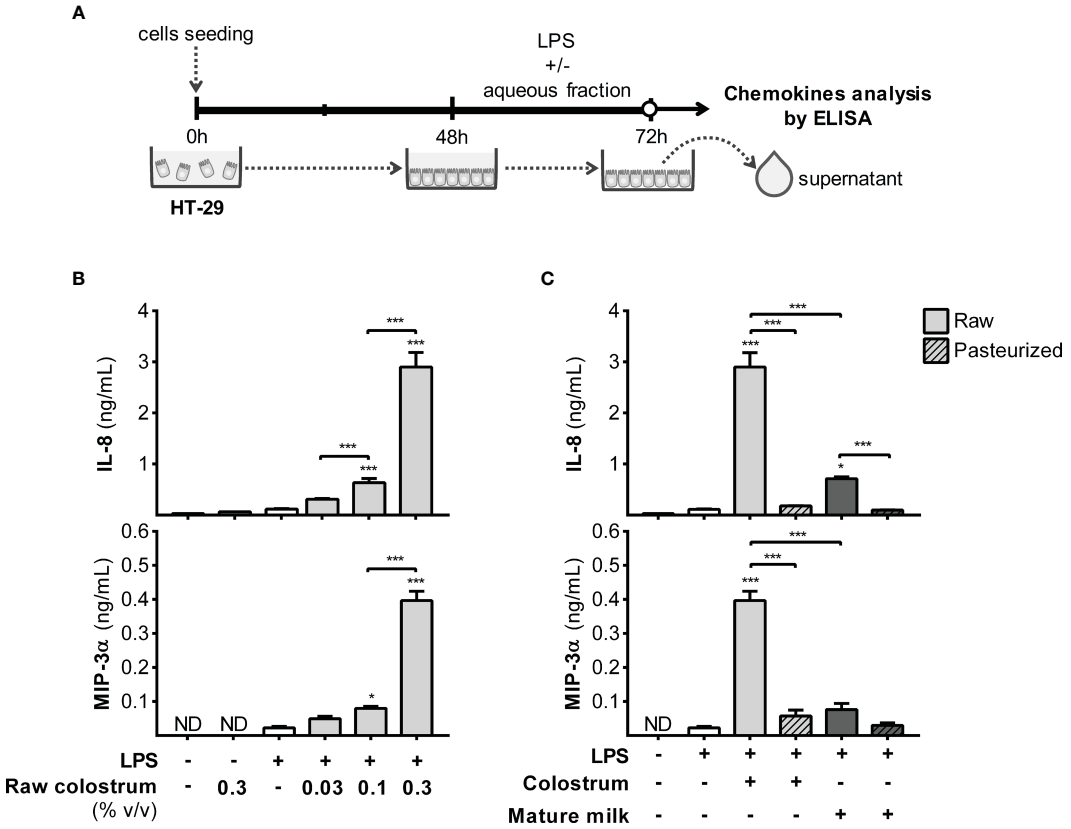

FIGURE 5
Effect of human milk AF on the LPS-response of gut epithelial cells. **(A)** Experimental design. **(B)** Dose-dependent effect of AF from raw colostrum on HT-29 chemokine response to 1 μg/mL LPS. **(C)** Comparative effect of 0.3% AF from raw or pasteurized colostrum and mature milk on HT-29 cells in response to 1 μg/mL LPS. The bars represent the mean ± SEM of three analytical replicates corresponding to one representative of three independent experiments. The asterisks on the bars indicate significant differences from the control condition (open bar, only LPS addition), as determined by one-way ANOVA (*p ≤0.05, ***p ≤0.001). ND, not detected.

presence of 0.3% AF from raw colostrum displayed a dose-dependent reduction in cytokine production compared to the control condition. Specifically, the fold decreases were 2.8 ± 0.4 for IL-6, 3.7 ± 0.3 for IL-10, and 1.6 ± 0.1 for TNF-α (mean ± SEM) (Figure 9B). The addition of 0.3% AF from mature milk resulted in a reduced response to LPS, similar to that observed in colostrum. Moreover, the effect of AF from raw colostrum and mature milk on the LPS response remained unaffected by pasteurization (Figure 9C).

4 Discussion

Numerous *in vivo* and *in vitro* experimental studies have demonstrated the beneficial effects of certain bioactive components in human milk (26, 27, 30, 44, 45). Nonetheless, limited research has delved into the mechanisms involved in the protective effects of donated HM within the neonatal gastrointestinal tract (4, 36, 37). The present study aimed to further investigate the biological significance of compositional changes in the activities of gut epithelial and innate immune cells upon exposure to bacterial stimuli. To mitigate the influence of inter-individual variability in milk composition on outcomes, we employed pooled samples of colostrum or mature milk collected the six-month after parturition, both in untreated and pasteurized conditions, as representative specimens of these lactation stages. Overall, our findings demonstrate that colostrum exerts more pronounced effects in most assessments than mature milk, consistent with its higher concentration of extensively documented bioactive components (2–4). Conversely, diverse effects of pasteurization were noted depending on the specific cell type and function under examination. These variations might be attributed to discrepancies in the interplay between the receptor profiles of distinct cell types and AF composition. The results are summarized in Figure 10.

Given the association between inadequate epithelial restoration and an immature gut, we initially investigated the impact of AF on the rates of proliferation and migration of HT-29 cells. Remarkably, AF from all milk sources triggered equivalent enhancement in cell proliferation. One potential bioactive constituent accounting for this effect could be Epidermal Growth Factor (EGF) (46), which maintains a consistent concentration throughout the first year of lactation and demonstrates resilience to the influence of Holder pasteurization (4, 35). AF from raw colostrum exhibited the capacity to stimulate cell migration. Within the wealth of bioactive constituents present in AF, lactadherin (MFG-E8) is a pivotal mediator of the wound healing process (25, 47–49).

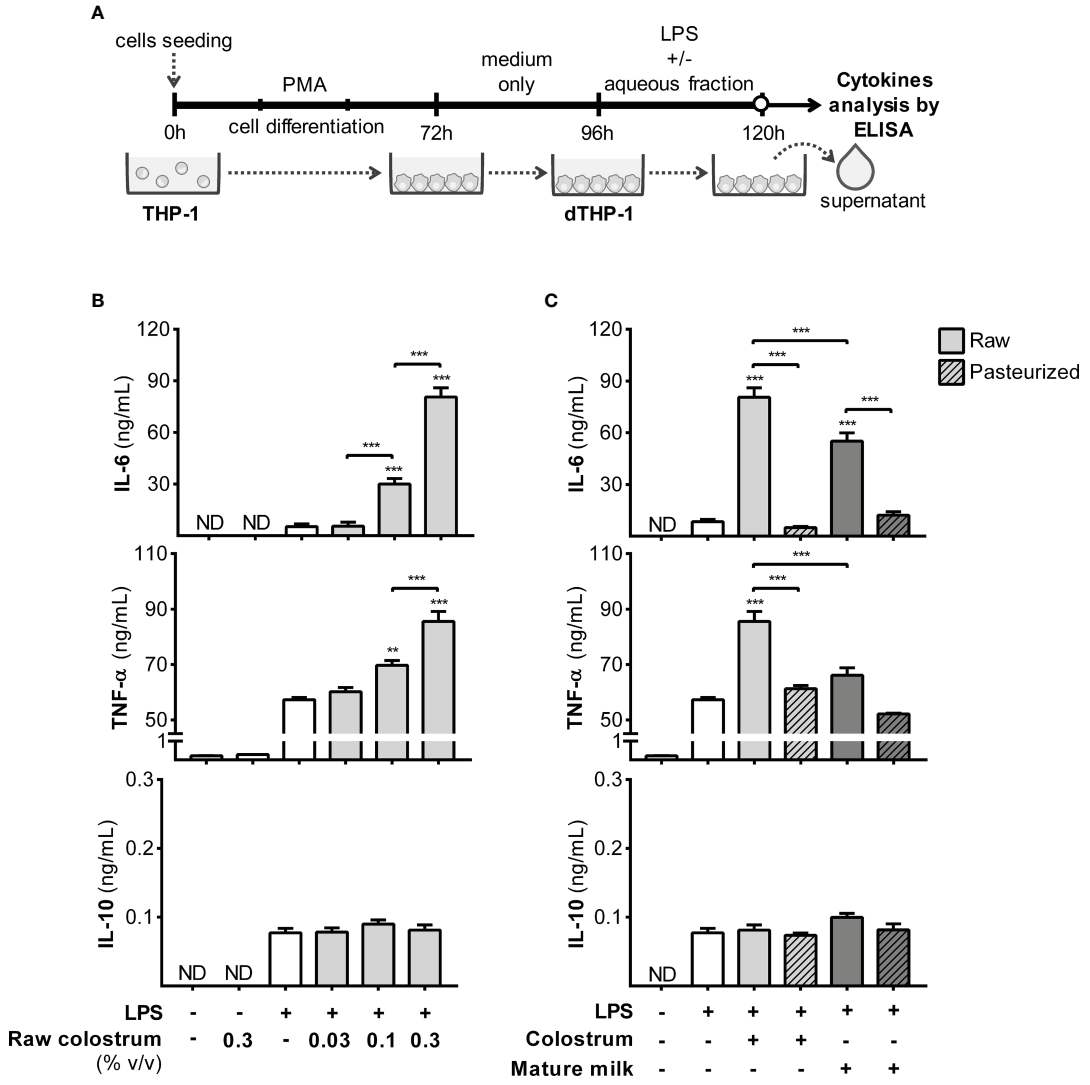

FIGURE 6
Effect of human milk AF on the LPS response of macrophages. **(A)** Experimental design. **(B)** Dose-dependent effect of AF from raw colostrum on dTHP-1 cytokine response to 1 µg/mL LPS. **(C)** Comparative effect of 0.3% AF from raw or pasteurized colostrum and mature milk on the dTHP-1 response to 1 µg/mL LPS. The bars represent the mean ± SEM of three analytical replicates, corresponding to one representative of three independent experiments. Asterisks on the bars indicate significant differences from the control condition (open bar, only LPS addition), as determined by one-way ANOVA (**p ≤0.01, ***p ≤0.001). ND, not detected.

Consistent with our findings, lactadherin was more abundant during the early stages of lactation (50), and its levels were significantly affected by the Holder pasteurization process (Figure S6).

The attachment of bacteria and their subsequent invasion into epithelial cells plays a pivotal role in gut inflammatory pathologies. Our study revealed that only the AF of raw colostrum affected the interaction between *E. coli* and HT-29 cells. This effect can be attributed to the presence of various heat-sensitive bioactive components, including antibodies. Notably, we found that all anti-*E. coli* and anti-LPS antibodies decreased after pasteurization. Given that secretory IgA (sIgA) is the most abundant antibody isotype in human milk, we investigated the effects of purified colostrum sIgA on bacterial adhesion. Thermal treatment reduced the neutralizing effect of sIgA on bacterial adhesion to epithelial cells (Figure S2). These variations in the effects observed between the pasteurized and raw samples can be attributed to these underlying factors. Lactoferrin, a significant protein in AF, could also play a role in these outcomes (51), considering its sensitivity to thermal treatment and its decline over the course of lactation (52, 53).

The pathophysiology of NEC is closely linked to increased reactivity of the preterm gut epithelium to LPS. This reactivity is characterized by elevated TLR4 expression, translocation of LPS to the lamina propria, and the subsequent activation of inflammatory cells (18, 20). Globally, milk exhibits anti-inflammatory properties, while also playing a role in the effector mechanisms involved in defense against pathogens (54, 55). The ultimate outcome *in vivo* depends on the functional characteristics of resident cells in the lamina propria, signaling within the microenvironment, and the

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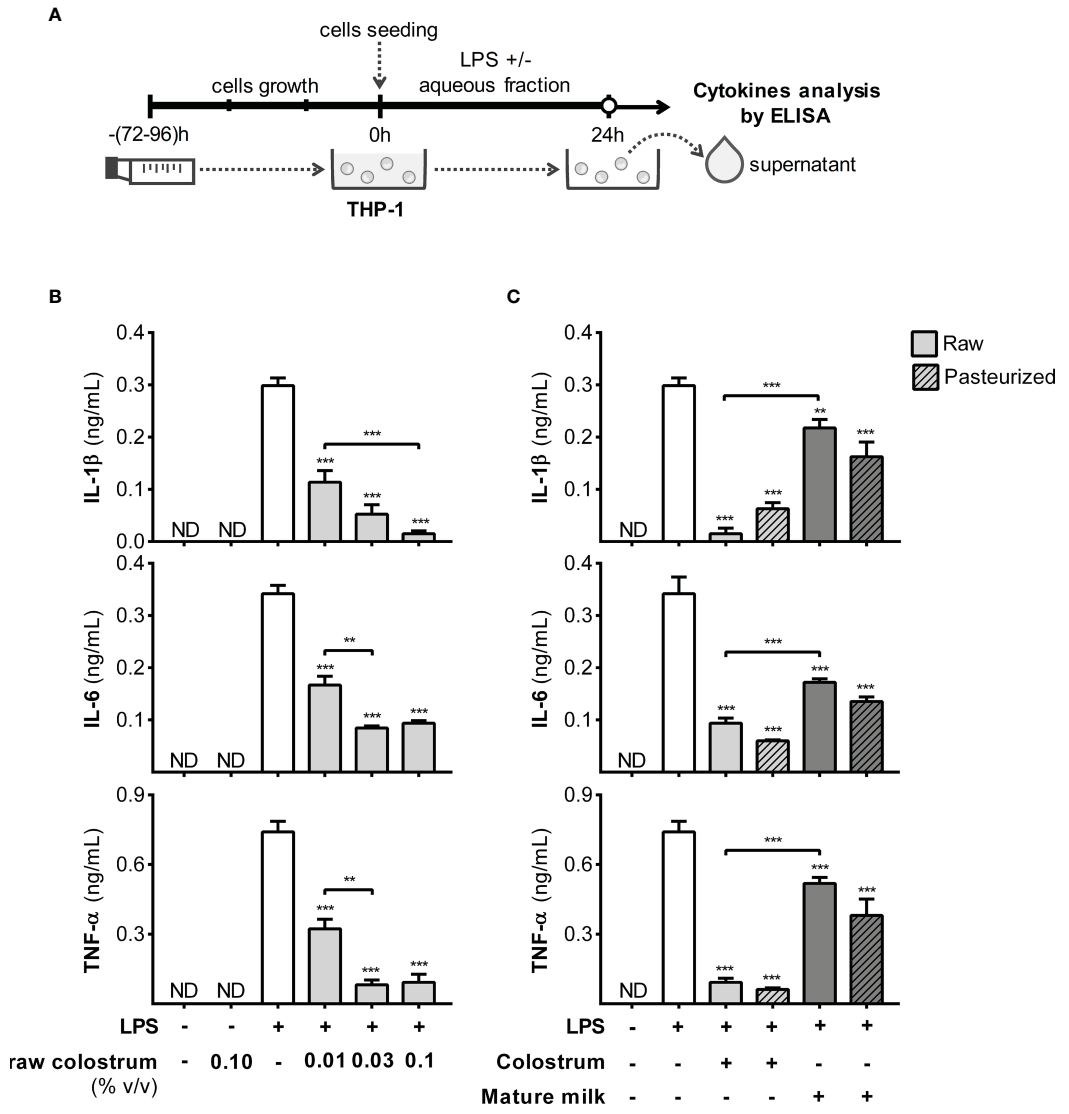

FIGURE 7
Effect of human milk AF on the LPS response of monocyte-like cells. **(A)** Experimental design. **(B)** Dose-dependent effect of AF from raw colostrum on the THP-1 cytokine response to 1 μg/mL LPS. **(C)** Effect of 0.1% AF from raw or pasteurized colostrum and mature milk on THP-1 response to 1 μg/mL LPS. The bars represent the mean ± SEM from three analytical replicates corresponding to one representative of three independent experiments. The asterisks on the bars indicate significant differences from the control condition (open bar, only LPS addition), as determined by one-way ANOVA (**p ≤0.01, ***p ≤0.001). ND, not detected.

maturity of both the immune system and gut epithelium. In our *in vitro* model of gut epithelial cells and macrophages, we successfully replicated the role of raw milk in augmenting the inflammatory response to bacterial stimuli. In a study by LeBouder et al., it was observed that the response of HT-29 cells to LPS was reversed by employing a blocking antibody against sCD14 (23). CD14 exists in both the cell membrane-bound form (mCD14) and soluble form (sCD14), both of which can modulate the TLR4 signaling pathway (56, 57). In our previous study, we demonstrated that sCD14 is present in higher concentrations in colostrum than in mature milk, and its levels are drastically diminished by the Holder pasteurization process (4). We investigated the influence of thermal treatment on the functionality of recombinant sCD14 and found that the augmented LPS response in HT-29 and dTHP-1 cells was abolished by Holder pasteurization (Figure S7).

The severity of NEC is correlated with a decrease in circulating monocyte levels because of their recruitment into the gut (58); this parameter was proposed as a biomarker of NEC pathology (59). Consequently, we assessed the effect of AF on the response of THP-1 monocyte-like cells to LPS. Unlike epithelial and macrophage cells, this response was dampened by AF and remained unaffected by thermal treatment. However, the effect of purified sCD14 mirrored that observed in the other cell types (Figure S7). This distinct pattern indicated the involvement of other milk constituents in the LPS response. To investigate this further, we examined the effect of purified colostrum sIgA with reactivity to LPS on the response to this stimulus. No variation was observed across any of the cell types (data not shown). Lactoferrin is a crucial thermosensitive component that interacts with LPS and may contribute to modulating the response. In addition to the

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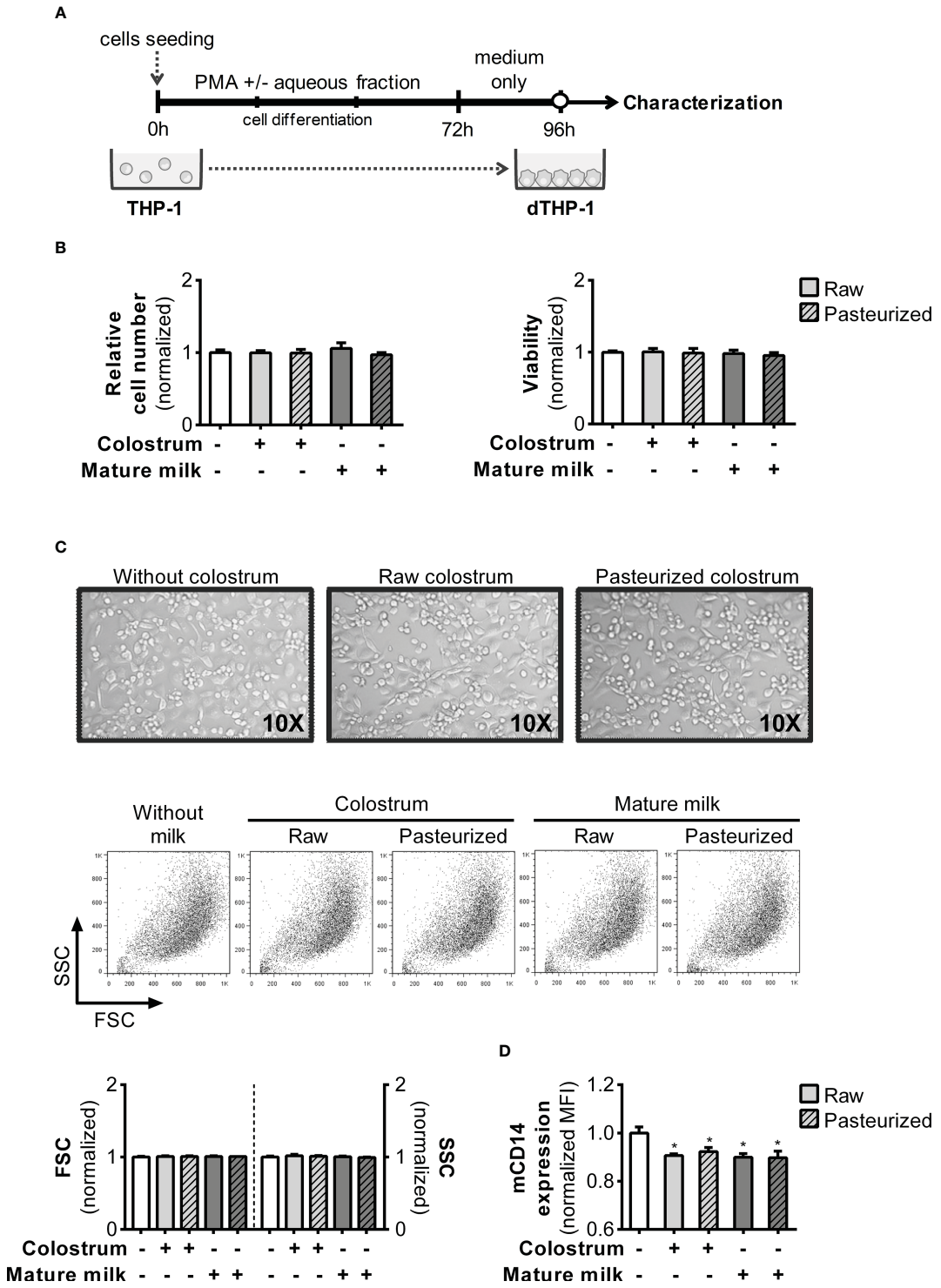

FIGURE 8
Effect of human milk AF on macrophage differentiation of PMA-treated monocyte-like cells. **(A)** Experimental design. **(B)** Effect of AF from raw or pasteurized colostrum and mature milk on the relative cell count and viability of macrophages (dTHP-1) on day 4. The effects were determined using the crystal violet assay and MTT method. **(C)** Representative photographs (×10 magnification) of cells and FSC/SSC Scatter on day 4 of culture, where cells were treated with PMA in the absence or presence of 0.3% AF from raw or pasteurized milk. **(D)** Flow cytometric analysis of mCD14 expression in dTHP-1 cells differentiated in the absence or presence of 0.3% AF from raw or pasteurized milk. The bars represent the mean ± SEM of four analytical replicates corresponding to one representative of three independent experiments. Asterisks on the bars indicate significant differences from the control condition (open bar), as determined by one-way ANOVA (*p ≤0.05).

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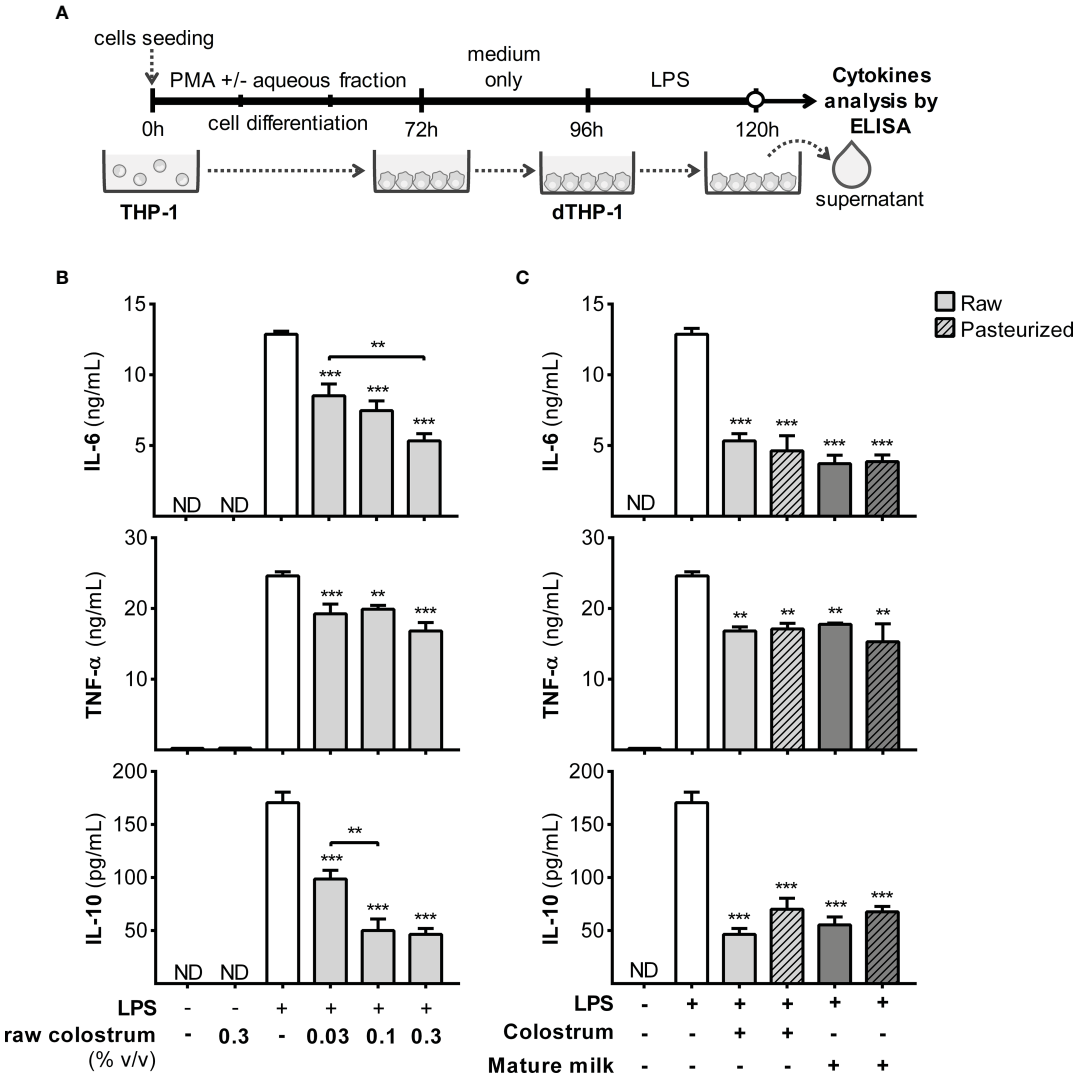

FIGURE 9
Effect of human milk AF on the LPS response of dTHP-1 during PMA-driven macrophage differentiation. **(A)** Experimental design. **(B)** Dose-dependent effects of AF from raw colostrum during macrophage differentiation on the cytokine response of dTHP-1 cells to 1 µg/mL LPS. **(C)** Comparative effect of 0.3% AF from raw and pasteurized milk during macrophage differentiation on the cytokine response of dTHP-1 cells to 1 µg/mL LPS. The bars represent the mean ± SEM of three analytical replicates corresponding to one representative of three independent experiments. Asterisks on the bars indicate significant differences from the control condition (open bar, differentiation without AF), as determined by one-way ANOVA (**p ≤0.01, ***p ≤0.001). ND, not detected.

components that can directly interact with LPS, numerous bioactive molecules present in HM can either enhance or inhibit TLR expression and its associated signaling pathways (60). Ultimately, the distinct intrinsic properties of cell types, such as activation thresholds, patterns of receptor expression, and functional attributes, could elucidate the variations observed in the response to LPS.

Macrophages exhibit remarkable plasticity, and their responses can pivot between inflammatory and anti-inflammatory states based on cues from the microenvironment, such as M1- and M2-like phenotypes, in a simplistic view (61). We investigated whether AF might have an impact on macrophage behavior, potentially altering their responsiveness to LPS-induced inflammation. Interestingly, when AF was present during PMA-induced differentiation, a subdued inflammatory reaction to LPS was observed. While this outcome deserves more in-depth exploration of the relationship between macrophages and a potential M2-like phenotype, our findings are in line with prior research that showed the potential of pasteurized AF to redirect murine macrophages to an M2 phenotype (62). The milk constituents responsible for this effect remain unclear. Our findings suggest the involvement of bioactive components that remain unaffected by thermal processing, as evidenced by the similar outcomes in both raw and pasteurized samples.

Among these components, HMOs comprise a complex array of multifunctional elements that can potentially influence the outcomes of our *in vitro* experiments (63). The overall quantity of HMO is most abundant in colostrum, although the contribution of specific molecules within this group tends to vary throughout the lactation period (5). HMO are not affected by thermal treatment

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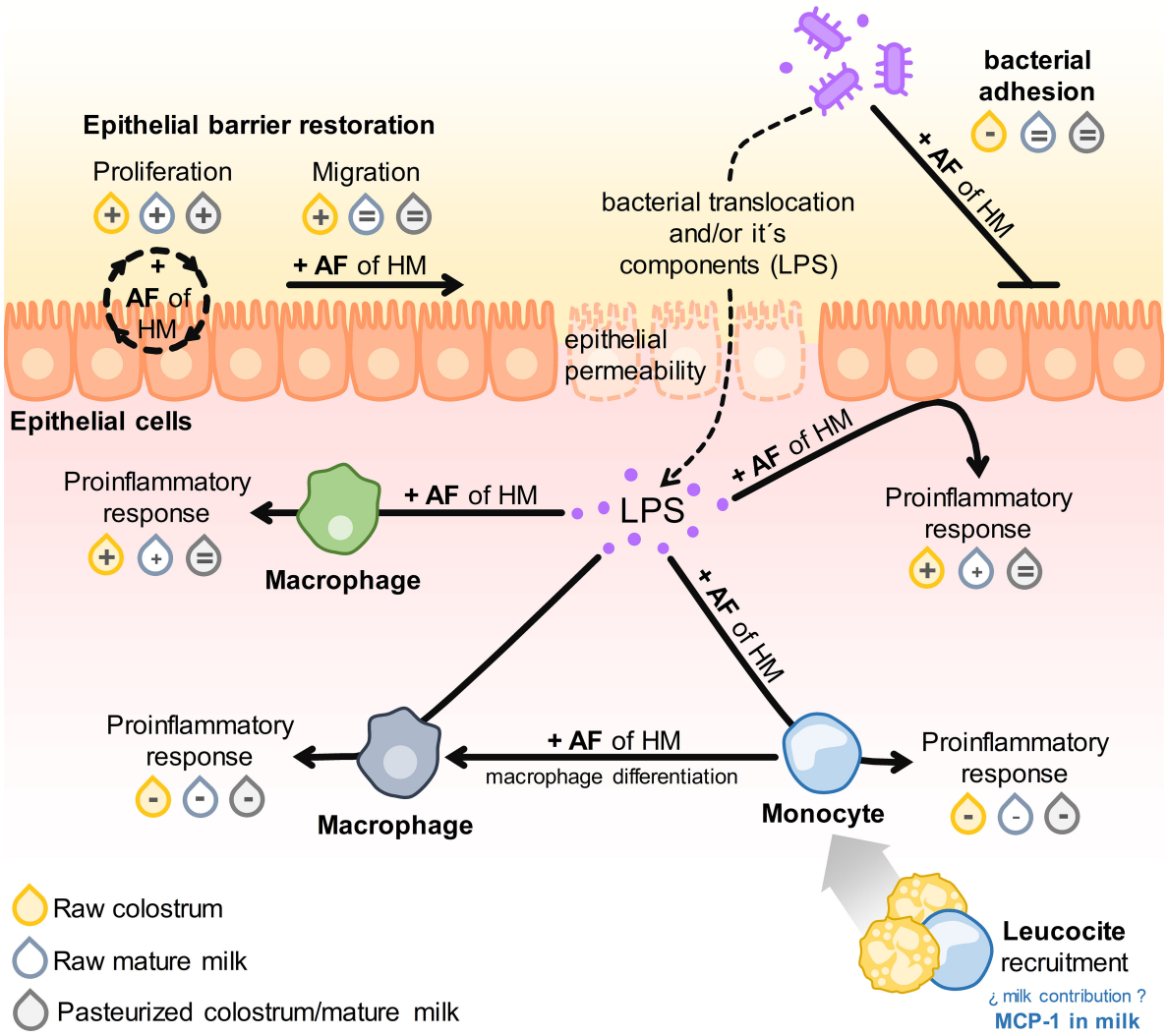

FIGURE 10
Schematic illustration of the results obtained from all assays. The impact of adding AF from raw or pasteurized colostrum and mature milk on the respective parameters for each assay is denoted as: enhancement (+), inhibition (–), or no difference (=) compared to the conditions without AF.

(64), which could explain some similar effects between the AF of raw and pasteurized milk. In addition, exosomes are another class of bioactive components present in the AF of milk that have shown *in vitro* and *in vivo* effects (30, 65, 66). However, their concentration decreases as lactation progresses, and they are influenced by Holder pasteurization (67). These and other components might potentially contribute to some of the effects observed in the AF; however, additional research is necessary to unravel their specific roles.

4.1 Limitations of this study

This study had several limitations from the perspective of knowledge translation. First, it employs simplistic *in vitro* models using transformed cell lines, which lack the complexity of tissue-microenvironment signals. Incorporating microbiota and mucus could provide a more accurate representation of the intricate gastrointestinal tract environment. Second, the observed *in vitro* effects may differ when the impact of gastrointestinal digestion on bioactive components is considered. Third, the study focused solely on the bioactive constituents of AF, excluding the potential effects of other milk components.

In conclusion, this study sheds light on the principal biological processes influenced by milk, which exhibit varying responses depending on lactation stage and thermal treatment. Further studies are underway to assess the relevance of our results in more complex *in vitro* and *in vivo* systems. This basic knowledge is essential for understanding the mechanisms underlying diverse clinical outcomes in preterm NB who are nourished with donor milk. Moreover, it provides insights into enhancing the strategies of Human Milk Banks in cases where fresh maternal milk is not accessible.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Comité de Ética en Investigación, Centro Hospitalario Pereira Rossell, Montevideo, Uruguay. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CR-C: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing, Data curation. AP: Investigation, Methodology, Resources, Validation, Writing – review & editing. PA: Investigation, Methodology, Resources, Validation, Writing – review & editing. CS: Investigation, Methodology, Resources, Validation, Writing – review & editing. LF: Resources, Writing – review & editing. GS: Resources, Writing – review & editing. AH: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1282144/full#supplementary-material

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Identification of omega-3 oxylipins in human milk-derived extracellular vesicles with pro-resolutive actions in gastrointestinal inflammation

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Introduction: Premature infants (PIs) are at risk of suffering necrotizing enterocolitis (NEC), and infants consuming human milk (HM) show a lower incidence than infants receiving formula. The composition of HM has been studied in depth, but the lipid content of HM-derived small extracellular vesicles (HM sEVs) remains unexplored. Identifying these molecules and their biological effects has potential for the treatment of intestinal disorders in PIs and could contribute to the development of HM-based fortified formulas.

Methods: We isolated HM sEVs from HM samples and analyzed their oxylipin content using liquid chromatography coupled to mass spectrometry, which revealed the presence of anti-inflammatory oxylipins. We then examined the efficacy of a mixture of these oxylipins in combating inflammation and fibrosis, *in vitro* and in a murine model of inflammatory bowel disease (IBD).

Results: HM-related sEVs contained higher concentrations of oxylipins derived from docosahexaenoic acid, an omega-3 fatty acid. Three anti-inflammatory oxylipins, 14-HDHA, 17-HDHA, and 19,20-DiHDPA (ω3 OXLP), demonstrated similar efficacy to HM sEVs in preventing cell injury, inducing re-epithelialization, mitigating fibrosis, and modulating immune responses. Both ω3 OXLP and HM sEVs effectively reduced inflammation in IBD-model mice, preventing colon shortening, infiltration of inflammatory cells and tissue fibrosis.

Discussion: Incorporating this unique cocktail of oxylipins into fortified milk formulas might reduce the risk of NEC in PIs and also provide immunological and neurodevelopmental support.

KEYWORDS

oxylipins, small extracellular vesicles (sEVs), human milk (HM), inflammatory bowel disease (IBD), necrotizing enterocolitis (NEC)

1 Introduction

Human milk (HM) has several nutritional and immunological benefits that favor the clinical evolution and neurodevelopment of premature infants (PIs) in the short- and long term (1). PIs fed HM, especially their own mother's milk, are at significantly less risk of serious diseases such as necrotizing enterocolitis (NEC), neonatal sepsis, bronchopulmonary dysplasia and retinopathy of prematurity (2). However, PIs have higher nutrient requirements than full-term infants, and need enriched milk formulations to meet their nutritional needs, and it is challenging to fulfil their high and variable nutrient requirements during hospitalization (3).

HM consists of 87% water, 1% protein, 4% lipids, and 7% carbohydrates (including 1 to 2.4% oligosaccharides) (4). It also contains many minerals and vitamins. HM is unique in its high abundance of long-chain polyunsaturated fatty acids (LC-PUFAs), which are derived from two essential fatty acids: linoleic acid (LA, omega-6 [ω6]) and alpha-linolenic acid (ALA, ω3). Elongation of these two LC-PUFAs gives rise to arachidonic acid (AA, ω6) and eicosapentaenoic acid (EPA, ω3), respectively, with the latter further metabolized to docosahexaenoic acid (DHA, ω3) (5). LC-PUFAs are important for regulating growth, immune function, vision, cognitive development, and motor systems in newborns (6–8). There is accumulating evidence that milk-derived bioactive lipids have multifunctional properties (6). Oxylipins are a diverse class of specialized signaling molecules derived from LC-PUFAs that regulate neonatal intestinal development and protect PIs against intestinal injury (9, 10). Both ω-3 and ω-6 oxylipins are involved in the initiation and resolution of inflammatory processes (10). In addition, some oxylipins are precursors of specialized pro-resolving and cytoprotective mediators (SPMs), in particular, ω3-derived oxylipins (resolvins, maresins, and protectins) have anti-inflammatory effects and are involved in the resolution process following tissue injury (11–13).

Bioactive compounds of HM can also be transferred from mother to child *via* small extracellular vesicles (sEVs) (8), which are lipid bilayer membrane vesicles (50 to 200 nm) containing myriad signaling molecules including proteins, lipids, microRNAs, mRNAs and other biomolecules protected from degradation (14). sEVs are present in high concentrations in HM and play an important role in inflammation and immune response of the newborns through intracellular communication (15). It has been reported that sEVs can escape degradation during digestion, reach gut cells, and be transferred to circulation through lymphatic vessels (16, 17). Moreover, HM sEVs have been reported to enhance gut cells migration and inhibit $CD4^+$ T cell activation *in vitro* (18), and restore intestinal barrier homeostasis in a mouse model of ulcerative colitis (19, 20). The use of HM sEVs in fortified formulas is, however, controversial due to obvious ethical and logistical reasons (21), and well-defined formulations are needed. A better understanding of the composition of sEVs is essential to identify key molecular players and their mechanism of action. While many characteristics of sEVS are under active investigation, such as surface markers (22), protein cargo (18, 23), and miRNA content (24), little is known about the lipid composition of sEVs from HM (25).

In the present study, we used targeted lipidomic analysis to profile oxylipins in HM sEVs purified from 15 breast milk samples donated by healthy volunteers. We then evaluated and compared the efficacy of a combination of the three most abundant oxylipins in HM sEVs in reducing inflammation in a mouse model of colitis with HM sEVs, which confirmed the potent therapeutic value of 14-HDHA, 17-HDHA, and 19,20-DiHDPA (hereafter referred to as ω3 OXLP). Our findings suggest that the ω3 OXLP formulation could serve as a promising dietary supplement for early and intensive nutrition in PIs to prevent NEC.

2 Materials and methods

2.1 Ethical statements

For inclusion in the study, all donors gave their informed consent. The research was carried out in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the *Hospital Universitari i Politècnic La Fe*, Valencia, Spain (approval numbers 2021-071-1, 2022-748-1 and 2019-289-1).

Ethics Committee of the *Hospital Universitari i Politècnic La Fe* (protocol N° 2021/VSC/PEA/0060) approved animal procedures by according to guidelines from Directive 2010/63/EU of theEuropean Parliament on the protection of animals used for scientific purposes.

2.2 Human samples

HM samples were obtained from lactating women (28–42 years of age). Fifteen volunteers were enrolled at the Human Milk Bank of the University & Polytechnic Hospital La Fe (Valencia, Spain). Buffy coats of healthy donors were from human blood obtained from the Centro de Transfusión de la Comunidad Valenciana (Valencia, Spain), and were used to obtain peripheral blood mononuclear cells (PBMCs).

2.3 Cell culture

Caco-2 intestinal epithelial cells (isolated from human colonic cancer) were maintained in Dulbecco's modified Eagle's medium (DMEM)-high glucose (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Corning, Glendale, AZ, USA) and 100 U/mL penicillin and 100 µg/mL streptomycin (P/S, Sigma-Aldrich, Saint Louis, MO, USA). Caco-2 cells were stimulated with 60 µg/mL of lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Sigma-Aldrich, Darmstadt, Germany) in DMEM-high glucose supplemented with 0.5% FBS and 1% P/S for 24 h in the presence or not of HM sEVs or ω3 OXLP. For differentiation experiments, Caco-2 cells (1×10^5 cells/cm^2) were added to 8 µm-pore size Transwell® polycarbonate membranes (Corning® Inc., Corning, NY, USA) in complete medium. Upon reaching a confluent monolayer, Caco-2 cells differentiate spontaneously, and after 21 days they show dense microvilli on the apical side, characteristic of small intestinal enterocytes (26).

Fibroblasts were isolated from human skin biopsies and were cultured in DMEM/F12 (Gibco, Thermo Fisher Scientific) supplemented with 10% FBS and 1% P/S. One day before stimulation, cells were seeded in serum-free medium supplemented with 1% P/S. Fibroblasts were then stimulated with LPS (10 ng/mL) for 24 h in the presence or not of HM sEVs or ω3 OXLP in the same medium.

Both fibroblasts and Caco-2 cells were cultured under oxygen/glucose deprivation (OGD) conditions in some experiments. OGD conditions were induced by culturing the cells with DMEM medium without glucose, glutamine, nor phenol red (Thermo Fisher Scientific) in a cell culture incubator at 1.5% O_2, creating a hypoxic environment.

PBMCs were isolated from healthy blood donor buffy coat by density gradient centrifugation with Histopaque (Sigma-Aldrich, Darmstadt, Germany), and were cultured in the Rosewell Park Memorial Institute medium (RPMI, Gibco, Thermo-Fisher Scientific) supplemented with 10% FBS, 1 mM pyruvate, 2 mM glutamine and 1% P/S (all from Sigma-Aldrich). Monocytes were isolated as described (27). To generate monocyte-derived type 1 or type 2 macrophages (Mφ1 or Mφ2, respectively), cytokine stimulation was added to the cells in complete RPMI medium: 5 ng/mL recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF, Peprotech) or 20 ng/mL recombinant human macrophage-colony stimulating factor (rhM-CSF, Peprotech). Cytokines were fed back every two days. On the fifth day of differentiation, 10 ng/mL of LPS and 20 ng/mL of IFNγ (R&D Systems, Minneapolis, MN, USA) were added to Mφ1, whereas 10 ng/mL of LPS and 40 ng/mL of IL4 (PeproTech, London, UK) were added to Mφ2, for 16 h. Under Mφ1 conditions, HM sEVs or ω3 OXLP were added on day 0 of the differentiation protocol.

2.4 sEV isolation and characterization

sEVs were isolated using a serial ultracentrifugation protocol (25). Briefly, HM was centrifuged three times at 3000×g for 10 min at 4°C to remove milk fat and fat globules. After removing the upper fat layer, the liquid was transferred to a 25-mL polycarbonate bottle and centrifugated twice at 10,000×rpm for 1 h at 4°C. Supernatants were filtered manually through a 0.45-µm filter using a syringe. HM sEVs were then concentrated by three of rounds ultracentrifugation at 30,000 rpm for 2 h at 4°C. Samples were filtered through a 0.22-µm filter to maintain sterility. To ensure equal amounts of protein were used for experiments, a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) was used to determinate protein concentration. For western blotting, sEVs were suspended in RIPA buffer, Sigma-Aldrich). For characterization and functional analysis, sEVs were suspended in PBS. Nanoparticle tracking analysis (NTA) and electron microscopy were performed as described (28). Dynamic light scattering (DLS) was performed to determine the size, distribution, surface charge and stability of sEVs. HM sEVs were placed in a cuvette filled with PBS. The zeta potential magnitude (ζ) and the polydispersity index (PDI) of the samples were measured using a DLS detector (Zetasizer Nano ZS DLS detector, Malvern, UK), which was operated in both continuous and discontinuous modes, employing laser doppler micro-electrophoresis. The instrumental conditions for the DLS system, including temperature, acquisition time, measurement position, and attenuator settings, were optimized for accurate measurements. The specific details of the DLS system setup are described in Table 1, which summarizes the parameters for continuous DLS, discontinuous DLS, and Z-potential measurements. The temperature was maintained at 25°C throughout the experiments, and an equilibration time of 120 s was allowed before each measurement. The acquisition time and attenuator settings were automatically adjusted to seek the optimum conditions for data acquisition. The Smoluchowski model with a correction factor of 1.50 F(ka) was employed for zeta potential calculations, and the voltage was set to auto with a maximum value of 150 V.

2.5 Western blot analysis

Equal amounts of HM sEVs were lysed in RIPA buffer containing protease and phosphatase inhibitors (Complete Mini and PhosSTOP, Sigma-Aldrich), then were mixed with non-reducing Laemmli sample buffer (BioRad) and denatured at 96°C for 5 min. Proteins were separated on 10% SDS-polyacrylamide gels. Human primary antibodies used were: anti-calnexin (dilution 1/1000, Santa Cruz Biotechnology, H-70), anti-Hsp70 (dilution 1/500; Cell Signaling Technology; D69), anti CD63 (dilution 1/500; Santa Cruz Biotechnology; H-193), anti-TSG101 (dilution 1/200; Santa Cruz Biotechnology; C-2), anti-CD81 (dilution 1/500; Santa Cruz Biotechnology; B-11) and anti-CD9 (dilution 1/500; Santa Cruz Biotechnology; C-4). Peroxidase-conjugated secondary antibodies were anti-IgG rabbit (dilution 1/4000; Dako; P0448)

TABLE 1 Instrumental conditions of the DLS system.

Continuous DLS	Acquisition time	3.0 s
	Measurement position	4.2 mm
	Attenuator	11
Discontinuous DLS	Equilibration time	120 s
	Measurement angle	173° (NIBS default)
	Acquisition time	10.0 s
	Position	Automatic seek for optimum conditions
	Attenuator	Automatic seek for optimum conditions
Z-Potential	Model	Smoluchowski (1.50 F(ka))
	Equilibration time	120 s
	Acquisition time	Automatic seek for optimum conditions
	Attenuation selection	Automatic seek for optimum conditions
	Voltage	Auto (Max. 150 V)

and anti-IgG mouse (dilution 1/10000; Sigma-Aldrich; A9044). Proteins were detected with ECL Plus Reagent (GE Healthcare, Chicago, IL, USA) or SuperSignal West Femto (Thermo Fisher Scientific). Visualization was carried out using an Amersham Imager 600 (GE Healthcare) and quantified with ImageJ software (NIH, Bethesda, MD, USA).

2.6 Uptake of labeled HM sEVs

HM sEV uptake by Caco-2 cells was performed after labeling EVs with carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher Scientific) (29). HM sEVs were stained with 5 µM of CFSE in PBS for 15 min at 37°C in darkness, then were washed with PBS in an Amicon Ultra-0.5 Centrifugal Filter 100 kDa (Merk, Darmstadt, Germany) and suspended in filtered PBS. 30µg/mL of dyed HM EVs were added to 1×10^5 Caco-2 cells seeded in a 48-well plate. CFSE mixed with PBS was used as a negative control to normalize the amount of unincorporated dye. CFSE-positive cells were detected by flow cytometry after 24 h incubation.

2.7 Extraction of the HM-sEVs lipid fraction and oxylipin quantification

Sample preparation and oxylipin quantification were adapted as described elsewhere (30). In short, HM sEVs were extracted using a solid phase extraction Oasis® MAX 96 well plate from Waters (Taunton, MA, USA). Recovered sample extracts were evaporated using a miVac centrifugal vacuum concentrator (Genevac Ltd., Ipswich, UK) and then dissolved in 60 µL methanol:acetonitrile (50:50, v/v).

Sample extracts were analyzed using an Acquity-Xevo TQ-XS system (Waters, Milford, MA, USA) operating in negative electrospray ionization mode. Separations were performed on a Waters Acquity UPLC BEH C18 (2.1×100 mm, 1.7 µm) column using a 0.1% v/v acetic acid and acetonitrile: isopropanol (90:10 v/v) binary gradient. Mass spectrometry (MS) detection was carried out by multiple reaction monitoring. Oxylipins quantified were as follows: 12,13-DiHOME, 9,10-DiHOME, 14,15-DiHETRE, PGE2, PGF2α, 19,20-DiHDPA, 17-HDHA, 14-HDHA, 17,18-DiHETE, 14,15-DiHETE, Resolvin D5, Maresin 2, and 8(S),15(S)-DiHETE.

2.8 T-cell proliferation assay

T-cell proliferation assays were performed as described (28). PBMCs were labeled with 5 µM CFSE and activated with Dynabeads™ Human T-Activator CD3/CD28 (Thermo Fisher Scientific). As T-lymphocytes of PBMCs become activated and divide, CFSE staining is diluted. Immunosuppressive potential was evaluated by adding 30 µg/mL of HM sEVs to 1×10^5 CFSE-labeled and activated PBMCs seeded in a 24-well plate. After 5 days of activation, proliferation of T-cells was evaluated by flow cytometry to quantify CFSE dilution. The Flowjo® software (FlowJo LLC, BD, Franklin Lakes, NJ, USA) was used in order to analyze flow cytometry data and the expansion index (EI) (31). The percentage of immunosuppression was calculated using the following formula, where EI of untreated activated PBMCs (Act) represents 0% of immunosuppression and EI of non-activated PBMCs (No act) represents 100%:

$$\%\,Immunosuppression = \frac{(EI_{Act} - EI_{treated})}{(EI_{Act} - EI_{No\ act})} \times 100$$

2.9 Flow cytometry

PBMCs or macrophages were incubated with a blocking solution for 10 min and incubated with fluorochrome-conjugated antibodies for 1 h at 4°C. Human antibodies used were: anti-CD3 (PerCP-Cy, BD Biosciences; SK7), anti-CD14 (RPE, Dako, TUK4, Santa Clara, CA, USA), anti-CD163 (PerCP-Cy, BD Biosciences, GHI/61), anti-CD80 (APC, BD Biosciences, FUN-1), anti-CD86 (V450, BD Biosciences, L307.4) and anti-HLA-DR (FITC, Miltenyi Biotec, AC122) at concentrations recommended by the manufacturers. The BD FACSCANTO II flow cytometer was used for cellular analysis and the data were processed using Flowjo® software.

2.10 Cell viability assay

To test whether HM sEVs or ω3 OXLP affected cell viability, Caco-2 cells were cultured at a density of 1×10^4 cells/cm^2 on a 96-well plate and were then stimulated with LPS or cultured under OGD conditions and treated with HM sEVs or ω3 OXLP. After 24 h the Cell Counting Kit-8 (CCK-8) assay was used to measure proliferation. After 4h of incubation with CCK-8 solution, the optical density (450 nm) was measured.

2.11 Lactate dehydrogenase assay

Caco-2 cells were seeded at 1×10^4 cells/cm^2 in complete medium. On the next day, cells were stimulated with LPS or cultured under OGD conditions and treated with HM sEVs or ω3 OXLP. After 24 h the for lactate dehydrogenase was tested using the Cytotoxicity Detection KitPLUS (LDH) (Roche, Indianapolis, IN, USA). Following manufacturer's instructions, 50 µL of cell supernatant was mixed with 50 µL of reaction mix (1:45 catalyst in dye solution), incubated for up to 30 min at room temperature and measured the absorbance at 492 nm.

2.12 Oxidative stress assay

LPS- and OGD-treated cells were washed with PBS and stained with 5 µM 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich) for 20 min at 37°C to detect cell reactive oxygen species (ROS). After staining, cells were washed three times with PBS, and were detached with trypsin for flow cytometry, DCF fluorescence was detected at λ_{ex} of 488 nm and λ_{em} of 525 nm.

2.13 Scratch assay

Caco-2 cells and fibroblasts were seeded in a 24-well plate at 2×10^5 cells/well. Caco-2 cells were stimulated with LPS and treated with HM sEVs or ω3 OXLP for 48 h. To develop scratch assays under OGD conditions, the medium was replaced after 24 h with complete medium and cells were cultured under standard oxygen conditions. Caco-2 cells were then stimulated with LPS and treated with HM sEVs or ω3 OXLP for 48 h. A 20-μL pipette tip was used to generate a thin line in the monolayer culture. After 48 h with treatments, the cultures were imaged using a Leica DM600 inverted microscope at 10× magnification. ImageJ software was used to measure the scratch wound area.

2.14 Real time quantitative PCR

RNA was extracted using a guanidine-thiocyanate–containing lysis buffer (RLT; Qiagen, Dusseldorf, Germany) and purified with the RNeasy Plus Mini Kit (Qiagen). For quantified RNA, NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) was used. PrimeScript RT Reagent Kit (Takara, Kusatsu, Japan) was used to obtain cDNA. Human- or mouse-specific sense and antisense primers and RT-SYBR™ Green PCR Master Mix (Applied Biosystems) were used to performed th RT-qPCR. 384 multiwells plates were run on a Viia 7 PCR System (Applied Biosystems). The primers used were:

hGAPDH CCCCTCTGCTGATGCCCCA (F) and TGACCTTGGCCAGGGGTGCT (R)

hTNF-α CCCTCTGGCCCAGGCAGTCA (F) and ATGGGTGGAGGGGCAGCCTT (R)

hCOX2 GAATCATTCACCAGGCAAA (F) and TCTGTACTGCGGGTGGAACA (R)

hOCLN GGACTGGATCAGGGAATATC (F) and ATTCTTTATCCAAACGGGAG (R)

hCLDN CCGGGTTGCCCACCTGCAAA (F) and CGTACATGGCCTGGGCGGTC (R)

hTGF-β GAGTGTGGAGACCATCAAGGA (F) and CTGTTTTAGCTGCTGGCGAC (R)

hIL-1β AGGCACAAGGCACAACAGGCT (F) and AACAACTGACGCGGCCTGCC (R)

hIL6 CATTCTGCCCTCGAGCCCACC (F) and GGCAGCAGGCAACACCAGGA (R)

hIL8 CGTGGCTCTCTTGGCAGCCTTC (F) and TTCCTTGGGGTCCAGACAGAGCTC (R)

hTLR4 CCCTGCGTGGAGGTGGTTCCTA (F) and CTCCCAGGGCTAAACTCTGGATGGG (R)

hMMP1 GTGTCTCACAGCTTCCCAGCGAC (F) and GCACTCCACATCTGGGCTGCTTC (R)

mActβ GCCAACCGTGAAAAGATGACC (F) and GAGGCATACAGGGACAGCAC (R)

mArg1 GTGGGGAAAGCCAATGAAGAG (F) and TCAGGAGAAAGGACACAGGTTG (R)

mCd206 TGTGGAGCAGATGGAAGGTC (F) and TGTCGTAGTCAGTGGTGGTTC (R)

mCcr2 GTAGTCACTTGGGTGGTGGC (F) and TACAGCGAAACAGGGTGTGG (R)

mCx3cr1 ACTCCGGTCTCATTTGCAGG (F) and GGGACCTCTGTAGGAGCAGA (R)

mTnf-α CCCTCACACTCAGATCATCTTCT (F) and GCTACGACGTGGGCTACAG (R)

mIl-4 GTACCAGGAGCCATATCCACG (F) and CGTTGCTGTGAGGACGTTTG (R)

mIl-10 GGACAACATACTGCTAACCGAC (F) and CCTGGGGCATCACTTCTACC (R)

2.15 Immunofluorescence analysis

Caco-2 cells were cultured on Transwells® for differentiation. After 21 days, cells were cultured under LPS or OGD conditions and treated with HM sEVs or ω3 OXLP for 24 h. The next day, cells were fixed in 4% paraformaldehyde for 10 min and after washing with PBS, cell were permeabilized and blocked with 5% BSA and 0.1% Triton X-100 in PBS for 1 h. Mouse anti-human occludin (Santa Cruz, E-5) and rat anti-human E-cadherin (EMD Millipore, DECMA-1) were used at a concentration of 1/200 overnight. Secondary antibodies used were: goat anti-mouse IgG (1:500, Alexa Fluor® 488, Abcam) and goat anti-rat IgG (1:500, Alexa Fluor® 555, Abcam). DAPI (4',6-diamidino-2-fenilindol) was used for stain nuclei. Quantification of mean fluorescence intensity (MFI) was performed using ImageJ.

2.16 Pyrogen test assay

An *in vitro* pyrogen test using PBMCs was used to detect substances that activate human immune cells to express pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IL-8 by qPCR. PBMCs (4×10^6 cells/mL) were incubated with HM sEVs and ω3 OXLP for 5 h. LPS at concentration of 1 μg/mL was used as a positive control.

2.17 Mice

Adult male Balb/c mice (6 weeks old, 18–22 g) were purchased from Envigo (Inotiv Inc., Indianapolis, Indiana, USA), and maintained under standard laboratory conditions. All animal procedures were approved by institutional ethical and animal care committees.

2.18 TNBS-induced colitis

Colitis, a type of IBD, was induced using 2,4,6-trinitrobenzenesulfonic acid (TNBS) by an intrarectal administration of 3.5 mg/mice of TNBS (Sigma-Aldrich) dissolved in 100 μL of 40% ethanol, as described (32). The sham group received 100 μL of 40% ethanol. Mice were treated by oral gavage with 50 μg of HM sEVs or 0.5 μg of ω3 OXLP prepared in 100 μL of PBS. The untreated TNBS group only received 100 μL of PBS. Treatment was administrated just

after colitis induction and at day 1 and 2 thereafter. After 4 days of colitis induction, mice were sacrificed by cervical dislocation. Colons were removed and their length was measured. Tissue was fixed in 4% paraformaldehyde acid and embedded in paraffin for immunohistochemistry or frozen in liquid nitrogen for protein and RNA extraction.

2.19 Production of oxylipins preparation for *in vivo* assays

Oxilipins can be conjugated with albumin to make them more accessible for cellular uptake. For *in vivo* assays oxylipins were prepared as described before (33). First, 10% fatty acid-free bovine serum albumin (FAF-BSA, Sigma-Aldrich) was dissolved into PBS, shaken for 3 h at room temperature and filtered through a 0.22-µm filter. For every ω3 OXLP dose, 0.5 µg of a mixture of 14 HDHA, 17 HDHA and 19-20 DiHDPA at the same concentration each, was prepared together on 100 µL of PBS supplemented with 10% of FAF-BSA and stirred for 16 h at 37°C. Oxylipins were freshly prepared before experiments. Mice received three doses of ω3 OXLP, a cumulative dose of 1.5 µg/mouse.

2.20 Myeloperoxidase activity

For detection of myeloperoxidase (MPO) activity, protein was extracted by homogenizing colon tissue and Colorimetric Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) was used according to manufacturer's instructions. Optical density was measured at 412 nm in a micro-plate reader. MPO activity was expressed as U/µg protein.

2.21 Cytokine protein array

Colon samples were homogenized in PBS with protease inhibitors. Samples from each group were pooled and then a BCA assay was performed. A normalized protein content was analyzed with the Proteome Profiler Mouse Cytokine Array Kit, Panel A, (R&D systems, Inc., Minneapolis, Minnesota, USA). The array membrane was blocked for 1 h and then washed. Colon samples and the array detection antibody cocktail were mixed and added to the blocked membrane followed by overnight shaking at 4°C. Membranes were washed and incubated for 30 min with streptavidin-HRP buffer. After washing, a chemiluminescence reagent mix was added and measurements were performed using an Amersham Imager 600 (GE Healthcare) and quantified with ImageJ.

2.22 Measurement of cytokines by ELISA

Supernatants from *in vitro* macrophage differentiation, supernatants from colonic tissue homogenized, and the mice plasma were collected and used to measure the levels of TNF-α and IL-10. Commercial ELISA kits (Invitrogen, Waltham, MA, USA) were used to quantify these cytokines, according to the manufacturer's instructions.

2.23 Mouse histology and immunofluorescence

Paraffin-embedded colon samples were cut into 5-µm-thick sections and stained with hematoxylin-eosin (Sigma-Aldrich) to evaluate inflammatory infiltrates, the presence of ulceration and the lesion of crypts. In addition, a blind pathological examination was carried out and tissues were scored using the histological colitis scoring method described before (34–36). This score tests for three tissue characteristics: inflammation severity, crypt damage and colon wall thickness; all three relativized to the percentage involvement. The score pathology was calculated as the sum of each characteristic multiplied by the percent involvement. The total maximum score is 40. To evaluate fibrosis, a Picro-Sirius Red stain (Direct Red 80 and Picric Acid, Sigma-Aldrich) was developed. Slides were visualized on a Leica DMD108 Digital Microscope (Leica Microsystems). For immunofluorescence, slides were blocked with 5% normal goat serum and 0.1% Triton X-100 in PBS for 1 h. Slides were then incubated with rabbit anti-MUC2 (dilution 1/200, Invitrogen, PA5-21329), rat anti-F4/F80 (dilution 1/200, Abcam, ab6640), rabbit anti-CD206 (dilution 1/200; Abcam, ab64693) or rabbit anti-CD274 (dilution 1/200, AB Clonal A11273) overnight in a humidified chamber at 4°C. After washing with PBS, slides were incubated with secondary antibodies: anti-rat IgG Alexa 555 or anti-rabbit IgG Alexa 488 for 1 h. After washing, DAPI was used to stain cell nuclei and FluorSave™ Reagent (Merck Millipore) to mount the slides. The sections were observed and visualized on a Leica DM2500 fluorescent microscope (Leica Microsystems). Final image processing and quantification were performed with ImageJ by counting green and red spots in the fixed area.

2.24 Statistical analysis

Data are expressed as mean ± SD (standard deviation) or standard error of the mean (SEM), as specified. Student's t-test was used for unpaired samples in the comparison between groups. To compare means of more than two groups, one-way analysis of variance (ANOVA). To study the effect of two factors simultaneously, a two-way ANOVA was used. Analyzes were conducted with GraphPad Prism 8 software (San Diego, CA, USA). Differences were considered statistically significant at $p< 0.05$ with a 95% confidence interval.

3 Results

3.1 Isolation and characterization of HM sEVs

sEVs were isolated from HM by sequential centrifugation and filtration (25). Purified sEVs showed a median number of particles of 1.3 x 10^{11} and a median size of 158 nm, as determined by NTA (Figure 1A). We also used DLS to measure the size of vesicles, the ζ potential (which gives an indication of the potential stability of the colloidal system), and the PDI, which is used to characterize the size distribution of sEVs. The ζ potential was -7.7 ± 1.0 mV, which

represents an incipient instability of the system, so it cannot be stored for a long time, or the particles will tend to aggregate. The PDI was 0.390 (Figure 1B), indicating a relatively even size distribution of sEVs. WB revealed that the sEVs expressed the typical markers Hsp70, CD63, TSG101, CD81 and CD9 (Figure 1C), but were negative for the endoplasmic reticulum protein calnexin. Finally, transmission electron microscopy analysis of sEVs revealed a round or cup-shaped morphology and the size was consistent with the findings of NTA (Figure 1D).

3.2 Quantification and comparison of oxylipins in HM sEVs

Quantification of oxylipins from HM sEV samples was performed by means of a validated LC-MS and multiple reaction monitoring. Of the different oxylipins identified, the following could be quantified both in HM sEVs: 9,10-DiHOME, 12,13-DiHOME, 14-HDHA, 17-HDHA, 19,20-DiHDPA. Moreover, 14,15-DiHETE, 14,15-DiHETrE, 17,18-DiHETE, PGE_2, and $PGF_{2\alpha}$ (Table 2). The most abundant oxylipins were 9,10-DiHOME, 12,13-DiHOME, 19,20-DiHDPA, 14-HDHA and 17-HDHA (Figure 1E). In general, HM-derived sEVs showed higher concentration of DHA-derived oxylipins than LA-derived oxylipins. The former have been reported to have anti-inflammatory activity, and the latter show pro-inflammatory activity (Figures 1E, F) (10). Based on these results, we investigated whether the protective effects of HM-derived sEVs could be partly attributed to the presence of the three ω3-derived oxylipins – 19,20-DiHDPA, 14-HDHA and 17-HDHA – hereafter referred to as ω3 OXLP.

3.3 Protective effects of HM sEVs and ω3 OXLP on intestinal epithelial cells under stress and ischemic conditions

The main risks for developing NEC are known to be a weak immune system, which increases the presence of infection, and lack of blood flow reaching the colon to supply intestinal cells with oxygen and nutrients, preventing their maturation (37). To emulate these conditions *in vitro*, Caco-2 intestinal epithelial cells were stimulated with LPS at 60 µg/mL or were cultured in

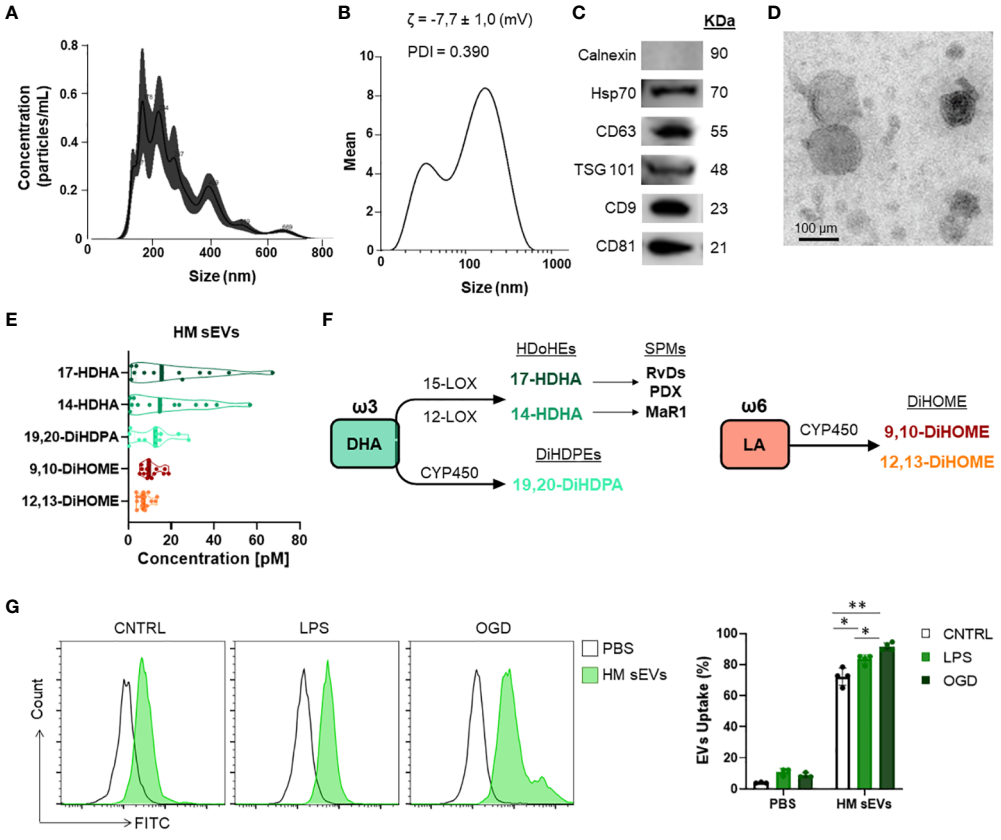

FIGURE 1
Characterization of HM sEVs and oxylipin content. **(A)** Representative images of HM sEVs assessed by nanoparticle tracking and **(B)** DLS analysis; **(C)** representative western blots of Hsp70, CD63, TSG101, CD81 and CD9 proteins in 30 µg of HM sEVs; absence of calnexin signifies a pure sEVs preparation **(D)** representative transmission electron microscopy images of HM sEVs. Scale bar: 200 nm; **(E)** concentration [nM] of the more abundant oxylipins in HM sEVs isolated from 25 mL of HM; **(F)** scheme of oxylipin synthesis; **(G)** Intestinal epithelial cells were incubated with CFSE-labeled HM sEVs for 3 h at 37°C and sEV internalization was assessed by flow cytometry. As a negative control, PBS was mixed with CFSE and added to cells in parallel. Representative histograms are shown. sEV internalization was measured by fluorescence intensity and is represented as the percentage of sEV uptake. Graphs represent mean ± SD of four independent experiments. Two-way ANOVA was used for statistical analysis. *p< 0.05, **p< 0.01.

TABLE 2 Quantification of oxylipins. Calibration range, linear coefficient of determination (R2), limit of detection (LOD), lower limit of quantification (LLOQ), mean concentration in HM sEVs.

Oxylipin	Calibrated range (nM)	R^2	LOD (nM)	*LLOQ (pM)	HM sEVs Mean ± SD (pM)
17-HDHA	0.29 - 300	0.996	0.09	0.9	21.02 ± 19.64
14-HDHA	0.15 - 300	0.996	0.04	0.5	18.65 ± 17.51
19,20-DiHDPA	0.07 - 300	0.995	0.02	0.2	11.32 ± 8.50
9,10-DiHOME	0.29 - 300	0.998	0.09	0.9	10.61 ± 4.16
12,13-DiHOME	0.15 - 300	0.995	0.04	0.5	7.46 ± 3.09
17,18-DiHETE	0.15 - 300	0.994	0.04	0.5	1.8 ± 1.1
14,15-DiHETRE	0.07 - 300	0.994	0.02	0.2	0.8 ± 0.3
$PGF_{2\alpha}$	0.07 - 300	0.995	0.02	0.2	0.7 ± 0.3

*referred to HM sEV encountered in the HM sample.

OGD to mimic an ischemic environment. We used an internalization assay with CFSE-stained HM sEVs to question how stress and ischemic conditions affected the uptake of HM sEVs by intestinal cells. Uptake of HM sEVs was observed in 72.3 ± 5.5% of intestinal cells 3 h after their addition to cultures (Figure 1G), and this was increased by 11.2% and 19.3%, respectively, when cells were treated with LPS and OGD (Figure 1G). Notably, cell death increased in Caco-2 cells treated with LPS or OGD, likely due to an increase in cytotoxicity and oxidative stress (ROS) (Figure 2). To assess the protective effect of HM sEVs and ω3 OXLP, Caco-2 cells were treated with 7.5 μg/mL of HM EVs or 0.5 nM of each of the three oxylipins. Both HM sEVs and ω3-OXLP protected Caco-2 cells from LPS-induced damage, improving cell viability over non-treated cells (Figure 2A). Treatment with HM sEVs and ω3 OXLP also decreased cytotoxicity (Figure 2B) and oxidative stress (Figure 2C). Similar results were found under OGD conditions with respect to cell death (Figure 2D). However, only ω3 OXLP treatment had a significant protective effect against cytotoxicity (Figure 2E) and oxidative stress (Figure 2F) generated by OGD. We next tested whether the cell injury triggered by LPS and OGD also affects migration. Indeed, a major concern of PIs with NEC is the presence of "wounds" in the intestine due to the lack of tissue maturation. If the wounds are not repaired the prognosis for the PIs is poor (38). To investigate whether HM sEVs and ω3 OXLP modulate the migration of intestinal epithelial cells, we used an *in vitro* scratch-wound assay. Results showed that wound closure was slower in cells treated with LPS and OGD *vs* control cultures. Treatment with HM sEVs or ω3 OXLP restored their migratory capacity and proliferation rate, promoting the development of a continuous monolayer (Figures 2G, H).

3.4 Modulation of pro-inflammatory genes and tight junction proteins by HM sEVs and ω3 OXLP in inflammatory conditions

Inflammatory responses triggered by LPS or hypoxia in the intestinal epithelium trigger the upregulation of pro-inflammatory genes such as tumor necrosis factor alpha (*TNF-α*) and cyclooxygenase-2 (*COX-2*). TNF-α is involved in the pathogenesis of IBD by increasing intestinal cell death and detachment in the gut, which damages the integrity of the epithelial barrier (39). COX-2, an enzyme that accelerates inflammation, also plays a role in the pathophysiological processes of intestinal inflammation (40). As expected, both LPS and OGD increased the expression of these genes in Caco-2 cells, whereas co-treatment with 7.5 μg/mL of HM sEVs or 0.5 nM of each of the three ω3 OXLP significantly reduced their expression (Figures 3A, B). The intestinal epithelium contains tight junctions that link neighboring cells to create a barrier preventing the free flow of substances between cells (38). Tight junctions are made up of proteins such as occludins (OCLN) and claudins (CLND). Results showed that stimulation of the intestinal epithelium with LPS or OGD decreased the expression of *OCLN* and *CLND*, whereas co-treatment with HM sEVs or ω3 OXLP increased their expression (Figures 3A, B). We validated this by immunofluorescence. LPS and OGD treatment decreased the expression of tight junction proteins (E-cadherin (E-CADH) in red and occludin in green), whereas co-treatment with HM sEVs or ω3 OXLP restored their expression and the architecture and cohesion of the intestinal epithelium (Figures 3C, D).

3.5 Modulation of pro-fibrotic genes and inhibition of fibroblast migration by HM sEVs and ω3 OXLP

Fibrosis is a pathological feature of most chronic inflammatory diseases, whereby fibroblast proliferation and migration lead to the excessive deposition of fibrous connective tissue, reducing its functionality (41). LPS activates fibrosis, modulating the release of inflammatory cytokines and increasing fibroblast proliferation and migration (42, 43). Results showed that the expression of the pro-inflammatory genes *TNF-α*, transforming growth factor beta (*TGF-β*), interleukin (*IL*)*-1* and *IL-6* increased significantly 24 h after LPS stimulation of fibroblasts. Treatment of LPS-activated fibroblasts with 7.5 μg/mL of HM sEVs or 0.5 nM of each of the three ω3 OXLP decreased the expression of these genes significantly (Figure 4A). In

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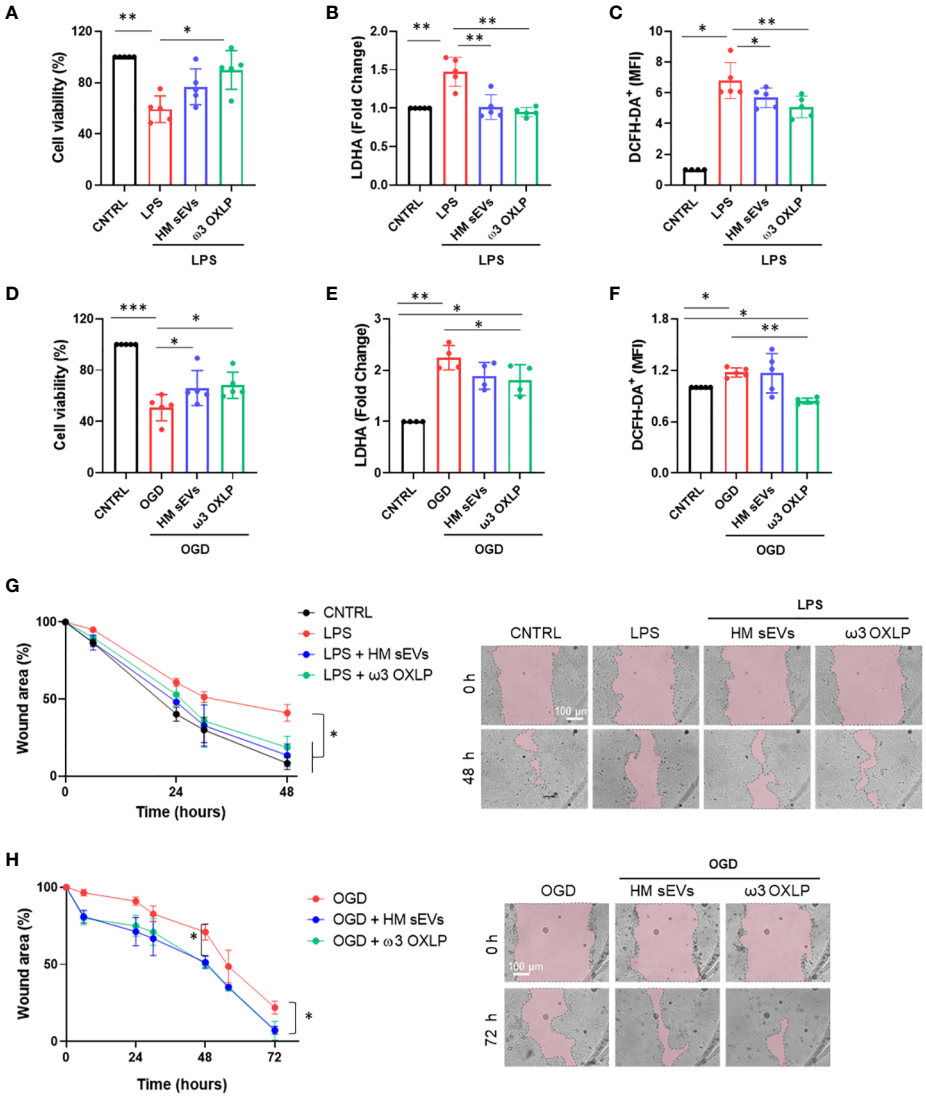

FIGURE 2
HM-derived sEVs and ω3 oxylipins protect intestinal epithelial cells from damage. **(A)** Quantification of cell viability measured by CCK8 assay **(B)**; cell cytotoxicity measured by LDH assay and **(C)** Reactive oxygen species (ROS) production measured by DCFH-DA oxidation in intestinal cells stimulated with lipopolysaccharides (LPS) (100 ng/mL) or oxygen/glucose deprivation (OGD) **(D–F)**. One-way ANOVA was used for statistical analysis. Quantification of intestinal cell wound area **(G)** after LPS (100 ng/mL) or OGD **(H)** treatment. Data were normalized to initial wound area and represented as mean percentage ± SD. One-way ANOVA was used for statistical analysis at different points. Representative brightfield images of wound healing assay at different times (0 and 48 or 72 h) are shown (pink area represents opened wound). Images were taken at 10× magnification. Scale bar: 100 µm. Experiments were performed in triplicate. *p< 0.05, **p< 0.01, ***p<0.001.

addition, the levels of other classical pro-fibrotic genes, toll-like receptor (*TLR*)-*4* and matrix metallopeptidase (*MMP*)*1*, were higher after LPS stimulation, and their expression was normalized after HM sEVs or ω3 OXLP treatment (Figure 4A). To test whether the changes in gene expression correlated with an anti-fibrotic response, the effect of HM sEVs and ω3 OXLP on fibroblast migration was assessed in scratch-wound assays. Stimulation with LPS promoted fibroblast migration and wound closure (37.8 ± 8.4%) of free area in LPS-treated cultures *vs* (57.5 ± 4.5%) in control cultures at 24 h. Contrastingly, the addition of HM sEVs and ω3 OXLP to LPS-activated fibroblasts reduced their migration, reaching levels similar to control cultures (Figure 4B).

3.6 Effects of HM sEVs and ω3 OXLP on inflammatory signaling pathways, T-cell activation, and macrophage polarization

Immune system cells, and more specifically macrophages, play a pivotal role in the pathogenesis of NEC, orchestrating both the inflammatory response and tissue repair processes (44, 45). To study the effect of HM sEVs or ω3 OXLP on immune system cells, we performed different *in vitro* assays. First, 7.5 µg/mL of HM sEVs or 0.5 nM of each of the three ω3 OXLP were added to PBMCs to test whether they generated an immune response, activating the upregulation of pro-inflammatory cytokines genes *TNF-α*, *IL-1β*,

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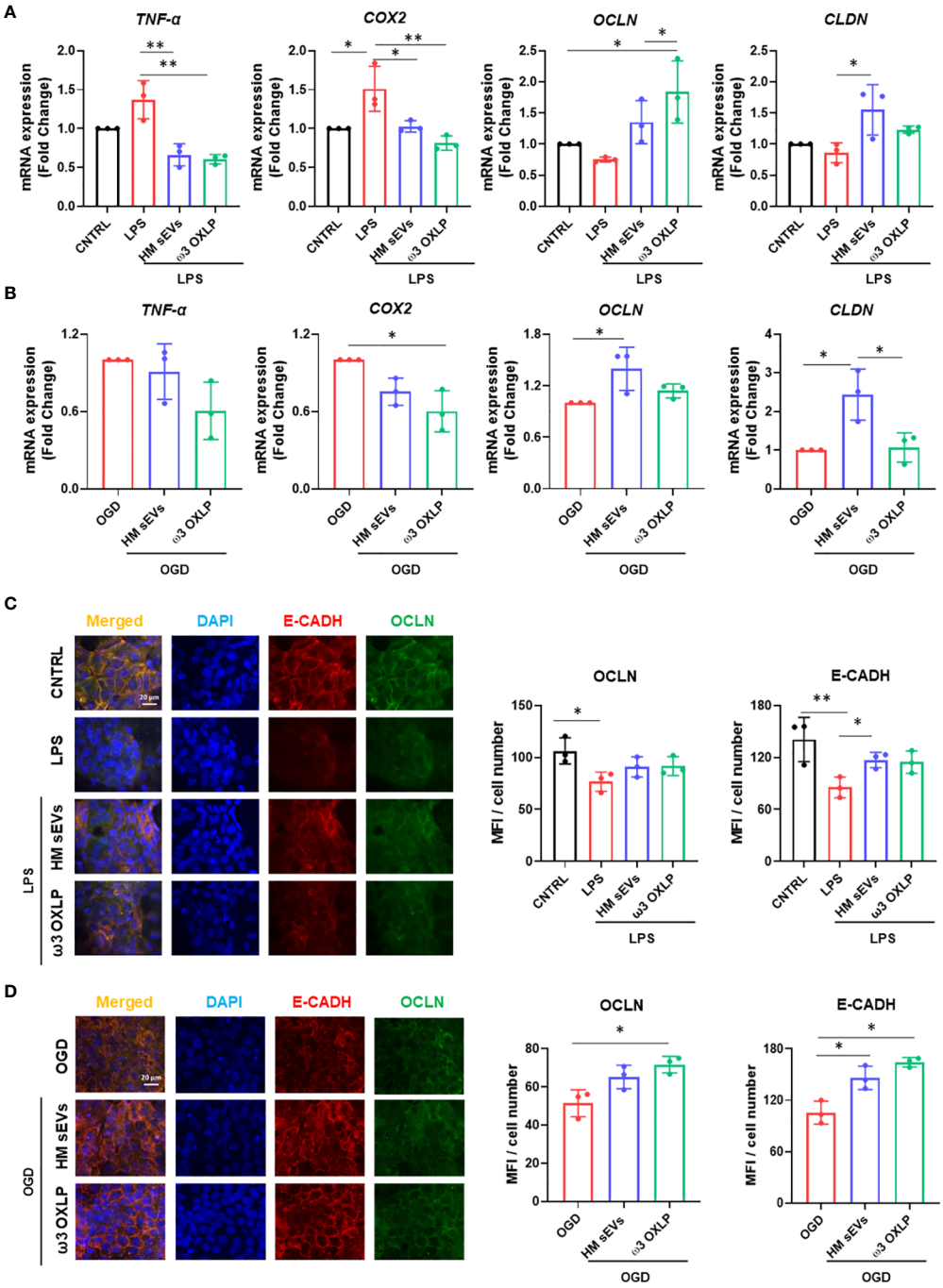

FIGURE 3
HM sEVs and ω3 OXLP dampen inflammatory responses in the inflamed epithelium. **(A)** Expression levels of *TNF-α*, *COX-2*, *OCLN* and *CLND* quantified by RT-qPCR in intestinal cells stimulated with lipopolysaccharides (LPS) and/or treated with 7.5 μg/mL sEVs or 0.5 nM of each of the three ω3 OXLP. **(B)** E-cadherin (E-CADH, red) and occludin (OCLN, green) immunofluorescence and nuclei staining (blue) show the distribution of tight junctions in the cell membrane. Unstimulated intestinal cells were used as controls. **(C)** Expression levels of *TNF-α*, *COX-2*, *OCLN* and *CLND* quantified by RT-qPCR in intestinal cell cultures under oxygen/glucose deprivation (OGD) condition and/or treated with 7.5 μg/mL sEVs or 0.5 nM of each of the three ω3 OXLP. The expression level of the target gene in each sample was normalized to *GAPDH* expression. **(D)** E-cadherin (E-CADH, red) and occludin (OCLN, green) immunofluorescence and nuclei staining (blue) show the distribution of tight junctions in the cell membrane. Scale bar: 20 μm. The bar graph shows the quantification of the mean fluorescence intensity (MFI). The graph represents the mean ± SD of three independent experiments. One-way ANOVA was used for statistical analysis. *p< 0.05, **p< 0.01.

IL-6, and *IL-8*. Results showed that ω3 OXLP did not activate proinflammatory signaling pathways with respect to non-stimulated control PMBCs, indicating that they are not immunogenic. However, the addition of HM sEVs resulted in a slight increase in the expression of *IL-1β*, *IL-6* and *IL-8* in PBMCs, although to a lesser extent than LPS (positive control) (Figure 5A). Second, we developed a T-cell activation and proliferation assay. Addition of ω3 OXLP to T-cells caused a slight reduction in their

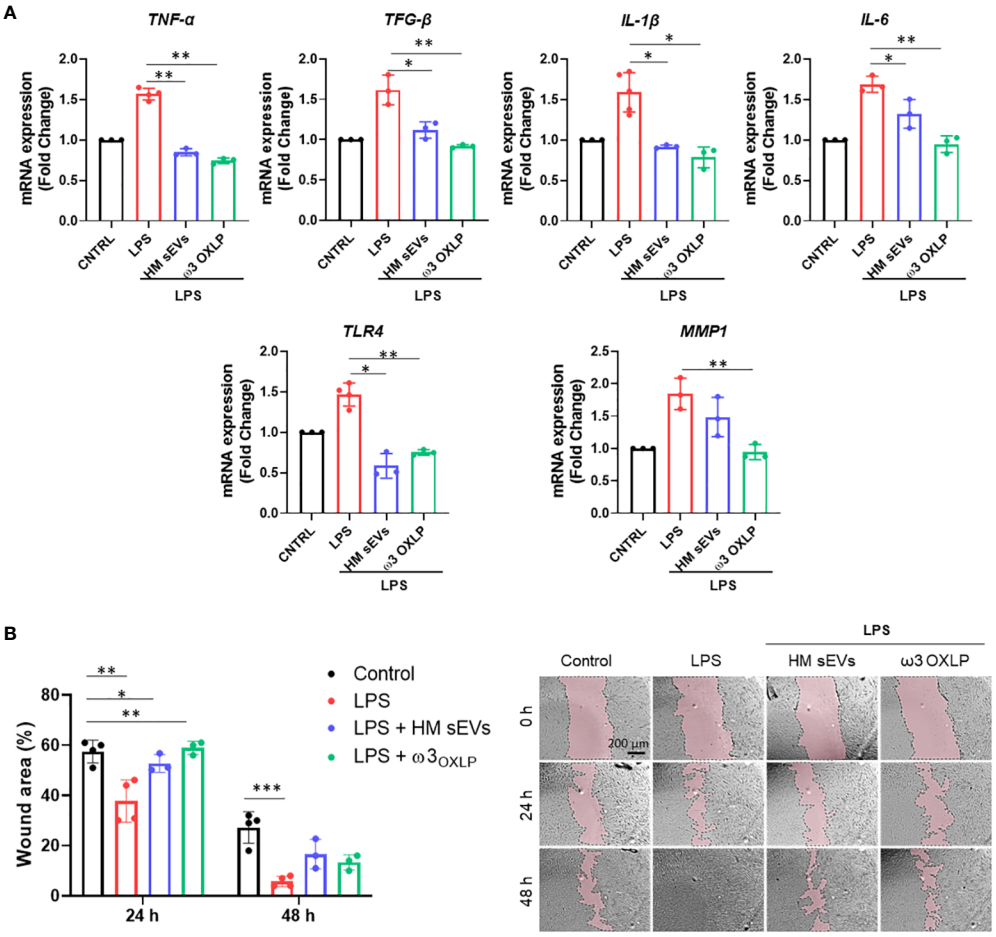

FIGURE 4
HM sEVs and ω3 OXLP prevent LPS-induced fibrosis. **(A)** Expression levels of *TNF-α, TGF-β, IL-1β, IL-6, TLR4* and *MMP1* quantified by RT-qPCR in fibroblasts stimulated with lipopolysaccharides (LPS) and/or treated with 7.5 µg/mL sEVs or 0.5 nM of each of the three ω3 OXLP. Unstimulated fibroblasts were used as controls. The expression level of the target gene in each sample was normalized to *GAPDH* expression. represented as mean percentage ± SD. **(B)** Quantification of fibroblast wound closure at 24 and 48 h. Data were normalized to initial wound area and are represented as mean percentage ± SD. Representative brightfield images of wound healing assay at different times (0, 24 and 48 h) after wound generation on a monolayer fibroblast culture stimulated with LPS alone or treated with 7.5 µg/mL sEVs or 0.5 nM of each of the three ω3 OXLP. Images were taken at 10× magnification. Scale bar: 200 µm. Experiments were performed in triplicate. One-way ANOVA was used for statistical analysis. *p< 0.05, **p< 0.01, ***p< 0.001.

proliferation, whereas HM sEVs treatment appeared to increase proliferation (Figure 5B). Third, to study the ability of HM sEVs or ω3 OXLP to modulate Mφ polarization, we differentiated monocytes to Mφ type 1 (Mφ1, pro-inflammatory) or type 2 (Mφ2, pro-resolutive). During the differentiation to Mφ1, some cultures were treated with HM sEVs or ω3 OXLP and surface markers were compared against non-treated Mφ1 and Mφ2 by flow cytometry. Results showed that the percentage of $CD14^{+}CD163^{+}$ cells, representative of a classical Mφ2 phenotype, was not modified by HM sEVs or ω3 OXLP treatment (Figure 5C). Contrastingly, when the expression of cell surface receptors on differentiated and LPS-stimulated Mφ1 were analyzed, we observed that treatment with HM sEVs significantly reduced the expression of the co-stimulatory molecules CD80 and CD86, and also HLA-DR expression to levels seen in Mφ2. Treatment with the ω3 OXLP also reduced the expression of all three markers, although to a lesser extent (Figure 5C). To confirm the ability of HM sEVs and ω3 OXLP to induce Mφ polarization, we measured the levels of proinflammatory TNF-α and anti-inflammatory IL-10 cytokines in the culture medium of Mφ. Mφ1 released a large amount of TNF-α and low levels of IL-10, and the opposite occurred with Mφ2 (Figure 5D). Treatment of Mφ1 with HM sEVs resulted in a profile more similar to Mφ2, with a reduced amount of TNF-α and a higher amount of IL-10; and treatment with ω3 OXLP significantly reduced released TNF-α but failed to alter IL-10 release by Mφ1 (Figure 5D).

3.7 Therapeutic potential of HM sEVs and ω3 OXLP in an experimental model of inflammatory bowel disease

The evident beneficial effects of HM sEVs and ω3 OXLP *in vitro* motivated us to test their therapeutic potential in an IBD model

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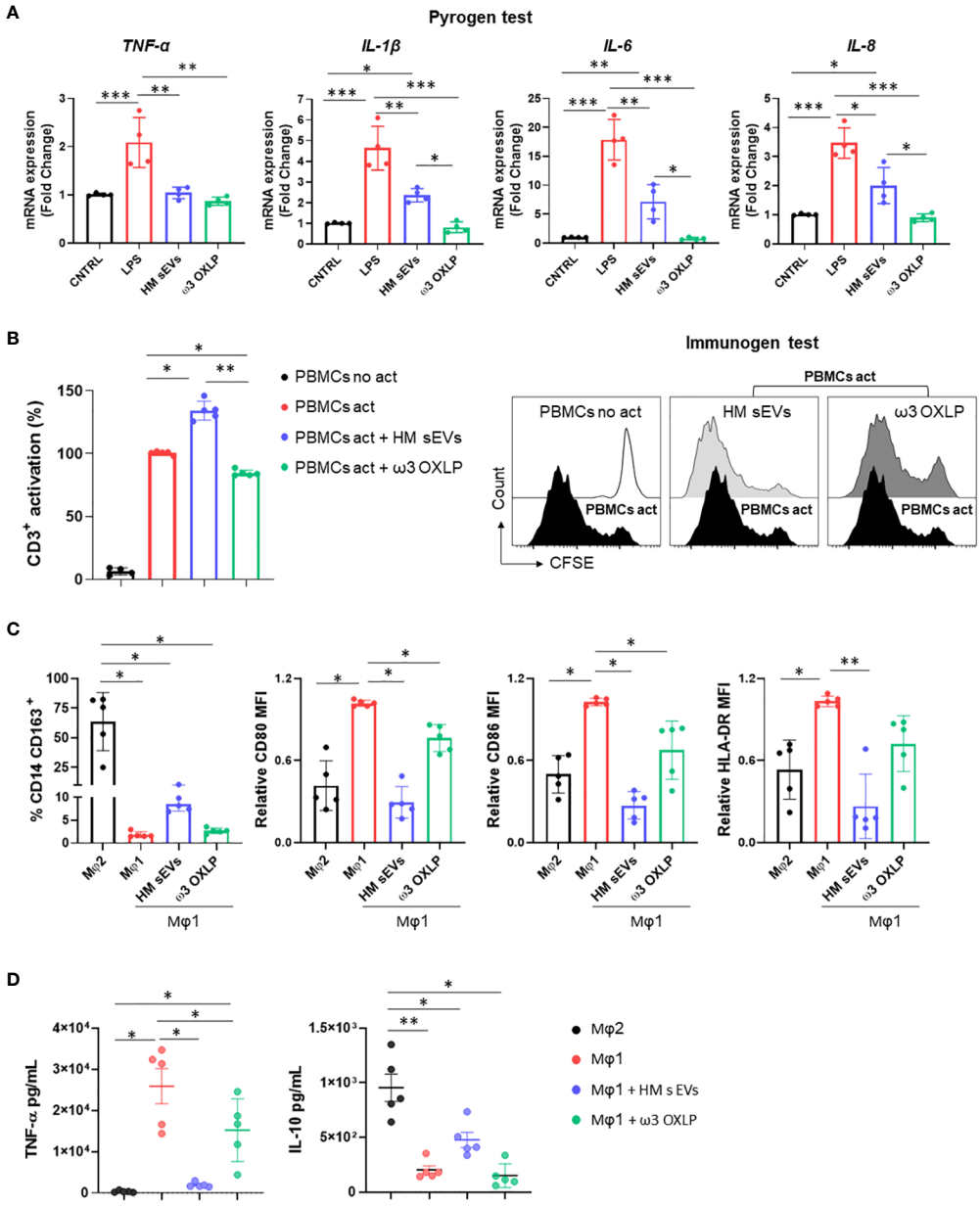

FIGURE 5
Response of HM sEVs and ω3 OXLP on immune system cells. **(A)** Expression of proinflammatory genes (*TNF-α*, *IL-1β*, *IL-6* and *IL-8*) in peripheral blood mononuclear cells (PBMCs) cultured for 6 h with treatments (HM sEVs and ω3 OXLP). Unstimulated and lipopolysaccharides (LPS)-stimulated PBMCs were used as negative and positive controls, respectively. The expression level of the target gene in each sample was normalized to *GAPDH* expression. The graphs represent the mean ± SD of four independent experiments. **(B)** PBMCs were stained with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with anti-CD3 and anti-CD28 in the presence or absence of HM sEVs or ω3 OXLP. After 5 days, cells were stained with anti-CD3 antibody and T-cell proliferation was determined by flow cytometry measuring CFSE dilution. Suppression (percentage) was calculated from the expansion index. The graphs represent the mean ± SD of four independent experiments. Representative histograms are shown. **(C)** Monocytes were differentiated to Mφ1 with treatment (HM sEVs and ω3 OXLP). Differentiation to Mφ1 and Mφ2 was used as a reference of pro-inflammatory and pro-resolving macrophages, respectively. After 5 days of differentiation, the percentage of $CD14^+$ and $CD163^+$ cells was assessed by flow cytometry. After LPS activation, CD86, CD80 and HLA-DR expression was assessed by flow cytometry. The mean relative fluorescence intensity (MFI) was calculated by dividing all individual data by the mean expression in Mφ1. **(D)** TNF-α and IL-10 production by Mφ was determined by ELISA 16 h after LPS stimulation. Graphs represent the mean ± SD of five independent experiments. One-way ANOVA was used for statistical analysis. *p< 0.05, **p< 0.01, ***p<0.001.

using TNBS administered intrarectally to induce severe colonic inflammation in mice (46). Balb/c mice were divided into four groups: a healthy sham group, an untreated TNBS group, a treated TNBS group with 50 μg of HM sEVs and a treated TNBS group with a cumulative dose of 1.5 μg of ω3 OXLP. Treatments were dissolved in 100 μL of PBS and were orally administered by gavage just after induction of acute colitis by TNBS and at 24 and 48 h later. The sham group was treated with 100 μL of vehicle (PBS). On the fourth day, mice were sacrificed, and the regenerative and anti-inflammatory effects of the treatments were assessed.

We monitored weight loss of mice across the experiment (Figure 6A). The sham group showed no weight loss, whereas the

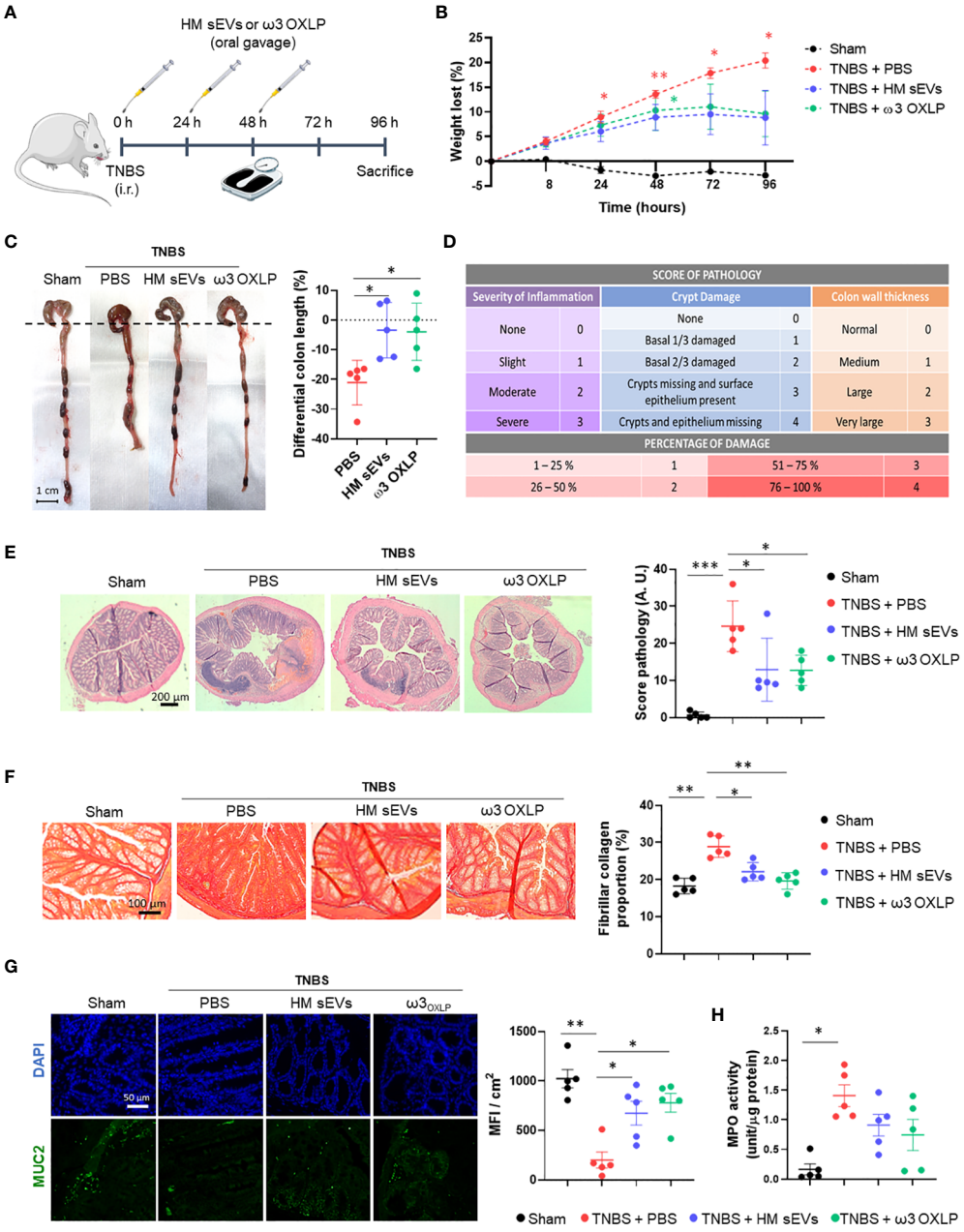

FIGURE 6
HM sEVs and ω3 OXLP attenuate disease in mice with TNBS-induced colitis. **(A)** Scheme of the *in vivo* experimental design. **(B)** Measurement of the weight loss of mice throughout the experiment (4 days). **(C)** Macroscopic images of colon tissue on day 4 after 2,4,6-trinitrobenzenesulfonic acid (TNBS) administration. Scale bar: 1 cm. Percentage differential length of the colon compared with the healthy group (horizontal dotted line). **(D)** Histology score table based on grade of pathology and percentage of damage. **(E)** Hematoxylin and eosin staining of representative histological sections of the colon of mice in the healthy group and in the PBS, sEVs and ω3 OXLP groups after TNBS administration. Scale bar: 200 µm. **(F)** Sirius Red staining was used to detect collagen fibers. Scale bar: 200 µm. Fibrillar collagen proportion (%) was calculated by dividing the area stained with red by the total tissue area. **(G)** Immunofluorescence of MUC2 (green) and nuclei staining (blue). Scale bar: 50 µm. Bar graph shows quantification of green mean fluorescence intensity (MFI) per cm^2. **(H)** Myeloperoxidase (MPO) activity was measured in colon homogenates. Values were relativized by µg of protein tissue. The graph represents the mean ± SEM of five mice in each group. One-way ANOVA was used for statistical analysis. *p< 0.05, **p< 0.01, ***p< 0.001.

TNBS group lost almost 20% of their weight. Mice treated with HM sEVs and ω3 OXLP also showed weight loss; however, this stabilized on the third day, reaching a maximum of 10% loss at sacrifice (Figure 6B). Colon length was shorter in the TNBS group than in the sham group, whereas TNBS-induced mice treated with HM sEVs and ω3 OXLP showed protection against colon shortening (Figure 6C).

Examination of colonic histology revealed severe mucosal damage in the TNBS group, characterized by fewer intestinal glands, distortion of crypts and a huge inflammatory cell infiltration. By contrast, the TNBS group treated with HM sEVs and ω3 OXLP showed significant protection against histopathological damage and a preserved tissue architecture (Figures 6D, E). To investigate the pathways underlying colitis

recovery after treatment with HM sEVs and ω3 OXLP, we analyzed the presence of collagen fiber by Sirius Red staining. Chronic inflammation leads to intestinal fibrosis, causing tissue damage and difficulty in tissue regeneration with high deposits of extracellular matrix (47). As expected, the percentage of collagen in the colon was significantly higher in the TNBS group than in the sham group, whereas the groups treated with HM sEVs or ω3 OXLP showed significantly lower levels of collagen (Figure 6F), indicating that treatment with HM sEVs and ω3 OXLP alleviated intestinal fibrosis in colitis.

The intestinal mucosa is protected by a variety of glycoproteins known as mucins (MUC), which play a role in the mucociliary transport system by trapping pathogens in a mucin gel layer (48). To further explore the protective effects of ω3 OXLP from HM sEVs in experimental colitis, we investigated the expression of mucin-2 (MUC2) by immunofluorescence. Results demonstrated that treatment with HM sEVs or ω3 OXLP maintained MUC2 expression in TNBS-induced mice (Figure 6G). Because a correlation between disease severity in IBD patients and neutrophil infiltration has previously been reported (49), we used a MPO assay to assess neutrophil activity. MPO activity was significantly higher in the TNBS group than in the sham group, and treatment with HM sEVs or ω3 OXLP resulted in a trend for decreased neutrophil activity (Figure 6H).

3.8 Modulation of immune response and cytokine expression by HM sEVs and ω3 OXLP in TNBS-induced colitis

An imbalance between proinflammatory and anti-inflammatory immune cells and cytokines is a key characteristic of IBD, which hinders the resolution of inflammation. To assess the modulation of immune responses by HM sEVs and ω3 OXLP, we examined cytokine expression in colon tissues of treated mice. Cytokine protein arrays revealed that the levels of several cytokines were higher in the untreated TNBS group than in the sham group, including intercellular adhesion molecule (ICAM)-1, tissue inhibitors of metalloproteinase (TIMP)-1, CC motif chemokine ligand (CCL)2, CXC motif chemokine ligand (CXCL) 9, CXCL13, CXCL1, IL-1β, triggering receptor expressed on myeloid cells (TREM)-1, IL-1α, CXCL11, IL-17, and TNF-α. By contrast, the TNBS group treated with HM sEVs or ω3 OXLP showed significantly lower levels of these cytokines, with some approaching the levels seen in the sham group. Notably, the colitis-induced group treated with HM sEVs or ω3 OXLP had elevated levels of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (IL-1Ra) (Figure 7A).

To further evaluate the immune response, we examined immune cell infiltrates in colon tissue. mRNA expression levels of pro-inflammatory cytokines (*Tnf-α* and *Il-6*) were significantly lower in the groups treated with HM sEVs and ω3 OXLP than in the untreated TNBS group, whereas the opposite pattern was seen for the anti-inflammatory cytokine *Il-10*. Analysis of Mφ2-associated genes: *Arginase* (*Arg*1), *Cd206, CC motif chemokine receptor (Ccr2), and C-X3-C motif chemokine receptor* (*Cx3cr1*) (50), also revealed an increase in the groups treated with HM sEVs and ω3 OXLP (Figure 7B). We also measured IL-17A and IL-10 in plasma and colonic tissue by ELISA. The pro-inflammatory cytokine IL-17A was elevated in the TNBS group, but its levels were lower in mice co-treated with HM sEVs and ω3 OXLP. Conversely, IL-10 levels were lower in the TNBS group but were increased in TNBS mice co-treated with HM sEVs or ω3 OXLP, both in plasma and colon extracts (Figure 7C).

To gain further insight into the impact of the treatments on macrophage infiltration at the injury site during the disease, we performed an immunofluorescence assay using the classical macrophage marker F4/F80, combined with CD274 or CD206 to distinguish Mφ1 and Mφ2, respectively. The results demonstrated that the ratio of Mφ1 to Mφ2 was significantly higher in the untreated TNBS group than in the sham group, whereas treatment with HM sEVs and ω3 OXLP reversed this ratio, decreasing Mφ1 and increasing Mφ2 (Figure 7D). Overall, our findings indicate that HM sEVs and ω3 OXLP can mitigate the inflammatory response in TNBS-induced colitis by regulating immune cell infiltration and cytokine expression.

4 Discussion

HM is the best food for newborns and PIs, as it provides them with all the necessary nutrients in the right measures. Indeed, the World Health Organization recommends mothers to breastfeed infants for the first six months of life to achieve optimal growth, development, and health (51), and HM is an essential member of the complex biological system between mother and infant (52). In cases where breastfeeding is not possible or not chosen, infant formula may be a suitable alternative. However, while milk formula may provide adequate nutrition, it does not contain the immunological factors and other bioactive components present in HM, which provide additional protection against illness and promote optimal development. Recently, there has been renewed interest in bioactive lipids, as oxidized metabolites of PUFAs (oxylipins) have been detected in HM (53). Several oxylipins, especially those derived from ω-3 fatty acids (ω-3-PUFAs), have been found to have anti-inflammatory properties and might be protective against chronic diseases and inflammatory conditions (54, 55).

Recent clinical studies have demonstrated that formula feeding might constitute a risk for NEC in PIs (56). In this sense, supplementing HM is warranted. Here, we comprehensively investigated the presence of oxylipins derived from HM-sEVs and their therapeutic potential in the setting of intestinal inflammation. Our results support the idea of incorporating a combination of pro-resolving lipid mediators in milk formulations.

We show that HM-derived sEVs are loaded with 14-HDHA, 17-HDHA and 19,20-DiHDPA, that are pro-resolutive metabolites derived from the ω-3 fatty acid DHA and, in addition, both 14-

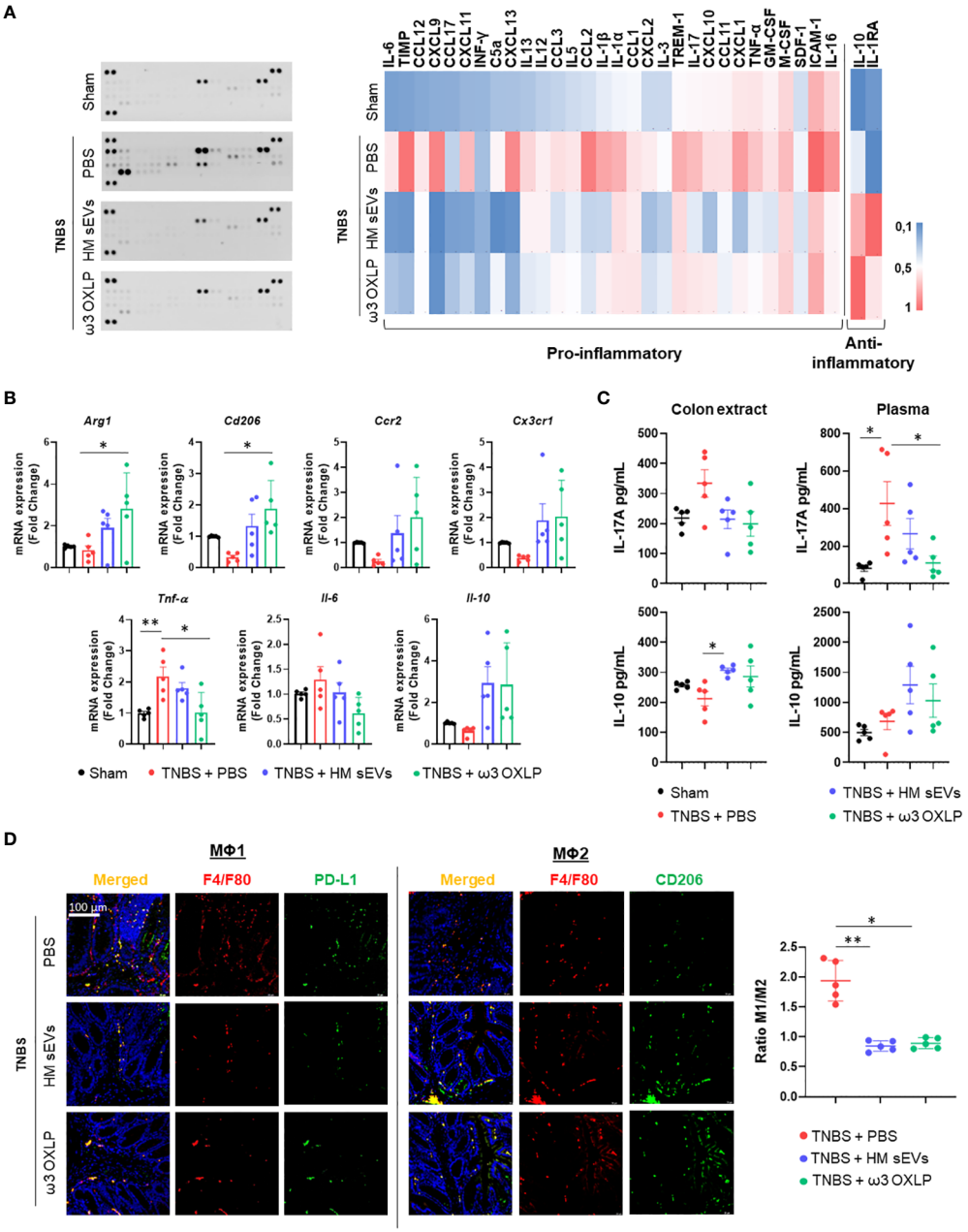

FIGURE 7
HM sEVs and ω3 OXLP change the ratio of infiltrating Mφ1/Mφ2. **(A)** Levels of inflammation-related cytokines were analyzed in colonic tissues by immunoblot array (left). Different time exposition was used to reveal different amounts of protein. The relative expression of each cytokine was quantified and represented in a heat map (right); data are representative of a pool of five animals per group. **(B)** *Arg1*, *Cd206*, *Ccr2*, *Cx3cr1*, *TNFα*, *Il-6*, and *Il-10* mRNA expression levels quantified by RT-qPCR in colon. Sham group was used as a control. Expression level of the target gene in each sample was normalized to *β-actin* expression. Graphs represent mean ± SEM of fold change of five independent experiments. **(C)** ELISA assay to assess IL-17A and IL-10 production (pg/mL) in colon extracts and plasma. **(D)** Immunodetection of F4/F80 (pan-macrophage marker, red) and PD-L1 (Mφ1, green) or CD206 (Mφ2, green) in colon samples 4 days after TNBS-induced colitis. Scale bar: 100 µm. Quantification of double-positive cells per mm^2. Ten sections of 0.14 mm^2 per mouse were analyzed. Graphs represent the Mφ1/Mφ2 ratio ± SEM of five mice. One-way ANOVA was used for statistical analysis. *p< 0.05, **p< 0.01.

HDHA and 17-HDHA are precursors of SMPs; specifically, maresins and D-series resolvins, respectively (57). This may have a biological significance when considering HM-sEVs as therapeutic vehicles. Pizzinat et al. (58) previously reported the presence of lipid mediators in EVs derived from cardiomyocytes and mesenchymal stromal cells. However, they find a different oxylipin profile to the one found in HM-sEVs described in this work, probably because the source of EVs is different. Also, Chen et al. identified a total of 395 lipids in term and preterm HM-derived EVs (59), but no studies on oxylipins have thus far been reported.

We corroborated the utility of HM-sEVs for treating inflammatory disorders (60). Since their discovery (61), the interest in the role of HM-derived EVs in early development has gained increasing interest, particularly with regards to their role in

the gastrointestinal tract (14), and the contribution of HM-EVs to the maturation of the intestinal barrier has been studied in both physiological and pathological models (62, 63). Moreover, recent studies have shown that HM EVs are resilient to digestion and can be endocytosed by intestinal epithelial cells (16). In the present work, we show that HM-sEVs are taken-up by intestinal cells and that different damage stimuli (LPS or OGD) increase this process, pointing to a potential role for HM-sEVs in rescuing injured tissue from damage. In this regard, several studies have reported that milk derived EVs can ameliorate IBD in different *in vivo* models by suppressing immune cell infiltration and fibrosis, modulating MUC2 expression, reducing neutrophil activity, and promoting a pro-resolutive cytokine environment (19, 60, 64). However, the potential use of milk-derived EVs is limited by the need for donors and the lack of scale-up procedures that would allow cost-effective commercialization.

We then tested whether ω3 OXLP present in HM-sEVs could reproduce the four main effects that are exerted by HM-EVs their selves in *in vitro* and *in vivo* models: (i) cell survival and proliferation, (ii) integrity (cell-cell junctions), (iii) resolution of inflammation and (iv) mucin production (additional defence) (14, 18, 59). We demonstrate that ω3 OXLP present in HM-sEVs ameliorates oxidative stress and cytotoxicity in intestinal cells, resulting in improved cell viability and wound healing. Moreover, ω3 OXLP restored tissue integrity, increasing the expression of cell junction proteins including occludin, claudin and E-cadherin and halting fibrosis. ω3 OXLP was not immunogenic, endorsing its suitability for *in vivo* administration. Moreover, ω3 OXLP reduced T-lymphocyte proliferation and Mφ1 polarization *in vitro*. It has been previously described that different SPMs can stimulate a switch in macrophage phenotype from a proinflammatory to a pro-resolving M2-like phenotype (65).

Several studies have addressed the potential beneficial effects of PUFAs in inflammatory diseases. For example, RvD1 administration (17-HDHA-derived) was found to reduce intestinal fibrosis in a colitis animal model (66). In another study, Borsini et al. combined the ω3-PUFAs, EPA and DHA, to stimulate the production of lipid mediators, including 14-HDHA and 19,20-DiHDPA, which had neuroprotective effects. Also, treatment with ω3-PUFAs prevented neurogenesis loss and reduced apoptosis induced by pro-inflammatory cytokines in human hippocampal progenitor cells (67). Regarding this latter strategy, increasing the intake of EPA and DHA provides the necessary substrates for the body to produce SPMs, which can be effective in boosting SPM levels indirectly and may have broader effects beyond the administration of specific SPMs. In this context, several studies have indicated that increasing the consumption of EPA and DHA can lead to higher concentrations of specific SPMs in human plasma or serum (68). However, the relationship between the intake of EPA and DHA and the augmentation of particular SPMs remains unclear. The impact of EPA and DHA on SPM levels may be influenced by the minimum intake threshold of ω3-PUFAs required to stimulate significant endogenous biosynthesis of SPMs. While the availability of free EPA and DHA is crucial as substrates for endogenous SPM production, most of the EPA and DHA in the bloodstream, cell membranes, and intracellular compartments is esterified within complex lipids (69). For this reason, the administration of ω3 OXLP rather than their precursors might overcome this problem. Interestingly, two of these OXLP are present in a commercial marine oil formulation, whose pro-resolutive properties have been demonstrated by our research group and others (33, 70).

The present study has several limitations that should be addressed. First, we did not use a NEC mouse model. NEC is the most common life-threatening gastrointestinal emergency experienced by PIs (71), affecting 7–8% of patients in the neonatal intensive care units and with mortality rates approaching 20–30% (72). Nonetheless, despite the differences in their clinical presentation and affected demographics, emerging evidence suggests commonalities in the underlying inflammatory processes and molecular mechanisms between NEC and IBD, including dysregulated immune responses, mucosal barrier dysfunction, and altered gut microbiota composition, which contribute to intestinal inflammation in both NEC and IBD. While NEC primarily affects PIs, IBD encompasses a group of chronic inflammatory disorders that can occur in both children and adults. Moreover, the alterations in immune response, intestinal necrosis and fibrosis seen in the NEC model are relatively non-specific clinical manifestations that can be easily conflated with other gastrointestinal diseases, such as Crohn's disease (73). For this reason, we used the TNBS-induced mouse colitis model, as it shares common functional alterations with NEC (74).

A second major limitation is that although we detected other oxylipins, such as 9,10-DiHOME and 12,13-DiHOME (ω6-PUFAs), we did not analyze their therapeutic potential in our preclinical models. Nonetheless, the role of γ-linolenic acid (GLA), another ω6-fatty acid, was investigated recently in an elegant study on cardiac physiology (63). The findings of these authors support the significance of ω-6 fatty acids in maternal milk, highlighting the complex interplay between specific fatty acids, such as GLA, retinoid x receptors, and the metabolic switch towards fatty acid utilization for energy production in cardiac myocytes after birth (75). Further research exploring other ω6-PUFA-derived oxylipins in HM-sEVs and their therapeutic role in intestinal inflammation could provide valuable insights into the usefulness of these molecules in the resolution of inflammation.

Finally, although differential ultracentrifugation has been considering the gold standard for sEVs isolation, critical drawbacks of this technique include vesicle aggregation (especially originating from highly viscous solutions such as milk) and lipoprotein contamination, where high density lipids (HDLs) could sediment alongside HM-sEVs due to similar densities (76). If the suspension has a large negative ζ potential, vesicles will tend to repel each other and there will be no tendency for be added (77).

In conclusion, oral administration of ω3 OXLP attenuates intestinal inflammation *via* inhibiting pro-inflammatory signaling pathways, restoring M2/M1 macrophage balance and preventing collagen deposition, preserving tissue integrity. Our findings support that a diet formula supplemented with this cocktail of ω3 OXLP may have great potential in protecting and preserving the gut health of PIs and adults with IBD.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV230969) (78).

Ethics statement

The studies involving humans were approved by Ethics Committee of the Hospital Universitari i Politècnic La Fe (Approval Number 2021-071-1 & 2022-748-1). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Ethics Committee of the Hospital Universitari i Politècnic La Fe. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MG-F: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. EA-P: Investigation, Methodology, Writing – review & editing. AA-D: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing. IT-D: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing. JK: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. PS: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

Authors MG-F, AA-D, IT-D, JK and PS are inventors of the patent with application number No. 23382313.7 ref C002270EPO001MAT, named Composition comprising oxylipins present in human milk derived small extracellular vesicles and its use in the prevention and treatment of intestinal diseases.

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

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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.2023.1293737/full#supplementary-material

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Breast milk immune composition varies during the transition stage of lactation: characterization of immunotypes in the MAMI cohort

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Background: Breast milk is a complex and dynamic fluid needed for infant development and protection due to its content of bioactive factors such as immunoglobulins (Igs). Most studies focus primarily on IgA, but other types of Ig and even other immune components (cytokines and adipokines) may also play significant roles in neonatal health. As a first step, we aimed to characterize the Ig profile, many cytokines, and two adipokines (leptin and adiponectin) at two sampling time points within the transitional stage, which is the least studied phase in terms of these components. The secondary objective was to identify different breast milk immunotypes in the MAMI cohort substudy, and finally, we further aimed at analyzing maternal and infant characteristics to identify influencing factors of breast milk immune composition.

Methods: Breast milk samples from 75 mothers were studied between days 7 and 15 postpartum. The Igs, cytokines, and adipokine levels were determined by a multiplex approach, except for the IgA, IgM, and leptin that were evaluated by ELISA.

Results: IgA, IgM, IgE, IgG2, IL-1β, IL-5, IL-6, IL-10, and IL-17 were significantly higher on day 7 with respect to day 15. The multiple factor analysis (MFA) allowed us to identify two maternal clusters (immunotypes) depending on the breast milk immune profile evolution from day 7 to day 15, mainly due to the IgE and IgG subtypes, but not for IgA and IgM, which always presented higher levels early in time.

Conclusion: All these results demonstrated the importance of the dynamics of the breast milk composition in terms of immune factors because even in the same lactation stage, a difference of 1 week has induced changes in the breast milk immune profile. Moreover, this immune profile does not evolve in the same way for all women. The dynamic compositional changes may be maternal-specific, as we observed differences in parity and exclusive breastfeeding between the two BM immunotype groups, which could potentially impact infant health.

KEYWORDS

breastfeeding, breast milk, transitional stage, immunoglobulins, cytokines, adipokines

1 Introduction

Breast milk (BM) is considered the gold-standard food for infants since, in addition to the basic nutrients (carbohydrates, lipids, proteins, vitamins, and minerals), it also contains a huge variety of bioactive compounds, including growth factors and anti-infective molecules such as immunoglobulins (Ig), cytokines (CKs), and human milk oligosaccharides. All of these components contribute to the proper growth of the baby, both anatomically and neurologically, while also providing defenses and collaborating in the microbiota colonization and the immunity development of the offspring (1–3).

Among other anti-infective compounds in BM, Igs are the most studied, mainly IgA (1), whose presence has been known for a long time (4–12). Although less information is available about the presence of other Igs (IgM, IgG, and IgE), they have been gaining attention in the past 10 years (13) because they can also be influenced by maternal factors and infant requirements. It is reported that an infant and/or maternal infection induces an increase in secretory IgA (sIgA) and IgG in human BM (14). Among these bioactive compounds, CKs, which are in low concentrations (15), have also been generating considerable interest in recent years. Detectable CKs in BM are mainly transforming growth factor (TGF)-β, interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, IL-10, IL-5, IL-4, IL-13, IL-12, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF) (16–18). Moreover, adipokines can also be found in BM, such as leptin and adiponectin, which are involved in fetal development and growth (19–23) and have immunomodulatory actions (24). In this regard, leptin and adiponectin regulate both innate and adaptive immune responses (25, 26).

It is well known that BM components change along the phases of breastfeeding, leading to three different types of milk: colostrum (from birth to 5–6 days), transitional milk (from 7 to 15 days), and mature milk (since 15 days), with colostrum being the richest in immunological components (13, 27). It is important to highlight that the transition stage is the least studied in terms of the immune composition of BM, mainly for IgG, their subtypes, and many cytokines and adipokines. In addition to compositional changes due to the time of milk sampling (18, 28–33), BM composition can be influenced by maternal prenatal and postnatal factors including diet (34, 35), vaccination (36–39), geographic location, antibiotics (40), smoking (41), maternal pathologies and infections (42), and maternal psychological stress (43). BM composition also seems to be associated with gestational age (31, 44, 45). The BM of mothers of preterm neonates has immune compensatory mechanisms to accelerate their development, such as increased concentrations of IL-6, TGF-β1, and TGF-β2 (31) and IgA, IgM, and IgG (31, 44, 46). Moreover, BM components also change by neonatal requirements during infant infections (14, 47).

In this study, we first aimed to characterize the BM Ig and immune-related compounds, including CK and growth factors, at two sampling time points within the transitional stage. The secondary objective was to identify immunological profiles in BM. Finally, we aimed to identify associations among maternal and infant characteristics able to influence BM immune composition.

2 Materials and methods

2.1 Cohort and study subjects

The present study was performed in a subgroup of 75 healthy mother–infant pairs within the MAMI birth cohort, which is a clinical prospective mother–infant birth cohort from the Mediterranean area (48) (Clinical Trial Registry NCT03552939). The subgroup of the MAMI cohort was chosen based on breast milk sample availability at both time points (days 7 and 15) within the transition stage. Women with accessible milk samples who adhered to exclusive breastfeeding practices at the specified time points (7 and 15 days) were considered for inclusion. Our study participants were in good health, with no reported chronic health conditions. Exclusion criteria encompassed non-compliance with any of the inclusion criteria, the use of medications or drugs, the presence of chorioamnionitis, mastitis, or health complications during the gestational and breastfeeding periods, and the presence of any chronic diseases or medication usage for chronic conditions. All mothers filled out a clinical questionnaire about them and their children throughout the study (49). Maternal and neonatal clinical records, including mode of birth, neonate gender, maternal BMI, antibiotic exposure, and others, were recorded. The weight and height of the mothers and the infants were measured in medical consultation at different time points: during pregnancy, at birth, at 7 and 15 days, and at 1, 6, 12, 18, and 24 months postpartum.

Furthermore, the mothers also filled out a 140-item Food Frequency Questionnaire (FFQ) about their regular diet during pregnancy (50). The FFQ information was analyzed to obtain data on the intakes of macronutrients and micronutrients per day. For that, FFQ was previously validated in this same cohort by a 3-day questionnaire (51). The analysis of this information permitted the identification of two different dietary patterns in the MAMI cohort: Diet I and Diet II groups, by clustering using the Jensen–Shannon distance and partitioning around medoid clustering methods (51). Diet I was characterized by a higher intake of total dietary fiber, vegetable protein, and polyunsaturated fatty acids, while Diet II was based on a higher intake of animal protein and saturated fatty acids.

Moreover, 72 of the 75 mothers had their secretor status analyzed. For this, total DNA was obtained as described previously (51) and used to test the secretor status by FUT2 genotyping with the polymerase chain reaction (PCR)-random fragment length polymorphisms (RFLPs) as detailed elsewhere (52). Secretor mothers were those with the positive detection of the FUT2 gene, which encodes for the galactoside 2-alpha-L-fucosyltransferase 2.

2.2 Collection of human milk samples

Human milk samples for this study were obtained between 7 and 15 days after birth. All the mothers were asked to collect the samples in a specific way: in the morning before lactation, cleaning the breast skin with a solution of 0.5% chlorhexidine, and using a sterile pumper. In addition, the first 2–3 drops were discarded to normalize the collection, and approximately 10 ml were collected. Milk samples were sent to the biobank and kept at −80°C until the lactic serum was obtained by centrifugation at 2,000 × *g* for 15 min at 4°C. Finally, lactic serum was frozen again at −80°C in different aliquots to carry out all the determinations in one freeze/thaw cycle.

2.3 Determination of immunoglobulin, cytokine, and adipokine concentrations

The quantification of Igs (IgE, IgG1, IgG2, IgG3, IgG4), CKs (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, TNF-α), and adiponectin was performed by ProcartaPlex™ Multiplex immunoassay (Thermo Fisher Scientific, Vienna, Austria) using an Antibody Isotyping 7-Plex Human ProcartaPlex™ panel, a Th1/Th2/Th9/Th17 Cytokine 18-Plex Human ProcartaPlex™ panel, and an Adiponectin Human ProcartaPlex™ Simplex Kit, respectively, following the manufacturer's instructions as in previous studies (49, 53–58). The plates were run on a Luminex instrument and analyzed in ProcartaPlex Analyst Software (MAGPIX® analyzer, Luminex Corporation) at the Flow Cytometry Unit of the Scientific and Technological Centers of the University of Barcelona (CCiT-UB). Assay sensitivity was as follows: 2.11 ng/ml for IgG1; 16.07 ng/ml for IgG2; 0.08 ng/ml for IgG3; 0.56 ng/ml for IgG4; 0.003 ng/ml for IgE 1.2 pg/ml for GM-CSF; 0.2 pg/ml for IFN-γ; 0.2 pg/ml for IL-1β; 0.8 pg/ml for IL-2; 1.5 pg/ml for IL-4; 0.3 pg/ml for IL-5; 0.4 pg/ml for IL-6; 0.5 pg/ml for IL-9; 0.1 pg/ml for IL-10; 0.04 pg/ml for IL-12p70; 0.1 pg/ml for IL-13; 0.1 pg/ml for IL-17A; 0.4 pg/ml for IL-18; 0.6 pg/ml for IL-21; 8.2 pg/ml for IL-22; 0.9 pg/ml for IL-23; 5.1 pg/ml for IL-27; 0.4 pg/ml for TNF-α; 4.6 pg/ml for adiponectin.

The quantification of leptin was performed by a Quantikine® Colorimetric Sandwich ELISA Kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions as in previous studies (41). Data were analyzed by Multiskan Ascent v2.6 software (Thermo Fisher Scientific, Vienna, Austria). Assay sensitivity was 7.8 pg/ml.

The quantification of IgA and IgM was performed by an ELISA kit from Bethyl Laboratories (Montgomery, TX, USA) and an ELISA kit from Cloud-Clone Corp. (Houston, TX, USA), respectively, following the manufacturer's instructions as in previous studies (31, 59). These kits included the quantification of the secretory form of both types of immunoglobulins. Assay sensitivity was 14.1 ng/ml for IgM, and 1.03–750 ng/ml was the assay range for IgA. Data were analyzed by Multiskan Ascent v2.6 software (Thermo Fisher Scientific, Vienna, Austria).

2.4 Data processing and statistical analysis

The results are expressed as mean ± SEM (standard error of the mean) unless otherwise specified. Box plots showed the interquartile range, with the error bars representing the lowest and highest values. Shapiro–Wilk and Levene's tests were used to determine the normality and homogeneity of the data variance, respectively. When variables were not normally distributed, a logarithmic normalization of the data was made or non-parametric tests were used. Multivariate general linear model (GLM) and repeated measures GLM analyses were conducted to assess the variance in multiple dependent variables using two-factor variables across measurements taken at different time points. For example, this approach was applied to analyze the tracking of infant weight and height up to the first month of life in the two study groups (BM immunotypes). Spearman's correlation coefficient was used to search for correlation between variables that were not normalized. Student's t-test and Mann–Whitney U-test were used to assess significant differences between groups, while the chi-square test (X^2) compared frequencies, such as the detectability of CKs. For cytokines, a left-censored Tobit model with a threshold of 0 was fitted to test differences between times using the R software package AER (60). The *p*-values were adjusted for multiple comparisons using the false discovery rate (FDR) correction. A *p*-value of <0.05 was considered significant in all the tests.

The MFA was performed to integrate the two measurements on days 7 and 15 with the aim of providing a comprehensive

TABLE 1 Mother characteristics.

Maternal characteristics	ALL	*N*	BM-I	*N*	BM-II	*N*	*p*-value
Pre-gestational BMI (Kg/m^2),[1] mean ± SEM	22.00 ± 0.40	75	23.20 ± 0.64	39	22.00 ± 0.40	36	0.286
Pregnancy weight gain (Kg),[1] mean ± SEM	11.80 ± 0.69	75	11.86 ± 0.71	39	11.80 ± 0.69	36	0.966
Antibiotic during pregnancy,[2] yes (%)	24 (32.00)	75	15 (38.46)	39	15 (41.67)	36	0.211
Intrapartum antibiotic,[2] yes (%)	29 (38.67)	75	14 (35.90)	39	9 (25)	36	0.608
[a]Perinatal antibiotic,[2] yes (%)	42 (56.00)	75	22 (56.41)	39	20 (55.55)	36	0.940
Gestational age (weeks),[1] mean ± SEM	39.33 ± 0.22	75	39.73 ± 0.19	39	39.33 ± 0.22	36	0.177
Mode of delivery: vaginal birth,[2] yes (%)	49 (65.33)	75	26 (66.67)	39	23 (63.89)	36	0.801
Primipara,[2] yes (%)	41 (54.67)	75	16 (41.02)	39	25 (69.44)*	36	0.014
Animals,[2] yes (%)	17 (26.98)	63	8 (25.81)	31	9 (28.13)	32	0.836
Exclusive maternal breastfeeding until 15th day,[2] yes (%)	64 (85.33)	75	37 (94.87)	39	27 (75.00)*	36	0.015
Exclusive maternal breastfeeding until 6-month,[2] yes (%)	57 (76.00)	75	33 (84.62)	39	24 (66.67)	36	0.069
Diet I cluster,[2] yes (%)	28 (37.33)	75	12 (30.77)	39	16 (44.44)	36	0.221
Secretor mother,[2] yes (%)	55 (76.39)	72	25 (71.43)	35	30 (81.10)	37	0.335

Maternal characteristics associated with the total and with the BM immunotype groups. BM-I, breast milk immunotype I; BM-II, breast milk immunotype II; BMI, body mass index; SEM, standard error of the mean. Exclusive maternal breastfeeding refers to the provision of human milk and excludes the use of formula milk.

[1]*p*-values were calculated with Student's t-test.

[2]*p*-values were calculated using X^2 test.

*$p < 0.05$ comparing two BM immunotype groups.

[a]Perinatal antibiotic includes both antibiotics during pregnancy and/or intrapartum antibiotics.

TABLE 2 Infant characteristics.

Infant characteristics	ALL	*N*	BM-I	*N*	BM-II	*N*	*p*-value[1,2]	*p*-value[3]
Gender: Female,[2] yes (%)	42 (56.00)	75	21 (53.85)	39	21 (58.33)	36	0.696	
Antibiotic intake,[2] yes (%)								
0–1 month	5 (6.67)	75	3 (7.69)	39	2 (5.55)	36	0.711	
0–6	9 (12.33)	73	6 (16.22)	37	3 (8.33)	36	0.306	
0–12	17 (26.56)	64	11 (34.38)	32	6 (18.75)	32	0.157	
0–24	23 (71.88)	32	14 (87.50)	16	9 (56.25)*	16	0.049	
Atopy (0–24 month),[2] yes (%)	11 (34.38)	32	5 (31.25)	16	6 (37.50)	16	0.710	
[a]Number of infections,[1] mean ± SEM								
0–6 months	0.42 ± 0.13	73	0.38 ± 0.11	37	0.42 ± 0.13	36	0.827	
0–12 months	0.72 ± 0.20	72	0.61 ± 0.13	36	0.72 ± 0.20	36	0.648	
0–24 months	6.82 ± 0.85	33	8.00 ± 1.43	16	6.82 ± 0.85	17	0.485	
Weight (kg),[1] mean ± SEM								Group: 0.033
Birth	3.15 ± 0.08	75	3.29 ± 0.07	39	3.15 ± 0.08	36		Time: <0.001
7th day	3.08 ± 0.06	72	3.31 ± 0.07	39	3.08 ± 0.06	34		Int: 0.76
15th day	3.33 ± 0.07	71	3.57 ± 0.08	37	3.33 ± 0.07	34		
1st month	3.98 ± 0.09	73	4.16 ± 0.09	37	3.98 ± 0.09	36		
Height (cm),[1] mean ± SEM								Group: 0.01
Birth	49.26 ± 0.41	75	50.08 ± 0.35	39	49.26 ± 0.41	36		Time: <0.001
7th day	49.73 ± 0.32	72	51.24 ± 0.34	39	49.73 ± 0.32	34		Int: 0.52
15th day	51.10 ± 0.35	71	52.30 ± 0.37	37	51.10 ± 0.35	34		
1st month	53.44 ± 0.40	73	54.61 ± 0.40	37	53.44 ± 0.40	36		
BMI z-score,[1] mean ± SEM								Group: 0.46
Birth	−0.46 ± 0.15	75	−0.29 ± 0.15	39	−0.46 ± 0.15	36		Time: 0.030
7th day	−0.65 ± 0.13	72	−0.55 ± 0.16	39	−0.65 ± 0.13	34		Int: 0.59
15th day	−0.70 ± 0.15	71	−0.47 ± 0.16	37	−0.70 ± 0.15	34		
1st month	−0.66 ± 0.11	73	−0.65 ± 0.20	37	−0.66 ± 0.11	36		

Maternal characteristics associated with the total and BM immunotype groups. BM-I, breast milk immunotype I; BM-II, breast milk immunotype II; BMI, body mass index; SEM, standard error of the mean.

[1]*p*-values were calculated with Student's t-test comparing two BM immunotype groups.

[2]*p*-values were calculated using a X^2 test comparing the two BM immunotype groups.

[3]*p*-values were calculated using repeated measures GLM for the BM immunotype group variable, the time variable, and the interaction (Int) between these two independent variables.

*$p < 0.05$.

[a]Number of infections includes both respiratory and gastrointestinal.

understanding of the contribution of the different variables and times to the variability of the data. The MFA models were graphically displayed by plotting the projections of the samples onto the bidimensional space defined by the two first dimensions. All the data were normalized by means of log transformation prior to carrying out these statistical analyses using the R software packages FactoMineR and factoextra (61).

To discover the relationships between milk composition variables and immunotype groups, a mixed graphical model (MGM) was used. MGMs are undirected probabilistic graphical models, where each node corresponds to one variable, and the edges between two nodes represent a conditional dependency between them given all other variables in the graphical model (62). We have used 'mgm' R-package to estimate the network of dependencies (63). Specifications were set to allow the maximum number of interactions in the network.

All statistical analyses were performed using IBM SPSS Statistics 22 (IBM, USA) and R version 4.1.2 (R Foundation, Austria) (64).

3 Results

3.1 Study population

The mean maternal weight gain during pregnancy was in the range recommended by the Institute of Medicine (65). Overall, approximately 50% of them used antibiotics during pregnancy and/or on delivery day. More than half of the participants' deliveries were vaginal, and approximately 50% of the total participants were first-time mothers (Table 1). The table includes the breast milk (BM) immunotype classification, depending on the later results.

With regard to the infant characteristics, slightly more than half of the infants were females, 23% took antibiotics from birth to 2 years, and 11 of 32 infants developed atopy. As expected, the intake of antibiotics and the number of infections increased over time (Table 2).

3.2 Changes in the immune composition of breast milk from day 7 to day 15

The description of some immune components, particularly the characterization of the IgG isotype profile and IgE levels found in transition milk, is described for the first time in this study. The immune compounds present in milk on days 7 and 15 of lactation were analyzed and compared.

Although there were no differences in adipokine levels (Supplementary Figure 1), the immunoglobulin and cytokine concentrations showed some differences between days 7 and 15 (Figure 1; Table 3, respectively). IgA, IgM, and IgE showed their highest values on day 7. However, IgG levels did not change between milk from day 7 to day 15, and with respect to the IgG isotypes associated with the Th1 or Th2 response, only IgG2 displayed a significant decrease with time ($p < 0.05$) even though it did not affect the Th1/Th2 ratio. With regard to the cytokine levels, day 7 presented a higher concentration of IL-1β, IL-6, IL-9, IL-10, and IL-17 ($p = 0.021$, $p = 0.020$, $p = 0.0021$, $p = 0.020$, and $p = 0.048$, respectively) than day 15. On the contrary, IFN-γ and IL-18 levels were higher on day 15 than on day 7 ($p = 0.021$ and $p = 0.028$, respectively). Overall, the cytokine concentration in the samples analyzed was variable, and some cytokines were not detected in some subjects, similar to what has been previously described (66) (Table 3). The components were grouped depending on their functions to study the immune response (pro-inflammatory, anti-inflammatory, Th1, Th2, Th9, Th17, hematopoietic factors, innate immunity, and acquired immunity) (27, 58, 67–75) (Supplementary Figure 2). Some of the calculated variables were also significantly higher on day 7 than on day 15, such as anti-inflammatory response, Th2, Th17, innate immunity, and acquired immunity (Supplementary Figure 3).

3.3 Description of BM immunotypes

A multivariate factor analysis (MFA) was performed to observe different profiles regarding the BM immune composition. The distribution of the mothers taking into consideration each day separately, on days 7 and 15 (Figures 2A,B, respectively), did not show any particular aggrupation. However, when the MFA was generated considering the two types of milk from the same mother, two clusters were identified with strong similarities among individuals (Figure 2C). It was also observed that the mothers from the same cluster had similar dynamic behavior regarding their BM immune components from d7 to d15. The clusters were based on the BM immune components; therefore, they were named BM immunotype I (BM-I)

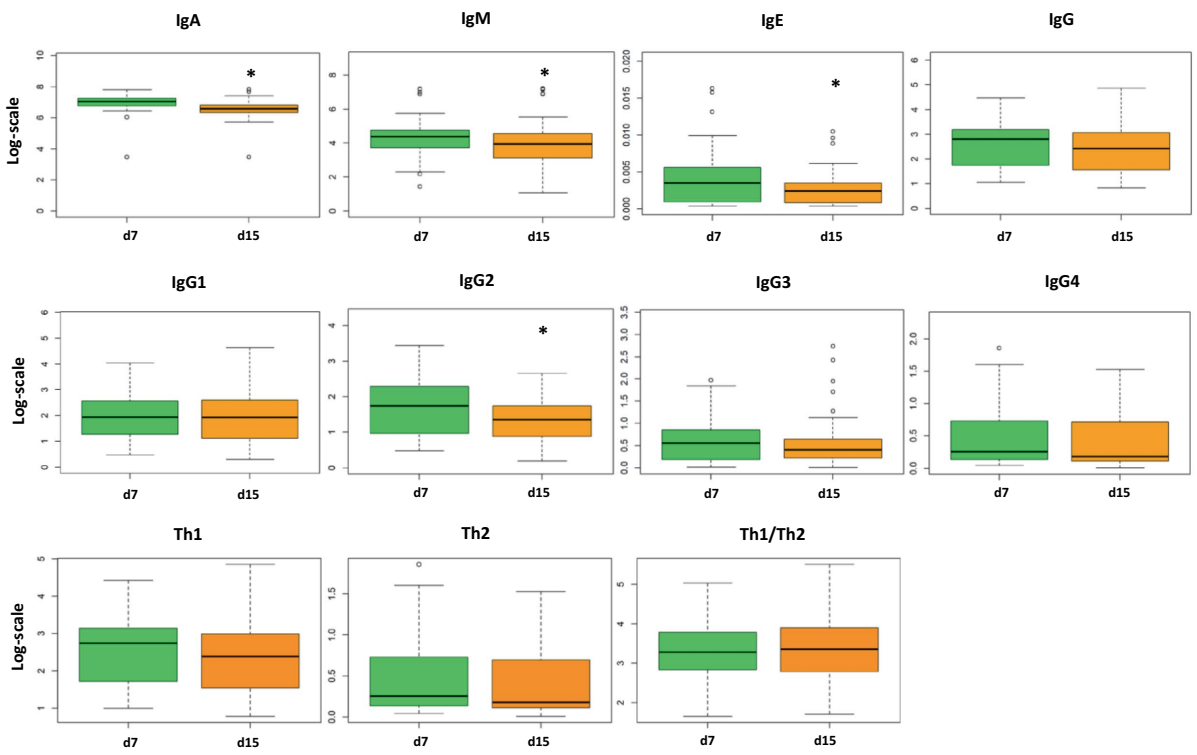

FIGURE 1
Concentration of immunoglobulin (Ig) levels in breast milk on days 7 and 15 from the same mothers ($N = 75$). The concentration of each analysis (pg/ml) was normalized logarithmically, and the mean from each group is expressed in box plots (interquartile range). Student's t-test was used to determine significant differences between sampling days. The p-values were adjusted for multiple comparisons using the FDR correction. *$p < 0.05$. IgG1, IgG2, and IgG3 (Igs associated with Th1 response); IgG4 (Ig associated with Th2 response).

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TABLE 3 Concentrations and detectability of cytokines in breast milk.

Cytokines	d7, $N = 75$			d15, $N = 75$		
	pg/ml	%det	IQR	pg/ml	%det	IQR
GM-CSF	0.07 ± 0.03	6.67 (5/75)	0.00–0.00	0.01 ± 0.01	1.33 (1/75)	0.00–0.00
IFN-γ	0.08 ± 0.03	20 (15/75)	0.00–0.00	0.13 ± 0.03*	21.33 (16/75)	0.00–0.00
IL-1β	0.32 ± 0.06	45.33 (34/75)	0.00–0.56	0.19 ± 0.05*	26.67 (20/75)*	0.00–0.08
IL-2	0.14 ± 0.04	13.33 (10/75)	0.00–0.00	0.03 ± 0.02	4 (3/75)*	0.00–0.00
IL-4	0.04 ± 0.02	6.67 (5/75)	0.00–0.00	0.00 ± 0.00	1.33 (1/75)	0.00–0.00
IL-5	0.09 ± 0.03	13.33 (10/75)	0.00–0.00	0.06 ± 0.03	6.67 (5/75)	0.00–0.00
IL-6	0.92 ± 0.10	61.33 (46/75)	0.00–1.74	0.60 ± 0.09*	45.33 (34/75)*	0.00–1.31
IL-9	0.08 ± 0.03	14.67 (11/75)	0.00–0.00	0.03 ± 0.01	12 (9/75)	0.00–0.00
IL-10	0.14 ± 0.03	45.33 (34/75)	0.00–0.16	0.11 ± 0.03*	45.33 (34/75)	0.00–0.06
IL-12	0.06 ± 0.02	33.33 (25/75)	0.00–0.04	0.04 ± 0.01	45.33 (34/75)	0.00–0.06
IL-13	0.01 ± 0.01	1.33 (1/75)	0.00–0.00	0.01 ± 0.01	2.67 (2/75)	0.00–0.00
IL-17	0.09 ± 0.03	16 (12/75)	0.00–0.00	0.03 ± 0.02*	8 (6/75)	0.00–0.00
IL-18	0.94 ± 0.06	94.67 (71/75)	0.53–1.32	1.09 ± 0.07*	92 (69/75)	0.73–0.74
IL-21	0.64 ± 0.06	85.33 (64/75)	0.22–0.97	0.56 ± 0.05	85.33 (64/75)	0.31–0.74
IL-22	0.68 ± 0.06	74.67 (56/75)	0.00–1.14	0.66 ± 0.06	74.67 (56/75)	0.00–1.03
IL-23	0.07 ± 0.03	8 (6/75)	0.00–0.00	0.03 ± 0.01	4 (3/75)	0.00–0.00
IL-27	0.03 ± 0.02	2.67 (2/75)	0.00–0.00	0.03 ± 0.03	1.33 (1/75)	0.00–0.00
TNF-α	0.41 ± 0.04	88 (66/75)	0.14–0.58	0.35 ± 0.03	85.33 (64/75)	0.14–0.52

Data show the concentration of cytokines (CKs) in breast milk on day 7 and day 15 from the same mothers ($N = 75$). The concentration of each analysis (pg/ml) was normalized logarithmically and expressed as mean ± S.E.M, detectability frequencies (%det), and interquartile ranges (IQRs). Tobit model was used to determine significant differences between sampling days. X^2 test compared detectability. The p-values were adjusted for multiple comparisons using the FDR correction. GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor. *$p < 0.05$.

and BM immunotype II (BM-II). Furthermore, as represented in Supplementary Figure 4, considering each day separately and the existence of the two BM immunotypes, the two clusters could already be observed, but without a good separation, as noticed in Figure 3C.

The next step was to characterize these clusters, or BM immunotypes, to define the differences in composition between them and to study the association with maternal and infant characteristics to find potential sources of variability in the composition of milk immune molecules.

The variables that influenced the most separation among the participants were the Ig levels, mainly IgG subtypes, and total IgG. Observing the importance of the variables in each dimension (Dim) defining the MFA, it can be established that while Dim-1 was described mainly by IgG-15d, IgG1-15d, and IgG3-15d, Dim-2 was linked to levels of IgG-7d, IgG1-7d, and IgG2-7d (Figures 2D,E, respectively). In line with these results, these components displayed differing temporal evolutions when compared between clusters, as was observed in the interaction of variables (group and time) by conducting a multivariate general linear model (GLM; Supplementary Table 1). Additionally, differences between groups were also observed when fixing the sampling time for those components that exhibit distinct temporal evolutions. For instance, the BM immunotype II showed higher levels of IgG, IgG1, and IgG2 on day 15 with respect to the BM immunotype I, even though on day 7 they were higher in the BM immunotype I (Supplementary Table 1). The influence of these components in clustering, and of others such as IgE and IL-22 was confirmed when strong relations were directly found in the mixed graphical model (MGM) with the BM immunotype group (Supplementary Figure 5a). In addition, strong correlations were also observed among the predominant variables that described Dim-2 (IgG1-7d, IgG2-7d, IgG3-7d, IgG4-7d, IgG-7d, and IgE-7d; Supplementary Figure 5b). In addition, IgG1-15d and IgG-15d, which are important in Dim-1 were also highly correlated (Supplementary Figure 5b).

Moreover, to investigate the relations between all the components, Spearman's correlations were made with the non-transformed data. The study of the impact of adipokine levels on the other BM immune components can be observed in Figure 3. Leptin and adiponectin showed significant positive correlations with several BM CKs and Igs (Figure 3).

To evaluate the dynamic behavior of the BM immune components within the BM immunotype group, we calculated the increase of each immune variable with the transformed data from d7 to d15 (Figure 4). Each BM immunotype group had a different dynamic pattern. Importantly, the most prominent components were the strongest in the dimensions of the MFA. As all the IgG subtype levels increased from d7 to d15 in BM-II, the total IgG concentration was also boosted in the BM-II group (Figure 4A). IgE and adiponectin concentrations also showed the same pattern (Figures 4C,D, respectively). The opposite behavior was observed in the BM-I cluster for the same components. On the contrary, while leptin levels increased after 1 week in the BM-I, they decreased in the BM-II (Figure 4D). Importantly, by assessing the IgA and IgM changes, we found that they behaved the same in both groups. Thus, these Igs

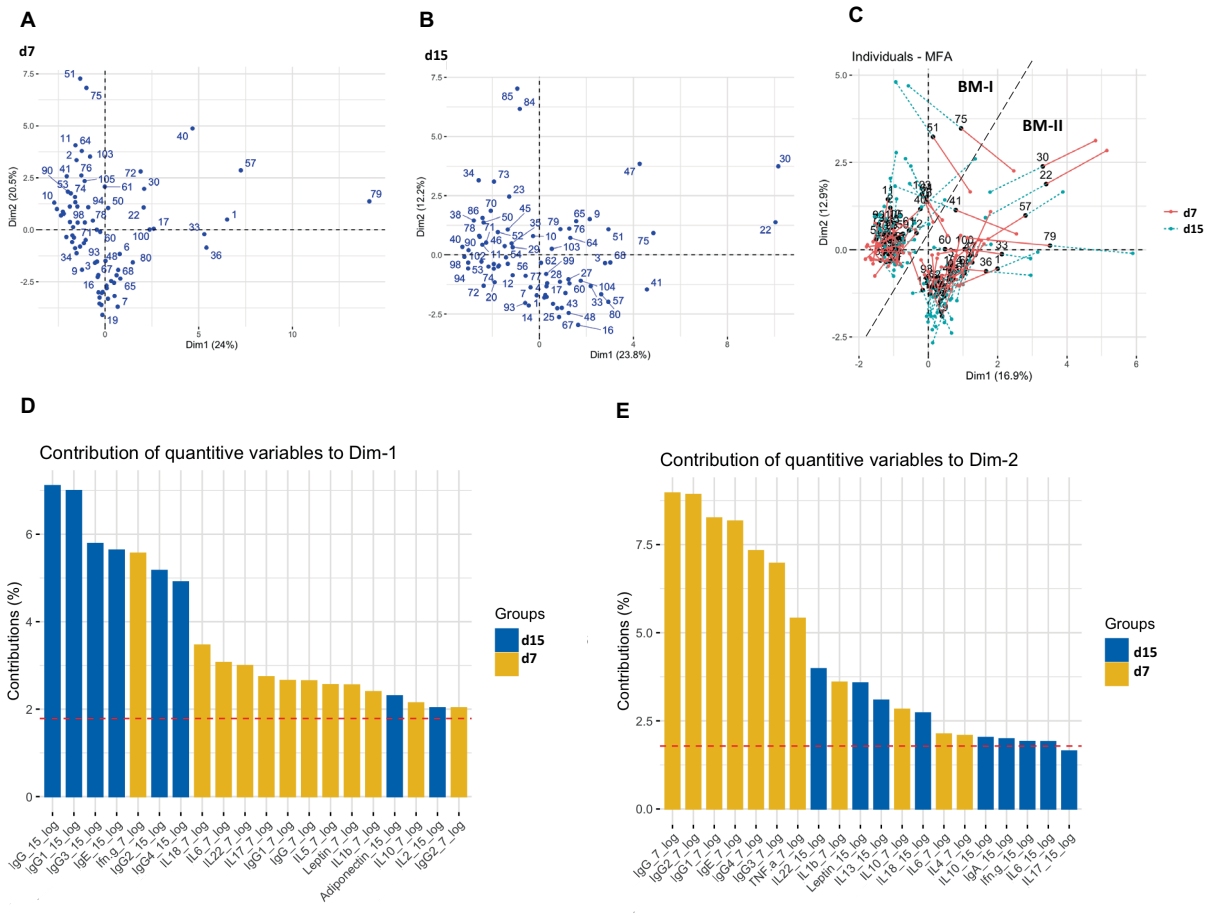

FIGURE 2
Multiple factor analysis (MFA) for the concentration of immune factors in the breast milk of 75 mothers (MAMI cohort) on days 7 **(A)**, 15 **(B)**, and taking into consideration the two sampling time points **(C)**. The contribution of quantitative variables to both dimensions (Dim): Dim-1 **(D)** and Dim-2 **(E)**.

decreased from d7 to d15 in all mothers (Figure 4B). In terms of cytokine concentration, IL-18, IL-22, IL-23, IFN-γ, and TNF-α also showed significantly different dynamic behaviors when comparing the two groups. For instance, IL-5, IL-18, IL-22, and IFN-γ increased over time in the BM-I group, while they decreased over time in the BM-II group.

3.4 Potential contribution of maternal factors to the breast milk immune composition

To further investigate the potential source of variability between clusters, the maternal characteristics within each group were compared in relation to the BM immunotype. The maternal variables (e.g., pre-gestational BMI, weight gain, the secretory classification, and the presence of animals at home) and the gestational aspects (i.e., gestational age, health complications during pregnancy, and antibiotic use) were similar between the two groups, allowing us to discard these factors as contributors to the clustering of the BM immunotypes. However, mothers belonging to BM-I displayed a lower frequency of being first-time mothers (Table 1) and a tendency to increase the number of mothers practicing exclusive breastfeeding ($p = 0.069$; Table 1) with respect to BM-II mothers. In line with these results, more mothers started a mixed feeding before day 15 in the BM-II group ($p = 0.015$) than in the BM-I group. To investigate the impact of the mother's genetic background with respect to HMO production in the BM immunotype, it was also possible to classify the mothers according to their secretor gene (secretor mother and non-secretor mother). The results showed that the genetic background did not influence the BM immune composition of the studied mothers (Supplementary Figure 6A).

Mothers were also classified into two dietary groups (Supplementary Figure 6B) according to their dietary records, as done in previous studies with the same cohort and as described in the MM section (51, 78). While the Diet I group ate foods richer in fiber and vegetable protein, the Diet II group ate foods richer in animal protein and saturated fatty acids. Although a clear association of the dietary group with the BM immunotype was not found (Supplementary Figure 6B), plant-derived metabolites, such as total polyphenols, tended to increase in the mothers belonging to the BM-II group ($p = 0.066$; Supplementary Figure 6C); however, vitamin D levels were lower in BM-II with respect to the BM-I group (Supplementary Figure 6D).

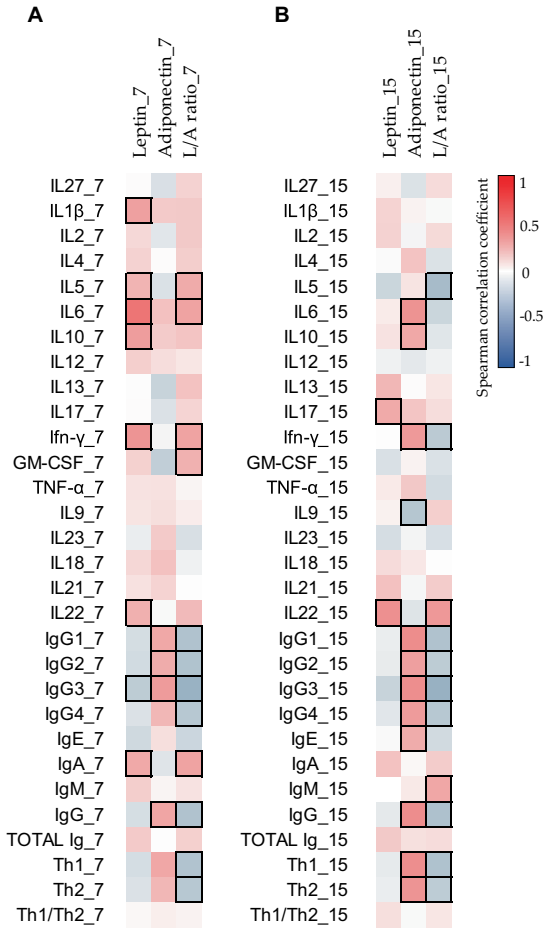

FIGURE 3
Correlations between the CK and Ig compositions present in BM on days 7 **(A)** and 15 **(B)** with the adipokine composition at the same sampling time. The Spearman correlation coefficient is represented in the heatmap following the color in the legend. Bold frames represent correlations with statistical significance ($p < 0.05$). GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; L/A ratio, leptin/adiponectin ratio; TNF, tumor necrosis factor. IgG1, IgG2, and IgG3 (Igs associated with Th1 response); IgG4 (Ig associated with Th2 response) (76, 77).

3.5 Effect of immune components and immunotypes on infant growth and infection incidence

To investigate the impact of having different immune dynamic profiles in the transition stage, the infant characteristics between the two groups (i.e., gender, weight, height, BMI z-scores, atopy, antibiotic use, and number of infections) were also analyzed (Table 2). Although children of mothers belonging to the BM-I group seemed to exhibit higher weight and height during the first month of life, the weight trajectory over time did not differ between clusters (Table 2), and the increase in weight and height from birth to the first month of life is similar when comparing the two groups (data not shown).

To assess the influence of each BM immune component on infant growth, the Spearman correlations between these parameters were also studied (Figure 5). Surprisingly, different correlations were found in the two BM sampling days (Figures 5A,B). While negative significant correlations between leptin at 7 days and the weights and heights from birth to 1 month were found (Figure 5A), the leptin levels at 15 days only showed significant negative correlations with the weight and the height at 1 month of age (Figure 5B). Additionally, more correlations between these infant parameters and CKs were found in the BM at 7 days than in the BM at 15 days. Contrary to what happened at 7 days, at 15 days, many important negative correlations were observed between Ig types and infant growth from birth to 1 month (Figure 5B). In addition, it is important to note that most of the correlations with infant parameters were concentrated in the first month of life.

Continuing with the infant characteristics, the offspring of the BM-I group required more antibiotics from birth to 24 months (Table 1), although it is worth noting that the data decreased throughout the follow-up time. In line with these results, it was also observed that the three children who had more infections from birth to 2 years belonged to the BM-I group (Figure 6D). However, there was no association between the presence of infections and the clusters of BM immunotypes (Figures 6A–C).

4 Discussion

The composition of human milk dynamically changes throughout breastfeeding (27, 79). Our results provide the characterization of the Ig profile, 18 cytokines, and 2 adipokines in transitional milk, which is the least studied at the immunological level. Moreover, we demonstrate the presence of different profiles of BM immunotypes for the first time.

In line with other studies, including those from other stages of BM, IgA is the principal Ig found in human milk (80–90% of total Ig) (80, 81), followed by IgM, IgG, and finally IgE (42, 67, 80, 82–87). Indeed, we observed that IgA, IgM, IgG, and IgE were present at a percentage of 91/8/1/0.0001%, respectively, on day 7 and 85/11/4/0.0005%, respectively, on day 15. Little is known about the concentration and impact of the IgG subtypes in BM and even less in the transitional stage; however, they are gaining attention due to the observed functions of IgG in the neonates at an intestinal level in dampening mucosal T helper cell responses and inducing oral tolerance (88–90). As found in the literature, the relative abundances of the IgG subtypes in colostrum and mature milk showed a predominance of IgG1, followed by IgG2, IgG3, and IgG4 (13), in agreement with what we have found in transitional milk in this study. In addition, very few research studies have focused on IgE levels in BM until now, and to our knowledge, this is the first study displaying IgE concentrations in BM during the transition period.

As it has been previously described, transition BM contains a broad variety of cytokines, including IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, and TNF-α (18, 66, 91, 92). Moreover, we also found good detectability for IL-18, IL-21, and IL-22 (94.67, 85.33, and 74.67%, respectively), which are cytokines less studied in BM. These CKs may play significant roles in infant immune defenses as they are key players in maintaining intestinal epithelial homeostasis and host defense (93–95). Therefore, they require further attention regarding their concentration in BM and their impact on the neonate. Finally, the cytokines least detected were GM-CSF, IL-4, IL-13, and IL-27, as also observed in other studies (30, 96).

It is well established that Ig concentrations, mainly IgA, tend to decrease from colostrum to mature milk (30–32). Furthermore, CK

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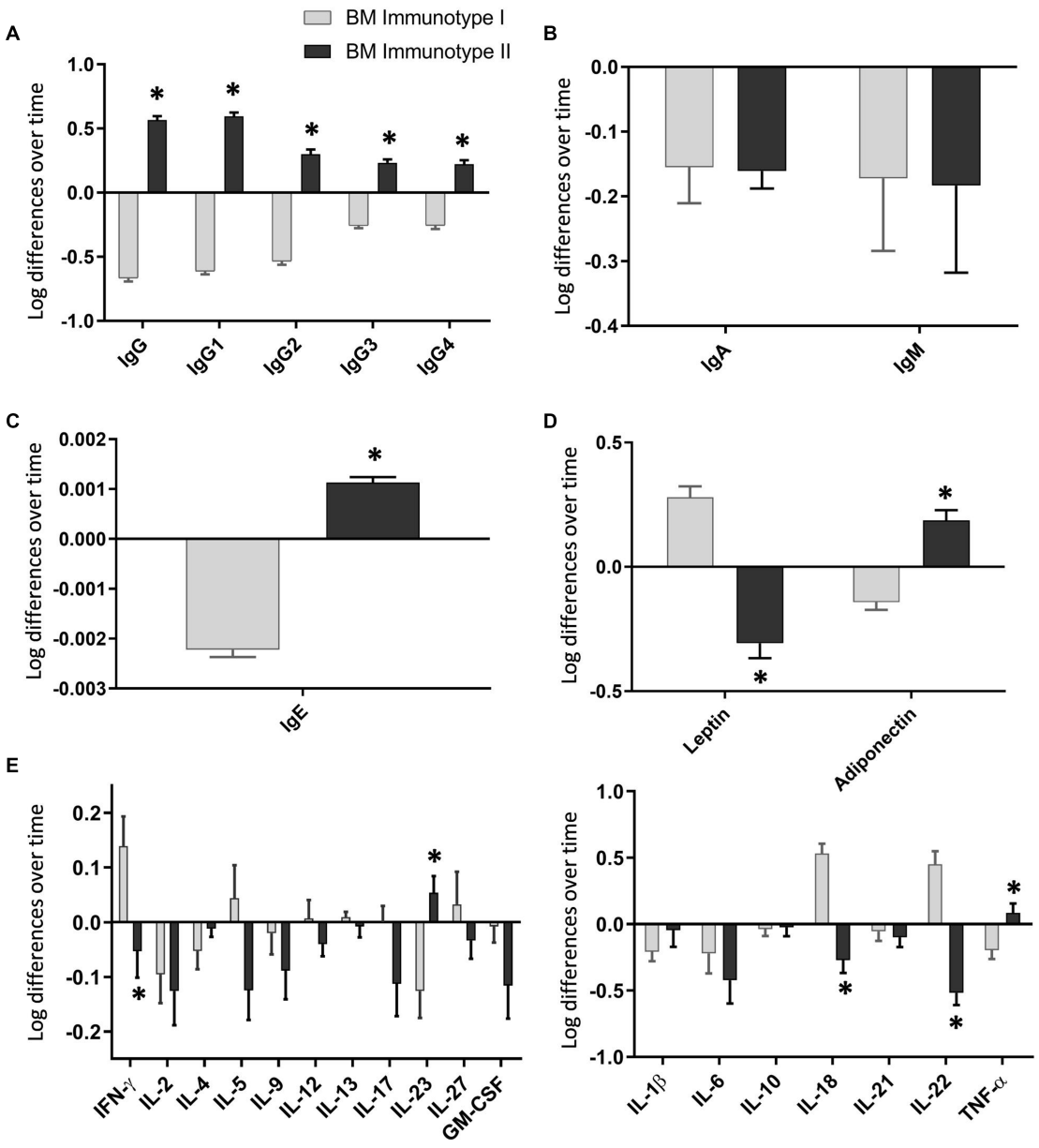

FIGURE 4
Changes in the breast milk immunoglobulin **(A–C)**, adipokine **(D)**, and cytokine **(E)** levels over time in each breast milk immunotype group. BM-I, breast milk immunotype I ($n = 39$); BM-II, breast milk immunotype II ($n = 36$). The normalized data from day 15 were subtracted from the normalized data from day 7 and are expressed as mean ± SEM. Student's t-test was used to determine significant differences between groups. The p-values were adjusted for multiple comparisons using the FDR correction. *$p < 0.05$.

levels also change throughout the lactation period (18, 31). Comparing the BM immune composition from day 7 to day 15, many changes appeared, indicating that BM is a highly dynamic source of these components in the transitional stage, with day 7 being the richest one in immune component levels. In this regard, IgA, IgM, IgE, IgG2, IL-1β, IL-6, IL-10, and IL-17 were significantly higher on day 7 with respect to day 15. However, IFN-γ and IL-18 increased from day 7 to day 15. Overall, these results allow us to conclude that the differences between day 7 and day 15 reinforce the idea that BM is a highly dynamic source of these immune factors, particularly in this period. Moreover, these results highlight the importance of milk collection timing in interventional and descriptive studies, as just a few days lead to dramatically different levels of BM immune components (Figure 7).

After performing the multivariate factor analysis (MFA), two clusters of mothers were observed with different profiles regarding the BM immune composition throughout the transitional milk stage: BM immunotype I (BM-I) and BM immunotype II (BM-II). While the IgA and IgM were not influencing factors in the ordination of the mothers because in both cases their levels decreased from day 7 to day 15, the immune components that drove the separation of the clusters the most were the IgG subtypes. Specifically, IgG1, IgG2, IgG3, and IgG4 increased from day 7 to day 15 in the BM-II mothers and decreased

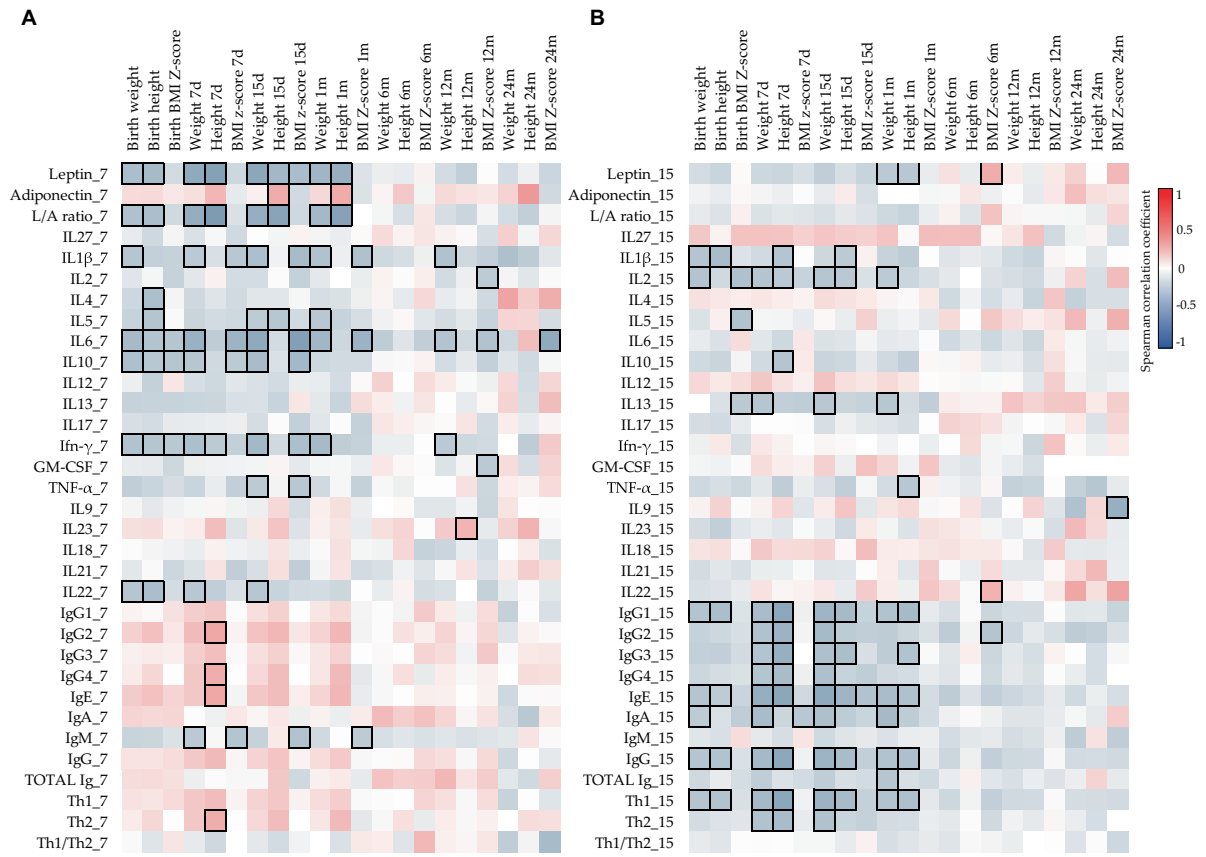

FIGURE 5
Correlations between CK, Ig, and adipokine levels present in BM on days 7 **(A)** and 15 **(B)** with the infant growth parameters. The Spearman correlation coefficient is represented in the heatmap following the color in the legend. Bold frames represent correlations with statistical significance ($p < 0.05$). GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; L/A ratio, leptin/adiponectin ratio; TNF, tumor necrosis factor. IgG1, IgG2, and IgG3 (Igs associated with Th1 response); IgG4 (Ig associated with Th2 response) (76, 77).

in the BM-I mothers. Furthermore, the same behavior was observed for IgE, IL-23, and TNF-α.

With regard to adipokine levels during breastfeeding, previous studies have reported that leptin levels in BM decrease from colostrum to mature milk. However, our results show that leptin levels were similar on days 7 and 15. When analyzing the data by clustering, we observed that leptin levels increased over time in BM-I but decreased in BM-II. Regarding adiponectin, some studies have shown an increase over time, while others have shown a decrease (97–100). Therefore, we observed that there were groups of mothers with different adiponectin dynamics, which explains the contradictory results in the literature. Furthermore, these adipokines are significant in distinguishing the BM immunotype groups and may have an impact on offspring. In addition, adiponectin levels were positively correlated with IgG1, IgG2, IgG3, and IgG4 on day 7 and with IgG1, IgG2, IgG3, IgG, and IgE on day 15. Consequently, these associations could suggest the potential immunomodulatory role of adipokines in early life, although additional research is required to draw more robust conclusions.

Many maternal and infant variables can impact the composition of breast milk, as observed mainly with IgA, adiponectin, and leptin (14, 31, 82, 101, 102). Further studies are needed to explore more in-depth these influencing factors. The second objective of this study was to explore the variables that could influence the clustering of breast milk into two immunotypes, aiming to identify any variables associated with these distinct milk evolution profiles among mothers. We analyzed the contribution of maternal and infant characteristics in the two clusters and found that there were more first-time mothers and more mixed breastfeeding before the 15th day in BM-II than in BM-I. These two characteristics are related since the breastfeeding experience is different between primiparous mothers and multiparous mothers, since the second ones have more successful initiation of exclusive breastfeeding and are more likely to breastfeed through 6 months (103–105). In addition, parity is a maternal characteristic that seems to have an influence on the BM composition. For instance, it was found that the parity number is positively associated with the lipid concentration but not with the total proteins and sIgA levels in transitional and mature milk samples (106). However, higher levels of IgA, IgG isotypes, IgM, TGF-α, and other factors in colostrum samples from primiparous mothers than from multiparous mothers have been reported (107, 108). In addition, different BM compositions of immune components during the transition stage do not seem to be the strongest factor influencing the infection episodes in early life, but they deserve to be further studied in future. In this line, the maternal diet does not seem to influence the dynamics of the immune composition of the transitional stage BM, contrary to what happens in the case of the infant and BM microbiota (51, 109). Secretor status is critical for HMO composition in BM; however, it does not seem to have an impact on the immune components analyzed here.

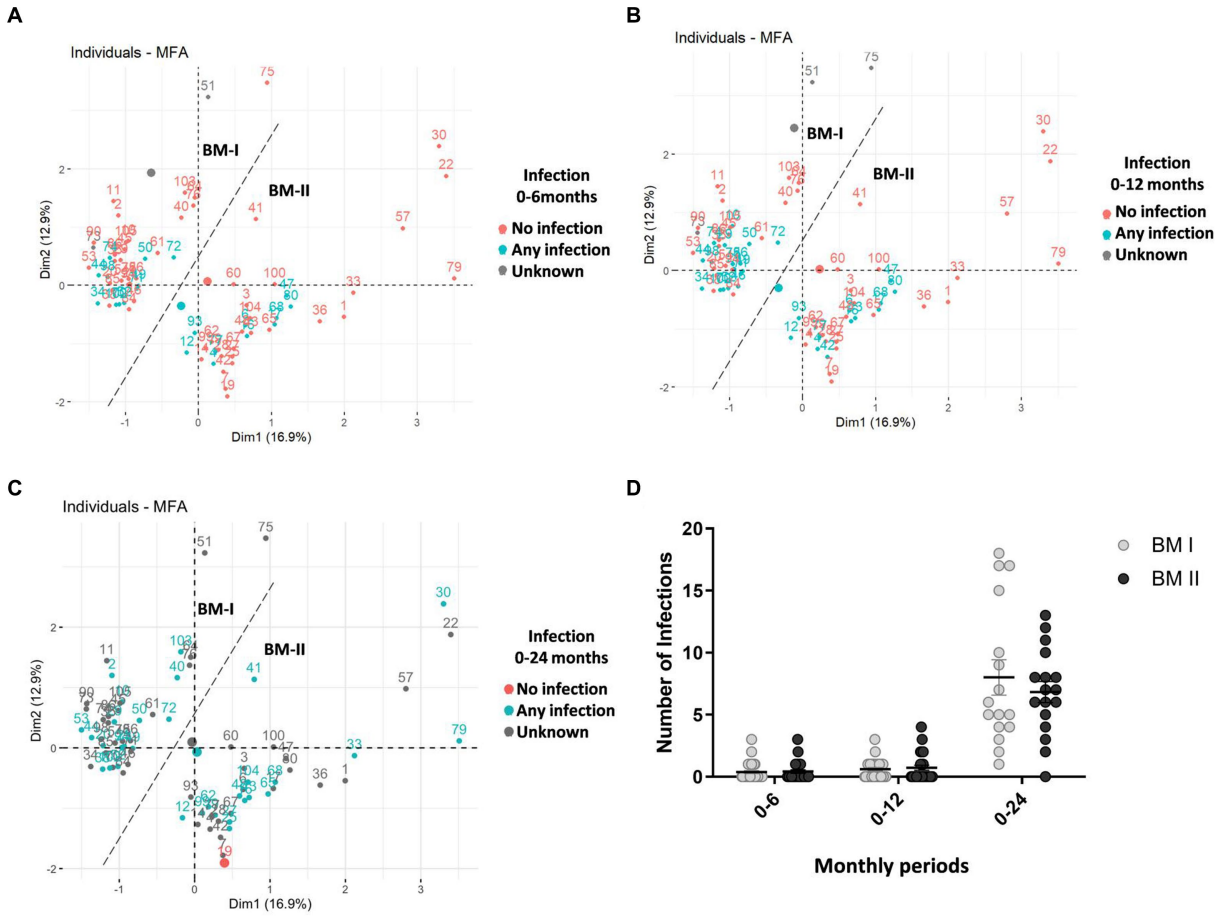

FIGURE 6
Multiple factor analysis (MFA) for the concentration of immune factors in the breast milk of 75 mothers (MAMI cohort), taking into account the two sampling days (days 7 and 15), considering Dim-1 (x-axis) and Dim-2 (y-axis). In blue, the mothers whose child had any infection, in red, the mothers whose child had no infection, and in gray are the mothers with unknown infection data **(A–C)** from birth to 6 months old **(A)**; from birth to 12 months old **(B)**; from birth to 24 months old **(C)**. The number of total infant infections over the three periods. BM-I, breast milk immunotype I ($n = 39$); BM-II, breast milk immunotype II ($n = 36$); Student's t-test was used to determine significant differences between groups. $^*p < 0.05$ **(D)**.

Regarding infant characteristics, it is worth noting that the distinct profile of BM immune composition does not appear to impact infant growth or predisposition to infections. However, specific associations have been found with certain immune components. Therefore, further studies with a larger sample size and longer follow-up are needed to make more robust conclusions.

5 Conclusion

Two different BM immune profiles among Mediterranean mothers have been described. This study highlights the importance of the time of sampling of lactation when analyzing BM composition since the BM immunotypes can only be observed after the global analysis of the two sampling points. The results characterizing the BM immunotypes indicate that not all the mothers have the same evolution in terms of BM immune components from day 7 to day 15, except for IgA and IgM, which always had higher levels early in life (Figure 7).

The dynamically composed changes could be maternal-specific since we found differences in parity and exclusive breastfeeding and could induce different infant growth (Figure 7). It would be of great interest to add more sampling times to the study to follow-up on the BM evolution and to find out whether the different dynamic profiles are associated with infant characteristics because having only two sampling time points could be insufficient to draw strong conclusions about infant health later in life.

Data availability statement

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

Author contributions

MR-L, MCo, and FP-C: conceptualization. KR-A and AF-B: data curation, methodology, and formal analysis. KR-A: writing—original draft preparation. EV-L, AM-A, MCa, MS-R, CM-C, MR-L, MCo, and FP-C: writing—reviewing and editing. MCo and FP-C: funding acquisition. All authors contributed to the article and approved the submitted version.

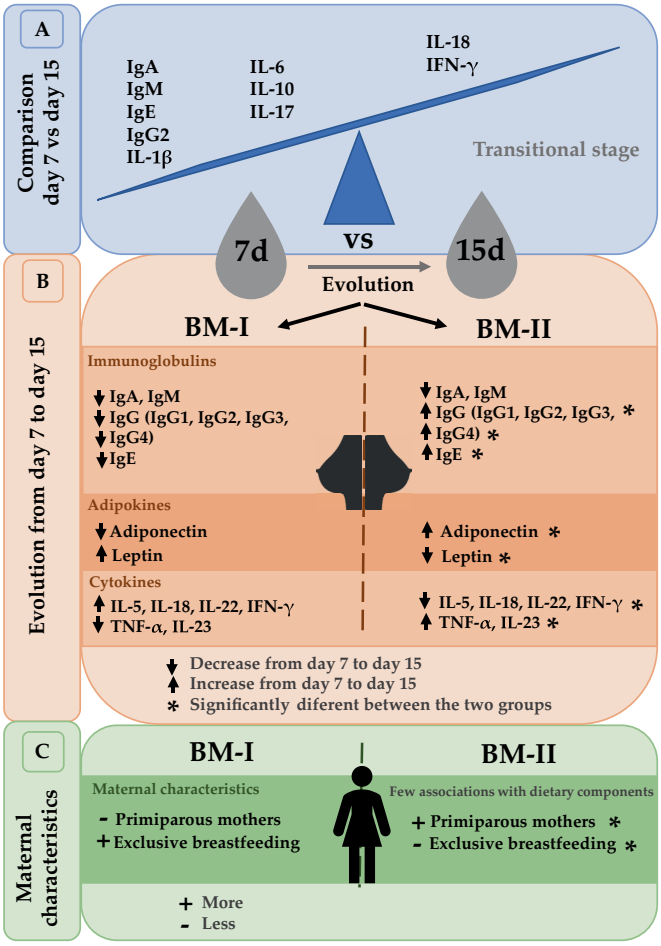

FIGURE 7
Summary of the comparison between day 7 and day 15 **(A)**. The evolution from day 7 to day 15 led to the separation of the mothers into two groups depending on the dynamical behavior of immunoglobulins, adipokines, and cytokines **(B)**. The maternal characteristics associated with these two clusters **(C)**. BM-I, breast milk immunotype I (n = 39); BM-II, breast milk immunotype II (n = 36); IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1252815/full#supplementary-material

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Milk fat globule membrane promotes brain development in piglets by enhancing the connection of white matter fiber trace

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Introduction: Brain development during infancy is crucial for later health and development. Although Milk Fat Globule Membrane (MFGM) has been demonstrated to enhance brain development, further investigation is needed to determine the optimal dose.

Methods: In this study, 80 piglets aged 2 days were randomly assigned to four groups: Control group, MFGM-L (1.74 g MFGM per 100 g diet), MFGM-M (4.64 g MFGM per 100 g diet), and MFGM-H (6.09 g MFGM per 100 g diet). Daily body weight and milk intake of the piglets were recorded until 31 days postnatal. Learning and memory abilities were evaluated using the spatial T-maze test on day 15. MRI analysis was conducted to assess functional and structural changes in brain tissues. Additionally, mRNA and protein expression of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NTF-3) in the hippocampus and prefrontal cortex were evaluated.

Results: The results indicated that the MFGM supplemented diet significantly improved the accuracy of the piglets in the T-maze test, with the MFGM-L group exhibiting the best performance. MRI showed no volumetric differences in the gray and white matter between the groups. However, the fractional anisotropy in the left and right hippocampus of piglets in the MFGM-L group was significantly higher than in the other three groups. Furthermore, there was a strong correlation between the accuracy of the T-maze test and hippocampal fractional anisotropy.

Discussion: The MFGM supplemented diet also increased the expression of BDNF in the cerebral cortex. However, the changes in BDNF were not consistent with the results of the T-maze test. In conclusion, adding 1.74 g MFGM per 100 g diet can significantly improve neonatal piglets' learning and memory abilities, potentially by enhancing the connection of white matter fiber bundles in the brain.

KEYWORDS

MFGM, memory-improvement, fractional anisotropy (FA), BDNF, infant diet

1 Introduction

The human brain develops rapidly in the last weeks of gestation and the first 2 years of life; brain development is highly dynamic during the fetal stage and the first 2 years after birth (1). Although WHO recommends exclusive breastfeeding up to 6 months of age, 55.8% of infants need formula feeds (2) when breastfeeding is not possible, suitable, or adequate, such as lack of breast milk, maternal and infant disease, and maternal separation (3). There was reported poorer cognitive development in formula-fed infants than in breastfed infants (4). Compared with the formula, these differences may be partially associated with the high concentration of phospholipids, sphingolipids, and ganglioside in the breast formula (5). There is a need to develop formula milk that closely mimics the benefits of breast milk (6).

The milk fat globule membrane (MFGM) has the structure of lipids surrounding every fat globule in breast milk (7). It contributes to 0.2–2% of total fat in breast milk, is the primary source of phospholipids, and plays an essential role in fat delivery (8). The MFGM added to infant formula was demonstrated to be safe (9), and it had potential benefits for brain development (10), such as improving spatial learning (11), promoting reflex development (12), and increasing hippocampal expression of genes related to neurodevelopment (13). However, the production cost of MFGM is relatively high due to its unique composition, which can result in higher prices for MFGM-enriched products.

To achieve a balance between efficacy and affordability, it is crucial to determine the most effective formulation of MFGM that can provide significant functional benefits while falling within a reasonable price range. Additionally, optimizing the composition of MFGM may have environmental benefits, such as reducing food waste and minimizing resource depletion (14). Thus, studying the optimal blend of MFGM is vital for striking a balance between functionality and pricing, which has the potential to benefit both consumers and the environment (15). In addition, the mechanisms underlying optimal MFGM proportion have yet to be fully explored.

Therefore, the purpose of this study is to test the learning and memory, as well as brain development, in neonatal piglets, fed a nutritionally complete diet of the MFGM supplemented diet of three different concentrations. As a large and gyrencephalic species, there are striking similarities between piglets and humans, making the piglet the ideal model for pediatric nutrition and metabolism research (16). The hypothesis was that The brain development of piglets varied depending on the dosage of the MFGM diet. One certain concentration of MFGM-enriched diet would promote brain growth and improve learning and memory.

2 Materials and methods

2.1 Animals and housing

Eighty male ternary hybrid piglets (Landrace × Yorkshire × Duroc, 2 days postnatal, 1.3–2.0 kg each) used in this study were provided by Chengdu Shepherd Boy Village Agricultural Development Co., Ltd., Sichuan Province, China. Two piglets were excluded because of poor growth state. Due to the facility space limitations, the animal experiments were conducted in five separate sessions, each consisting of 16 piglets obtained from three to six different sows. All animals were housed and kept in separate cages at the KangCheng facility [Sichuan KangCheng Biotech Co., Ltd.; animal production license number: SYXK (Chuan) 2019–215] in pig cages for 30 days under controlled temperature: 22 ± 2°C, humidity: 60% ~ 70% under a 12 h-alternate light/dark cycle (lights on at 8:00 am and off at 8:00 pm). Fresh milk powder was provided to the piglets every 2 h a day. At the facility, the standard room temperature was set at 24.5°C. Initially, the temperature in the cage was higher than the standard temperature (33–35°C). Then, the piglets were housed in cages with decreased temperatures as they grew. If a piglet developed diarrhea, it was given a saline rehydration solution. At the facility, 16 two-day-old newborn male piglets per entry were randomly divided into four groups according to body weight when piglets entered the facility, four piglets for each group in every session, and the piglets were reared until 31 days postpartum (Figure 1). All experiments followed the guidelines for the Care and Use of Laboratory Animals provided by the Ministry of Science and Technology of the People's Republic of China (PRC). All animal care and experimental procedures were performed according to the Animal Research: Reporting *in Vivo* Experiments (ARRIVE) guidelines (17) and were approved by the Institutional Animal Care and Use Committee (IACUC) of West China Hospital Sichuan University. The environmental breeding conditions followed the PRC national standard GB14925-2010.

2.2 Dietary

In this study, all artificially reared piglets were fed milk powder provided by Inner Mongolia Dairy Technology Research Institute Co., Ltd. Piglets in the control group ($n = 19$) were fed the control diet, the other groups were provided with diet supplemented with milk fat globule membrane (MFGM; Lacprodan® MFGM-10, Arla Foods Ingredients) of 1.74 g/100 g MFGM (MFGM-L, $n = 19$), 4.64 g/100 g MFGM (MFGM-M, $n = 20$), and 6.09 g/100 g MFGM (MFGM-H, $n = 20$). The specific diet in each group are listed in Table 1. The difference in the four groups mainly comes from the concentration of total phospholipids, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidylcholine, as indicated by the bold part in Table 1. According to the nutritional needs of piglets (nutritional requirement criterion, NRC 2022), the amount of piglets' daily dietary intake is 285 mL/kg*fasting body weight + 50 mL. The milk was freshly prepared with warm water (37–40°C) every 4–5 h, in a 1:4 (powder-to-water) ratio, and the prepared milk was placed in a feeder.

2.3 Spatial T-maze test

Spatial learning and memory were assessed by the spatial T-maze test, the task first used to compare the performance of different pigs by Mendl et al. in 1997 (18). The behavioral device comprises a 45 cm wide cross maze with a removable acrylic clapboard. Piglets can identify the orientation from all four corners using visual cues. Two bowls were placed at T-maze's left (west) and right (east) arms, one with milk as a reward and another empty (Supplementary Figure 1A). Topscan software (Cleversys Co. Ltd. United States version 8, 2020) assessed the piglet's motion trails. The nine-day T-maze behavioral test

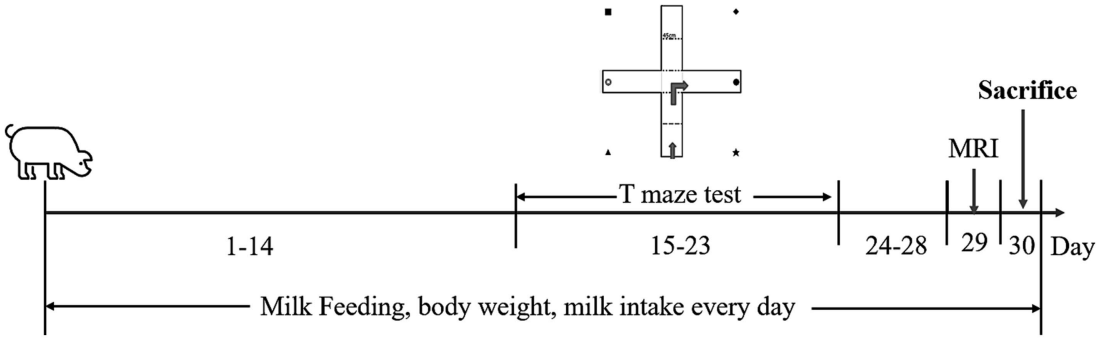

FIGURE 1
Timeline of the experimental procedure. Piglets aged 2 days postnatal (day 1) were brought to the animal facility. They were fed with a nutritionally complete, customized milk replacer for a month. The T-maze tests were performed from day 15 to 23 for spatial learning and memory assessment. MRI was conducted on day 29. The piglets were all sacrificed on day 30.

TABLE 1 The nutritional diet in each group.

	Control	MFGM-L	MFGM-M	MFGM-H
Energy KJ/100 g	464	466	465	464
Protein, g/100 g	23.8	23.6	23.5	23.6
Fat, g/100 g	21.3	21.7	21.4	21.3
Calcium, mg/kg	14,000	14,000	14,000	15,000
Phosphate, mg/kg	846	855	880	880
Zinc, mg/kg	88.8	87.5	87.8	89.3
VE mg/100 g	113	115	113	111
Total phospholipids, g/100 g	0.28	0.36	0.48	0.59
Phosphatidyl serine, g/100 g	0.03	0.04	0.06	0.08
Phosphatidyl ethanolamine, g/100 g	0.07	0.09	0.12	0.14
Phosphatidyl inositol, g/100 g	0.02	0.03	0.04	0.04
Phosphatidylcholine, g/100 g	0.08	0.1	0.13	0.16
Sphingomyelin, g/100 g	0.08	0.1	0.13	0.17

The piglets' daily dietary intake is 285 mL/kg*body weight + 50 mL (fasting weight, ranging from 1.7 to 10 kg). The daily dietary intake of a 1.5 kg piglet is 285 mL/kg*1.5 kg + 50 mL = 477.5 mL, which equals 0.2 g/mL = 95.5 g; MFGM intake is 95.5 g*1.74 g/100 g = 1.66 g. The recommended milk volume for infants aged 0–6 months is 750 mL/day. The corresponding MFGM intake is equivalent to 1.66 g*750 mL/477.5 mL = 2.60 g in infants.

included a six-day acquisition and three-day reversal phases (19). On the morning of day 15, the piglets were fasted (a nightly 6-h food deprivation period), removed from their home cages, and carried to the maze in the adjacent behavioral room until the behavior assessments were completed. In the acquisition phase, they were randomly placed at the northern or southern entrance of the maze (Supplementary Figure 1). They aimed to find the reward milk bowl via visual cues in 60 s. Each piglet was assessed 10 times daily for 6 days between 0900 and 1700 by the same two trained experimenters, and the correct rate was recorded. Within the 3-day reversal phase, the reward milk bowl and visual cues were swapped to assess the piglets' memory and to ensure that the piglets' orientation discernment was not self-centered. The number of incomplete/completed tasks within 60 s and the correct latency were also recorded. All behavioral technicians were blinded to the grouping information of the intervention dietary. The test indices collected include the correct rate (piglets choose reward milk bowl to represent correct), qualification (correct rate reaching 80%), non-qualified rate (the whole rate of animal in correct rate not reaching 80% across the entire test), and the earliest qualified day (the first-day piglets achieving qualification). The trace of pigs in the T-maze was shown in Supplementary Video.

2.4 Magnetic resonance imaging

2.4.1 Data collection

MRI was conducted on day 29 using SIGNA™ Architect AIR™ Edition 3.0 Tesla, 70 cm MRI scanner (GE, United States) with a 64-channel head coil to evaluate the piglet's brain development. Piglets were anesthetized by intramuscular injection of 0.1 mg/kg Zoletil 50 (Virbac, France). Vital signs were monitored after anesthesia. The 3D T1-weighted image (3D-T1) and diffusion tensor imaging (DTI) were scanned sequentially.

The parameters of 3D-T1 scanning were as follows: 0.7 mm thick; Frequency coding direction: front and back; Repetition time 9.1 s; Echo time 3.6 s; Layer number: 100; Signal-to-noise ratio

100%; Matrix: 256 × 256, excitation times: 2; Flip Angle: 12; The bandwidth: 31.25; Scan time: 6:29.

The procedure setting of DTI scanning is as follows: slice thickness 2 mm; Frequency coding direction: right–left; Repetition time: 6826 s; Echo time: 122.3 s; Slices: 26; Signal-to-noise ratio 70%; Matrix:128 × 128, T2 excitation times: 1; The bandwidth: 166.7; B value: 1000; Diffusion direction: 32; Scanning time: 3:52.

2.4.2 Postprocessing

3D-T1 Post-imaging analysis with GE imaging workstation (GE, United States) generated axial, coronal, and sagittal images to calculate the gray and white matter volume. Then, the post-imaging analysis of piglet images, previous DTI, and voxel-based morphological (VBM) measurements was also conducted. Based on DTI, the fractional anisotropy (FA) of four regions of interest (ROIs), hippocampus (left and right, respectively), corpus callosum, and whole brain, were calculated by MRI postprocessing station. The gradient echo T1 MRI file was a 2D tomography file, which was converted into a 3D file for Voxel-based morphological processing using the Neuroimaging Informatics Technology Initiative. Brain tissue was segmented based on the Trusted Platform Module in the Statistical Parametric Mapping software (version 12); 3D files spatially distributed images of gray matter and white matter were obtained by smooth processing. Finally, voxel-based statistics were performed using the images of gray matter and white matter.

2.5 Quantitative RT-PCR

All the piglets were anesthetized by intramuscular injection of Zoletil 50 (Vibrac, French, 0.2 mg/kg). The brains were dissected and weighed. The hippocampus and prefrontal cortex (PFC) were excised from the fresh brain tissue and frozen in liquid nitrogen. Total RNA was isolated from 40 to 100 mg of hippocampal and prefrontal cortex tissues. Tissues were dissolved with 1 mL Trizol (Beyotime Biotechnology) and purified with organic solvent (Chloroform, isopropyl alcohol, and alcohol). The isolated total RNA was then diluted to 500 ng/μL. RNA was converted into cDNA with a reverse transcription kit (Vazyme Biotech Co., Ltd., R323-01). BDNF content was measured with a qPCR kit (Vazyme Biotech Co., Ltd., Q711-02). The purity and concentration of the RNA were evaluated by spectrophotometry (Thermo-Nano Drop 2000c-spectrophotometer) by measuring the mean absorbance ratio and optical density of all RNA at 260/280 nm. BDNF and β-actin primer sequences were as follows: BDNF primer sequence (F: 5'-AGCATTAGCGAGTGGGTGAC-3′; R: 5'-GGGACTTTTTCGAGGACCGT-3′; β-actin primer sequence: F: 5'-GACTGCGCCCATAAAACCC-3′, R: 5'-CACGAGCGCCAGCAATATCGT-3′). The qPCR parameters were as follows: pre-denaturation at 95°C, 30 min, 40 cycles reaction procedure: denaturation 95°C, 10 s; Annealing 55°C, 30 s; The fusion curve program was 95°C 10 s, 55°C 30 s, and 95°C 10 s. (The relative quantification of the transcription levels of the copy melting curve genes was performed by the $^{-\Delta\Delta}$CT method).

2.6 Western blot

We weighed 40 mg of porcine hippocampal tissue and lysed in 1 mL buffer Radio Immunoprecipitation Assay (RIPA; Beyotime, P0013B) with 10 μL phenylmethylsulfonyl fluoride (PMSF; Servicebio, G2008); added 400 μL RIPA for lysis tissue (the RIPA concentration was 10 μL/mg). The tissue lysates were homogenized and lysed on ice for 20 min and centrifuged at 15000 g for 20 min at 4°C. The supernatant was collected as a total protein extract. BCA Protein Detection Kit (Beyotime, P0010S) measured the protein concentration. The total protein was added to 1 × SDS-loading buffer and boiled for 5 min at 100°C. Thirty microgram protein was loaded on 12% SDS polyacrylamide gel (GenScript, M01210C). The separated protein was transferred to the polyvinylidene fluoride (PVDF) membrane (Beyotime, FFP32) and washed with Tris Buffered saline Tween (TBST). At room temperature, the membrane was blocked in a 5% nonfat milk powder blocking buffer for 2 h. The membrane was then incubated with primary antibody [Anti-BDNF antibody (Bioss, bs-4989R, 13 kDa, China); neurotrophin-3 (NTF3) antibody (Affinity, DF7235, 31 kDa); Rabbit anti-beta-actin antibody (Bioss, bs-0061R, 42 kDa)] at 4°C overnight followed by the incubation with secondary antibody HRP-conjugated affinipure goat anti-rabbit IgG (Proteintech, SA00001-2) 1:5000 for 2 h at room temperature. For imaging, they were treated with a chemiluminescence detection kit (Millipore, P90719) and exposed to a chemistry luminescence imaging system (Fluor chem. Co. model protein simple FC3, United States). Each band was quantified using ImageJ software (version 8).

2.7 Statistical analysis

In this study, all data were normally distributed and presented as Mean ± Standard Error of the Mean (SEM). GraphPad Prism 8 software (version 3.1) was used to plot the data. The data were analyzed by SPSS 22.0 statistical software (IBM Corporation, version 26). The parameters were evaluated for variance homogeneity by the Kolmogorov–Smirnov test. Repeated measures analysis of variance (ANOVA) was performed to determine the main effects and interaction effects of body weight, food intake, correct rate, correct latency, and proportion correct at different time points. Differences among the single index, such as white matter volume and FA, were analyzed by one-way ANOVA, followed by Bonferroni *post-hoc* tests. The correlations between correct rate and FA in different regions were analyzed statistically by Pearson's correlation. A $p < 0.05$ was considered statistically significant.

3 Results

3.1 Dietary MFGM was well-tolerated and supported growth of piglets in 31 days

In this study, the piglets were fed with a diet containing different percentages of MFGM. In 30 days, the body weight of piglets was not affected (Supplementary Figure 2A); all body weight gain remained at normal levels of growth [$F_{(3,\ 74)} = 0.295$, $p = 0.829$, Supplementary Figure 2B]. Milk intake showed no difference between groups and stayed at average increases as the piglets' development changed [Supplementary Figure 2C; $F_{(3,\ 74)} = 0.348$, $p = 0.790$, Supplementary Figure 2D]. As part of the total body weight, the brain weight also showed no difference between the groups. These results indicated that dietary MFGM was well-tolerated for piglets and

supported growth. Besides, it was reported that formula feeding increased the risk of diarrhea (20). Unfortunately, two piglets had to be eliminated from the study due to the severity of their diarrhea. For the remaining piglets, the incidence of diarrhea was not alarmingly high. On average, about two or three piglets would experience bouts of diarrhea during each round of experimentation. Notably, this was particularly prevalent within the first 10 days after birth. It is worth mentioning that there were no discernible differences in the incidence of diarrhea among the various groups studied. Overall, dietary MFGM was well-tolerated and supported piglet growth and milk intake in 31 days.

3.2 MFGM improved piglet performance in the T-maze task

The spatial T-maze test was performed on day 15 of feeding, and the trial was divided into two phases: the acquisition and reversal phases. The difference between the acquisition and reversal phases was the direction of visual cues (Supplementary Figure 1). The acquisition phase lasted 6 days, while the reversal phase lasted 3 days. The most important indicator of the T-maze is the correct rate in the 10 trials every day. During the acquisition phase, the correct rate of the piglets improved steadily over time. They completed the learning process by day 3, defined as achieving an accuracy rate of 80%. By day 5, most of the tested piglets met the specified criterion. On day 7, when the reversal phase started, the accuracy rate dropped sharply due to the changed position of the visual cues. Although the accuracy rate improved on the following days (8 and 9), it did not return to the comparable performance level achieved during the acquisition phase (Figure 2A). Repeated measures revealed a day effect on correct rate ($F_{(8,\ 68)} = 10.822$, $p < 0.001$) and no interaction effects between days and groups [$F_{(24,\ 210)} = 0.889$, $p = 0.617$]. Difference existed among four group [$F_{(3,\ 75)} = 3.049$, $p = 0.034$]. The *post-hoc* Bonferroni analysis showed that the MFGM-L group had the highest correct rate, much higher than the control ($p = 0.011$) and MFGM-H groups ($p = 0.044$; Figure 2A). There was a day effect of latency to choice [$F_{(8,\ 68)} = 10.822$, $p < 0.001$] and no interaction effects between days and groups. Significant differences in latency to make a choice existed in each group [$F_{(3,\ 75)} = 3.049$, $p = 0.034$]. However, the *post-hoc* Bonferroni analysis showed only a trend of difference between Control and MFGM-L ($p = 0.083$), as well as MFGM-L and MFGM-H ($p = 0.054$; Figure 2B). The correct proportion demonstrated that in neither the acquisition nor the reversal phase, the MFGM-L had the highest proportion correct (Figure 2C).

The qualification days of the MFGM-L group also showed the best results in the T-maze test's acquisition and reversal phases (Figure 2D). In the MFGM-L group, all piglets were qualified (achieving a correct rate of 80%) in both the acquisition and reversal phases. However, 20% of piglets were non-qualified in 9 days. The other two groups had the same non-qualified rates, higher than MFGM-L but lower than the control (Figure 2E). The other index of T-maze was the earliest qualified day; piglets in the MFGM-L group learned faster than the other groups, but no difference in learning rate was identified among the four groups [$F_{(3,\ 75)} = 1.199$, $p = 0.316$; Figure 2F].

3.3 MFGM promoted the growth of white matter fiber bundles

On day 30, all the piglets were recruited for an MRI scan to measure the gray and white matter volume. The results showed that the representative white and gray matter images were based on VBM, the standard accepted test method (Figures 3A,B). There were no significant differences in white and gray matter volume calculated based on VBM [$F_{(3,\ 57)} = 0.442$, $p = 0.724$; $F_{(3,\ 57)} = 0.760$, $p = 0.521$, respectively; Figures 3C,D]. The other index of brain development was the trace of fiber bundles, with the representative images of fractional anisotropy (FA) determined by DTI in the whole brain, corpus callosum, and hippocampus shown in Figure 3E. No differences in FA were found among each group both in the whole brain [$F_{(3,\ 74)} = 2.237$, $p = 0.091$; Figure 3F] and corpus callosum [$F_{(3,\ 74)} = 1.665$, $p = 0.182$; Figure 3G]; However, there was a statistically significant difference in FA in the left hippocampus among the four groups [$F_{(3,\ 74)} = 5.882$, $p = 0.001$]. A Bonferroni post-hoc analysis revealed significant differences between Control and MFGM-L ($p < 0.001$), MFGM-M ($p = 0.040$), and MFGM-H ($p = 0.005$; Figure 3H). The same trend could be seen in the right hippocampus [$F_{(3,\ 74)} = 6.269$, $p < 0.001$]. A Bonferroni *post-hoc* analysis showed that MFGM-L fiber bundles were much better than control ($p < 0.001$), MFGM-M ($p = 0.006$), and MFGM-H ($p = 0.003$; Figure 3I). The MRI results were consistent with T-maze tests, indicating that MFGM-L contributed to memory-improving and white matter fiber growth.

3.4 The correct rate of the piglet was related to fa in the hippocampus

We performed a correlation test to find the relation between FA and the correct rate in spatial T-maze tests. Specifically, the FA values of the whole brain, corpus callosum, left and right hippocamps white matter fiber bundles were correlated with the area under the curve (AUC) of the correct rate. The correlation between FA in the whole brain and correct rate (AUC) was no difference ($r = 0.344$; $p = 0.665$; Figure 4A). Similarly, the correlation between FA in the corpus callosum and correct rate (AUC) was also no statistical difference but more trend than the whole brain ($r = 0.750$; $p = 0.250$; Figure 4B). However, compared to the above results, the correlation between FA in the left hippocampus and correct rate (AUC) was another story. More correlation and statistical differences were observed ($r = 0.998$; $p = 0.003$; Figure 4C). Analogously, the right hippocampus also correlated with FA, and the correct rate was observed ($r = 0.986$; $p = 0.013$; Figure 4D). This figure shows that the FA value in the hippocampus correlates with the correct rate in the T-maze task.

3.5 The dose-dependent effect of BDNF expression in brain tissue

We further studied the mechanism of MFGM function by examining the expression of BDNF, which plays a role in the growth, maturation (differentiation), and maintenance of the neurons (21). Neurotrophin-3 (NT-3), including BDNF, nerve growth factor, and neurotrophin-4/5, play an important role in neurogenesis (22). The

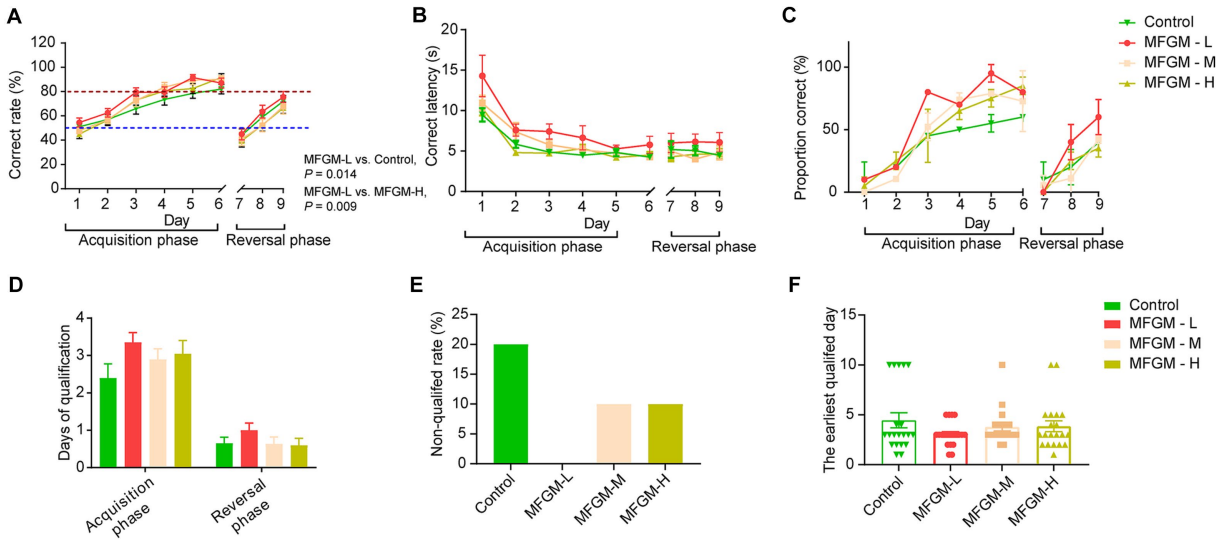

FIGURE 2
T-maze performance of piglets aged 16–24 days. Correct rate **(A)**; Correct latency **(B)**; Proportion correct rate **(C)**; Days of qualification **(D)**; Non-qualified rate **(E)**; The earliest qualified days **(F)**. $n = 20$ in Control and MFGM-H groups; $n = 19$ in MFGM-L and MFGM-M groups. Data were presented as mean ± SEM.

acquisition and consolidation of spatial memory depend on the correct functioning of both the hippocampus (23) and prefrontal cortex (24), where the highest levels of BDNF are found in the central nervous system (25), so we analyzed changes in BDNF in the hippocampus and PFC. There was a significant difference in relative BDNF mRNA expression in the hippocampus among each group [$F_{(3, 58)} = 7.332$, $p < 0.001$]. A Bonferroni post-hoc analysis showed that the BDNF mRNA expression was higher in MFGM-H than in control ($p < 0.001$) and MFGM-L ($p < 0.001$; Figure 5A), indicating that a high dose of MFGM promoted the expression of hippocampal BDNF and the development of the neurons. However, the expression of BDNF in PFC was no statistical difference but only a trend [$F_{(3, 70)} = 1.590$, $p = 0.200$; Figure 5B]. The protein expression of BDNF and NTF -3 was determined by Western blotting, mixing each sample of one group as a new sample. It showed that BDNF expression was the highest in MFGM-L and MFGM-M and lowest in control, while NTF-3 expression was the highest in MFGM-M and most lacking in control (Figures 5C,D). These results demonstrated that after the addition of MFGM, regardless of the dosage, the expression of BDNF and NTF-3 in brain tissues would increase.

4 Discussion

This study investigated the effect of various concentrations of MFGM-enriched diet on memory improvement and brain development in piglets. The concentration of MFGM did not show a dose-dependent relationship in spatial T-maze tests, and the 1.74 g MFGM per 100 g diet was the best choice, especially in memory-improving and white matter fiber bundle enhancement. This was confirmed for the first time through behavioral and imaging analysis.

Piglets were used in this study due to their similarities to infants in terms of brain structure, function, and digestive system function (26). During 30 days of suckling, piglets grow equivalent to an average six-month-old infant (16). Additionally, piglets are advantageous in short growth cycles, fast breeding, and low cost compared to other laboratory animals, such as nonhuman primates (27). The physiological superiority of nonhuman primates is undeniable; however, their limited availability, specialized management requirement, ethical considerations, and high costs significantly restrict their practical applications (28). Besides, the typical litter size of piglets can exceed 10, which is suitable for our large-scale experiments. Due to ethical considerations, exploring the effects of early life insults, specifically infection or nutritional deficiencies, on the development of the nervous system and cognitive abilities in human subjects is infeasible. Consequently, neonatal piglets offer an unparalleled translational animal model that can effectively tackle some of these concerns above.

The spatial T-maze test, widely recognized in the industry, assessed piglet memory. This choice was based on existing literature (29) and its ease of use. The test was designed to evaluate both "place" and "direction" learning, with piglets being trained to locate milk rewards consistently in a designated spot using visual cues (19). In our study, the piglets improved their ability to find the milk reward over time, as indicated by reduced latencies in making choices. By day 5 of acquisition, the piglets reached the criterion of 80% correct choices. However, when the location of the reward was reversed, the correct rate significantly dropped compared to the last acquisition day. Nonetheless, the correct rate gradually increased over 3 days in the reversal phase, which aligns with previous findings (11, 30). While the control group performed reasonably well in the T-maze test, there was still room for improvement, making it a sensitive measure of study and memory. We observed instances where piglets would only turn left or right in the T-maze test without relying on visual cues, resulting in a precise correct rate of 50%. These observations suggest that these piglets might be utilizing egocentric processing, which is self-centered and defined relative to the subject, a mechanism dependent on the striatum, as opposed to allocentric processing, which is

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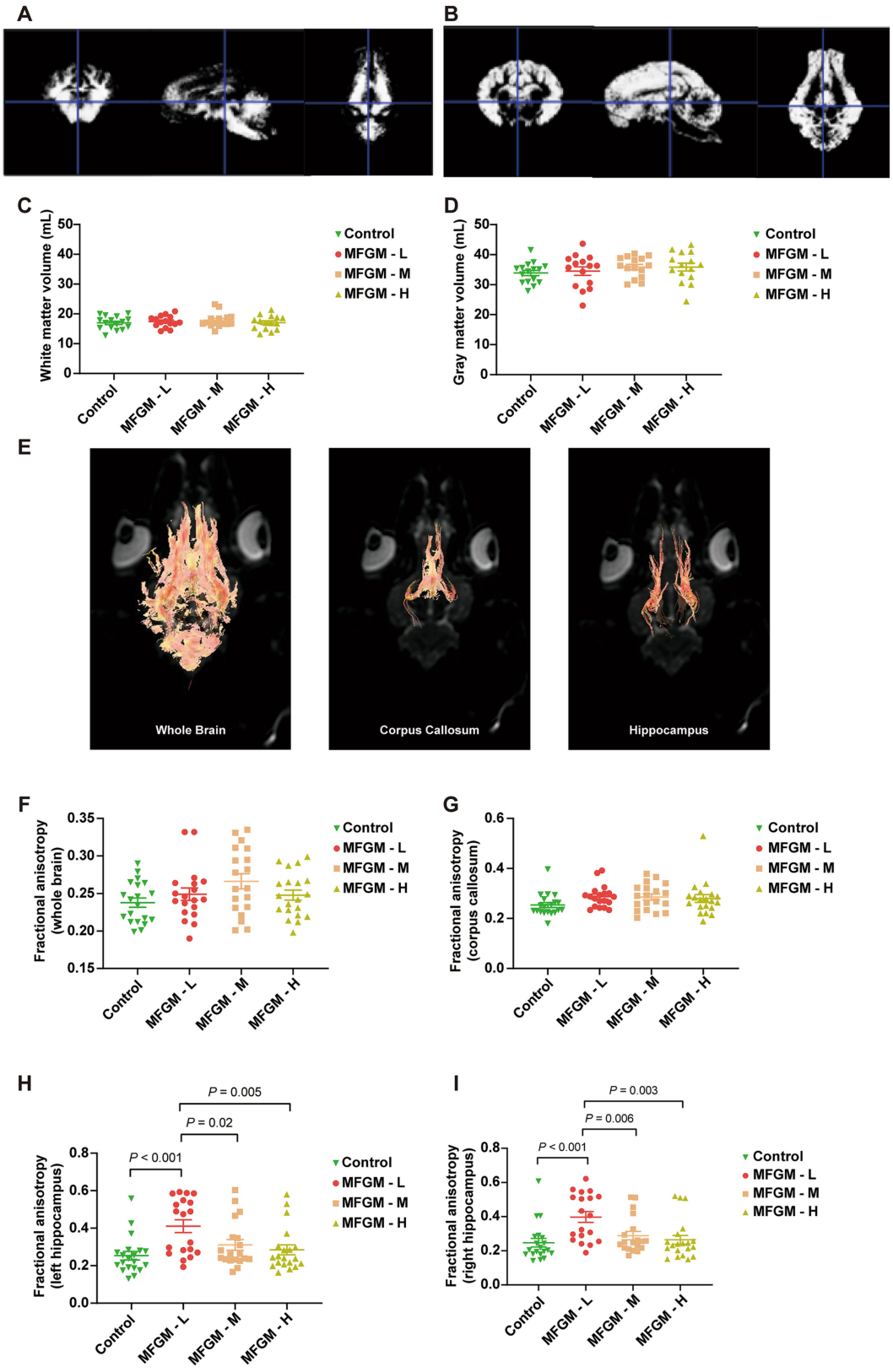


FIGURE 3
The volume and fractional anisotropy of white matter and gray matter determined by MRI. Representative images of white matter **(A)** and gray matter **(B)** determined by VBM. The volume of White matter **(C)**, Gray matter volume **(D)**, Representative image of Fractional anisotropy in of the whole brain, corpus callosum, and hippocampus **(E)**. Fractional anisotropy analysis of the whole brain **(F)**, corpus callosum **(G)**, left hippocampus **(H)**, and right hippocampus **(I)**. $n = 20$ in Control and MFGM-H groups; $n = 19$ in MFGM-L and MFGM-M groups. Data were presented as mean ± SEM.

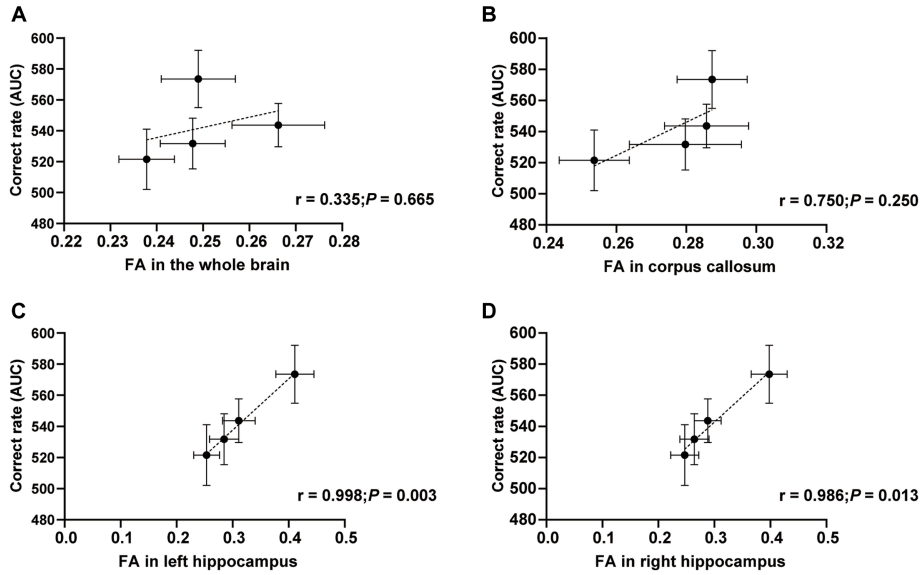
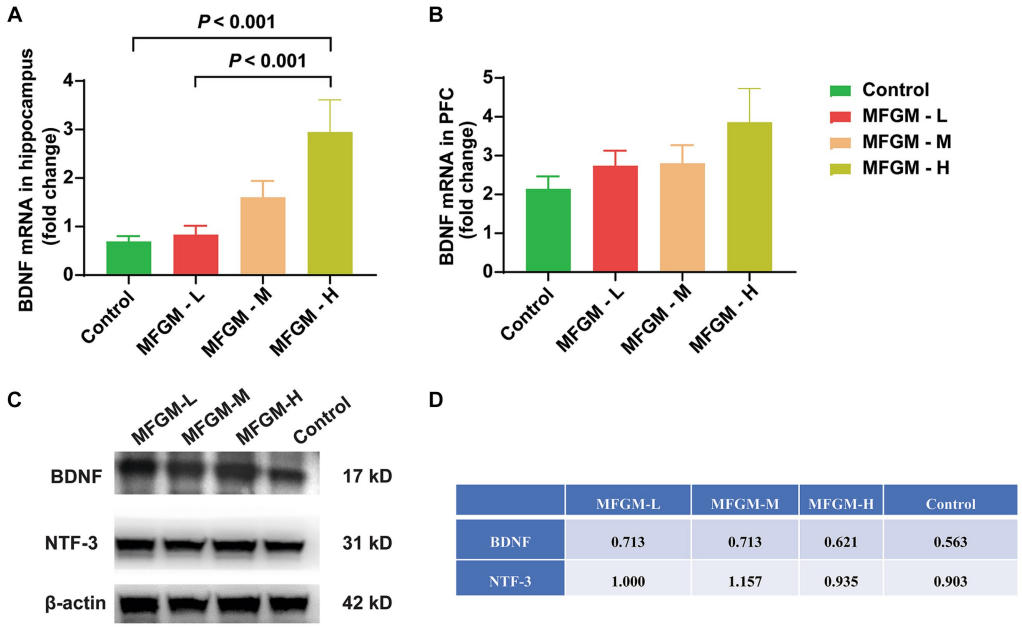


FIGURE 4
Correlations between FA and correct rate. Correlations between FA in the whole brain **(A)**, corpus callosum **(B)**, left hippocampus **(C)**, right hippocampus **(D)**, and correct rate (AUC). *n* = 20 in Control and MFGM-H groups; *n* = 19 in MFGM-L and MFGM-M groups. Data were presented as mean ± SEM.



	MFGM-L	MFGM-M	MFGM-H	Control
BDNF	0.713	0.713	0.621	0.563
NTF-3	1.000	1.157	0.935	0.903

FIGURE 5
The relative expression of BDNF and NTF-3 in the brain. The expression of BDNF mRNA in the hippocampus **(A)** and Prefrontal cortex (PFC) **(B)** relative to β-actin. Control (*n* = 15), MFGM-L (*n* = 18), MFGM-M (*n* = 13), MFGM-H (*n* = 16); The protein expression of BDNF and NTF-3 in the hippocampus in each group **(C,D)**. Data were presented as mean ± SEM.

world-centered and reliant on the hippocampus (19, 31). Notably, when we impaired spatial memory using the anticholinergic drug Scopolamine, the performance of piglets in the T-maze task sharply declined. This demonstrates the effectiveness of the T-maze task in evaluating learning and working memory (19).

MFGM supplementation did not affect the gray and white matter volume. Although total brain size is the most established neuroanatomical predictor of general intelligence, it only accounts for approximately 5% of the variation in individuals' intelligence quotients (32). Early brain development depends on various functional factors, including energy metabolism, myelination, neurotransmission, and synaptic plasticity, which require further investigation (33).

Imaging analysis, the FA value based on DTI, was used to demonstrate the white matter fiber trace and represent brain development and neurodevelopment (34, 35). It was confirmed that DTI was a powerful tool for the study of early brain development, and

higher FA was associated with maturational trajectories of primary and heteromodal association fibers (36). We found that changes in FA of the hippocampus in MFGM groups were much more significant than FA in the whole brain or corpus callosum. Mudd et al. confirmed that dietary sialyl lactose influenced brain development by increasing the diffusion tensor in the corpus callosum (37–39). We did not find the FA changes in the corpus callosum, which suggested that the corpus callosum and hippocampus may be differentially sensitive to MFGM supplementation (40, 41). The selection of cognitive domains – episodic memory, working memory, and information processing speed are contingent upon the pivotal role played by the hippocampus in memory consolidation and information processing. By administering a comprehensive battery of cognitive tests, Morris Moscovitch et al. explored potential links between alterations in hippocampal morphology and changes across multiple cognitive domains (42). Previous investigations into the hippocampus have attested to its heightened involvement in episodic, as opposed to other types of memory. Prior research has also demonstrated a positive association between higher FA level measurement in hippocampal volume and enhanced working memory and verbal memory (43). In accordance with previous literature, our study aligns with the anticipated outcomes.

The lack of a dose-dependent relationship for MFGM in the low-dose group may be attributed to the saturation of MFGM at low doses. Since phosphatidylcholine existed in the control diet, and control piglets did not perform poorly in the T-maze task, it was not easy to discover better T-maze results as the increasing of MFGM concentration in the diet. The inability of the organism to absorb higher doses of MFGM may result in rapid metabolism, thereby hindering the manifestation of a dose-dependent relationship. The MFGM dosage design is based on the effective dosage reported in previous literature and the content of MFGM in both domestic and foreign breast milk. We may reduce the MFGM concentration and redesign the MFGM dose gradient in the following studies to determine the optimal MFGM dose for brain development in neonatal piglets.

The mechanism of MFGM efficacy remains unclear, and previous research aimed to determine the influencing factors in brain development through the expression of BDNF and NTF-3, the important factors in neurodevelopment. In the present study, MFGM-H was found to perform the best in terms of BDNF mRNA expression in the hippocampus, while NTF-3 protein showed only a trend and no statistical difference. After excluding experimental error in PCR and WB, we still observed inconsistent levels of BDNF mRNA and protein expression across different groups. The disconnection might be due to the post-transcriptional modifications, such as mRNA splicing, processing, degradation, RNA editing, and RNA interference, which worth further investigation. It was observed that the positive impact of MFGM on BDNF mRNA enhancement in hippocampus was solely evident at high dosages. This result, however, contradicts the spatial T-maze accuracy outcomes. We discovered conflicting evidence regarding the relationship between BDNF and postnatal neuronal development upon conducting an in-depth investigation. For instance, decreased BDNF has been associated with reduced neuroplasticity, compromised neuronal health, and impaired recovery (44). Studies on BDNF knockout mice have demonstrated impulsive behavior, hyperactivity, and learning deficiencies (45). Conversely, another study suggests that dysregulation of BDNF contributes to the development of intellectual disability, and BDNF levels could serve as an early biomarker for identifying such disabilities (46). Yeom et al. have found a negative correlation between high peripheral BDNF levels and intelligence, behavioral problems, and intellectual disability in preschool children (47). In summary, BDNF at physiological levels supports learning and memory. However, both elevated and reduced levels of BDNF can disrupt inhibitory and excitatory neurotransmission in the brain, leading to a decline in synaptic refinement and memory impairment (48).

Moreover, it is essential to consider that BDNF is a downstream effector protein that works at the cellular level by modulating synaptic plasticity, promoting differentiation and survival of neurons (49, 50). Although BDNF levels only showed significant improvement in the high-dose group, the crucial indicator for improved neural development remains the enhancement of behavioral functions. Evidence suggests that the relationship between changes in behavioral processes and BDNF levels is non-linear. Therefore, future research will further investigate the pathways through which MFGM improves brain development (51). Thus, other factors beyond the presence or absence of MFGM may have played a role in BDNF expression.

Despite the lack of significant differences in our study, it is important to continue exploring the potential effects of MFGM on brain development and function, given its complex composition and its demonstrated benefits for enhancing the richness and orderliness of white matter fiber bundles and behavioral benefits such as improved decision-making and spatial discrimination (52). BDNF research should remain a vital aspect of this investigation, as it is essential for understanding the mechanisms underlying neural plasticity, learning, and memory formation (44). Further studies are necessary to elucidate the interaction between MFGM and BDNF, among other factors, and their impact on cognitive and behavioral outcomes (53). Ultimately, the findings from such research could inform the formulation of more optimized infant formulas that promote optimal brain development and improve long-term health outcomes.

This study presents certain limitations. Firstly, the MFGM concentration did not ascertain the ideal dosage for promoting brain development. It is suggested that the MFGM concentration should be systematically reduced for future investigations to determine the optimal dosage. Secondly, a wider range of biomarkers other than BDNF may be explored to monitor the progress of brain development. Moreover, it is crucial to account for changes in the blood–brain barrier in forthcoming studies.

Overall, this research highlights the dosage of MFGM in the diet, the need for further research in this area, and the potential impact of this research on infant nutrition and development. This study contributes to the market promotion of MFGM dairy products and the development of the Chinese dairy industry.

5 Conclusion

The study investigated the impact of diet with 1.74 g/100 g MFGM on memory improvement in piglets. The effect was possibly due to white matter connection and through the modulation of the BDNF-independent pathway.

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 animal study was approved by Institutional Animal Care and Use Committee (IACUC) of West China Hospital Sichuan University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YZ, ZhZ, and TL conceived and designed the experiments. YZ, BZ, LG, QL, CT, and YW performed the animal experiments. ZP was responsible for imaging. XT was in charge of animal welfare. ZiZ, JH, SD, and SM-Y prepared reagents and materials. YZ, BZ, and ZZ wrote the manuscript. YY polished up the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

ZiZ, JH, SM-Y, YY, SD, and TL are affiliated with Inner Mongolia Yili Industrial Group Co., Ltd. and Inner Mongolia Dairy Technology Research Institute Co. Ltd.

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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1248809/full#supplementary-material

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Setting up the first human milk bank in Uganda: a success story from Nsambya hospital

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Background: The World Health Organization (WHO) strongly recommends the use of donor human milk (DHM) for low birth weight infants when mother's own milk is unavailable or insufficient. However, the use of DHM requires the availability of human milk banks (HMBs), the majority of which are in middle and high-income countries. Developing countries offer multiple opportunities and challenges regarding the establishment and operationalization of HMBs. This study describes the experience in setting up the HMB in Uganda at St. Francis Hospital Nsambya.

Methods: The establishment of the first HMB in Uganda followed a step-wise approach using the PATH's Resource Toolkit for Establishing and Integrating Human Milk Banks. The steps included: performing a facility readiness assessment, implementing quality control measures, forming and training a committee for the Baby Friendly Hospital Initiative, establishing a monitoring and evaluation system, developing a communication strategy, engaging with the Ministry of Health, providing staff training by a Human Milk Bank consultant, and maintaining regular coordination by a dedicated technical team.

Results: A total of 170 donors have been screened and of these 140 have donated milk with a mean age of 26 years since the establishment of the bank in November 2021. A total of 108 admitted neonates have received the milk; majority (88%) are preterm infants with a mean gestational age of 34 weeks. A total of 90 liters have been collected and 76 distributed. The challenges in establishment of the Human Milk bank included: lack of guidelines on human milk banking, use of unpasteurized milk, lack of communication strategy, lack of clear model infrastructure and lactation training. We addressed the challenges: by drafting guidelines, set up a human milk bank and had training on use donor pasteurized milk, designed communication messages through videos and brochures, visited Pumwani hospital and remodeled the Human Milk Bank according to the model at Pumwani, all the health workers in the human milk bank had a training on Lactation. Assessing the experiences and attitudes of mothers, donors,

healthcare providers, and hospital leaders revealed concerns about milk safety and fear about potential attachments or acquired traits through the donated milk to the babies that may receive it. Donors viewed milk donation as a life-saving act, although fears of breast cancer and lumps arose from misconceptions. To address these perspectives, creative media, such as videos and messages, were designed to raise awareness, promote behavioral change, and create demand for the HMB services.

Conclusion: The establishment and integration of HMB services at hospitals in Uganda is feasible.

KEYWORDS

breastfeeding, milk banking, milk bank, human milk, lactation

Introduction

Globally, about 20% of all births, an estimate of more than 20 million infants are born with a low birth weight (LBW) making them 20 times more likely to die that their counterparts (1). Developing countries are disproportionately affected accounting for the majority of LWB babies and highest infant mortality rates in the world (1). In Uganda, LBW babies and premature deliveries account for 1 in 10 deliveries but both accounting for about 3 in 10 infant deaths (2). To avert the high mortality, the World Health Organization (WHO) recommends prioritization of early initiation of breastfeeding for preterm and LBW infants (3). Human milk contains bioactive components that help to protect the medically fragile infant from the development of complications such as sepsis, retinopathy of prematurity, and necrotizing enterocolitis (4, 5). This helps to avert infant mortality, 40% of which occurs within the first month of birth (1). However, the provision of human milk through breastfeeding from mother-to-child is not always a practical option. Many babies may not receive breast milk in the first 48 h of life due to a lack of breast milk, inappropriate lactation and mother's morbidity or mortality (6, 7). In this event, WHO recommends the provision of pasteurized donor human milk (PDHM) from a human milk bank (HMB), as superior alternative to infant formula or any other breastmilk substitutes (3). The establishment of HMBs has gained increasing recognition as a vital intervention for promoting optimal infant nutrition, and health in complex situations (8). Human breast milk is considered the gold standard for infant nutrition as providing essential nutrients, antibodies, and growth factors that support their healthy development (9).

HMBs are a critical as they facilitate the collection, processing, and distribution of donor human milk and also strengthen breast feeding practices as well (10). They act as a vital link to appropriate infant feeding by providing a safe and reliable supply of donated human milk to infants who cannot access their mother's milk. These banks ensure that infants receive the benefits of human milk, promoting optimal nutrition, supporting healthy development, and reducing the risks associated with formula feeding (4, 11). They also offer support to mothers who are unable to breastfeed, fostering a sense of community and promoting knowledge about proper breast feeding practices. However, in many parts of the world, access to safe and sufficient breast milk is a significant challenge, especially for vulnerable infants born prematurely or with medical complications (12, 13). This can lead to serious health problems for babies, including poor postnatal growth, infection, developmental delay and even death (5, 14, 15). The absence of HMBs in these regions poses a significant barrier to providing essential, lifesaving breast milk to infants in need. Without access to a reliable and safe source of donor milk, healthcare providers are often compelled to resort to the use of infant formula and breast milk substitutes as alternative feeding options. Unfortunately, this reliance on artificial substitutes can have detrimental effects on both the initiation and sustenance of breastfeeding practices (3, 16–19). The lack of accessible and affordable donor breast milk through HMBs only exacerbates this issue, as it limits the availability of a vital resource that can be instrumental in overcoming breastfeeding challenges.

In 2021, the first human HMB in Uganda was established at St. Francis Hospital Nsambya in Kampala. The milk bank aims to provide a safe and reliable source of breast milk for babies who are unable to get it from their mothers for the first few days until they attain a regular milk supply with adequate lactation support. St. Francis Hospital Nsambya admits about 400 preterm infants annually of which 60 to 70% of these have very low birth weight and are at higher risk of poor postnatal growth, development and death attributable to lack of breast milk (4, 11, 14, 20). Therefore, the establishment of the first HMB in Uganda is a significant milestone in improving survival for preterm infants. This qualitative study aims to explore the process and experiences of initiating the first human breast milk bank in Uganda. The study findings of this study provide valuable insights into the experience of establishing the first HMB in Uganda. By sharing our journey, challenges, and successes in setting up the HMB, this study contributes to the existing body of knowledge on HMB establishment in low-resource settings. The findings highlight the importance of careful planning, collaboration with stakeholders, and the provision of high-quality training and mentorship to overcome the unique challenges faced in such settings. The establishment of the HMB in Uganda serves as a significant milestone in improving the health and well-being of newborns, and it offers a blueprint for other regions to follow in implementing similar initiatives. This study emphasizes the feasibility and potential impact of establishing HMBs in low-resource settings, ultimately aiming to save and improve the lives of vulnerable infants.

Setting up the first HMB in Uganda

Location

This study was conducted at St. Francis Hospital Nsambya, a private not-for- profit hospital in Kampala, Uganda, which serves as a tertiary referral centre for Makindye division with a total population of 398,800. The following services are available: obstetrics, paediatrics, internal medicine and surgery. Each of the departments have specialists and medical officers and nurses. It conducts 4,000 deliveries per year and provides 24 h comprehensive emergency obstetric and neonatal care services. The obstetric department has 9 obstetricians, 12 postgraduate doctors and 40 midwives. The neonatal unit admits 2,500 neonates annually, has a bed capacity of 50 and 1 neonatologists, 1 pediatrician, 2 medical officers, 25 nurses and 7 postgraduate students. The unit is currently divided into three levels: (1) Neonatal intensive care unit, (2) Neonatal observation unit, and (3) Kangaroo and isolation unit. The neonatal intensive care unit was opened in 2015 and includes 10 beds with access to oxygen therapy, artificial surfactant, low-cost bubble CPAP and mechanical ventilation. The neonatal observation unit opened in 2006 and has a total capacity of 30 beds with access to phototherapy and bubble CPAP. Together, the kangaroo and isolation unit have a total capacity of 10 beds. The feeding protocol ensures that all preterm infants less than 32 weeks or less than or those that sick are started on trophic feeds at 24mls/kg/day and these are increased by 24mls per kilo day till full freed attainment of 160mls/kg/day. The infants above 1.5Kgs and above and are stable are initiated on breast feeding or Cup feeds or Nasogastric feeds with breast milk at 60mls/kg/day. The nurses on the ward offer lactation support to the mothers to ensure that they have enough breast milk through education and counseling and assisting them to express breast milk on the Ward. Additionally, kangaroo mother care is encouraged for all mother's even those whose babies are on Bubble CPAP. Overall, the selection of St. Francis Hospital Nsambya as the site for the HMB takes into account the hospital's role as a tertiary referral centre, its well-equipped neonatal unit, and its existing support structures for breastfeeding and lactation. These factors collectively contribute to the successful implementation and functioning of the HMB, ultimately improving the care and well-being of newborns in the hospital's catchment area.

Setting-up process

Step 1: Learning visit and document review (January 2019)

A team consisting of a neonatologist, nurse, and technician visited Pumwani Hospital, the first HMB in Kenya (21). The purpose of the visit was to learn about the establishment of HMBs, challenges, lessons learned, and the processes involved. Due to the lack of established Ugandan guidelines on HMB establishment, the team relied on guidance provided by the Pumwani Hospital HMB and an international HMB expert and experts from PATH. All HMB processes were shared, including quality control, collection, screening, storage, pasteurization, prioritization, appropriate use of PDHM in the neonatal unit, donor recruitment, and lactation support. The international HMB technical expert and experts from PATH continued providing mentorship through remote support. The team reviewed project documents and made modifications to the project plan based on what they learned at Pumwani Hospital. They also identified lessons learned throughout the project, such as the importance of early initiation of breastfeeding, milk expression, and frequent expression for building maternal milk supply. The information and guidance received from Pumwani Hospital and the international HMB expert was essential for the successful establishment of a HMB in Uganda. Overall, the learning visits and document review at Pumwani Hospital played a critical role in laying the foundation for the successful establishment of the HMB in Uganda. It provided the team with essential knowledge, guidance, and practical insights from an established HMB, enabling them to adapt and apply these learnings within the unique context of Uganda. The collaboration with experienced professionals and the lessons learned from the visit were instrumental in ensuring the effective implementation of the HMB project in Uganda.

Step 2: PATH toolkit adaptation (January 2020)

The PATH HMB Toolkit is an essential resource for anyone involved in the establishment or operation of a human milk bank (22). It is a comprehensive, evidence-based resource that can help organizations ensure that their HMBs are safe, effective, and sustainable. It provides comprehensive guidance on all aspects of HMB operations and is freely. Using the toolkit as a framework, a facility assessment readiness was conducted to assess the existing infrastructure and resources within the newborn unit, which would be transformed to accommodate the HMB. This assessment aimed to identify the strengths and weaknesses of the facility to inform the remodeling process. By adapting the PATH HMB Toolkit and conducting HMB-specific facility assessment readiness, the team gained a thorough understanding of the specific strengths and weaknesses of the facility. This information guided the remodeling efforts to create a suitable and functional space for the HMB. The adaptation of the toolkit and the facility assessment were crucial steps in ensuring that the establishment of the first HMB in Uganda was well-planned, aligned with best practices, and tailored to the local context. The facility assessment enable the team to leverage the strength and mitigate the weakness:

Leveraging strengths

Overall, leveraging strengths such as current feeding practices, the recognition of the need for donor human milk, the presence of local leadership, existing quality control systems, and building networks with nearby hospitals, was essential for establishing a successful and sustainable HMB. By building upon these strengths, the HMB was able to effectively support breastfeeding, provide safe donor human milk to infants in need, and create a supportive network of healthcare providers and facilities. The hospital was able to overcome many of the challenges that could have arisen.

- *Current Feeding practices:* Alternative feeds were used, including expressing milk for preterm infants and milk sharing between mothers. These practices indicated the willingness and ability of mothers to provide breast milk for their babies, even in challenging circumstances. Leveraging this strength is crucial for

promoting and supporting breastfeeding, as well as creating a culture of milk sharing and donation within the community.

- *Identifying need for donor human milk:* Adequate and timely help provided to new mothers to collect their colostrum and initiate lactation when their infants were unable to breastfeed. Health workers also encouraged mothers to do skin-to-skin contact and stay with their babies. This indicated a proactive approach to supporting breastfeeding and encouraging maternal involvement in infant care. Leveraging this support helped facilitate the acceptance and utilization of donor human milk among mothers and healthcare providers.
- *Identifying local leadership:* The presence of a neonatologist, head of the neonatal unit, who was identified as the leader and local champion to establish the HMB. A multidisciplinary leadership team was also identified, consisting of the administrator, the in-charge of the neonatal unit, a microbiologist, a nutritionist, and pediatricians working at the hospital. These leaders possess the necessary expertise, knowledge, and influence to drive the implementation of the HMB and ensure its successful integration within the hospital and the wider healthcare system.
- *Quality Control Systems:* The hospital facility had available appropriate hospital cleaning, disinfection, and equipment maintenance services that were necessary once the human milk bank was established. Leveraging these existing quality control systems ensured that the necessary infrastructure and processes are already established, reducing the burden of implementation and facilitating the smooth functioning of the HMB.
- *Building Networks:* Identified HMB sites to serve as scaling satellite lactation centers, such as the nearby hospitals of Lubaga, Mengo, Kibuli and Naguru. These existing facilities allowed for the establishment of a broader network of support, collaboration, and knowledge sharing, ultimately enhancing the effectiveness and sustainability of the HMB initiative. We anticipate to scale up the Human Milk banking activities to these units in Future.

Mitigating our weaknesses

The overall importance of mitigating weaknesses in the establishment of an HMB cannot be overstated. By taking steps to address these weaknesses, the hospital was able to ensure that the HMB was established in a safe and effective manner. The initiatives aimed at improving feeding practices, establishing a safe and sustainable supply of donor human milk, engaging stakeholders, ensuring quality control, and fostering collaboration and knowledge sharing. These efforts collectively contribute to improving infant health outcomes, promoting breastfeeding, and addressing the healthcare needs of the community.

- *Current Feeding practices:* Alternative feeds involved the use of unpasteurized milk highlighting the importance of starting a HMB. This addressed the need for appropriate feeding practices and emphasized the importance of promoting donor human milk as an alternative bridge when breastfeeding is not immediately feasible. All the health workers were also trained on the use and safety of pasteurized human milk.
- *Identifying need for donor human milk:* The hospital lacked of well-trained lactation support staff, nurses being too busy to assist with breastfeeding, insufficient privacy and facilities for expressing milk, limited availability of breast pumps, inadequate education on expressing milk, storage facilities, and a lack of counseling and support regarding donor breast milk for mothers. Efforts were made to train all health workers on lactation support and to provide comfortable rooms with privacy for expression with in the human milk Bank premises. These measures aimed to improve breastfeeding practices and create a conducive environment for both donor milk and maternal milk expression.
- *Identifying pool of donors:* The lack of HMB-related infant feeding guidelines made it difficult to establish and use donor human milk. The development of infant feeding guidelines were essential steps toward establishing processes for the identification of donors and utilizing donor human milk effectively. This ensures that there is a reliable supply of safe donor milk and promotes the implementation of evidence-based guidelines for infant feeding.
- *Identifying local leadership:* The establishment of the HMB required involvement of stakeholders beyond the hospital. Approval was obtained from the hospital and regional/national leaders for the integration of the HMB. Potential religious and cultural barriers related to the collection, donation, and acceptance of donor milk were identified or examined. A series of important meetings took place to explore the establishment of a HMB. Initially, a stakeholder meeting was convened between St. Francis Hospital Nsambya and ELMA to discuss the potential of having a HMB. Following this, another meeting was held involving St. Francis Hospital Nsambya, the Ministry of Health (MOH) Assistant Commissioner for Child Health, and the Assistant Commissioner for Nutrition, and other hospitals such as Lubaga Hospital and Mengo Hospital, to discuss the scaling of the HMB initiative. The engagement of the local champion played a key role in these discussions. To ensure effective implementation, an advisory committee consisting of 15 members was carefully selected. This committee held regular meetings to assess local needs, provide guidance, and oversee the implementation of the HMB. Additionally, a Technical Working Group was formed, comprising the champion, three local pediatricians, a microbiologist, ward in-charges, ward assistants, and the hospital engineer. This group was responsible for the day-to-day activities involved in setting up the HMB. The formation of advisory committees and technical working groups allowed for collaborative decision-making, guidance, and oversight in setting up and operating the HMB.
- *Quality Control Systems:* A plan for quality assurance, such as hazard analysis and critical control point (HACCP) and HACCP training, was not in place and this was installed after the training using the PATH tool kit and the international Human Milk Banking expert.
- *Building Networks:* No communication platforms were identified to facilitate the sharing of information and best practices between the learning sites established. Therefore, we are in the process of improving networks with the nearby hospitals. Scaling up of the networks and communication networks will be done subsequently in the years 2023–2025.

Step 3: Ensuring quality assurance control (January–December 2020)

First, a Consultant with expertise in HMB was hired in collaboration with PATH to provide guidance and support. With the Consultant's guidance, the HMB guidelines were developed along with the involvement of a neonatologist from Kenya with expertise in the field. These guidelines covered various components such as infant and child mortality, the importance of breast milk as a life-saving intervention, the process of establishing a Human Milk Bank, handling and processing of the milk, criteria for selecting recipients of donor milk, quality control measures, and monitoring and evaluation protocols. The development of these guidelines involved consultations with the advisory committee of the Human Milk Bank, the Ministry of Health, and the Nutritional departments of Uganda. Following the completion of the guidelines, standard operating procedures (SOPs) and registers were drafted to ensure proper execution of activities within the bank. These included procedures related to donor milk culture, identification, tracking, and tracing, as well as allocation and prioritization of human milk. The registers included various records such as equipment cleaning checklists, stock book data dictionary, stock book for the milk bank, pasteurization log, informed consent for receipt of donor human milk, register of human milk donors, thawing and pooling log, and donor milk daily order forms.

To ensure proper utilization of the SOPs and registers, training was provided to all health workers involved in the Human Milk Banking. Additionally, a workshop on Hazard Analysis Critical Control Points (HACCP) Plan, Lactation support was conducted in collaboration with a lactation consultant and the use of the PATH tool kit. The HMB team received training on the HACCP process, which is a quality assurance planning process for food systems. By utilizing standard guidelines for Human Milk Banking, a HACCP plan was developed, identifying critical control points and interventions to reduce hazards. This process highlighted areas where system strengthening was required to ensure optimal quality and safety within the local context. To promote a holistic approach, a Baby Friendly Hospital Initiative Committee was formed to enhance breastfeeding support and practices within the facility. Additionally, monitoring and evaluation activities were implemented to track progress and measure outcomes. An advisory committee was created to provide expert advice and guidance on the operations of the Human Milk Bank. Overall, the importance of these initiatives lay in creating a well-structured, standardized, and safe HMB that adheres to best practices, guidelines, and quality control measures. By establishing clear protocols, providing training, and engaging with expert advisors, the HMB can effectively support breastfeeding, provide safe donor human milk, and contribute to improving infant health outcomes within the facility and the broader community (Table 1).

TABLE 1 List of registers and standard operating procedures.

Register/SOP	HMB	Neonatal unit
Donor milk culture	√	
Human milk identification, tracking and tracing	√	
Allocation and prioritization of human milk	√	
Cleaning of human milk bank equipment	√	
Discarding of donor milk	√	
Donor screening	√	
Donor recruitment	√	
Handling and storage of donor human milk	√	
Milk expression	√	
Processing and testing of donor human milk	√	
Transportation of donor human milk	√	
Donor screening register	√	
Donor screening questionnaire	√	
Donor informed consent form	√	
Register of human milk donors	√	
Received donor milk log	√	
Thawing and pooling log	√	
Pasteurization log	√	
Human milk stock book	√	
Informed consent for receipt of donor human milk		√
Donated human milk daily order form		√
Donor milk dispensing register	√	
Recipient donor human milk log		√
Donor milk recipient record		√
Human milk bank equipment cleaning checklist	√	

Step 4: Procurement, installation, training, and mentorship (January 2021–October 2022)

This period involved the procurement of the necessary equipment, training, and mentorship of the health workers on the use of HMB and lactation. Under the guidance of the HMB consultant and experts from PATH, the facility requirements for the HMB were established by benchmarking them against Pumwani Hospital HMB in Kenya. The hospital administration, along with the HMB team, supervised the construction process. ELMA philanthropies and St. Francis Hospital Nsambya facilitated the procurement, importation, and installation of essential equipment and supplies, including the human milk automated pasteurizer Sterifeed, pasteurization bottles, autoclave, freezers, and refrigerators. A suitable space near the Kangaroo Mother Care room was identified to accommodate the Human Milk Bank, ensuring integration within the health facility. The suppliers of the

TABLE 2 Cost of items procured for the HBM.

Item	Estimated cost (Ugx)	Estimated cost (USD) (1$ = 3,693 Ugx)
Pasteurizer	100,000,000	27,078.26
Refrigerators (2)	48,000,000	12,997.56
Freezers	72,000,000	19,496.34
Autoclaves	5,000,000	1,353.91
Washing machine	3,000,000	812.35
Sterile Glass bottles	3,000,000	812.35
Infrastructure remodeling	85,000,000	23,016.52
Training and Stationary used	500,000	135.39
Consultant Human Milk Banking	28,157,322	7,624.51
Training and stationary used	10,000,000	2707.83
Overall cost	**354,657,322**	**96,035**

equipment provided guidance on the proper functioning and maintenance requirements, which would be monitored locally to ensure smooth operations of the HMB. Table 2 shows the cost of equipment. The most expensive item on the list was the pasteurizer, which was estimated to cost 27,078 USD. The other major expenses were the refrigerators (12,997 USD), freezers (19,496 USD), and infrastructure remodeling (23,016 USD). The total estimated cost of procuring the items required for setting up a HMB was 96,035 USD. The initial cost of setting up the HMB was significant, but it is important to note that this was just the one-time cost of purchasing the equipment and supplies needed to start the HMB. The ongoing costs of operating the HMB, such as the cost of staff salaries, are much lower. The majority of the items listed in the table are one-off items, meaning that they were only needed to be purchased once. This includes the pasteurizer, refrigerators, freezers, autoclaves, washing machine, and sterile glass bottles. The infrastructure remodeling cost is also a one-off cost. The only ongoing costs that was incurred after the HMB is set up are the cost of staff salaries (not included in the table), the cost of milk testing, and the cost of consumables, such as gloves, masks, and gowns. These costs are much lower than the initial cost of setting up the HMB. For example, the cost of staff salaries depends on the number of staff employed by the HMB and their salaries yet these salaries can be offset by integration of the HMB responsibilities within existing staff. The Health Workers working were included on the hospital pay roll as well moreover, these some cost can be off-set by applying affordable patient fees for the service. The cost of milk testing depends on the number of milk samples that need to be tested and the cost of the tests. The cost of consumables also depends on the number of consumables used and their cost (22).

Step 5: Launch (November 2021)

The St. Francis Hospital Nsambya HMB, the first HMB in Uganda encompassing activities such as the collection, processing, storage, and allocation of Pasteurized Donor Human Milk (PDHM) to the neonatal unit. A highly visible official opening ceremony was organized to highlight the crucial role of the HMB in saving newborn lives across Uganda. The MoH officials, Commissioner for Clinical Services, Commissioner for Child Health, Academia including the school of Public Health and many stakeholders will invited for the launch. To create awareness and encourage the use of PDHM, donor recruitment, and breastfeeding promotion, a range of demand generation activities were implemented, including events, one-on-one engagements, mass media campaigns, and social networking. To ensure the smooth functioning of the Human Milk Bank, a nurse from Pumwani Hospital, who is experienced in HMB procedures, was identified to mentor the HMB nurses. Additionally, a systematic approach was taken to recruit donor mothers. A questionnaire was administered to screen potential donors based on age, health status, and their willingness to contribute milk. The establishment of the St. Francis Hospital Nsambya HMB as the first HMB in Uganda represented a significant milestone in improving newborn care and saving lives.

Operationalization of the human milk bank

A nurse from Pumwani Hospital's HMB was chosen as a mentor to provide guidance to the HMB Nurses regarding the necessary procedures. The mentor nurse assisted in training the HMB Nurses on how to utilize the registers and adhere to the SOPs that were developed. Additionally, the mentor nurse provided guidance on offering lactation support to mothers. The next phase of the process involved identifying and recruiting mothers who had an excess supply of human milk. A questionnaire was administered to assess their eligibility as milk donors, considering factors such as age, health status, and willingness to participate. The mentor nurse from Pumwani Hospital provided guidance throughout this process. Once a sufficient quantity of milk had been accumulated, the first batch underwent pasteurization, a crucial step to ensure the safety of the donated milk. The mentor nurse provided guidance during the pasteurization process, and samples were collected and sent to the laboratory for further analysis. Overall, the mentor nurse was important in guiding and supporting the HMB Nurses in various aspects, including using registers, following SOPs, providing lactation support, and ensuring the proper collection and processing of donated milk. Their expertise and guidance contribute to the successful establishment and operation of the HMB.

Collection and recipients of milk

Figure 1 shows the donor recruitment, milk collection and milk administration results from the first 10 months of the operation of the St. Francis Hospital Nsambya HMB. During the period, 170 mothers agreed to become donors. Of the donors, 140 were found eligible and subsequently donated milk to the HMB. The average age of the donors was 27 years and 66% of them gave birth St. Francis Hospital Nsambya. Overall, 89 liters of donor milk were collected, 95% of which passed the post pasteurization screening. During the period, the HMB served 108 infants and 72% of whom were preterm with a mean gestation age of 32 weeks. Inadequate mother's milk was the most common reason for the issuing of donor milk.

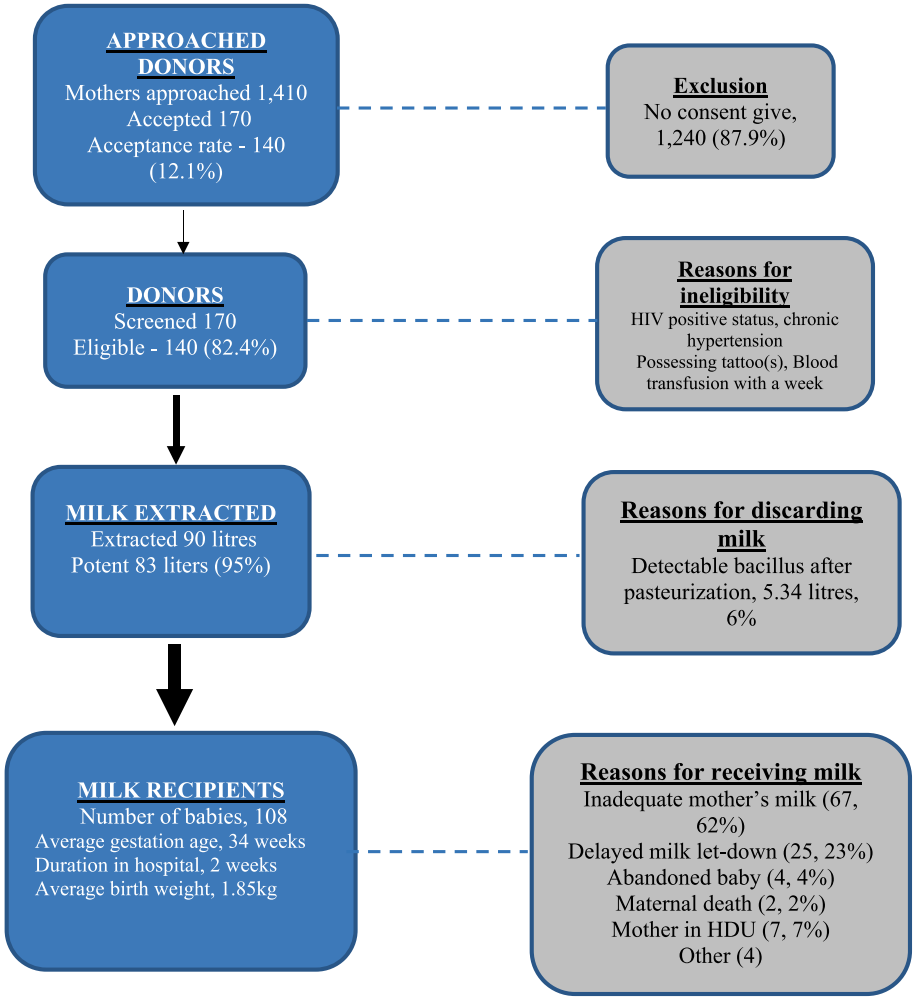

FIGURE 1
Donor recruitment, milk collection, and milk administration.

Perspectives regarding collection and use of human milk

A quick comprehensive assessment was conducted to explore the experiences, attitudes, and perceptions of various stakeholders involved in the HMB. The aim was to gather valuable insights to inform the planning, design of a behavioral change communication strategy, implementation of a demand generation strategy, and facilitate future scalability of the human milk bank. Qualitative methods, including focus group discussions and key informant interviews, were employed to gather data. The assessment revealed several noteworthy findings. Mothers whose babies received donated milk expressed concerns regarding the safety of the milk, particularly among those who had not received proper orientation prior to enrollment. Some mothers also expressed worries about their babies developing attachments to the donors or acquiring characteristics from them. These concerns highlight the importance of providing adequate information and reassurance to mothers about the safety and benefits of donated milk to alleviate their anxieties.

> *"how can I give my children some else' milk, I did not know I had only one week given so after delivery the nurses told me that I had to get a donor, I said aaaahh a donor? How am I going to bond with my kids if am going to give some else' milk?"* – *A recipient's mother.*

On the other hand, the donors perceived milk donation as a way to save lives and expressed happiness in knowing that they were contributing to someone else's well-being. This positive perspective among donors emphasizes the altruistic nature of milk donation and the potential for creating a supportive community of donors.

> *"For me when I gave birth to my baby, I delivered by caesarian section. On day 3, I had the…… the milk which I had wasn't too much but my neighbor had twins and she could suffer with milk so I was advised also to donate the little I had to share with her. This made me feel good because I was saving a life"* – A Milk donor.

Mothers also expressed concerns about their own well-being, with some expressing fear of developing breast cancer and breast lumps as

a result of continued milk donation. This highlights the need for comprehensive education and support for donors to address their concerns and ensure their physical and emotional well-being throughout the donation process.

> *"I told my mother about breast milk donation, do you know what she told me, she told me that "You, are you serious? Do you know what you are going to acquire? You are going to get cancer, better get serious. Do you know the machines they are using? She also told me that the electricity will enter into me…… Let me stop but on the way after donating, I felt things like a ball moving around in my breast so when I went back and told her that something was moving around in my breast, do you know what she told me, "You see, I told you not to go back there, now you have got cancer"* – A Milk donor.

Overall, the findings underscored the need for effective communication strategies to address the concerns and anxieties of both recipient mothers and milk donors. Providing accurate information, addressing misconceptions, and offering comprehensive support helped alleviate worries and promote a positive environment within the HMB. It is crucial to prioritize the well-being of both donors and recipients and ensure that they are well-informed and supported throughout their participation in the milk donation program. Based on these findings, the research team designed creative media, including short video clips and messages, to raise awareness and sensitize the community about the human milk bank. The aim is to create social behavioral change and generate demand for donated milk. These communication strategies can play a crucial role in dispelling misconceptions, addressing concerns, and promoting the benefits and safety of human milk donation. These findings provide valuable insights for the development and implementation of strategies to enhance the acceptance, utilization, and scalability of human milk banks. By addressing concerns, providing education, and creating awareness in the community, it is possible to foster a supportive environment that promotes milk donation and ensures the well-being of both recipients and donors, ensuring the successful implementation and scalability of the HMB.

Conclusion

The establishment of the first HMB in at St. Francis Nsambya Hospital in Uganda demonstrates a comprehensive and feasible approach to improving newborn health through the provision of safe and donor human milk. The feasibility of the project is evident in several key aspects. The initial learning visits to Pumwani Hospital in Kenya provided valuable insights and lessons that were adapted to the local context. The utilization of the PATH HMB Toolkit (1) further facilitated the understanding and implementation of best practices in HMB operations. The identification of local leadership, formation of multidisciplinary teams, and engagement of key stakeholders, including the hospital administration, MoH officials, and other hospitals, ensured a collaborative approach to the establishment of the HMB. This widespread involvement and support were instrumental in overcoming potential religious, cultural, and operational barriers. The development of guidelines, standard operating procedures, and a HACCP plan ensured the implementation of robust quality control measures. Training programs provided to health workers, including lactation support training, further enhanced the safety and effectiveness of the HMB operations. The successful procurement, importation, and installation of necessary equipment and supplies, as well as the identification of suitable space within the hospital, showcased the project's feasibility in terms of resource allocation and infrastructure development. The implementation of demand generation activities, including mass media campaigns, one-on-one engagements, and community sensitization, contributed to the recruitment of donors and the promotion of PDHM usage. The findings from the stakeholder perspectives highlighted the need for comprehensive education and support for both donors and recipients, which can be addressed to further enhance the feasibility and acceptance of the HMB. Overall, the establishment of the HMB at St. Francis Nsambya Hospital in Uganda demonstrates a feasible and sustainable approach, with careful consideration given to local contexts, stakeholder engagement, quality control, infrastructure development, and demand generation. This comprehensive and collaborative effort ensures that safe and donor human milk is available for infants in need, ultimately improving newborn health outcomes in Uganda.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Nsambya Hospital institutional review board (SFHN-2023-106). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

VN: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. JK: Data curation, Formal analysis, Project administration, Supervision, Writing – review & editing. RNas: Supervision, Writing – original draft, Writing – review & editing. SN: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. RNaz: Writing – original draft, Writing – review & editing. HM: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. CNu: Writing – review & editing. RK: Supervision, Writing – review & editing. RNan: Data curation, Formal analysis, Writing – review & editing. CNa: Data curation, Writing – review & editing. BNal: Supervision, Writing – review & editing. IN: Supervision, Writing – review & editing. MN: Supervision, Writing – review & editing. CK: Supervision, Writing – review & editing. ON: Supervision, Writing – review & editing. JN: Supervision, Writing – review & editing. BNam: Supervision, Writing – review & editing. PK: Project administration, Supervision, Writing

– original draft, Writing – review & editing. GW: Supervision, Writing – review & editing, Formal analysis.

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Conflict of interest

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

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A case series exploring the human milk polyclonal IgA1 response to repeated SARS-CoV-2 vaccinations by LC–MS based fab profiling

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Introduction: Upon vaccination against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) humans will start to produce antibodies targeting virus specific antigens that will end up in circulation. In lactating women such antibodies will also end up in breastmilk, primarily in the form of secretory immunoglobulin A1 (SIgA1), the most abundant immunoglobulin (Ig) in human milk. Here we set out to investigate the SIgA1 clonal repertoire response to repeated SARS-CoV-2 vaccination, using a LC–MS fragment antigen-binding (Fab) clonal profiling approach.

Methods: We analyzed the breastmilk of six donors from a larger cohort of 109 lactating mothers who received one of three commonly used SARS-CoV-2 vaccines. We quantitatively monitored the SIgA1 Fab clonal profile over 16 timepoints, from just prior to the first vaccination until 15 days after the second vaccination.

Results: In all donors, we detected a population of 89–191 vaccine induced clones. These populations were unique to each donor and heterogeneous with respect to individual clonal concentrations, total clonal titer, and population size. The vaccine induced clones were dominated by persistent clones (68%) which came up after the first vaccination and were retained or reoccurred after the second vaccination. However, we also observe transient SIgA1 clones (16%) which dissipated before the second vaccination, and vaccine induced clones which uniquely emerged only after the second vaccination (16%). These distinct populations were observed in all analyzed donors, regardless of the administered vaccine.

Discussion: Our findings suggest that while individual donors have highly unique human milk SIgA1 clonal profiles and a highly personalized SIgA1 response to SARS-CoV-2 vaccination, there are also commonalities in vaccine induced responses.

KEYWORDS

antigen binding fragment, immunoglobulin A, mass spectrometry, human milk, COVID-19

Introduction

Immunoglobulins (Ig), or antibodies, are a key part of the adaptive immune response capable of specifically recognizing and binding to antigens derived from bacteria or viruses initiating and aiding in their neutralization. Every individual has a unique antibody repertoire generated by a magnitude of distinct antibody-producing B cells, with estimates ranging from 10^{13} to 10^{18} (1, 2). Throughout our lives these repertoires are built up by encountering a huge variety of pathogens and other foreign stimuli, which we are exposed to daily or at specific moments in time, such as vaccines. However, at a given moment in time there are likely only hundreds to thousands of different detectable antibodies in human serum and milk, and typically the top 50 most abundant Ig clones account for up to 90% of the complete Ig repertoire (3–5).

In our first moments of life, we begin to build this repertoire and are provided passive immunity through breastfeeding, receiving in most cases our mother's own unique antibodies. After natural infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), SARS-CoV-2 specific antibodies with neutralizing capacity are present in human milk and are thought to provide immunity to infants (6–12). Due to the overwhelming health benefits of breastfeeding and the absence of vertical transmission of SARS-CoV-2 via human milk (6, 8, 13–15) have led to the advice of the WHO to encourage mothers to continue breastfeeding their infant during the COVID-19 pandemic (16). Recently, several SARS-CoV-2 vaccines have been widely administered to people around the world. While the accumulated evidence has shown that these vaccines are safe and effective also for pregnant and lactating women (17–22), this more vulnerable group was excluded from initial SARS-CoV-2 vaccine trials. Therefore, information regarding vaccine driven antibody development in lactating women is still rather limited. This information is beneficial for breastfeeding women to make a well-informed decision regarding vaccination to confer protection to not only themselves, but also their immune naïve infant (23). The most abundant Ig in human milk is IgA at a concentration of 1.0–2.6 g/L being 10 to 100 times greater than IgG and IgM, respectively, (24, 25). IgA comes in two subclasses IgA1 and IgA2, with IgA1 typically being the more abundant subclass in human milk. We recently developed methods to study IgA1 clonal repertoires in human serum and milk. After affinity-purification, all IgA (IgA1 and IgA2) molecules from human serum or milk (4, 5) become bound to the affinity resins, whereafter we use specific enzymes to cleave IgA1 molecules selectively, yielding the fragment antigen binding (Fab) domains that harbor the complementarity determining regions. These Fabs are then subjected to intact mass analysis by LC–MS clonal profiling. This yields a clonal profile that typically contains several hundred unique clones, each identified by a specific LC–MS signature based on mass and retention time. We can quantify the human milk concentrations of each Fab clone by spiking in recombinant IgA1 mAb standards (4), enabling us to monitor the abundance of individual clones over time. Monitoring the human milk secretory IgA1 (SIgA1) clonal repertoire of healthy individuals, we observed that they are relatively simple, being dominated by just a few hundred to thousand different clones at a given time. These repertoires are unique and highly personalized as we do not observe the same clones in more than one donor. Furthermore, we found the human milk SIgA1 repertoires of healthy donors to be very stable over time, with all SIgA1 Fab clones having Pearson correlations >0.8 (4). However, the clonal repertoires of individuals that experience serious illness, can undergo distinct and sudden changes (3, 26).

Mothers that were previously infected with SARS-CoV-2 have significantly higher concentrations of spike specific IgA in their breastmilk than negative controls (9), and using LC–MS we were able to detect spike specific SIgA1 Fab fragments in these donors. Interestingly, concentrations of spike specific IgA in human milk had little correlation with neutralization capability, and spike specific SIgA1 Fabs were of a relatively low concentration when compared to total SIgA1 in human milk. Other studies have also shown weak correlations between antibody titers and the frequency of recirculating memory B cells relative to a respective antigen (27). These findings suggest that high concentrations of antibodies may not be good predictors for effective viral recognition and binding. Detailed knowledge about the emergence and evolution of antibodies in response to vaccination could render better insights into the immunity they provide and thereby yield better predictors for its longevity and effectivity.

Here, we aim to expand the knowledge about the antibody response of lactating women following SARS-CoV-2 vaccination by investigating the SIgA1 profiles of six individuals that received repeated mRNA-based or vector-based SARS-CoV-2 vaccines. Donors and their samples for this observational longitudinal case series were selected from a previously described cohort, with the criteria of having high SARS-CoV-2-specific IgA titers in human milk (28). Using LC–MS Fab clonal profiling, we monitored the abundance of individual SIgA1 clones and studied the antibody response at a clonal level of detail. We used a novel computational approach in this study to detect SIgA1 Fab clonal populations emerging after vaccination by eliminating all clones that were present before a response to vaccination could be expected. The human milk SIgA1 clonal repertoires of six individual donors receiving one of three SARS-CoV-2 vaccines were quantitatively monitored over 16 timepoints. All six donors had unique SIgA1 clonal repertoires in which longitudinal changes were observed, with novel clonal populations emerging after both the initial and second vaccination. Our data reveals that antibody responses to vaccination are highly personalized traits and argues for monitoring antibody responses beyond the total Ig titer level, using a more detailed, personalized, and longitudinal approach.

Results

Vaccination results in a heterogeneous polyclonal response

In this observational longitudinal case series, we recorded the human milk SIgA1 clonal profiles of six individual donors receiving either Comirnaty, Spikevax or Vaxzevria vaccines (Figure 1; Supplementary Table S1). We detected a total of 2,553 clones across all donors, ranging between 229 and 505 unique clones per donor (Figure 2A), excluding clones that were only found at a single timepoint from all subsequent analysis to limit false discoveries. In line with our previous studies, there was virtually no overlap in the clonal repertoire between donors (only a single clone had an overlapping mass and retention time between donors). In contrast, overlap within each individual donor over the longitudinal sampling

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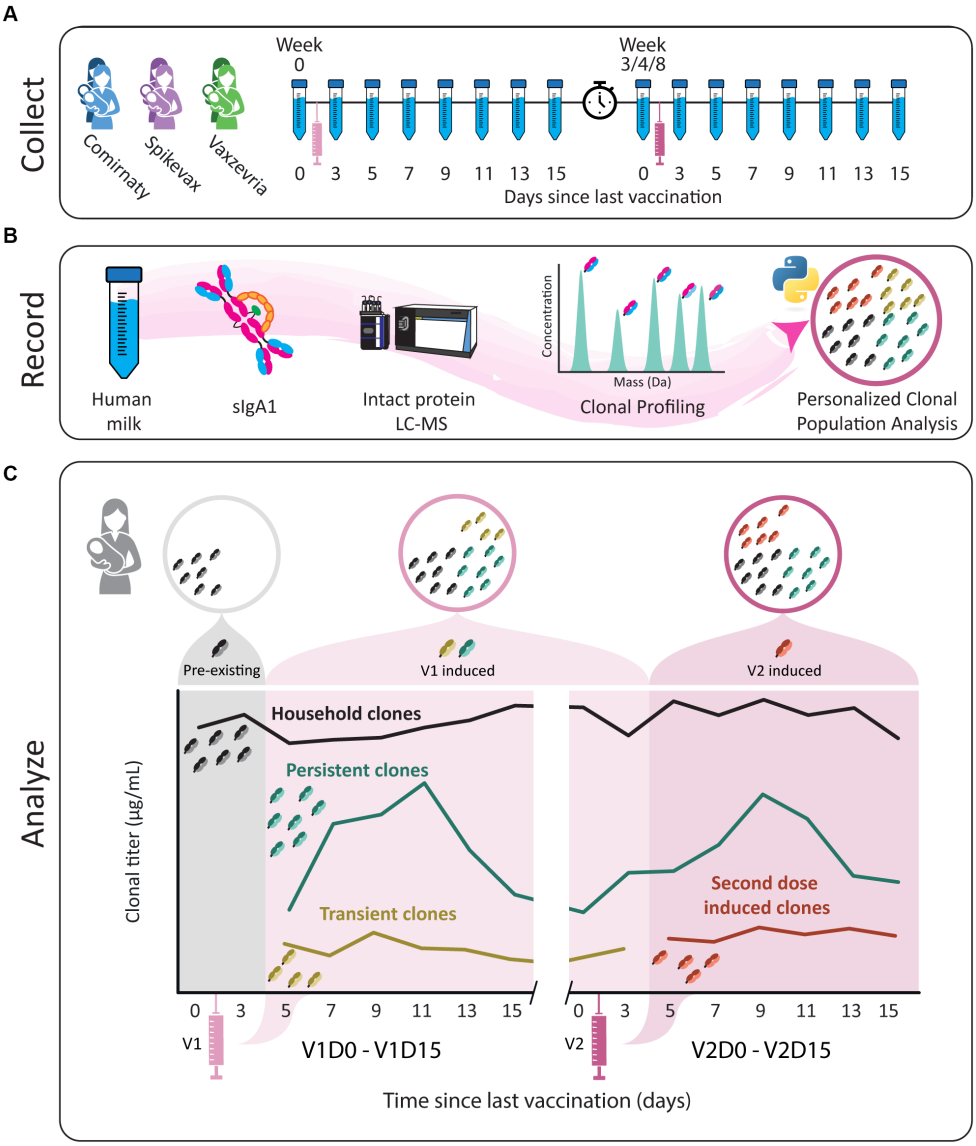

FIGURE 1
Study workflow. **(A)** Human milk samples were obtained from six donors across 16 timepoints, from just prior to the first vaccination until 15 days after the second vaccination. Individual donors received one of three vaccines, BNT162b2/Comirnaty (blue), mRNA-1273/Spikevax (purple) or AZD1222/Vaxzevria (green). The sample collections are indicated by the tubes and each vaccination with a syringe. The clock indicates the gap in time between vaccinations. **(B)** SIgA1 was affinity-purified from human milk. Subsequently, proteolytically formed SIgA1 Fab fragments were separated and analyzed by LC-MS to obtain a list of clones (i.e., Fab molecules with a unique mass/retention time pair). The concentration of each clone was retrieved at the sampled timepoints using two recombinant IgA internal standards. Clones were then assigned to populations based on their window of detection relative to vaccination, and these populations were analyzed for each donor individually. **(C)** Illustrative examples of abundance profiles of clonal populations over time. The y-axis shows the clonal titer (i.e., the summed concentrations of the clones) for each population over time. Timepoints are referred to as for example V1D3, where D3 indicates the number of days since the last vaccination and V1 indicates the last vaccination. Clones were assigned to one of four populations based on their detection window relative to vaccination. The black line represents *household* clones, SIgA1 clones that were detected in one of the first two timepoints, before a response to vaccination could be expected based on analysis of the parent cohort. All other clonal population were absent from these time points and are considered vaccine induced clones. The remaining three populations designated are *persistent* (teal), *transient* (mustard) and *second dose induced* (maroon) clones. The transient population consists of clones that are only detected in the window V1D5 - V2D3. The persistent clones are clones that arise in the window V1D5 - V2D3 and are also detected after V2D3. Clones in the second dose induced population are clones that were not observed until after V2D3.

was exceptionally high (over 95% of clones were detected at more than one timepoint).

For all donors, the SIgA1 clonal repertoire was dominated by abundant clones that were already detected in the first (V1D0) or second (V1D3) milk sample, before a clonal response is expected as it is prior to or too close to vaccination, as also confirmed based on ELISA data (28) (Supplementary Figure S1). These clones, which we term *household* clones (Figure 1C; Supplementary Figure S2), accounted for 92% of the total SIgA1 *clonal titer* (the summed abundance of all clones) of all samples combined (Figure 2B) and 83–99% of the total SIgA1 clonal titer in any single sample. In each donor, we detected between 89 and 191 clones that emerged more

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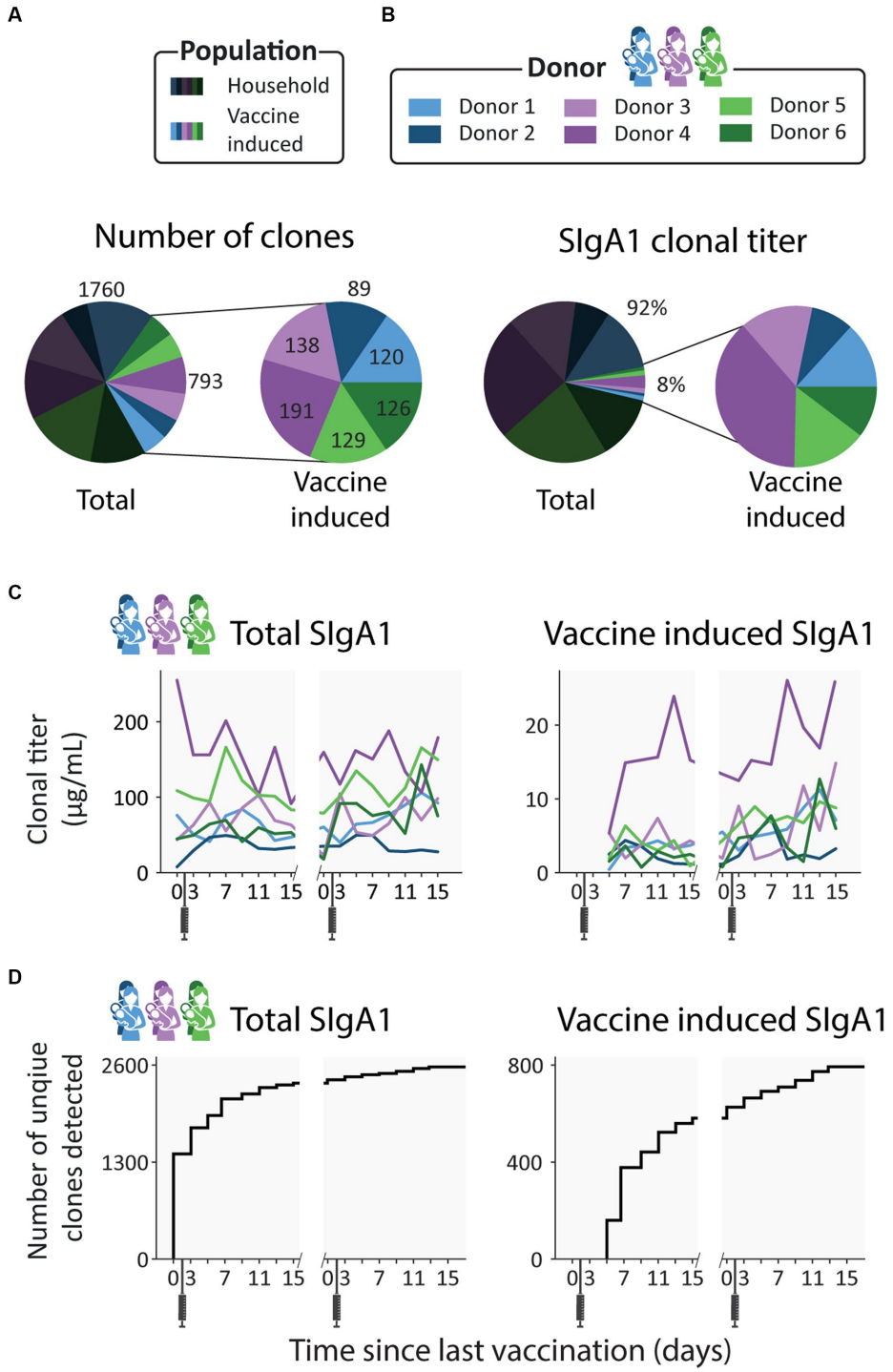

FIGURE 2
Emergence of novel clones after vaccination. **(A)** Pie charts showing the number of unique clones designated as household (dark) and vaccine induced (light), colored per donor. Vaccine induced clones made up 31% of all detected clones (793 out of 2553 clones). **(B)** Pie charts showing the percent total abundance the household clones (dark) and vaccine induced clones (light), colored per donor. **(C)** Longitudinal changes in total SIgA1 clonal titer (left) and vaccine induced SIgA1 clonal titers (right) for each donor. Vaccine induced SIgA1 clonal titers rise in response to the repeated vaccinations and make up an average of 7% of the total SIgA1 clonal titer. **(D)** Total number of unique clones detected over time (left) and number of unique vaccine induced clones (right). Novel clones emerge shortly after vaccination and by day 7 nearly half of all vaccine induced clones (377 out of 793) have been detected.

than 3 days after the first vaccination (V1D5 and later) (Figure 2A). These clones, which we termed *vaccine induced* clones (Figure 1C; Supplementary Figure S2), made up 31% of the total detected clones (793 out of 2,553 clones, Figure 2A). These vaccine induced clones were comparatively low in abundance and made up a relatively small portion of the total SIgA1 clonal titer per sample (Figure 2C). Most of

the vaccine induced clones emerged shortly after the first vaccination was administered: 47% of the vaccine induced clones (377 clones), were first observed between V1D5 and V1D7 (Figure 2D). This agreed with the ELISA findings for these same samples, where anti-spike SIgA titers started rising around day 5, and further sharp increases were observed 9 days after vaccination (Supplementary Figure S1).

Novel clonal populations emerge after the second vaccination in all donors

As the vaccines the donors received consist of two doses, we defined four clonal populations based on the window of detection relative to both vaccinations (Figure 1C; Supplementary Table S1). The first population we termed above as *household* clones. These are SIgA1 clones that were detected before a clonal response was expected, at V1D0 or V1D3. The previously described *vaccine induced* clones were categorized into three distinct populations: *transient*, *persistent*, and *second dose induced* clones. The transient and persistent populations are both made up of clones that were detected before timepoint V2D3 but were absent at the first two timepoints (V1D0 and V1D3). The transient clones were *only* detected in the time window from V1D5 to V2D3. Persistent clones arose in this same time window but were also detected after V2D3. Clones in the second dose induced population are clones that were first observed after V2D3.

These four populations were observed in all donors. Persistent clones were the largest population: 21% of all detected clones were persistent clones (539 clones, Figure 3A), and persistent clones made up between 50 and 80% of donor specific vaccine induced clones (Figure 3B). The transient and second dose induced populations were much smaller and more diverse. The transient and second dose induced populations each make up 5% of all clones (126 and 128 clones respectively, Figure 3A), and 5–20% and 5–27% of donor specific vaccine induced clones (Figure 3B) respectively. When looking at the fractional clonal titer (i.e., the proportion a population contributed to the total SIgA1 clonal titer at a single timepoint) over time, the behavior of these populations was remarkably similar between donors (Figure 3C). The persistent clones dominated here too, as they made up the bulk of the vaccine induced clonal titer at nearly all timepoints and on average of 5.9% of the total SIgA1 clonal titer. Transient and second dose induced populations accounted for a much smaller fraction (on average 0.7 and 1.7% respectively) of the total SIgA1 clonal titer for any single sample (Figure 3C).

Clonal titer fluctuations can be driven by highly divergent clonal populations

From the ELISA analysis by Juncker et al. (28), donor 4 was identified as the strongest responder in terms of spike-specific IgA. This prompted us to have a closer look at this donor. Our analysis confirmed the strong response, as the vaccine induced clonal titer reached a peak concentration of 26.3 µg/mL, higher than any other donor (maximum 14.7 µg/mL, Figure 2C), and featured 191 unique clones, more than any other donor (maximum 138 clones, Figure 2A).

Uniquely in donor 4, we observed that the second dose induced clonal titer increased comparably to the persistent clonal titer (Figure 3C), indicating that the clonal makeup of the response to the second vaccination was strongly divergent from the response to the initial vaccination. Despite looking very similar to the first phase of the biphasic response (Figure 2C), the second phase of the response was largely driven by the second dose induced population and not the persistent population that was induced by the first dose as the persistent population clonal titer remained relatively stable (Figure 3C). The second dose induced population that drives this second peak in the vaccine induced titer is the largest and most abundant in this study, consisting of 52 unique clones (Figure 3), peaking at over 10 µg/mL (Supplementary Figure S3). Additionally, the second dose induced population made up 45–50% of the vaccine induced clonal titer and 39–44% of vaccine induced clones during the last 3 timepoints (Figure 4), demonstrating how seemingly similar titer fluctuations can be driven by highly divergent clonal populations.

At these final timepoints, the persistent clonal titer had decreased to approximately half its peak value (Supplementary Figure S3). However, we did not observe a similar decrease in the number of detected persistent clones suggesting either a simultaneous drop in the intensity of the individual persistent clones or a strong decrease in abundance of one or more dominant clones from this population. Inspection of the individual persistent clones revealed that at its peak (V1D13), the persistent population included three highly abundant clones which together made up 60% of the persistent clonal titer (Figure 4). While initially these highly abundant clones almost completely dictated the persistent clonal titer fluctuations, they quickly declined in abundance after an initial peak and eventually disappeared while the persistent clonal titer remained relatively stable (Figure 4; Supplementary Figure S2), seemingly causing the persistent clonal titer drop between the first and second phase.

A similarly dominant second dose induced clone was observed to increase in concentration as the three dominant persistent clones were decreasing at V2D5 (Figure 4). The abundance profile of this clone mirrored the upward trending second dose induced titer (Supplementary Figure S3) and was the most abundant clone at the final timepoint, at 3.1 µg/mL (Figure 4). However, 37 other second dose induced clones are still detected at the last timepoint and as we saw with the persistent clones, it may be these lower abundant clones that persist in the long term.

These dominant clones demonstrate how clonal titers fluctuations can be strongly influenced by a limited number of abundant clones. Sometimes these clones only amplify the behavior of their parent population, as with the highlighted second dose induced clone from donor 4. However, they may paint a misleading picture by masking the cumulative behavior of the remaining, lower abundant, clones in the population. The clonal resolution of the LC–MS based Fab profiling method enables us to zoom in on individual clones and allows us to confirm the presence or absence of factors that drive polyclonal responses.

Discussion

The current body of knowledge about humoral immunity in response to infections and vaccinations are normally determined by ELISAs for total antigen-specific antibody titers and more recently by screening for antigen-specific B-cells. However, recent work by Wolf et al. (27) shows that the antibody titers are poor markers of the frequency of memory B cells after an infection. Therefore, alternative

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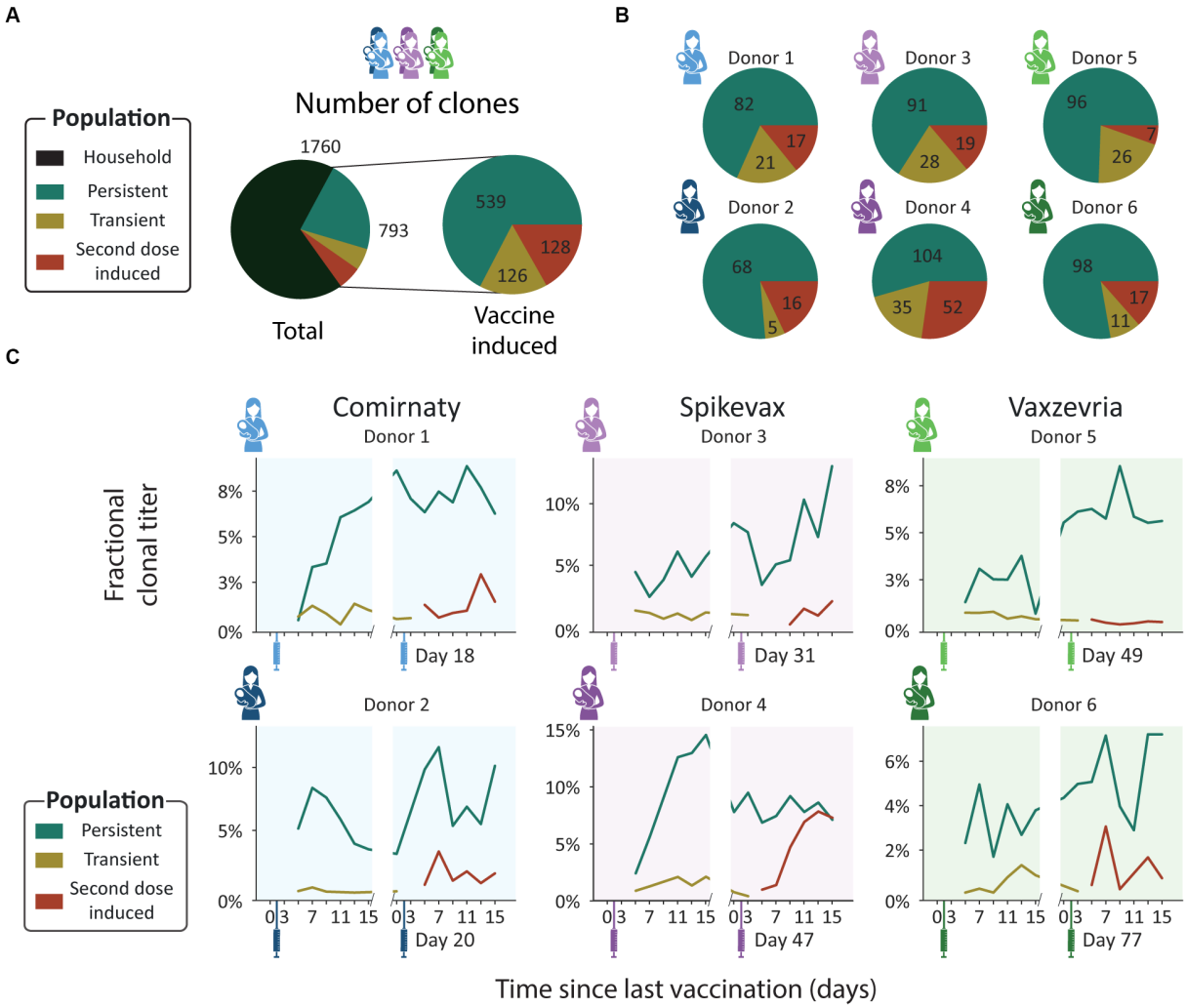

FIGURE 3
Clonal population analysis. **(A)** Pie charts showing the total number of unique clones in each population. Clones were assigned to populations based on their detection window relative to the vaccination moments: persistent (teal), transient (mustard), and second dose induced clones (maroon). **(B)** Pie charts showing the number of unique clones in each population per donor. Each pie chart shows data for a single donor [Comirnaty (2 blue donors), Spikevax (2 purple donors) and Vaxzevria (2 green donors)]. **(C)** Longitudinal changes in fractional clonal titer (i.e., fraction of the total clonal titer made up by each population) for the vaccine induced clonal populations. Vaccination moments are depicted as color-coded syringes. Each panel shows donor-specific, fractional clonal titers for the three vaccine induced populations. While all donors show a unique repertoire without overlapping clones, varying in number of clones and total clonal titer, when grouped into populations the responses are more consistent. Persistent clones make up the bulk of the vaccine induced SIgA1 clonal titer at nearly every timepoint. The clonal titers of the transient and second dose induced populations account for a much smaller fraction of SIgA1.

analyses are needed to assess the basics of humoral immunity. We may gain new insights by uncovering when, how and why specific antibodies come up after an infection or vaccination. A first step in doing this is by monitoring individual clones and extracting patterns from clonal populations, as we demonstrate here.

To date, many studies have been conducted to evaluate the antibody titers in human milk from either SARS-CoV-2 infected or vaccinated women. A recent systematic review by Nicolaidou et al. highlights that many different studies were able to detect SARS-CoV-2 specific IgAs and IgGs in human milk after vaccination, however there were inconsistent results of these studies regarding human milk vaccine induced IgAs and their ability to neutralize the virus (29). A possible explanation for this inconsistency could be due to the discrepancy in distinguishing between IgA and SIgA, as it is known for other vaccines like influenza that SIgAs are important for neonatal protection but require special consideration when analyzed (30). Additionally, like Influenza vaccines, it is possible that stronger antibody responses are elicited in the infant if the vaccine is administered during pregnancy, suggesting that also the type of antibody is important for greater protection (18, 31). To further support this, some studies showed no change in IgA in human milk related to neutralization, but showed an increase in anti-SARS-CoV-2 IgGs in human milk lead to neutralization (29). While some of the studies did find back neutralizing IgAs in human milk, these results were not as conclusive as for neutralizing IgGs. In the review by Nicolaidou et al. it was concluded that even though IgA, specifically SIgA, is the dominant Ig in human milk and mucosal surfaces, vaccination against COVID-19 in lactating mothers lead to a dominant IgG response (29). Nicolaidou et al. suggested that the dominant IgG response and low levels of IgA in human milk from the vaccinated mothers could be due to route of exposure to the viral

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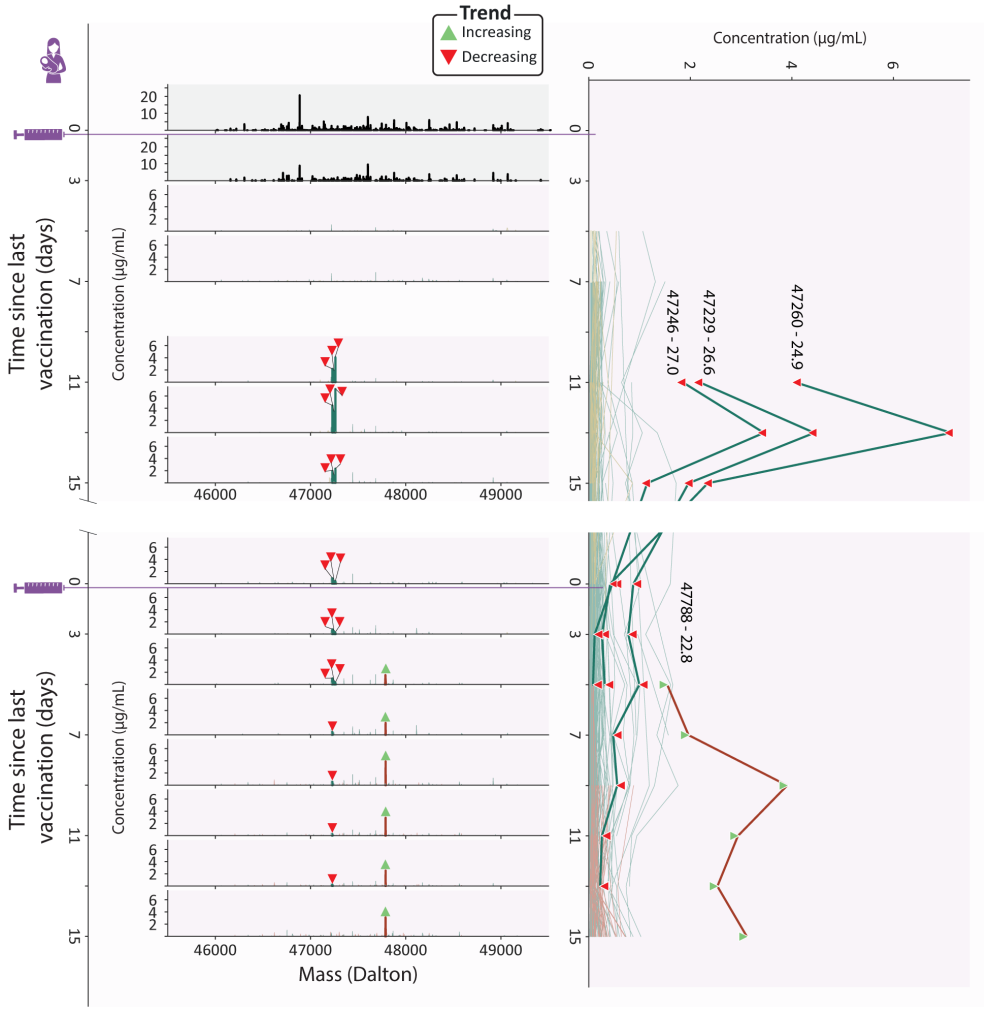

FIGURE 4
Clonal profile analysis for donor 4, a strong responder. Changes in the vaccine induced clonal profile for donor 4 are depicted, with the 4 most abundant vaccine induced clones annotated by their mass and retention time, highlighted in bold, with each timepoint annotated with a triangle indicating if the clone trends upwards or downwards in concentration over the course of this study. On the left we show mass profiles (SIgA1 clonal concentration in µg/mL) showing either household clones (top two profiles, in black) or vaccine induced clones [subsequent profiles, with individual clones colored according to their assigned population (persistent (teal), transient (mustard), and second dose induced clones (maroon))]. Each peak indicates a single clone and each row a single timepoint. The line plot on the right shows the abundance of individual vaccine induced SIgA1 clones over all timepoints, colored by their population, with the same clones as the mass plots highlighted in bold, labeled with their mass and retention times and annotated with triangles indicating if the clone trends upwards or downwards in concentration throughout the study duration. The highlighted persistent clones are initially highly abundant, but their abundance decreases rapidly, and at the final timepoint none of them are detected. The highlighted second dose induced clone is part of a large and abundant population of second dose induced clones, which at the final timepoints make up 45-50% of the vaccine induced clonal titer.

spike protein, via intramuscular vaccination and that IgA is typically an antibody which is important for initial stages of immune responses (29).

Even though the titer levels and neutralization results for human milk IgA were not as strong as for IgG, six studies also tested infant samples to see if SARS-CoV-2 antibodies could be detected in the breastfed infant of vaccinated mothers (31–36). All together the six studies, investigating different infant bio samples, found detectable levels of SARS-CoV-2 specific antibodies in the infant samples. One study by Narayanaswamy et al. assessed the infant stool samples and found back both Anti-RBD IgGs and IgAs suggesting both antibodies survived infant digestion and provided protection to the infant. One recent study showed the long-lasting importance of SIgA in human milk, as spike-specific SIgAs were found back in the human milk samples of COVID-19 infected women 10 months (37) and 1 year after the initial infection (38).

Beyond the evaluation of antibody titers, in this study we detected a heterogeneous polyclonal response to vaccination, as distinct populations of novel clones emerged in every donor after vaccination. We defined three populations of vaccine induced clones, which can be assigned based on their window of detection relative to vaccination: transient, persistent and second dose induced clones (Figure 1C; Supplementary Figure S2). These populations were not only observed in all donors, but also behaved remarkably similar relative to each other, as the persistent population was dominant both in terms of clonal titer and size, regardless of which vaccine the donor had received.

As the donors in this study had not been exposed to SARS-CoV-2 prior to vaccination, the clonal response to the first vaccination can be considered the primary response: a first generation of antibodies, having undergone little to no somatic hypermutation (39). The dominant persistent clones we observe might be the effective portion of the primary response, which are encoded in long lived plasmablasts or memory B cells that proliferate quickly in response to restimulation with the same antigen whereas the transient population could be the ineffective portion of the primary response.

The response to the second vaccination can then be considered the secondary response. In every donor, we observed a population of novel clones emerge during this secondary response. In at least one donor, donor 4, the secondary response had a strongly divergent clonal makeup from the primary response, as it was not predominantly driven by clones induced upon the first vaccine dose but largely by a completely novel clonal population. These novel clones may be the result of a completely new gene recombination but could also be the result of somatic hypermutation of transient or persistent clones, as even small mutations are likely to cause shifts in mass and retention time. Alternatively, they could be clones that escaped detection during the primary response or clones that were derived from previously undetected clones through somatic hypermutation or isotype switching, as our profiling method in this study was limited to SIgA1. While more extensive sequence information is needed to definitively determine the genetic and cellular origin of circulating clones, the second dose induced clones in this study seemingly did not emerge faster than the transient clones, possibly indicating they are not maturations of the first batch of B cells. However, given the small sample size in this study, we are unable to sufficiently answer these questions.

In the parent study of this cohort, spike specific IgAs were longitudinally monitored by ELISA. A biphasic antibody response to SARS-CoV-2 vaccination was observed for spike-specific IgA in these samples, with an accelerated response after the second vaccination, in line with expectations based on leading theories on humoral immune responses (39). We were able to confirm a number of these findings from our case series analysis of this cohort. From both the ELISA and Fab profiling data, donor 4 could be identified as a strong responder, with an observed biphasic response irrespective of analysis type. These confirmations, however, could only be made qualitatively. Quantitatively, we observed a discrepancy between the reported ELISA and measured clonal titers. We believe there are several factors contributing to these discrepancies. First, the applied ELISA measured IgA1 and IgA2, whereas our profiling method detects only IgA1 (9, 28). Secondly, the ELISA based methods may not be fully optimized for SIgAs whereas we know from our previous work that in our LC–MS based Fab method we detect little to no IgAs that are not secretory (5). Furthermore, the ELISA measured spike-specific IgA titers, using a pre-fusion stabilized variant of the spike protein sequence termed 2P (40). Thus, clones that do not bind to the 2P spike protein variant, but perhaps to other components of the vaccine, will not be detected. Similarly, weak binders may be underrepresented in affinity-based assays. As a recent study showed that low rather than high affinity antibodies delivered greater antibody-mediated receptor activity through increased receptor clustering (41), these low affinity clones may be of particular importance.

To date, it is often thought that highly efficient neutralizing antibodies would perhaps not be among the most abundant clones. However, at the current stage of implementation the most reliable detection and quantitation through LC–MS is limited to relatively abundant clones, and low abundant clones likely exist at concentrations below our limit of detection. One way to study these low abundant clones is through fractionation or purification. While there is value in retaining biological context by minimizing purification, simultaneous analysis of the sample in an enriched form can enable a more targeted look at clones of interest or provide us with contextual information about clones in our sample such as binding affinity. For example, in a recent study van Rijswijck et al. (26) analyzed serum samples of SARS-CoV-2 patients, with and without affinity purification, and combined the results to yield information about the cross-reactivity of individual clones to different SARS-CoV-2 variants of concern. This illustrates how the ability to identify and track clones between samples and experiments can be used to obtain functional information about individual clones, and how we can relate this information back to the original abundance profile. Future applications of LC–MS fab profiling hold the promise of high throughput characterization of antibody repertoires, allowing for a greater understanding of the mechanisms related to antibody mediated immunity and defining immune signatures that predict how an individual will respond to future encounters with similar antigens. We imagine this to have future applications similar to HLA phenotypes for organ transplants or genetic markers for cancer treatment. In addition to defining such "biomarkers" for individual patients, we could identify markers of efficacy for individual clones, potentially enabling the direct identification of potential therapeutic antibodies from polyclonal samples. We believe studies like this pave the way to elucidate the mechanisms involved in mounting an effective antibody response and can lead to future targeted efforts to find potential therapeutic candidates.

Methods

Study design

In this observational longitudinal case series we used samples from an existing prospective longitudinal study COVID MILK – POWER MILK (28). All participants were subjected to longitudinal analysis of specific antibodies against the SARS-CoV-2 spike-protein by ELISA and general SIgA1 Fab clonal profiling in human milk after vaccination against COVID-19 with BNT162b2/Comirnaty developed by Pfizer-BioNTech, mRNA-1273/Spikevax developed by Moderna or AZD1222/Vaxzevria developed by Oxford/AstraZeneca. Ethical approval was acquired from an Independent Ethics Committee (2020.425/NL74752.029.20). The study was conducted in accordance with the principles of the declaration of Helsinki and the ICH GCP Guidelines, and the Regulation on Medical Research involving Human subjects.

Subjects

Details concerning subjects have been extensively described (28), demographic details for the six donors in this case series are presented in

Supplementary Table S2 and reported symptoms after each vaccination in Supplementary Table S3. Briefly, lactating women in the Netherlands receiving one of the above-described SARS-CoV-2 vaccines were eligible to participate and were recruited through social media platforms. There were no exclusion criteria. All participants were requested to send their vaccination certificate, including the type of vaccination and lot number. From the larger study a subset of 2 women per vaccine group were selected based on the following criteria: (1) a pre-vaccine milk sample was available, (2) data from an enzyme-linked immunosorbent assay (ELISA) with the SARS-CoV-2 spike protein for human milk SIgA was available and indicated high spike-specific SIgA titers. Of the 6 donors in this case series, none had a SARS-CoV-2 infection prior to vaccination, as confirmed with a negative spike-specific ELISA at V0D0 (28). Written informed consent was obtained from all participants.

Sample collection

Sample collection was performed between January 2021 and July 2021. Human milk samples were collected longitudinally over a period of up to 95 days (Figure 1; Supplementary Table S1). In this study, 16 samples of human milk were analyzed per lactating woman. These samples were collected according to the following schedule: one sample before the first vaccination and one sample on days 3, 5, 7, 9, 11, 13, and 15 after the first vaccination. This schedule was the same for the second vaccination (Supplementary Table S1). Participants were instructed to empty one breast in the morning, before the first feeding moment, and collect 5 mL of milk after mixing the milk. Participants were requested to store the milk samples in the home freezer. Samples were transported back to the lab on dry ice and remained at −80 until analysis (9, 28).

Fab clonal profiling from human serum and milk

IgA enrichment, capture, and digestion

Methods for IgA1 Fab profiling have previously been extensively detailed (3, 4). Briefly, all IgA was captured using CaptureSelect IgA affinity matrix (Thermo Fisher Scientific). Human milk samples were assumed to contain 0.8 µg/µL SIgA and added to excess amount of bead slurry, PBS, and 200 ng of the monoclonals anti-CD20 mIgA1 (7D8-IgA1) and anti-cMET (5D5v2-IgA1). These monoclonals were used as internal standards for quantification, and were a gift from Genmab (Utrecht, NL). Samples were incubated followed by removal of the flow through, containing all non-IgA human milk components. The samples were then washed several times and IgA was digested overnight with the O-glycopeptidase from *Akkermansia muciniphila*, OgpA (OpeRATOR®, Genovis, Llund, Sweden). Digestion was performed using 40 U SialEXO (a sialidase cocktail to remove sialic acids from the O-glycans) and 40 U of OgpA enzyme, and incubated overnight at 37°C, in an Eppendorf thermal shaker (Eppendorf, The Netherlands). Following overnight digestion with OgpA, Ni-NTA agarose slurry was added to the samples to bind the enzyme and incubated for 30 min. Finally, the flowthrough, containing the IgA1 Fabs, was collected by centrifugation.

Fab profiling by LC–MS

The LC–MS and data processing approaches as described by Bondt et al. were applied (3, 4). In short, the collected Fab samples were separated by reversed phase liquid chromatography on a Thermo Scientific Vanquish Flex UHPLC instrument, equipped with a 1 mm x 150 mm MAbPac analytical column, directly coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, California, USA). The column preheater and the analytical column chamber were heated to 80°C during chromatographic separation. Fab samples were injected as 10 µL and subsequently separated over a 62 min gradient at a flow rate of 150 µL/min. The gradient elution was achieved using mobile phases A (0.1% HCOOH in Milli-Q HOH) and B (0.1% HCOOH in CH3CN), see previous publications for details (3, 4). The instrument was operating in Intact Protein and "Low Pressure" mode for the acquisition of MS data, with a spray voltage of 3.5 kV set from minute 2 to minute 50 of the gradient. The ion transfer tube temperature was set at 350°C, vaporizer temperature at 100°C, sheath gas flow at 15, auxiliary gas flow at 5, and source-induced dissociation (SID) was set at 15 V. Spectra were recorded with a resolution setting of 7,500 (@ 200 m/z) in MS1. Scans were acquired in the range of 500–4,000 m/z with an AGC target of 250% and a maximum injection time set to 50 ms. For each scan 5 µscans were recorded.

IgA1 clonal profiling data analysis

Intact masses were retrieved from the generated RAW files using BioPharmaFinder 3.2 (Thermo Scientific). Deconvolution was performed using the ReSpect algorithm between 5 and 57 min using 0.1 or 0.3 min sliding windows with a 25% offset, a merge tolerance of 30 ppm, and noise rejection set to 95%. The output mass range was set from 10,000 to 100,000 with a target mass of 48,000 and mass tolerance 30 ppm. Charge states between 10 and 60 were included and the Intact Protein peak model was selected.

Further data analysis was performed using Python 3.9.13 (with libraries: Pandas 1.4.4 (42), NumPy 1.21.5 (43), SciPy 1.9.1 (44), Matplotlib 3.5.2 (45) and Seaborn 0.11.2). Masses of the BioPharmaFinder identifications (components) were recalculated using an intensity weighted mean considering only the most intense peaks comprising 90% of the total intensity. Using the mAb standards, the intensity was normalized, a relative mass shift was applied to minimize the mass error and a retention time shift was applied to minimize deviation between runs.

Components between 45 and 53 kDa with the most intense charge state above m/z 1,000 and a score of at least 40 were considered Fab portions of IgA1 clones. The clones in samples of the same donor were matched between runs using average linkage (unweighted pair group method with arithmetic mean UPGMA) L∞ distance hierarchical clustering. Flat clusters were formed based on a cophenetic distance constraint derived from a mass and retention time tolerance which were 2 Da and 1 min, respectively. Clones within a flat cluster were considered identical between runs. Clones that were only detected at a single timepoint within a donor were excluded from the analysis. Clones were assigned to populations according to their detection window relative to vaccination as outlined in Supplementary Figure S2.

Trial registration

This research project was registered at the Dutch Trial Register on May 1st, 2020, number: NL 8575, https://onderzoekmetmensen.nl/nl/trial/23001.

Data availability statement

The datasets presented in this study can be found online in the MassIVE repository (https://massive.ucsd.edu), under the accession number MSV000092157.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Amsterdam University Medical Centre. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SG: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. Software. AB: Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Investigation, Supervision. DR: Formal analysis, Investigation, Writing – review & editing, Methodology. HJ: Formal analysis, Investigation, Methodology, Writing – review & editing, Data curation. SM: Writing – review & editing. MD: Writing – review & editing, Formal analysis, Investigation, Methodology. MH: Methodology, Writing – review & editing, Data curation, Software, Visualization. BK: Data curation, Methodology, Writing – review & editing, Investigation, Supervision. JBvG: Investigation, Supervision, Writing – review & editing, Conceptualization, Funding acquisition, Resources. AH: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing, Writing – original draft. KD: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Methodology, Visualization.

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Conflict of interest

JBvG is the founder and director of the Dutch National Human Milk Bank and a member of the National Health Council. JBvG has been a member of the National Breastfeeding Council from March 2010 to March 2020.

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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1305086/full#supplementary-material

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Development and large-scale production of human milk fat analog by fermentation of microalgae

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Background: Human milk contains a complex mixture of triacylglycerols (TAG), making it challenging to recreate using common ingredients.

Objective: The study aimed to develop an innovative fermentation technique to produce essential human milk TAG, effectively tackling a significant hurdle in infant nutrition.

Method: An in-depth analysis of the literature has been conducted to identify the specific TAG to be targeted. We used a microalgal oil production platform and a two-step procedure to modify its fatty acid and TAG composition. The palmitic acid (16:0) content has been increased by classical strain improvement techniques, followed by a step involving the expression of a lysophosphatidic acid acyltransferase (LPAAT) sequence capable of esterifying 16:0 specifically at the internal position (*sn*-2 palmitate) of TAG. Once the strain was stabilized, the fermentation was scaled up in a 50-L reactor to yield several kilograms of biomass. Subsequently, the oil was extracted and refined using standard oil processing conditions. Liquid chromatography-mass spectrometry was employed to monitor the TAG profile and the region specificity of 16:0 at the internal position (*sn*-2 palmitate) of TAG.

Results: The initial strain had a 16:0 level of 25% of total fatty acids, which was increased to 30% by classical strain improvement. Simultaneously, the oleic acid level decreased from 61% to 57% of total fatty acids. Upon expression of an exogenous LPAAT gene, the level of the 16:0 esterified in the internal position of the TAG (*sn*-2 palmitate) increased by a factor of 10, to reach 73% of total palmitic acid. Consequently, the concentration of oleic acid in the internal position decreased from 81% to 22% of total fatty acids, with TAG analysis confirming that the primary TAG species in the oil was 1,3-dioleoyl-2-palmitoyl-glycerol (OPO). The 50-L-scale fermentation trial confirmed the strain's ability to produce oil with a yield of >150 g of oil per liter of fermentation broth in a timeframe of 5 days, rendering the process scalable for larger-scale industrialization.

Conclusion: We have demonstrated the feasibility of producing a suitable TAG composition that can be effectively integrated into the formulations of infant nutrition in combination with other fats and oils to meet the infant feeding requirements.

KEYWORDS

human milk fat analog, infant nutrition, OPO, *sn*-2 palmitate, fermentation, structured TAG

1 Introduction

Human milk stands as an unparalleled, complex nutrient source, renowned for its intricate structural composition and diverse biological functionalities vital for infant development. Comprising 3%−5% of lipids, these nutrients serve as the primary caloric source for newborns, representing 50%−60% of the calories, or ∼70 kcal/dl, in human milk (1). In breast milk, lipids are found as globules, predominantly composed of triacylglycerols (TAG), which represent ∼98% of the total lipids (1). Human milk TAG contains a diverse range of fatty acids, spanning from caproic acid (6:0) to nervonic acid (24:1 n-9), with oleic, palmitic, and linoleic acids being the primary components (1). Notably, the TAG in human milk exhibits distinctive and conserved structures, characterized by the presence of palmitic acid at the internal position and unsaturated fatty acids like oleic and linoleic acids at the external positions (1–3). The specific configuration of palmitic acid in human milk TAG is also referred to as *sn*-2 palmitate, and this feature is largely unaffected by maternal diet variations worldwide and bestows several metabolic advantages, including enhanced absorption of TAG containing internal palmitic acid (4–6). This trait facilitates the absorption of essential nutrients such as calcium and liposoluble micronutrients while conferring immunological and anti-inflammatory properties in infants (5, 7, 8).

In situations where breastfeeding is not possible due to personal preferences and physiological or other constraints, providing safe alternatives with nutritional properties mirroring those of human milk becomes the alternative of choice for parents and caregivers. Over the years, numerous strategies have been explored to develop substitutes for human milk TAG. Enzymatic processes enabling the production of TAG rich in internal palmitic acid have been extensively employed in infant formula development (9). This method, categorized as a first-generation approach, has been in use for decades to improve the nutritional properties of infant formula (10–16). First-generation substitutes typically yield infant powders containing palmitic acid residing in the internal position at levels lower than 50% in commercial infant formula (9), which is a significant deviation from ∼67.8% ± 4.7% found in human milk (17). These substantial variations in the repartition's level of palmitic acid concentration potentially hold crucial nutritional implications. Consequently, continuous research endeavors are essential to augment these developments, striving to achieve higher levels of palmitic acid at the internal level and thereby enhance the nutritional quality of infant products.

In response to this challenge, this study delves into the realm of fermentation, pioneering a groundbreaking methodology aimed at producing key TAG components of human milk, such as 1,3-dioleoyl-2-palmitoyl-glycerol (OPO). Through a meticulous review of the literature related to the complex fatty acid and TAG composition found in human milk, this research identifies a specific TAG as the target for replication. An innovative approach utilizing an oleaginous microalgae strain, which undergoes a series of modifications, including classical strain improvement techniques and the incorporation of specific genetic sequences, has been used. The resulting strain was used to produce a human milk fat analog on a large scale.

2 Materials and methods

2.1 Strain development

Modification of wild-type *Prototheca moriformis* strain isolate UTEX 1533 was performed to alter the stereoisomeric structure of TAG obtained by fermentation of *P. moriformis* to produce TAG enriched with palmitic acid (16:0) esterified to the middle position (*sn*-2) on the glycerol backbone. First, *P. moriformis* was subjected to classical mutagenesis to increase the level of palmitic acid from 25% to 30% of the total fatty acids. The classically improved strain was then genetically modified by targeted integration of a cassette encoding a heterologous lysophosphatidic acid acyl transferase (LPAAT), which is responsible for the regioselective insertion of saturated fatty acids (e.g., palmitic acid) at the *sn*-2 position of TAG produced by *P. moriformis*.

2.2 Production of algae oil

Algae oil was produced by fermenting the genetically modified strain of *P. moriformis* essentially as described in Running (18) in a 50-L-scale reactor, followed by the drying of algal biomass and subsequent mechanical and solvent extraction to recover crude oil. The latter was subsequently processed using standard edible oil refining methods, including degumming, bleaching, and deodorization.

2.3 Fatty acid and triacylglycerol analysis

Fatty acids are measured as their fatty acid methyl esters (FAMEs), following a direct transesterification reaction with a sulfuric acid methanol solution (19). The sample is injected on an Agilent 8890 gas chromatograph system equipped with a split/splitless inlet and flame ionization detector (Agilent Technologies, Palo Alto, CA, United States). An Agilent DB-WAX column (30 m × 0.32 mm × 0.25 μm dimensions) is used for the chromatographic separation of the FAME peaks. A FAME standard mixture purchased from Nu-Chek Prep (Nu-Chek Prep Inc., Elysian, MN, United States) is injected to establish retention times. Response factor corrections were previously determined empirically using various standard mixtures from Nu-Chek Prep. Methyl non-adecanoate (19:0) is used as an internal standard for the quantitation of individual FAMEs.

2.4 Determination of the *sn*-2 fatty acid profile

The fatty acid composition at the *sn*-2 position was determined after incubating the TAGs with porcine pancreatic lipase, as described by Christie and Han (19). The lipase reaction results in deacylation of the TAG at the *sn*-1(3) positions, leaving *sn*-2-MAGs. The *sn*-2-MAGs were then isolated using Agilent Bond Elut amino propyl solid phase extraction cartridges (Agilent Technologies, Palo

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TABLE 1 Fatty acid composition of human milk samples ($N = 835$) collected in several countries* (20–22). Results are expressed in g/100 g of fatty acids.

Fatty acid	Mean	Median	Min	Max	SD	RSD%
8:0	0.20	0.20	0.16	0.28	0.04	20.0
10:0	1.73	1.66	1.44	2.35	0.27	15.6
12:0	6.02	5.25	4.24	13.82	2.65	44.0
14:0	6.06	5.84	3.61	12.12	2.21	36.5
16:0	20.58	19.91	18.62	23.28	1.84	8.9
18:0	5.84	6.07	4.75	6.77	0.59	10.1
18:1 n-9	31.39	32.23	21.85	36.49	4.10	13.1
18:2 n-6	14.11	14.78	7.90	22.80	4.12	29.2
18:3 n-6	0.15	0.15	0.10	0.17	0.02	13.3
20:3 n-6	0.33	0.33	0.25	0.44	0.06	18.2
20:4 n-6	0.42	0.42	0.36	0.50	0.05	11.9
18:3 n-3	1.18	1.14	0.43	2.02	0.39	33.1
20:5 n-3	0.11	0.09	0.05	0.26	0.06	54.5
22:6 n-3	0.40	0.30	0.17	0.99	0.26	65.0
Other FA	10.08	10.13	-	-	-	-

Dataset consolidated from 835 samples of mature human milk collected in 10 counties as follows: Ref. 20: Australia (n = 48), Canada (n = 48), Chile (n = 50), China (n = 50), Japan (n = 51), Mexico (n = 46), Philippines (n = 54), the United Kingdom (n = 44), the United States (n = 49); Ref. 21: Singapore (n = 50); and Ref. 22: China (n = 345). SD stands for standard deviation, and RSD% stands for relative standard deviation percentage.

Alto, CA, United States) and subjected to fatty acid composition analysis by gas chromatography as described earlier.

2.5 Triacylglycerol analysis

TAG profiles were determined as described previously using an Agilent 1290 Infinity II UHPLC system coupled to a 6470B triple quadrupole mass spectrometer and APCI ionization source according to the parameters described previously (23). Diacylglycerol (DAG) ion ratios, carried out as described by Byrdwell (24), were used to assess the relative abundance of TAG regio-isomers, and quantification was performed using a calibration curve of pure 1,3-dioleoyl-2-palmitoyl-glycerol (OPO), 1,2-dioleoyl-3-palmitoyl-glycerol (OOP), 1,2-dipalmitoyl-3-oleoyl-glycerol (PPO), and 1,3-dipalmitoyl-2-oleoyl-glycerol (POP) standards. For example, OPO (876.8 m/z) was monitored by the loss of oleic acid leading to the fragment at 577.5 m/z and the loss of palmitic acid leading to the fragment at 603.5 m/z.

3 Results

3.1 Identification of fatty acid and TAG compositional targets to guide the development of a new functional ingredient for infant nutrition

In human milk, lipids are primarily organized as globules known as milk fat globules (MFG), comprising a core of neutral TAG isolated by the layers of polar lipids, cholesterol, and transmembrane proteins (1, 25). TAG accounts for ∼98% of MFG and consists of fatty acids with chain lengths ranging from 6 to 24 carbons (1, 25). The analysis of the variations of the fatty acid concentration measured as relative standard deviation percentage (RSD%; Table 1) in a dataset comprising more than 800 samples (20–22) reveals minimal variations (RSD<10%) in palmitic (16:0) and stearic (18:0) acids, supporting the hypothesis that these saturated fatty acids primarily originate from an endogenous pool generated through *de novo* synthesis. Oleic (18:1 n-9), γ-linolenic (18:3 n-6), and arachidonic (20:4 n-6) acids exhibit intermediate variation (< 15%), indicating their relative insensitivity to ethnic and dietary differences. Conversely, medium-chain fatty acids like lauric (12:0) and myristic (14:0) acids, prevalent in certain tropical fats consumed significantly in the Philippines and Indonesia, have high coefficients of variation (>35%), as do linoleic (18:2 n-6) and γ-linolenic (18:3 n-3) acids, which are most abundant polyunsaturated fatty acids in vegetable oils, with coefficients of variation around 30%. Consistent with previous studies (26), concentrations of omega-3 long-chain polyunsaturated fatty acids, especially eicosapentaenoic (20:5 n-3) and docosahexaenoic (22:6 n-3) acids, vary considerably (coefficients of variation >50%) due to diverse marine product consumption patterns influenced by geographical origin and dietary habits of the studied populations (Table 1).

The examination of TAG molecular distribution, particularly the specific arrangement of fatty acids in internal (*sn*-2) or external [*sn*-1(3)] positions, revealed distinct asymmetry for palmitic, oleic, and linoleic acids, a characteristic observed not only in human milk but also in other mammals as reported decades ago (2, 3, 27). Advanced mass-spectrometry techniques have identified ∼300 individual TAG species (Table 2), with the prominent

TABLE 2 Main triacylglycerol (TAG) individual species determined by high-resolution mass-spectrometry techniques on 55 human milk samples (17).

TAG	Average (% of total TAG)	SD	Norm. Average (% of main TAG*)
sn-OPO	13.9	2.1	41.2
sn-OPL	6.0	1.5	17.8
sn-PPO	5.7	1.6	16.9
sn-OPS	5.2	1.4	15.4
sn-POO	2.9	1.0	8.6

O, P, S, and L stand for oleic, palmitic, stearic, and linoleic acids, respectively. The prefix *sn*- indicates that the TAG species reported has a defined structure and is not a mixture of isomers. *Normalized to the sum of *sn*-OPO, *sn*-OPL, *sn*-PPO, *sn*-OPS, and *sn*-POO as % of total TAG. Around 300 additional species have been reported in low numbers in this study. The normalized average has been estimated using the values for *sn*-OPO, *sn*-OPL, *sn*-OPP, *sn*-OPS, and *sn*-POO and used as indicative targets to guide the development of the new algae-derived human milk fat substitutes.

TABLE 3 The level of palmitic acid esterified in the internal (*sn*-2) and external [*sn*-1(3)] positions of the triacylglycerols (TAG) expressed as g/100 g of total palmitic acid determined by high-resolution mass-spectrometry techniques on 55 human milk samples (17).

Palmitic acid	Average	SD	Min	Max
sn-2 position	67.8	4.7	60.8	76.7
sn-1(3) position	32.2	4.7	23.3	39.2

ones being 1,3-dioleoyl-2-palmitoyl-glycerol (OPO) > 1-oleoyl-2-palmitoyl-3-linoleoyl-glycerol (OPL) > 1,2-dipalmitoyl-3-oleoyl-glycerol (PPO) > 1-oleoyl-2-palmitoyl-3-stearoyl-glycerol (OPS). Studies indicate regional differences, such as in China, where high vegetable oil consumption leads to elevated linoleic acid levels in human milk, resulting in higher concentrations of OPL compared to OPO (25, 28, 29). In all instances, palmitic acid is consistently esterified to the *sn*-2 position of TAG, constituting an average of 67.8 ± 4.7% of the total palmitic acid (Table 3), as determined through recent high-resolution mass spectrometry methods (17, 25, 28, 29).

Using the literature data presented in Tables 1–3, it is possible to establish compositional targets for the key fatty acids and the main TAG structures to guide the development of a new functional ingredient for infant nutrition. It is important to note that it is assumed that this ingredient's composition should emulate the structural complexity of TAG in breast milk when combined with other types of oils and fats to formulate a regulatory-compliant infant formula. Designing a strain capable of producing all the acids and TAG found in breast milk seems exceedingly challenging. Therefore, the concentration of the palmitic acid set is higher compared to human milk data to account for the effect of the dilution with other vegetable oils that might be used in the final oil mix. We assumed that the new ingredient might represent 40–60% of the total lipid composition of infant formula and used the following targets for strain development:

- OPO: 40 ± 3% of total TAG in accordance with literature data (Table 2).

- Palmitic acid: 30 ± 3% of total fatty acids, of which 67.8 ± 4.7% in the internal position of TAG in accordance with literature data (Table 3).

Due to significant fluctuations in the levels of polyunsaturated fatty acids, which are regulated differently in infant formula across countries, there are no fixed targets in place. We assumed that easily accessible sources like canola, soybean, DHA, and ARA microbial oils could be utilized to create a nutritionally complete and regulation-compliant oil mixture.

3.2 Development of the strain and production of the algae oil

A wild-type *P. moriformis* isolate (UTEX 1533) was mutagenized utilizing classical strain improvement to increase levels of palmitate beyond the 25% normally seen in this strain. The resulting mutant strain (named the high-palmitic acid strain) was capable of reproducibly elaborating over 30% palmitate, as shown in Table 4. However, regiospecific lipase treatment, followed by FAME analysis of the resulting monoacylglycerols, showed only modest changes in fatty acid distribution along the glycerol backbone. This was in contrast to the oil produced by the strain subsequent to the introduction of the heterologous LPAAT. While palmitate levels remained relatively constant between the classically improved isolate and the transgenic strain, regiospecific insertion of saturates shifted dramatically, with *sn-2* levels of palmitate increasing from 6% to 69.3% in the transgenic line. Not surprisingly, *sn*1–3 insertion of palmitate decreased over 10-fold in the transgenic line, dropping from 35% to 3.3%. The 50-L-scale fermentation trial conclusively demonstrated the strain's capacity for oil production, achieving a yield of over 150 g of oil per liter of fermentation broth within 5 days. This outcome underscores the strain's efficiency and establishes the feasibility of upscaling the process.

Table 5 illustrates the TAG profile of the resulting engineered strain vs. oils found in several commercially available OPO ingredients. *sn*-2 analysis shows that oil from the engineered microalgal strain has higher *sn*-2 palmitate levels than any of the commercial infant fat formulations tested (73.3% vs. 38.95% in the commercial fat containing the lowest level of palmitate at the *sn*-2 position). Similarly, regiospecific OPO levels in oil derived from the transgenic strain were higher than any of the commercial oils, at 38.3% vs. 21.7% in the commercial fat containing the lowest level of OPO. While levels of highly desirable OPO were higher in oil derived from the transgenic line, levels of less desirable TAG species, including OOP, PPO, and PPP, were lower in algae oil compared to any of the commercial OPO ingredient samples tested (Table 5).

4 Discussion

The design of infant formula presents a challenge in mimicking the composition and functional properties of human milk TAG. Understanding the structure-function relationships of human milk TAG is essential for formulating nutritionally optimized infant formulas that closely mimic the benefits of human milk (25).

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TABLE 4 Fatty acid profiles of total triacylglycerols (TAG) as well as *sn*-1(3) and *sn*-2 positions from the parent *P. moriformis* microalgae strain, the high palmitic acid strain generated by mutagenesis, and the OPO strain engineered by insertion of the LPAAT gene.

	Parent strain			High-palmitic acid strain			OPO-engineered strain		
Fatty acid	TAG	*sn*-1(3)	*sn*-2	TAG	*sn*-1(3)	*sn*-2	TAG	*sn*-1(3)	*sn*-2
16:0	25.3	36.5	3	30.3	35	6	31.5	3.3	69.3*
18:0	3.3	4.7	0.5	2.3	4.7	0.5	2.6	4.7	0.5
18:1 n-9	61.1	48.9	85.6	57.4	50.8	81.6	55.2	80.3	22.8
18:2 n-6	6.9	6.1	8.4	6.3	6	8.5	6.3	8.4	3.7
Other FA	3.4	3.9	2.5	3.8	3.5	3.3	4.4	3.3	3.7

*When expressed in g/100 g of total palmitic acid, the level of palmitic acid esterified in the internal (sn-2) position is 73.3%. Data are expressed in g/100 g of total fatty acids.

TABLE 5 Total *sn*-2 palmitate and main triacylglycerols (TAG) of individual species of the algae-derived human milk fat substitute developed in the present study (OPO-engineered strain) and in commercial human milk fat substitute samples (CS1–3). TAG results are expressed in g/100 g of TAG and *sn*-2 and *sn*-1(3) palmitate data in g/100 g of total palmitic acid.

TAG	OPO-engineered strain	CS1	CS2	CS3
sn-2 palmitate	73.30	65.44	55.93	38.95
sn-1(3) palmitate	26.70	34.56	44.07	61.05
sn-OPO	38.30	34.40	30.20	21.70
sn-OOP	2.40	3.30	4.70	5.90
sn-PPO	10.90	14.60	13.30	20.30
sn-POP	0.40	0.00	1.00	3.00
OOO	13.17	9.67	11.10	11.02
OPL	9.56	13.21	12.89	8.14
POS	3.91	5.88	4.81	6.44
OOM	2.66	0.00	0.00	0.42
OOL	4.88	4.81	6.23	3.28
MOP	2.14	0.00	0.00	0.00
LOL	1.31	0.92	1.47	0.69
MOL + POLn	1.22	0.00	0.00	0.00
OOS + POG	1.52	2.83	2.88	2.85
PPL	1.22	2.42	2.80	2.99
PPP	0.00	1.57	1.27	5.30
Other species	4.30	4.32	4.18	5.65

O, P, S, L, M, Ln, and G stand for oleic, palmitic, stearic, linoleic, myristic, a-linolenic, and gondoic acids, respectively. The prefix *sn*- indicates that the TAG species reported has a defined structure and is not a mixture of isomers.

Various strategies have been explored to incorporate human milk-mimicking TAG structures into infant formulas, including lipid blending, interesterification, and structured lipid synthesis (25).

In recent years, considerable research efforts have focused on unraveling the complexities of human milk TAG and elucidating their impact on infant nutrition and health (25). Havlicekova et al. (5) as well as Miles and Calder (8) summarized in a very comprehensive review all the health benefits that emerged from investigating in animals and premature as well as term-born infants the effects of higher *sn*-2 palmitate content in supplemental milk formula (Figure 1). The positional distribution of fatty acids within TAG is known to influence their digestion, absorption, and subsequent utilization by infants. The presence of palmitic acid at the *sn*-2 position (*sn*-2 palmitate) enhances the formation of stable emulsions during digestion, facilitating efficient lipid absorption. *sn*-2 palmitate improved fatty acid absorption and reduced the formation of insoluble calcium dipalmitate, leading to softer stools without increased volume (4, 12–15, 30–35). Studies also revealed a direct relationship between *sn*-2 palmitate content and calcium absorption, reducing calcium waste in stools and improving overall mineral balance (7, 36–38). Infants fed high *sn*-2 palmitate formula displayed significantly higher bone mineral mass compared to those fed standard formula, akin to breast-fed infants (13, 39, 40). While studies indicated a positive impact on bone health, further research is needed to assess long-term effects. Some studies suggest that infants fed formula enriched with *sn*-2 palmitate experienced reduced crying episodes and duration, especially during evenings and nights (10, 41–43). This effect may be partially attributed to a synergetic interplay between *sn*-2 palmitate, prebiotic oligosaccharides, and whey hydrolysate that have been used in combinations in these studies (41). *sn*-2 palmitate influenced the intestinal microbiome positively, increasing beneficial bacteria such as *Lactobacillus* and *Bifidobacteria in vitro* (25, 44–47) and *in vivo* settings (16, 33, 48, 49). This modulation of the microbiome had immunomodulatory effects and positively influenced intestinal maturation, counteracting the growth of pathogenic bacteria (44, 50–53). Experimental studies demonstrated that *sn*-2 palmitate might have a protective effect against intestinal inflammation (25, 54). Animal models showed reduced intestinal erosion and other morphological changes in mice fed a high *sn*-2 palmitate formula, suggesting potential anti-inflammatory properties (54). Further research is necessary to fully explore the long-term impact of this aspect.

All these studies were conducted using human milk fat substitutes produced enzymatically using vegetable oils, particularly palm oil fractions, as sources of oleic and palmitic acids (9). This method was historically developed by Bungee Loders Crocklaan and Enzymotec and has been scaled up to produce various formulations under commercial names such as Betapol™ and Infat™. With this approach, it is possible to obtain oils with palmitic acid content in the *sn*-2 position ranging from 39% to 65% of the total palmitic acid content [(25), Table 5]. These

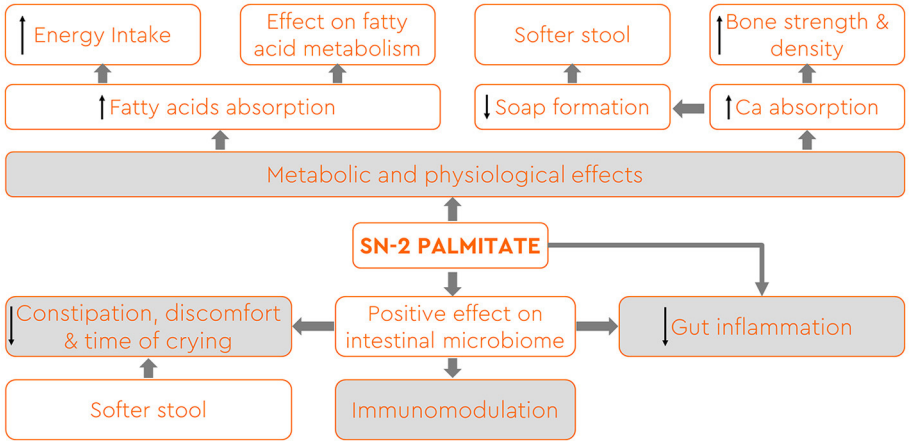

FIGURE 1
Health benefits associated with the occurrence of palmitic acid esterified in the internal position of TAG (*sn*-2 palmitate) in breast milk and infant formula (adapted from references 5 and 8).

concentrated oils are subsequently used as blends in combination with other oils such as coconut oil, high-oleic sunflower oil, and canola oil to achieve a nutritionally balanced composition in compliance with relevant regulations. The emergence of these *sn*-2 palmitate concentrates has enabled a deeper understanding of their nutritional effects and has contributed to advancements in our comprehension of the relationship between the complex TAG structures in human breast milk and their nutritional functions. Nonetheless, significant differences exist in nutritionally relevant clinical endpoints, such as fatty acid absorption, residual calcium in feces, and stool consistency, when comparing infant formulas containing these first-generation and breast-fed infants (4, 12–15, 31, 33, 36). Human milk fat substitutes produced by enzymatic methods contain significant levels of TAG, such as 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1,2-dipalmitoyl-3-oleoyl-glycerol (PPO), 1,2-dipalmitoyl-2-linoleoyl-glycerol (PPL), 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), and tripalmitin (PPP, Table 5), which are not present in such levels in breast milk (Table 2). The digestion of these TAGs, upon hydrolysis by various lipases active in newborns, results in the release of palmitic acid in the digestive tract (25). These free fatty acids can combine with divalent minerals, such as calcium, to form insoluble calcium dipalmitate that is excreted in feces (25).

To address the challenge of residual palmitic acid accumulation in the external position of TAG and the desire to achieve a high *sn*-2 palmitate content, it is crucial to minimize the use of palmitic-rich fractions derived from tropical fats for enzymatic human milk fat substitute synthesis. Instead, we explore an innovative approach by leveraging the potential of oleaginous microalgae. Although microalgae present a promising avenue for the controlled production of TAG oils, a significant challenge in developing a viable substitute for human milk fat lies in the inherent composition of TAG produced by microalgae as well as the low lipid productivity of these microorganisms (55). Unlike human breast milk and certain animal fats, microalgae and higher plants typically synthesize TAG with unsaturated fatty acids in the internal position and saturated fatty acids in the external position. To address this disparity, our research initially focused on enhancing the palmitate content, which was originally 25.3% in the parent strain (Table 4). Utilizing classical strain improvement techniques, we successfully increased it to slightly over 30% (Table 4). Subsequently, genetic transformations were employed to modify the esterification selectivity of saturated fatty acids on the glycerol backbone, favoring the production of TAG where palmitic acid predominantly occupied the internal position. This strategic approach led to the isolation of a strain consistently producing oil with a high palmitic acid concentration, reaching 69.3% (Table 4), esterified to the internal position. When normalizing the palmitic acid concentration in the *sn*-2 position to the total concentration of palmitic acid in the TAG, we achieved a level of 73.3% (Table 5). Notably, the primary TAG in this oil was identified as OPO, constituting ~38.3% of the total TAG produced, as illustrated in Table 5.

Microalgae species like *P. moriformis*, used in the present study, exhibit a doubling time compared to that of yeast (around 3–4 h) when cultivated in a defined medium containing suitable sugars and the same macro- and micronutrients used by terrestrial plants. When nitrogen becomes scarce in the presence of sugars, oleaginous microalgae halt their division and transition their metabolism toward oil production, as illustrated in Figure 2A. This process involves the conversion of carbon from sugars into fatty acids within the plastid, followed by their incorporation into TAG within the endoplasmic reticulum. These TAG accumulate within the cell in the form of large vesicles, leading to biomass, where ~80% of the dry cell weight consists of oil, as depicted in Figure 2B. Furthermore, this oil contains various micronutrients such as tocopherols, tocotrienols, and sterols, the composition of which varies based on the strain. Industrial-level oil production from microalgae involves specific fermentation conditions spanning 3–5 days (Figure 2A). After this period, the fermentation broth is harvested and dried to obtain biomass, from which oil can be extracted using conventional pressing or solvent extraction methods. The resulting crude oil is then refined using standard techniques to yield a neutral-tasting and

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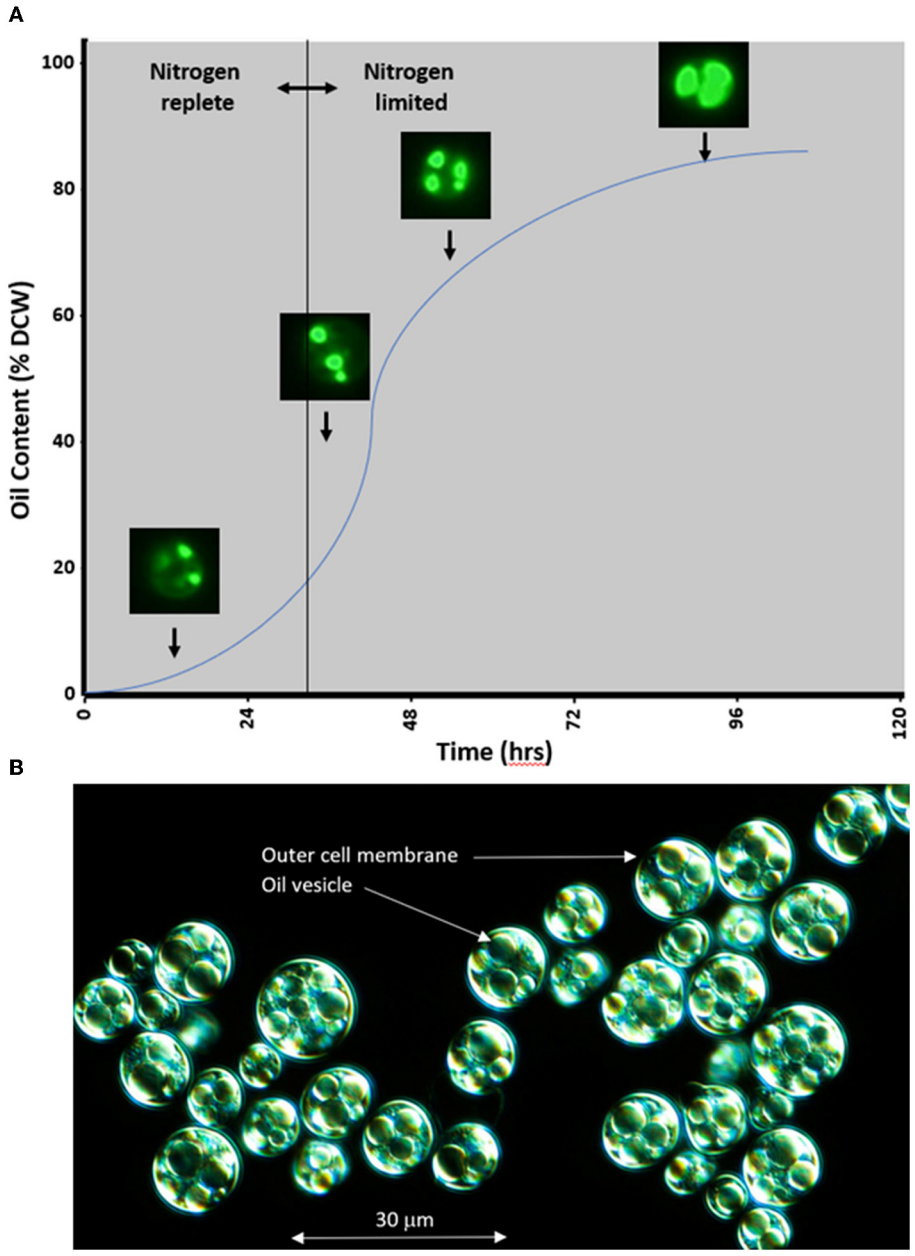

FIGURE 2
(A) Schematic representation of oil production kinetics in heterotrophic microalgae *Prototheca moriformis*. Microalgal cells stained with BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene) and imaged under fluorescence show increasing oil accumulation in response to nitrogen deprivation in a time-dependent manner. This process is reversible, and if nitrogen is returned to the system, algae will catabolize their lipid stores and re-enter a state of vegetative reproduction. **(B)** Micrograph of microalgae *P. moriformis* at the end of fermentation, displaying intracellular large oil vesicles.

neutral-colored oil suitable for the formulation of nutritional products. It is imperative to emphasize that microalgae oils are cultivated under controlled fermentation conditions, allowing for a highly traceable supply chain designed to minimize exposure to chemical, microbial, and allergenic contaminants that could pose adverse health effects. A comprehensive food safety evaluation will be essential before introducing this new ingredient to the market, ensuring compliance with regulatory approvals in various legislations.

5 Conclusion

The structural variations observed in TAG within human milk hold profound implications for infant health and development, particularly impacting processes like digestion, absorption, modulation of gut microbiota, immune function, and potentially long-term health outcomes. To emulate these structure-function relationships in human milk TAG, infant formulas aim to deliver optimal nutrition and promote the overall wellbeing of infants

who are not exclusively breastfed. The utilization of fermentation technology signifies a significant stride in the pursuit of creating nutritionally relevant and structurally analogous analogs for human milk fat. We demonstrated that this technology enables the precise targeting of specific TAG structures, such as OPO, while minimizing the presence of TAG species like PPO, POP, POS, and PPP, which produce calcium dipalmitate, an anti-nutritional compound that hinders the optimal utilization of fatty acids and minerals in infants.

Data availability statement

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

Author contributions

XZho: Writing—original draft, Formal analysis, Investigation, Methodology, Resources, Supervision. XZha: Formal analysis, Investigation, Methodology, Resources, Writing—original draft. LP: Formal analysis, Investigation, Methodology, Resources, Project administration, Supervision, Validation, Writing—original draft. PD: Formal analysis, Resources, Writing—original draft. MC: Formal analysis, Resources, Methodology, Writing—original draft. VB: Methodology, Resources, Writing—original draft. RM: Resources, Writing—original draft. DA: Formal analysis, Methodology, Resources, Writing—original draft. BD: Resources, Writing—original draft. GA: Writing—original draft, Resources. JW: Resources, Writing—original draft. DG: Writing—review & editing, Formal analysis, Methodology, Resources. FD: Conceptualization, Writing—review & editing, Writing—original draft. WR: Conceptualization, Writing—review & editing. SF: Conceptualization, Investigation, Methodology, Project administration, Writing—review & editing.

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Conflict of interest

XZho, XZha, LP, PD, MC, VB, RM, DA, BD, GA, JW, DG, FD, WR, and SF were employed by Checkerspot, Inc, Alameda, California (USA).

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Influence of microbially fermented 2′-fucosyllactose on neuronal-like cell activity in an *in vitro* co-culture system

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Scope: 2′-Fucosyllactose (2′-FL), the most abundant oligosaccharide in human milk, plays an important role in numerous biological functions, including improved learning. It is not clear, however, whether 2′-FL or a cleavage product could influence neuronal cell activity. Thus, we investigated the effects of 2′-FL, its monosaccharide fucose (Fuc), and microbial fermented 2′-FL and Fuc on the parameters of neuronal cell activity in an intestinal–neuronal transwell co-culture system *in vitro*.

Methods: Native ^{13}C-labeled 2′-FL and ^{13}C-Fuc or their metabolites, fermented with *Bifidobacterium (B.) longum* ssp. *infantis* and *B. breve*, which were taken from the lag-, log- and stationary (stat-) growth phases of batch cultures, were applied to the apical compartment of the co-culture system with Caco-2 cells representing the intestinal layer and all-*trans*-retinoic acid-differentiated SH-SY5Y (SH-SY5Y$_{ATRA}$) cells mimicking neuronal-like cells. After 3 h of incubation, the culture medium in the basal compartment was monitored for ^{13}C enrichment by using elemental analysis isotope-ratio mass spectrometry (EA-IRMS) and effects on cell viability, plasma, and mitochondrial membrane potential. The neurotransmitter activation (BDNF, GABA, choline, and glutamate) of SH-SY5Y$_{ATRA}$ cells was also determined. Furthermore, these effects were also measured by the direct application of ^{13}C-2′-FL and ^{13}C-Fuc to SH-SY5Y$_{ATRA}$ cells.

Results: While no effects on neuronal-like cell activities were observed after intact 2′-FL or Fuc was incubated with SH-SY5Y$_{ATRA}$ cells, supernatants from the stat-growth phase of 2′-FL, fermented by *B. longum* ssp. *infantis* alone and together with *B. breve*, significantly induced BDNF release from SH-SY5Y$_{ATRA}$ cells. No such effects were found for 2′-FL, Fuc, or their fermentation products from *B. breve*. The BDNF release occurred from an enhanced vesicular release, which was confirmed by the use of the Ca^{2+}-channel blocker verapamil. Concomitant with this event, ^{13}C enrichment was also observed in the basal compartment when supernatants from the stat-growth phase of fermentation by *B. longum* ssp. *infantis* alone or together with *B. breve* were used.

Conclusion: The results obtained in this study suggest that microbial products of 2′-FL rather than the oligosaccharide itself may influence neuronal cell activities.

KEYWORDS

2′-fucosyllactose, fermentation, microorganisms, neuronal-like cell activity, BDNF

1 Introduction

Human milk oligosaccharides (HMOs) are the third largest solid component in human milk, present to the extent of 20–25 g/L in colostrum and 10–15 g/L in mature milk (1–5). 2′-Fucosyllactose (2′-FL) belongs to the fraction of fucosylated neutral HMOs and is quantitatively the most prominent component in the breastmilk of women expressing fucosyltransferase-2 (FUT-2), a phenotype referred to as secretor positive and representing 70–80% of the Western population (3–8). 2′-FL is a well-known structural homolog to bacterial adhesion sites in the intestine and may act as a prebiotic, supporting colonization of the colon with bacteria that may be beneficial to the breastfed infant (9–11).

In infants, breast milk feeding is known to provide significant health benefits and may even improve cognitive development and intellectual performance (12–16). In this context, 2′-FL or Fuc has been shown to affect cognitive domains and improve learning and memory in animal studies (17–19). 2′-FL is also associated with improved cognition (18, 20) and changes in brain tissue microstructure in breastfed infants (21). The mechanisms behind these neuronal effects are largely unknown. For example, a continuous administration of 2′-FL increased the expression of several molecules involved in the storage of newly acquired memories, such as the postsynaptic density protein 95, phosphorylated calcium/calmodulin-dependent kinase II, and brain-derived neurotrophic factor (BDNF) in cortical and subcortical structures (17). BDNF and its isoforms are members of the neurotrophin family and are synthesized by both, neuronal and non-neuronal cells. They are involved in processes such as differentiation and regeneration (22, 23). It has also been shown that BDNF plays a key role as a mediator of activity-induced long-term potentiation (LTP) in the hippocampus as well as in other brain regions (24). The role of BDNF and its isoforms in LTP is best studied in the hippocampus where the neurotrophins act at pre- and post-synaptic levels and are mediated by Trk (tropomyosin-related kinase) and the tumor necrosis factor receptor family, which are known to be coupled to the activation of the Ras/ERK, phosphatidylinositol-3-kinase/Akt and phospholipase C-g(PLC-g) pathways, and proBDNF/p75NTR/sortilin pathways (24–26). In addition, BDNF is the most important modulator of glutamatergic and GABAergic synapses and is also associated with glutamate and GABA through TrkB signaling (24, 27).

However, it is unclear whether 2′-FL itself or its metabolites are responsible for the observed effects. To achieve neuronal effects, 2′-FL or its metabolites may need to accumulate in the relevant brain regions; however, as we have recently shown, ^{13}C-labeled 2′-FL administered orally to wild-type and germ-free mice was unable to cross the healthy blood–brain barrier (28). A subsequent study showed that even the Fuc moiety from 2′-FL, administered as ^{13}C-labeled Fuc, was also not able to cross the blood–brain barrier either, although it was rapidly absorbed. It was observed that ^{13}C was enriched in the brain at time points after the oral bolus had reached the lower gut (29). This points to the influence of the intestinal microbiota, which are shown to metabolize HMOs and selectively promote the growth of beneficial microbiota such as bifidobacteria (30, 31). Metabolic studies in infants have demonstrated that 2′-FL in milk from secretor mothers is excreted via infants′ urine (32), which was confirmed by endogenously ^{13}C-labeled HMOs in breastfeeding mothers and the urinary excretion of ^{13}C-labeled HMO in their infants (33–36). Low amounts of 2′-FL have also been detected directly in the plasma of breastfed infants of secretor mothers compared to infants fed milk from non-secretor mothers or in plasma from formula-fed infants only when 2'FL was added as a supplement (37, 38). Despite the absorption of intact 2′-FL into the circulation, HMOs are not digested by human enzymes and reach the colon where they are metabolized by the infant gut microbiota.

In general, HMOs are substrates for beneficial microbes such as species of the *Bifidobacterium* genus, but it seems that only a few strains use HMOs as a preferred carbon source (39–43). However, the uptake of HMOs by microbial ABC transporters and their degradation by glycosyl hydrolases result in the formation of monosaccharides, which could be further metabolized by the fructose-6-phosphate phosphoketolase pathway into ATP, acetate, and lactate as end products, which was observed in the case of *B. longum* ssp. *infantis* (44–47). In contrast, extracellular glycosyl hydrolases of *B. breve* and *B. bifidum* generate metabolites that may serve as substrates for *B. longum* ssp. *infantis*, which highlights the co-existing or cross-feeding effects influencing HMO metabolism (48, 49). This microbe–HMO interaction was supported by an accumulation of HMO building blocks such as Fuc and trisaccharides after fermentation of HMO by bifidobacteria and lactobacilli, suggesting a symbiotic interaction of HMOs and specific gut microbiota (50). Recently, the analysis of the development of the gut microbiota of infants during the first month of life shows that colonization of FL-utilizing *Bifidobacteria* species is associated with altered metabolite profiles and microbiota composition (44). Equal co-cultures of bifidobacteria in 2′-FL-containing media produced different ratios of metabolites such as acetate and lactate under steady-state conditions when compared to monocultures (45). Furthermore, it has recently been confirmed that HMOs such as 2′-FL selectively promote the formation of a bifidobacteria-rich microbiota (30), which may then increase their potential impact on neurological functions via the gut–brain axis.

The overall aim of our *in vitro* intestinal–neuronal transwell co-culture system was to investigate if and how ^{13}C-labeled 2′-FL as well as its monosaccharide Fuc were metabolized by different *Bifidobacterium* species, alone or in combination, and if intact or subsequent metabolites cross the monolayers of Caco-2 cells cultured on transwell inserts to affect neuronal-like parameters in neuronal-like ATRA-differentiated SH-SY5Y$_{ATRA}$ cells.

2 Results

2.1 Effects of 2′-FL and Fuc on neurogenesis markers in neuronal-like cells before and after passage through an intestinal epithelial cell layer

To investigate the effects of 2′-FL and Fuc on neuronal-like cell activity markers, we used the human cell line SH-SY5Y, which had been differentiated by all-*trans*-retinoic acid (ATRA) into cells with a significant expression of the well-known neuronal marker synaptophysin (SYP) (51, 52) determined by flow cytometry (Figures 1A–C). Figure 1D shows that in the cultured SH-SY5Y

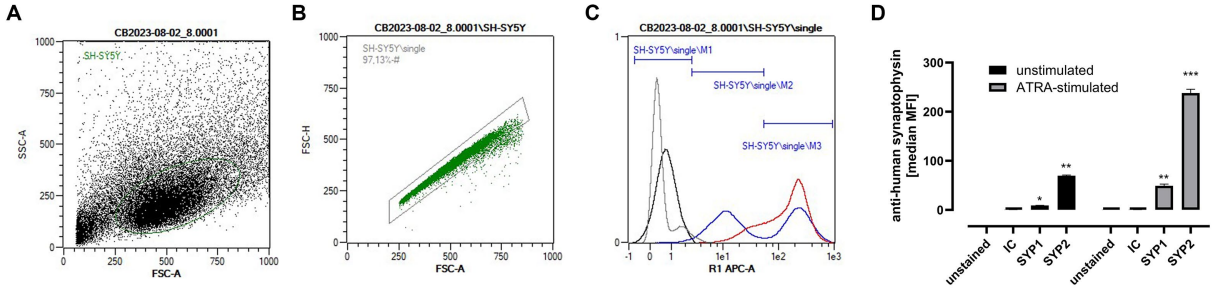

FIGURE 1
Synaptophysin (SYP) expression in SH-SY5Y and ATRA-differentiated SH-SY5Y cells. Differentiation of SH-SY5Y to SH-SY5Y$_{ATRA}$ was confirmed by measuring SYP expression by flow cytometry. Differentiated and non-differentiated cells were detached with accutase solution, centrifugated (500 × *g*, 5 min at RT) and stained according to the manufactures´ instructions (see Methods and Materials 4.1.2). The gating strategy for analyzing SYP expression is given for a representative staining in **(A–C)**. [**(A)** dotblot for cell gating (SSC-A vs. FSC-A), **(B)** dotblot for single cell inclusion (FSC-H vs. FSC-A), and **(C)** representative histogram of unstained cells (gray line), isotype control (IC) stained cells (black line), undifferentiated anti-human-SYP stained cells (blue line), and anti-human-SYP stained ATRA-induced cells (red line)]. **(D)** Quantification of the MFI (mean fluorescence intensity) was performed by setting histogram markers (M) for unstained and IC-stained cells (M1), low SYP (SYP1) expressing cells (M2), and high SYP (SYP2) expressing cells (M3). MFI data were performed using the MACSQuant 2.13.0 software and data analyses (medians with 95% CI) were performed with GraphPad Prism 10.0.2. Differences to IC-stained cells were significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.05$ (ANOVA with multicomparison test) for at least $n = 3$ (in duplicates).

cells, cell populations with both low and high SYP expression levels were present (Figure 1D). Incubation of these SH-SY5Y cells with ATRA over 10 days induced a significant enhancement of cells with high SYP expression, which is 2.6 times higher than in unstimulated cells.

These neuronal-like SH-SY5Y$_{ATRA}$ cells were used to investigate the effects of 2´-FL and Fuc with or without co-cultured Caco-2 cells (Figure 2A). Therefore, 2´-FL and Fuc were applied at non-cytotoxic concentrations (Figure 2B) to the apical side of the transwell (indirect incubation) or directly to SH-SY5Y$_{ATRA}$ cells. As shown in Figures 2C–F, incubation with ^{13}C-2´-FL and ^{13}C-Fuc (5 mM) at the apical side of the co-culture system did not result in any ^{13}C enrichment [δ^{13}C in $^{0}/_{00}$] in the basal compartments (Figure 2C, left *Y*-axis) compared to controls (5 mM glucose), nor did it induce BDNF release (Figure 2C, right *Y*-axis) in the supernatant or choline levels in the cells (Figure 2D). Consistent with these results, no changes in plasma membrane or mitochondrial potential were observed by direct or indirect incubation with 2´-FL or Fuc (Figures 2E,F).

These observations clearly indicate that neither 2´-FL nor Fuc had an influence on neuronal activity markers when they were applied to neuronal-like cells directly or indirectly. Due to the low concentration of intact 2´-FL or Fuc in systemic circulation and recently published data about the intense fermentation of 2´-FL and Fuc in the intestine of mice (28, 29), we aimed to investigate whether the fermentation of 2´-FL and/or Fuc by *Bifidobacterium* species had an influence on neuronal cell activity markers. Again, we used ^{13}C-labeled 2´-FL as well as Fuc. To gain further insight into the metabolic pathways of 2´-FL and/or Fuc during microbial fermentation, we used 2´-FL and Fuc either ^{13}C-labeled on C-atom 1 ($^{13}C_1$-Fuc) or 6 ($^{13}C_6$-Fuc).

2.2 Microbial fermentation of 2´-FL and Fuc

For fermentation studies, we used *B. longum* ssp. *infantis* and *B. breve* as bifidobacterial strains, as they are known to ferment HMOs by extra- and intracellular glycosyl hydrolases and have the potential for bifidobacterial cross-feeding (50). As shown in Figures 3A–C, all the bacterial strains grew well in media containing high concentrations of glucose (55 mM). *B. longum* ssp. *infantis*, *B. breve*, and co-cultured bifidobacteria grew rapidly and reached an optical density ($OD_{600\ nm}$) values of 1.58 ± 0.05, 1.39 ± 0.03, and 1.41 ± 0.05, respectively. When these strains were grown in media containing 5 mM glucose instead of 55 mM glucose, they still grew well, but with a lower maximum $OD_{600\ nm}$ values of 1.02 ± 0.07, 1.16 ± 0.05, and 1.25 ± 0.04 after 36 h of incubation, respectively (Figures 3A–C). Substitution of this lower glucose concentration of 5 mM with an isomolar concentration of 2´-FL as the sole carbohydrate source, *B. longum* ssp. *infantis* alone (Figure 3A) or in co-culture with *B. breve* (Figure 3C) grew to an optical density ($OD_{600\ nm}$) similar to that with 5 mM glucose (1.12 ± 0.07 and 1.16 ± 0.06). However, in media containing $^{13}C_1$-Fuc- or $^{13}C_6$-Fuc, *B. longum* ssp. *infantis* grew very slowly with maximum $OD_{600\ nm}$ values of 0.54 ± 0.02 and 0.52 ± 0.01. In contrast, *B. breve* showed better growth on $^{13}C_1$-Fuc and $^{13}C_6$-Fuc-containing media with a maximum optical density of 0.68 ± 0.01 and 0.63 ± 0.01, respectively, but did not grow in media containing ^{13}C-2´-FL as a carbohydrate source (Figure 3B).

To investigate the possible effects of fermentation products on neuronal cell activity makers, we collected growth media from 2´-FL- and Fuc-fermented batch cultures at three different time-points: lag-, log- and stat-growth phase. The collected supernatants were used in the intestinal–neuronal transwell co-culture system (Figure 4A).

2.3 Effects of bacterial fermentation products on SH-SY5Y$_{ATRA}$ cells in a co-culture model

In the first set of experiments, we aimed to investigate whether bacterial fermentation products collected at the three different time points during batch cultures passed an intestinal Caco-2 cell

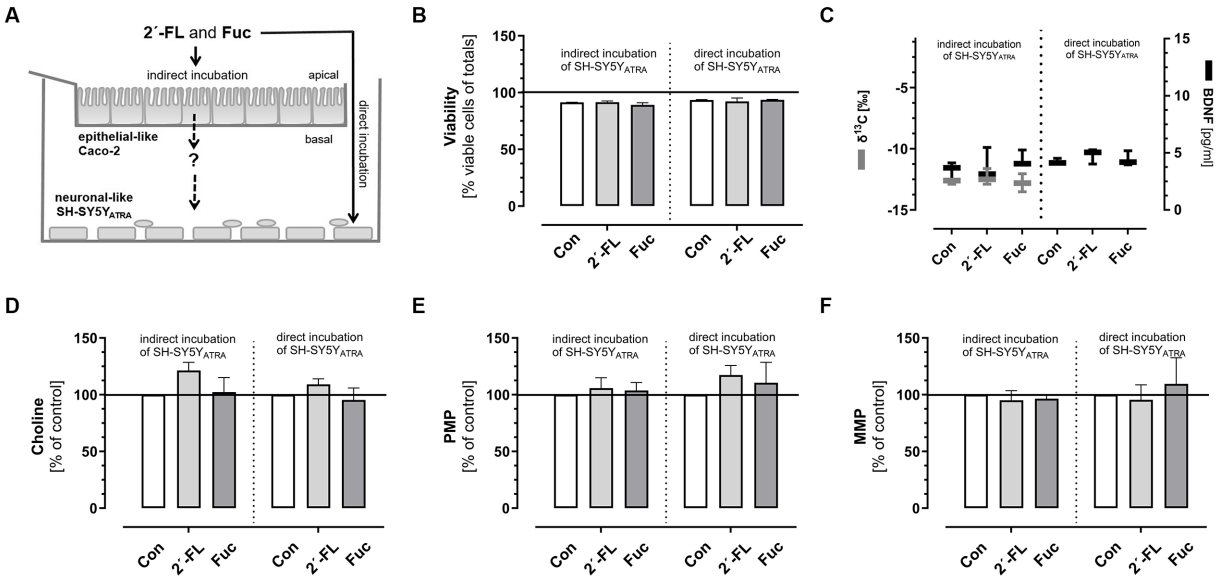

FIGURE 2
Determination of indirect and direct effects of 2´-FL and Fuc on ATRA-differentiated neuronal-like SH-SY5Y cells (SH-SY5Y$_{ATRA}$). Caco-2 cells, cultured on transwell inserts, were incubated with 2´-FL or Fuc (5 mM) for 3 h at 37°C. Thereafter, transwell inserts were removed and SH-SY5Y$_{ATRA}$ cells were further incubated with basal media (indirect incubation) or directly with 2´-FL or Fuc (0.5 mM) in comparison to controls (5 mM Glucose) **(A)**. Viability was measured by flow cytometry using the ViaCount Reagent® [% viable cells of total cells] **(B)**, ^{13}C enrichment [δ^{13}C in $^{0}/_{00}$] was determined by EA-IRMS [**(C)**, left *Y*-axis], BDNF concentrations [pg/mL] were measured in the supernatant by ELISA [**(C)**, right *Y*-axis], choline levels **(D)**, plasma membrane potential (PMP) and mitochondrial membrane potential (MMP) were determined fluorometrically and are given as % of controls **(E,F)**. Data are shown as box blots with min-max (whiskers) or as bars with means and standard deviation for $n = 3$ (each in duplicate). Significant differences were calculated by *t*-test comparing control with 2´-FL or Fuc.

monolayer and reached the basal compartment of the transwell system. In the second set of experiments, we measured BDNF secretion from SH-SY5Y$_{ATRA}$ cells after a 24 h incubation with the enriched basal media (Figures 4B–D).

Using cell-free media from different time points of bacterial growth, we observed significant ^{13}C enrichment and a concomitant BDNF secretion only with stat-growth phase 2´-FL metabolites from *B. longum* ssp. *infantis* in the basal compartments (Figure 4B) and, to a lesser extent, with 2´-FL metabolites from the stat-growth phase of *B. longum* ssp. *infantis* co-cultured with *B. breve* (Figure 4D). In the *B. breve* cultures, neither ^{13}C enrichment nor BDNF secretion by SH-SY5Y$_{ATRA}$ cells was observed with fermented 2´-FL metabolites (Figure 4C). However, when *B. breve* was incubated with ^{13}C-Fuc, ^{13}C enrichment was observed after the fermentation of Fuc (lag-growth phase) when C_6 atom of Fuc was ^{13}C-labeled, but not when $^{13}C_1$-Fuc was used. This was also observed when *B. breve* was *co*-cultured with *B. longum* ssp. *infantis*. Interestingly, no secretion of BDNF was observed by SH-SY5Y$_{ATRA}$ cells when Fuc metabolites were present in the basal media (Figures 4B–D).

In addition to BDNF, we could not detect any further effect on other potential neurotransmitters such as GABA (γ-aminobutyric acid) or the precursor molecule glutamate (see Supplementary Figures S1, S2). Further, it should be noted that the secretion of BDNF by differentiated neuronal-like cells was relatively low. Thus, the secreted amounts of BDNF in the co-culture system may not have been sufficient to influence further neurotransmitter release.

2.4 Effect of the calcium channel blocker verapamil on BDNF secretion from differentiated SH-SY5Y$_{ATRA}$ cells after incubation with bifidobacterial fermentation products

Based on the results with the stat-growth phase 2´-FL metabolites from *B. longum* ssp. *infantis* alone or grown together with *B. breve* on BDNF secretion by neuronal-like SH-SY5Y$_{ATRA}$ cells, we probed further with the aim of understanding the mechanism of the enhanced secretion. This secretion could be a result of increased mRNA expression or the release from secretory vesicles (53, 54). Therefore, we used Verapamil (VP) as a L-type calcium channel blocker to verify the effects on vesicular release and additionally RT-qPCR to measure mRNA expression. As shown in Figures 5A,B, BDNF release induced by 2´-FL metabolites from *B. longum* ssp. *infantis* was partially reduced by pre-incubation of SH-SY5Y$_{ATRA}$ cells with VP (Figure 5A). This effect was not observed for 2´-FL metabolites generated by *B. longum* ssp. *infantis* co-cultured with *B. breve* (Figure 5B). Due to the incomplete inhibition by VP, other calcium channels (e.g., N-, T-type) may also play a role. In contrast to the inhibiting effect of VP on BDNF release by *B. longum* ssp. *infantis*, we did not observe any changes in mRNA expression due to 2´-FL metabolites produced by *B. longum* ssp. *infantis* nor by *B. longum* ssp. *infantis* *co*-cultured with *B. breve* (see Supplementary Table T1).

Because of the well-known effect of neurotrophic factors on the Trk-signaling cascade (55), we measured the protein expression of the isoforms TrkA and TrkB on SH-SY5Y and SH-SY5Y$_{ATRA}$ cells. Both the isoforms were expressed on neuronal cells, but TrkB signaling is a

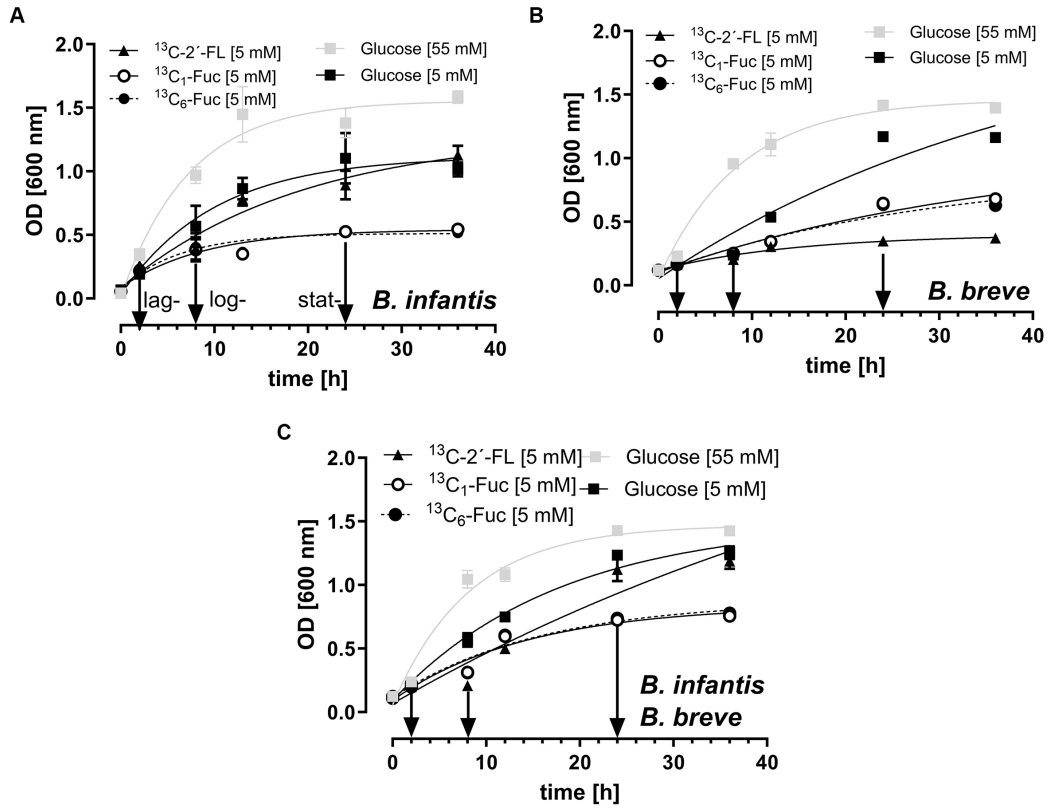

FIGURE 3
Growth of *B. longum* ssp. *infantis* **(A)**, *B. breve* **(B)**, and co-culture of *B. longum* ssp. *infantis with B. breve* **(C)** in high glucose-containing medium (55 mM), glucose-reduced media (5 mM), ^{13}C-2′-FL- and ^{13}C-Fuc-supplemented media (5 mM). Bacterial strains were anaerobically cultured at 37°C (see Methods and Materials 4.1.5) and growth was measured spectrophotometrically (600 nm). Data are given as means and standard deviation for $n = 3$. Arrows indicate the collection time of growth media at lag-, log- and stat-growth phase of the batch cultures.

well-known effect of BDNF, whereas TrkA signaling was induced by unprocessed BDNF (26, 56–58). Here (Figures 6A–H), we detected a slight but significant expression of TrkA (Figure 6F) and TrkB (Figure 6G) on unstimulated and ATRA-stimulated cells. Although BDNF has been found to increase the expression of TrkB as well as AChE (acetylcholine esterase) and ChAT activity (choline acyltransferase) (59, 60), we could not see any effect produced by the stat-phase supernatants of 2′-FL metabolized by *B. longum* ssp. *infantis* (Figure 6H).

3 Discussion

In the present study, we evaluated the effects of 2′-FL and Fuc, either as intact or fermented saccharides, on neuronal-like cell activity using an *in vitro* transwell co-culture model with intestinal Caco-2 cells, which reflect the intestinal cell layer in the gut, and ATRA-induced SH-SY5Y$_{ATRA}$ cells, which are used as a model of neuronal-like cells (61–63). These SH-SY5Y$_{ATRA}$ cells were either directly incubated with intact 2′-FL or Fuc or indirectly applied to a Caco-2 cell monolayer. In addition, 2′-FL and Fuc were fermented prior to the indirect incubation of SH-SY5Y$_{ATRA}$ cells to assess viability, neurotransmitter release, and changes in plasma membrane and also measure mitochondrial potential. While no effects on neuronal cell activities were detected on SH-SY5Y$_{ATRA}$ cells using intact 2′-FL or Fuc, metabolites from 2′-FL fermentation produced by *B. longum* ssp. *infantis* alone or together with *B. breve* showed an increase in BDNF secretion from SH-SY5Y$_{ATRA}$ cells in the *in vitro* co-culture model. Although only low levels of BDNF was secreted, it was a result of enhanced vesicular release and not a result of an induction of mRNA expression, as demonstrated by the use of the L-type calcium channel blocker Verapamil (VP).

HMOs are considered to exert effects in extra-intestinal tissues such as the brain (21, 64, 65). Many studies in this connection have reported that breastfeeding is associated with higher intelligence quotient (IQ), either at school age or in adulthood (20, 66, 67). Among the fucosylated oligosaccharides, *in vitro* administered 2′-FL and Fuc were able to enhance long-term potentiation (LTP) in the rat hippocampus (68, 69). In addition, Vázquez et al. (17) reported that synaptic plasticity in rodents was enhanced after oral supplementation with 2′-FL and Wu et al. (19) have recently shown that oral intake of 2′-FL improved locomotor activity and upregulated BDNF expression in rats. In another study, no significant differences were observed between 2′-FL-supplemented rats (age 4–6 weeks post weaning) and controls in behavioral tests such as the maze tests; however, significant differences were shown at age 1 year (20).

The underlying mechanisms are poorly understood and it has been speculated that a direct effect of 2′-FL in the brain or an indirect interaction with the vagus nerve at the intestinal level is possible (17, 70, 71). Despite the data showing that 2′-FL may reach the brain via

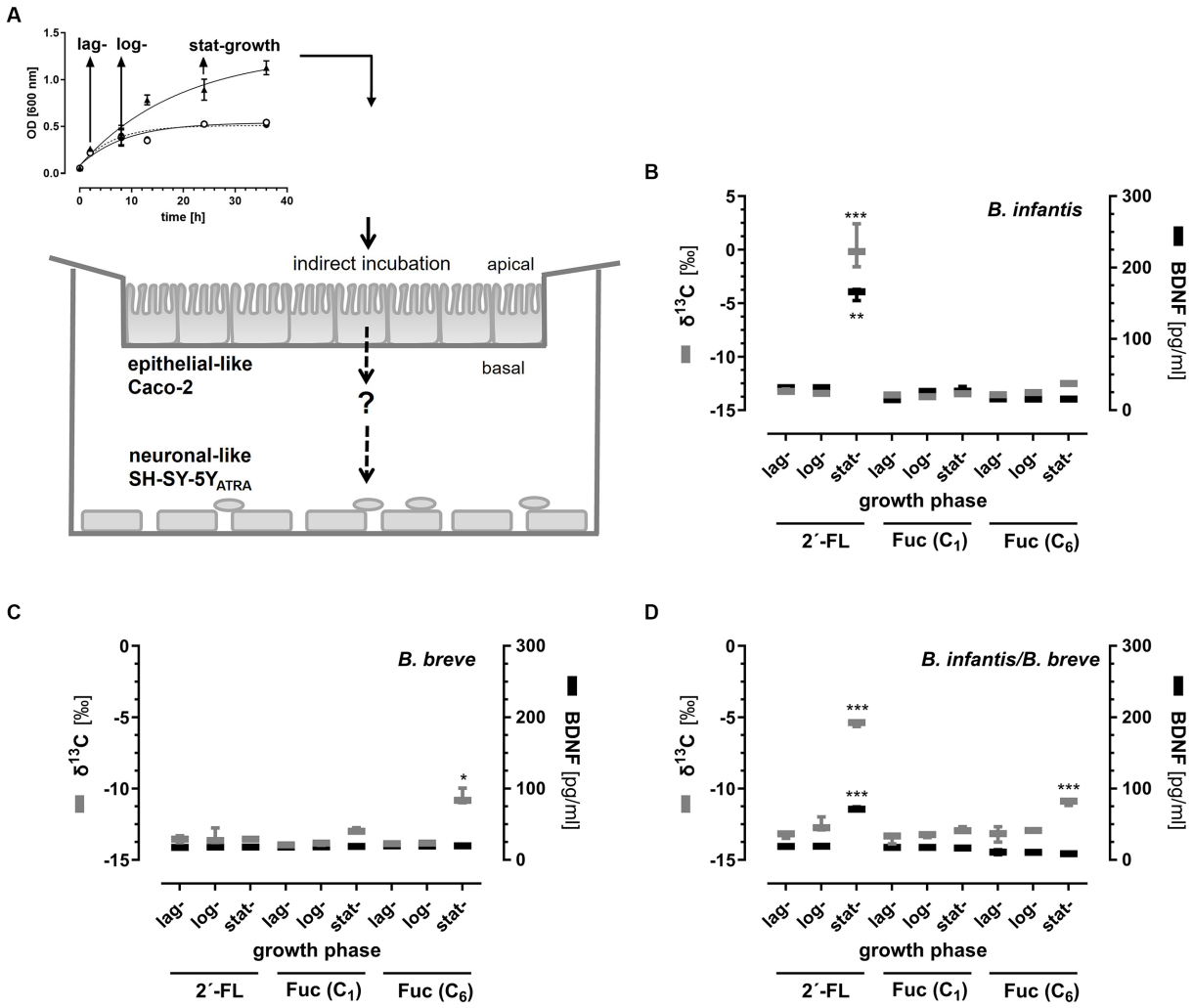

FIGURE 4
Enrichment of ^{13}C-2´-FL- and ^{13}C-Fuc-derived metabolites in the basal compartments of a transwell system and their influence on BDNF-secretion from neuronal-like SH-SY5Y$_{ATRA}$ cells. *B. longum* ssp. *infantis, B. breve*, and *B longum* ssp. *infantis* together with *B. breve* were grown in ^{13}C-2´-FL-, ^{13}C-Fuc (^{13}C$_1$-Fuc or ^{13}C$_6$-Fuc)-containing (5 mM) media. At the lag -, log-, and stat-growth phase, growth media were collected, centrifuged, filtered, and pH-adjusted (pH 7.4). Thereafter, supernatants were applied to the transwell inserts containing the Caco-2 cell monolayer (apical) and neuronal-like SH-SY5Y$_{ATRA}$ cells (basal) **(A)**. In the first set of experiments, basal compartments were collected after a 3 h incubation of Caco-2 cells to determine the ^{13}C enrichment by EA-IRMS being expressed as δ^{13}C [$^0/_{00}$] **[(B–D)** left *Y*-axis]. In a second set of experiments, SH-SY5Y$_{ATRA}$ cells were further incubated with basal media for 21 h to measure BDNF secretion by ELISA given as pg./mL **[(B–D)** right *Y*-axis]. Data are shown as box blots with min-max (whiskers) with n = 3 (each in duplicate). Significant differences between lag-, log-, and stat-growth phase values were calculated with one-way ANOVA with multi-comparison tests. Differences were significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

systemic circulation, we have recently shown that ^{13}C enrichment in the brain tissue does not occur when mice were given ^{13}C-labeled 2´-FL or Fuc via intravenous injection, indicating that none of these saccharides can cross the blood–brain barrier in mice. Furthermore, in germ-free mice orally fed with ^{13}C-labeled 2´-FL, the ^{13}C bolus remains in the intestinal content and was expelled via the feces, indicating that gut microbial metabolites of 2´-FL or Fuc could be responsible for the observed effects since ^{13}C enrichment of brain tissue occurred when the ^{13}C-2´-FL or ^{13}C-Fuc bolus had reached the lower gut containing microbiota (28, 29). In this context, it is well-known that bifidobacteria were able to utilize fucosylated HMOs to produce metabolites such as short-chain fatty acids (e.g., acetate) and lactate (72–74). During breastfeeding, *B. longum* ssp. *infantis* and *B. breve* are known to regularly colonize the infant gut and express several transport proteins and glycosidases directly involved in HMO utilization according to the HMO-degrading gene cluster. For example, *B. longum* ssp. *infantis* express transport proteins and intracellular 1,2-α-L-fucosidases or 1,3-1,4-α-L-fucosidases and therefore utilize HMO by transporting them from extracellular to intracellular sites and hydrolyzing them using glycoside hydrolases (74, 75).

In the present study, using our established intestinal–neuronal transwell co-culture system, we showed that intact ^{13}C$_1$-labeled 2´-FL or Fuc (^{13}C$_1$- and ^{13}C$_6$-labeled) were not able to cross the polarized Caco-2 cell layer as measured by EA-IRMS. Furthermore, using media in the basal compartment, we could not detect any effects on neuronal-like cell activities in SH-SY5Y$_{ATRA}$ cells when 2´-FL or Fuc was applied directly or indirectly via the Caco-2 layer. Based on these results,

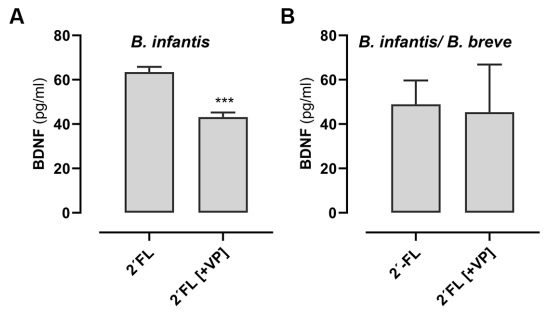

FIGURE 5
Effect of the calcium channel blocker verapamil (VP) on BDNF secretion from neuronal-like SH-SY5Y$_{ATRA}$ cells after incubation with stat-growth phase metabolite-enriched media. Neuronal-like SH-SY5Y$_{ATRA}$ cells were pre-incubated with 7.5 μM VP for 30 min or without VP. Thereafter, the cells were incubated for 24 h with the metabolite-enriched medium from stat-growth phase from *B. longum* ssp. *Infantis* **(A)** and *B. longum* ssp. Infantis co-cultured with *B. breve* **(B)**. After incubation with enriched media, BDNF secretion [pg/mL] was measured in the supernatant using ELISA (see Methods and Materials 4.4 and 4.9). Data are given as means ± standard deviation for $n = 3$ (in duplicates). Differences between cells with and without VP-treatment were analyzed with *t*-test and were significant at ***$p < 0.001$.

we used different *Bifidobacterium* strains to generate metabolites from 2´-FL or Fuc to further investigate their effects on neuronal-like cell activities. As expected, the bacterial strains *B. longum* ssp. *infantis* and *B. breve* alone or in combination showed different preferences with regard to 2´-FL and Fuc as carbohydrate growth substrates. *B. longum* ssp. *infantis* grew well on 2´-FL supplemented media very similar to an isomolar concentration of glucose, which was used as control. In contrast, *B. breve* preferred Fuc although to a much lower degree compared to the isomolar concentration of glucose; 2´-FL did not seem to be metabolized to support its growth. Co-incubation of *B. longum* ssp. *infantis* and *B. breve* revealed a more efficient fermentation of 2´-FL, when assessed by the pH levels (data not shown), achieved in the stationary phase of bacterial growth, suggesting an interaction of *B. longum* ssp. *infantis* and *B. breve* although a direct cross-feeding effect was not assessed.

To mimic the transport of bacterial metabolites from ^{13}C-labeled 2´-FL or Fuc across the intestinal epithelium, we collected supernatants at different time points (lag-, log- and stat-growth phase) of bacterial growth, applied them to a polarized Caco-2 cell monolayer, and measured ^{13}C enrichment in the basal compartment. While no ^{13}C enrichment was detected in lag- and log-growth phase supernatants, ^{13}C enrichment was observed in the stat-phase supernatants from *B. longum* ssp. *infantis* supplemented with 2´-FL and *B. breve* supplemented with Fuc, albeit in lower concentrations. Using supernatants from co-cultured *B. longum* ssp. *infantis* and *B. breve*, we also observed only an ^{13}C enrichment in the basal compartment with supernatants from the stat-growth phase. Interestingly, we detected the release of BDNF from SH-SY5Y$_{ATRA}$ cells only in stat-growth phase supernatants after 2´-FL fermentation from *B. longum* ssp. *infantis* containing batch cultures. Although we also observed a ^{13}C enrichment in stat-growth phase supernatants from $^{13}C_6$-Fuc-fermented bacterial strains, but not from $^{13}C_1$-Fuc, the ^{13}C enrichment was much lower than in the cultures with $^{13}C_1$-2´-FL. Nevertheless, it remains speculative whether the amount or type of metabolite was responsible for the BDNF releasing effect from SH-SY5Y$_{ATRA}$ cells. As mentioned above, *B. longum* ssp. *infantis* is able to degrade 2´-FL by several fucosidases, which may have released Fuc from 2´-FL. As the native Fuc applied directly to the differentiated cells did not produce any effect, it can be assumed that the effect was likely induced by metabolites. In this context, it has recently been shown that under anaerobic conditions Fuc was further metabolized to dihydroxyacetone-phosphate or lactate and/or 1,2-propanediole (1,2-PDO), which are intermediate productions for the generation of short chain fatty acids, i.e., lactate is a precursor of acetate and butyrate and 1,2-PDO of propionate (76). Keeping in mind that C_1 of Fuc was ^{13}C-labeled, ^{13}C enrichment may rather be derived from a dihydroxyacetone phosphate metabolite than from a lactate and/or 1,2-PDO metabolite, since lactate and/or 1,2-PDO are C4,5,6-backbone molecules, whereas dihydroxyacetone phosphate results from the C1,2,3-backbone (76–78). On the other hand, it has been shown that metabolites such as lactate play an important role in LTP. A pharmacological inhibition of MCT2 (monocarboxylate transporter 2), a transporter delivering lactate to neurons, irreversibly impairs long-term memory possibly by modulating the PGC1α/FNDC5/BDNF pathway (79–81). We expected that the use of Fuc labeled either on C_1 or C_6 of the molecule should enable us to gain further insight into the metabolic pathways of Fuc. However, we showed that 2´-FL labeled on C_1 of its Fuc moiety had been metabolized by *B. longum* ssp. *infantis*, but not ^{13}C-labeled compounds, which were able to pass an intestinal cell layer when ^{13}C-labeled Fuc was infused, labeled on either C_1 or C_6 of the molecule. In addition, we observed that Fuc degradation by *B. breve* led to soluble compounds containing the C_6-atom from Fuc; the C_1-ending of Fuc might have been completely metabolized, e.g., to CO_2 since no ^{13}C enrichment was seen in the basal compartment when $^{13}C_1$-Fuc was supplemented to bacterial media. Which metabolite is responsible for the ^{13}C enrichment in the basal compartment after incubation of Caco-2 cells with media from *B. longum* ssp. *infantis* or the mixture of *B. longum* ssp. *infantis* and *B. breve* needs further investigation. However, only metabolites from 2´-FL produced from *B. longum* ssp. *infantis* were able to induce secretion of BDNF in SH-SY5Y$_{ATRA}$ cells.

As mentioned above, BDNF and its isomers are members of the neurotrophin family and have been shown to play a key role as mediators of activity-induced LTP in neuronal cells. It has been shown that BDNF mRNA expression could be induced in SH-SY5Y cells by different stimuli (54, 82) and the released BDNF protein could act at auto- and paracrine levels. As such, it is an important modulator of glutamatergic and GABAergic synapses with glutamate and GABA release through TrkB receptor signaling (27). In this context, the released BDNF binds to TrkB and activates Ras/ERK, phosphatidylinositol3-kinase/Akt and phospholipase C-g(PLC-g) signaling cascades, which in turn stimulate glutamate and GABA release as neurotransmitters (24, 25, 80). BDNF release, however, is a highly regulated process in which ER (endoplasmic reticulum)- and Golgi-associated vesicles are released either constitutively or through regulated mechanisms. The secretion via Golgi-derived vesicles requires Ca^{2+}-sustained intracellular elevations and is associated with plasma membrane hyperpolarization. In addition, TrkB activation by BDNF triggers the PGC1α (peroxisome proliferator-activated receptor- γ coactivator 1-alpha) pathway, which in turn increases the expression of BDNF protein (80, 83). In our experiments, metabolites from 2´-FL produced by

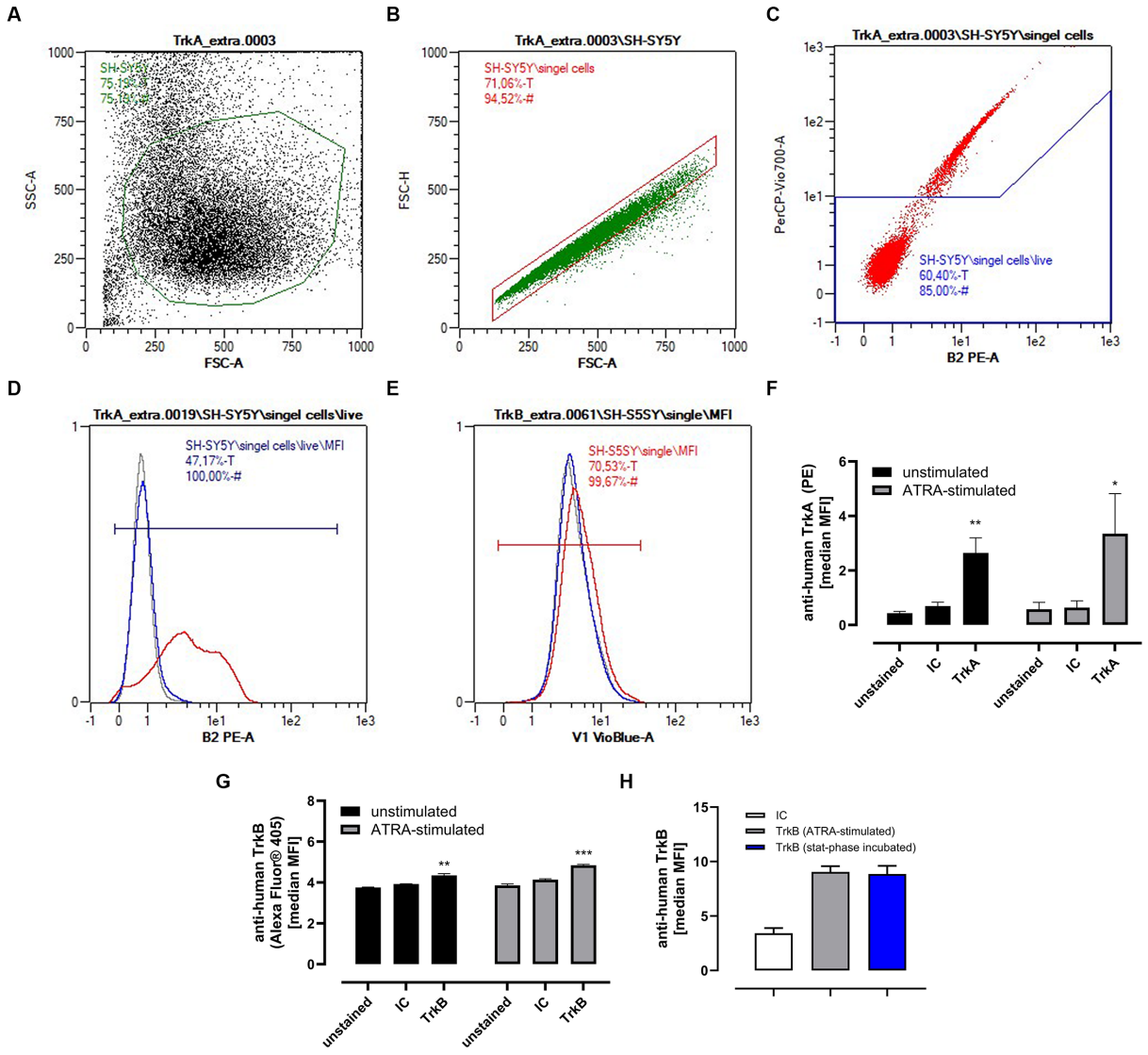

FIGURE 6
Expression of TrkA and TrkB in unstimulated SH-SY5Y and ATRA-stimulated SH-SY5Y$_{ATRA}$ cells and the effect of metabolites from stat-growth phase supernatant from 2´-FL fermentation by *B. longum* ssp. *infantis*. Cells were incubated with or without ATRA (see Section 4.1.2) and expression of TrkA and TrkB was analyzed by flow cytometry (see Section 4.10). Therefore, after incubation, cells were treated with accutase solution to ensure surface protein integrity. After centrifugation (500 × *g*, 5 min), harvested cells (1 × 10^5 cells/mL) were incubated with anti-human TrkA PE-conjugated, anti-human TrkB Alexa Fluor® 405-conjugated monoclonal antibody or with corresponding isotype control (IC) for 10 min in the dark at room temperature. Gating strategy for analyzing TrkA and TrkB with MQ10 Analyzer for a representative staining is given in **(A–C)**. [single cells (FSC-H vs. FSC-A) **(B)**, propidium iodide-stained cells (PerCP-Vio700A vs. PE) **(C)**, representative histogram overlay of unstained (gray), isotype stained (blue line) and ATRA-stimulated Trk stained cells (red line) **(D, E)**]. Quantification of TrkA **(F)** and TrkB, **(G)** expression and influence of 2´-FL metabolite enriched media on TrkB expression **(H)**. Data are given as MFI (main fluorescence intensity) medians with CI (95%) for $n = 3$ (each in duplicates) and quantification was performed using the MACSQuant 2.13.0 software. Data analysis were performed with GraphPad Prism 10.0.2. Significant differences between IC were calculated with One-way ANOVA with multi-comparison tests. Differences were significant at $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Bifidobacterium species did not affect the BDNF gene expression as confirmed by RT-qPCR but did induce a low, but significant BDNF release. Using Verapamil, a well-known L-type voltage-dependent calcium channel (VDCC) antagonist that inhibits BDNF release (84), we observed a significant, but not complete inhibition of BDNF secretion, suggesting that additional mechanisms are involved in the release of BDNF from SH-SY5Y$_{ATRA}$ cells. This was also reported for primary neuronal cells using Verapamil as a VDCC blocker (85). Other than the observed secretion of BDNF, no further influence on choline, glutamate, or GABA release was detected, possibly due to the low levels of secreted BDNF and an unexpectedly low TrkB expression on SH-SY5Y$_{ATRA}$ cells. Thus both the low BDNF secretion and the lack of signaling activation described above could be offered as an explanation, although TrkB receptor expression has previously been shown to be present in SH-SY5Y cells after differentiation with retinoic acid (86). In this context, it should be mentioned that several differentiation protocols for the neuroblastoma cell line SH-SY5Y into a neuronal-like cell type have been established using

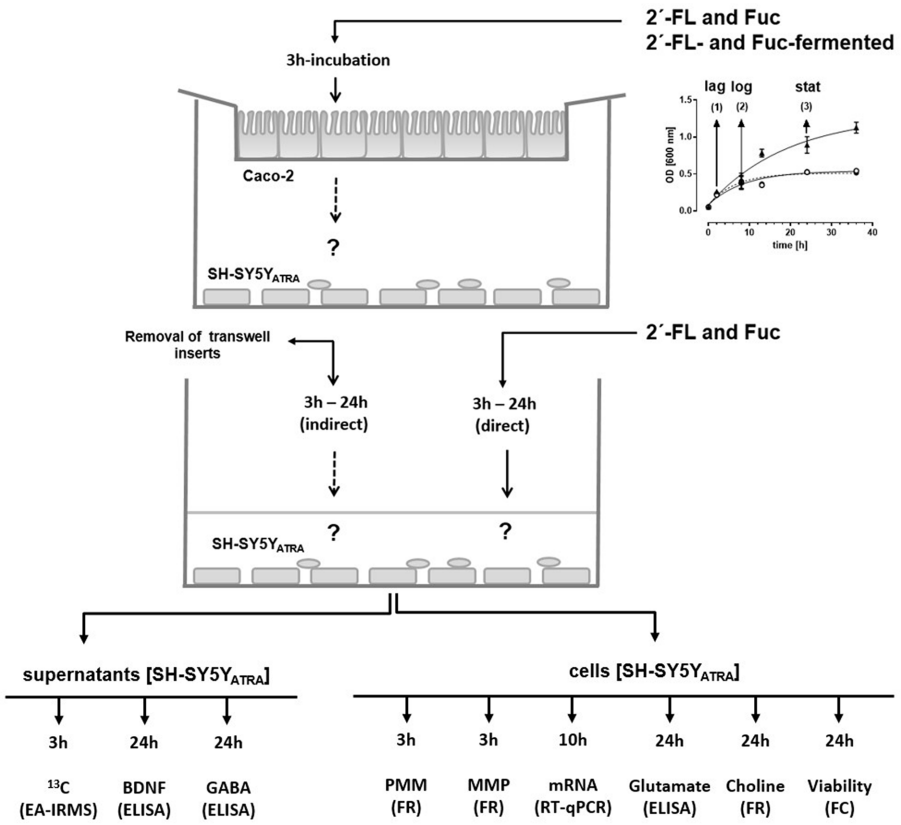

FIGURE 7
In vitro transwell intestinal-neuronal co-culture model. Non-fermented ^{13}C-2´-FL (5 mM) and ^{13}C-Fuc (5 mM) as well as ^{13}C-2´-FL or ^{13}C-Fuc fermented by *B. longum* ssp. *infantis, B. breve,* and *B. longum* ssp. *infantis /B. breve,* collected at the lag-, log-, and stat-growth phase, were applied to the transwell inserts cultivated with 22-day differentiated Caco-2 cells ("indirect incubation"). After a 3 h of incubation, Caco-2 inserts were removed and 10-day ATRA-differentiated SH-SY5Y cells (SH-SY5Y$_{ATRA}$) were used immediately or incubated for the times indicated to measure neuronal cell activities. For "direct" incubation, ^{13}C-2´-FL (0.5 mM) and ^{13}C-Fuc (0.5 mM) were incubated with SH-SY5Y$_{ATRA}$ cells. After indicated incubation times of SH-SY5Y$_{ATRA}$ cells, basal compartments (supernatants) were collected to measure ^{13}C enrichment by EA-IRMS, BDNF, and GABA concentrations by ELISA. Cells were used to measure membrane potential [plasma (PMM) and mitochondrial (MMP)] and choline levels by fluorescence kits using a fluorescence reader (FR), mRNA-expression of BDNF by real-time quantitative PCR (RT-qPCR), glutamate by ELISA, and viability by flow cytometry (FC).

ATRA, B27-supplement, and BDNF, alone or in combination (59, 62, 87).

In conclusion, our ATRA/B27-supplement treatment of SH-SY5Y cells revealed a neuronal-like phenotype with increased expression levels of synaptophysin, a well-known marker of neuronal cell differentiation. Using this neuronal-like cell model, we have shown that only 2´-FL, fermented by *B. longum* ssp. *infantis* induced BDNF secretion via vesicle-releasing mechanisms. However, it remains to be determined which metabolite may be responsible for ^{13}C enrichment and the effect of neuronal cell activity.

4 Methods and materials

4.1 Study design

In order to investigate the effects of non-fermented and fermented 2´-FL and Fuc on neuronal cell activity markers, we developed an *in vitro* transwell co-culture model in which human intestinal epithelial cells (Caco-2) and ATRA-differentiated SH-SY5Y neuronal-like cells (SH-SY5Y$_{ATRA}$) were able to impact each other (Figure 7) similar to our previously published *in vitro* epithelial-endothelial co-culture model (88). In order to mimic the absorption and metabolization sites in the intestine, Caco-2 cells were grown on semipermeable transwell filters over 22 days to differentiate and develop an enterocyte-like phenotype. After differentiation, transwell filters were inserted into a 24-well cavity where SH-SY5Y$_{ATRA}$ cells were cultivated at the bottom of the cavity. The upper compartment (transwell insert) with epithelial cells was exposed with non-fermented and fermented 2´-FL and Fuc for 3 h (indirect incubation). Thereafter, inserts were removed and basal media (supernatants) as well as SH-SY5Y$_{ATRA}$ cells were used immediately or after indicated times in order to determine neuronal cell activity markers. Direct incubation was done using intact 2´-FL and Fuc directly on SH-SY5Y$_{ATRA}$ cells.

4.1.1 Culturing intestinal Caco-2 cells

The human intestinal epithelial cell line Caco-2 (HTB37™) was derived from colon adenocarcinoma cells obtained from ATCC (Manassas, Virginia, United States). The cells were routinely grown in 75 cm^2 culture flasks using Dulbecco's Eagle's Minimum Essential Medium (DMEM) at pH 7.4 with 1% non-essential amino acids (NEAA), 1% sodium pyruvate, and 10% fetal calf serum (FCS,

TABLE 1 Antibodies for Synaptophysin staining of SH-SY5Y and SH-SY5Y$_{ATRA}$ cells.

Target	Primary recombinant antibodies (Ab)	Fluorochrome	Dilution	Incubation time	MQ10 channel
Synaptophysin (SYP), clone REA1121	REAfinity®	APC	1:50	10 min	R1 (APC)
Isotype control for SYP	Recombinant human IgG1	APC	1:50	10 min	R1 (APC)

Invitrogen, Germany). Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Stock passages were sub-cultured every 4 days until reaching 70–80% confluence. For incubation studies, pre-confluent cells were trypsinized with a 0.25% (w/v) trypsin/0.53 mM EDTA solution (Invitrogen, Darmstadt, Germany) and 1×10^4 cells per 0.5 mL^{-1} were seeded onto a 24-well transwell-insert with a polycarbonate membrane (0.4 µm pore size, Greiner-Bio-One GmbH, Frickenhausen, Germany) and placed in a 24-well cavity. Cells were allowed to grow to confluence (2 days) with DMEM (20% FCS) and thereafter to differentiate to absorptive enterocytes within 22 days. The culture medium was changed every 2–3 days at the apical (0.5 mL) and basolateral sides (1.5 mL). For incubation experiments at day 22, the transepithelial electrical resistance (TEER), a marker of the integrity of polarized epithelial cell monolayers, was determined before and after the experiments by using a Millicell® ERS volt-ohmmeter (Millipore Corporation, Bedford, MA, United States). TEER readings were taken at 37°C after equilibrium with the incubation media. A TEER value ≥800 Ohm × cm^2 was used as an indicator for an intact epithelial layer suitable to be used for incubation studies.

4.1.2 Culturing neuroblastoma SH-SY5Y cells

The human neuroblastoma cell line SH-SY5Y (ACC209) was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). Cells were cultured (5% CO_2; 37°C) in Ham's F12/DMEM (1:1; with GlutaMAX™, sodium bicarbonate and sodium pyruvate) and supplemented with 15% FCS (Invitrogen GmbH, Karlsruhe, Germany). Cells were routinely sub-cultured splitting sub-confluent cultures (70–80%) 1:10 with 0.5% (w/v) trypsin/0.25 mM EDTA solution (Invitrogen GmbH, Karlsruhe, Germany). Cells grow as undifferentiated, continuously proliferating cells and include both adherent and floating cells. For sub-cultivation, one third of the supernatant with floating cells was collected, centrifugated (500 × *g*, 5 min at RT) and taken up in fresh complete media. Pre-confluent adherent cells were trypsinized with a 0.5% (w/v) Trypsin/0.25 mM EDTA solution and after centrifugation (500 × *g*, 10 min at room temperature), 1×10^5 cells ml^{-1} were seeded into a new culture flask and combined with the pre-collected floating cells. For incubation studies (direct or indirect (co-culture system)), adherent and floating SH-SY5Y cells were cultured in serum-reduced medium (2.5% FCS) containing 10 µM all-*trans*-retinoid-acid (ATRA) (Merck, Darmstadt, Germany) on a 24-well-plate and allowed to differentiate within 8 days according to Teppola et al. (89) and Al-Maswary et al. (90) with slight modification. After 24 h of sub-culturing, serum reduced medium was replaced with a medium containing B-27™ supplement (ThermoFischer Scientific, Darmstadt, Germany) and 10 µM ATRA to promote differentiation into a neuronal-like phenotype. Stock solution of ATRA was diluted in 96% ethanol and the final ethanol concentration did not exceed 0.1% in cell culture medium. Control cells were treated with vehicle (0.1% ethanol). This treatment was replaced every 3 days to replenish ATRA in culture media and, after the differentiation protocol, SH-SY5Y$_{ATRA}$ differentiation was confirmed by flow cytometry with SYP as a well-known neuronal marker (51, 52). Therefore, after detachment of SH-SY5Y$_{ATRA}$ cells with accutase solution (PromoCell GmbH, Heidelberg Germany), cells were centrifugated (500 × *g*, 5 min at RT) and stained according the manufacturer's instructions with slight modifications. Centrifuged cells were resuspended in 100 µL MACS buffer (Miltenyi Biotec B.V. & Co. KG, Bergisch-Gladbach, Germany) and were fixed for 20 min in darkness with 150 µL Cyto Fast Perm FIX buffer (BioLegend®, Amsterdam, Netherland). After washing, step cells were permeabilized and stained with 98 µL Cyto Fast Perm solution with 2 µL anti-human Synaptophysin-APC REAfinity antibody (Miltenyi Biotec B.V. & Co. KG) for 10 min at room temperature (Table 1). Unbound antibodies were removed by washing the cells in 1 mL running buffer (Miltenyi Biotec B.V. & Co. KG). After centrifugation (500 × *g*, 10 min), cells were resuspended in 200 µL of MACS running buffer for final flow cytometry analysis in R1-APC channel. Cell gating strategy and quantification (Figures 1A–D) were performed using the MACSQuant 2.13.0 software (Miltenyi Biotec B.V. & Co. KG) by comparing the median fluorescence intensities (MFI) of unstained, isotype stained and SYP-stained cells with at least $n = 3$ (each done in duplicates).

4.1.3 Co-culturing intestinal and neuronal-like cells with a transwell system

In order to investigate the effects of non-fermented and fermented 2′-FL and Fuc on neuronal-like cells, we developed an *in vitro* transwell co-culture system with human intestinal epithelial cells (Caco-2) and SH-SY5Y$_{ATRA}$ cells (Figure 7). According to the experimental setting, differentiated Caco-2 cells on transwell filter inserts were placed onto a 24-well plate, where SH-SY5Y$_{ATRA}$ cells had been cultured as described above. In a first set of experiments, non-fermented 2′-FL and Fuc were exposed directly to SH-SY5Y$_{ATRA}$ cells in order to evaluate the direct effect on neural activity markers. In a second set of experiments, non-fermented and fermented 2′-FL and Fuc were exposed to the upper compartment with Caco-2 cells on transwell filters (indirect incubation) (see Section 4.1).

4.1.4 Isotope-labeled 2′-FL and Fuc

Stable isotope labeled 2′-FL containing the C-atom 1 in the fucose ring as ^{13}C ([1 -^{13}C$_1$]-2′-FL (^{13}C-2'FL)) was obtained from ELICITYL (Crolles, France). In addition, we used L-Fuc, which was ^{13}C-labeled either at C$_1$ [^{13}C$_1$-Fuc] or C$_6$ [^{13}C$_6$-Fuc] also with a ^{13}C enrichment of 99% (ELICITYL). Both were used either at a concentration of 0.5 mM for direct incubation or 5 mM for the fermentation studies (indirect incubation).

4.1.5 Bacterial fermentation of 2´-FL and Fuc

2´-FL or Fuc metabolites were generated by batch cultivation of 2´-FL or Fuc with *B. longum* ssp. *infantis* (DSM 20088) obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) and with *B. breve* (DSM 20213) as a gift from Prof. Dr. Sylvia Schnell (Department of Applied Microbiology, Justus-Liebig University Giessen, Germany). Rehydration of freeze-dried bacterial strains and −80°C stock cultures were done according to the manufacturer's instructions. *B. longum* ssp. *infantis* and *B. breve* were routinely cultured at 37°C in 'Bifidobacterium medium' containing 10 g/L casein peptone (tryptic digest), 10 g/L glucose, 5 g/L yeast extract, 5 g/L meat extract, 5 g/L bacto soytone, 2 g/L K_2HPO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $MnSO_4{\cdot}H_2O$, 1 mL/L Tween80, 5 g/L NaCl, 40 mL salt solution (0.25 g/L $CaCl_2{\cdot}2\ H_2O$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 1.0 g/L K_2HPO_4, 1.0 g/L KH_2PO_4, 10.0 g/L $NaHCO_3$, 2.0 g/L NaCl), and 4 mL/L resazurin (250 mg/L) dissolved in distilled water and autoclaved at 121°C for 40 min. Thereafter, the medium was left within the autoclave until reaching 98°C and was then further cooled down under oxygen-free gas (10% CO_2, 80% N_2, and 10% H_2) to avoid redissolving of oxygen. After autoclavation, pH was adjusted to pH 6.8 using NaOH (8 M) and supplemented with sterile filtered 0.5 g/L L-cysteine hydrochloride. Then, the medium was dispensed into Hungate anaerobic culture tubes under gas. Both strains were grown in independent triplicates under anaerobic condition at 37°C and growth was assayed by the determination of an increase in optical density (OD) at 600 nm using Shimadzu UV 1001 spectrophotometer (Shimadzu GmbH, Duisburg, Germany).

For incubation studies, corresponding bacterial growth media was prepared glucose-free and substrate utilization was determined by adding sterilized glucose or ^{13}C-labeled compounds to glucose-free medium. To obtain working cultures, cultivated stock cultures were incubated three times in carbohydrate-reduced medium to adapt microorganisms to the incubation media. After inoculation with bacterial suspensions for *in vitro* co-culture experiments, samples were taken at three different time points: the lag-growth phase, the logarithmic growth phase (log-growth phase), and stationary growth phase (stat-growth phase). After centrifugation (5 min, 13,000 rpm), the bacteria-free culture media were filtered through a 0.2 µm PES Whatman syringe filters (FisherScientific, Schwerte, Germany) and were used immediately for functional assays or stored at −80°C until for further analyses.

4.2 Determination of ^{13}C enrichment by elemental analysis isotope mass spectrometer

To analyze cell culture samples for ^{13}C enrichment, 0.15 mg liquid samples (apical cell culture samples) were weighted into tin capsules containing 5 mg of acid-washed Chromosorb W (IVA Analysentechnik e.K., Meerbusch, Germany). Triplicate samples were subjected to Elemental Analysis Isotope Ratio Mass Spectrometry (EA-IRMS) as described previously (28). Measurements and calculations were performed using the IonVantage Software v1.7 in combination with Ionos v4.2; both software applications were obtained from Elementar UK (Stockport, United Kingdom). Results are expressed as δ^{13}C enrichment [‰] with VPDB being the international standard obtained from the International Atomic Energy Agency (IAEA, Vienna, Austria).

4.3 Viability

A subset of cultured SH-SY5Y$_{ATRA}$ cells was used for measuring cell viability to ensure the viability of cells during co-cultivation by using the ViaCount™-assay (Luminex BV, MV ´s-Hertogenbosch, Netherland). Thus, cells were trypsinized using a 0.5% (w/v) trypsin/0.25 mM EDTA solution (Invitrogen) after 24 h incubation. After centrifugation (500 × *g*, 10 min), the pelleted cells were suspended in 500 µL of PBS. Following this, 20 µL of the cell solution was incubated with 480 µL ViaCount-Reagent™ and incubated for 10 min in the dark at 37°C. Immediately after Live/Dead-staining, cells were measured by flow cytometry on the Guava EasyCyte Mini Flow Cytometer (Guava Technologies, Merck Millipore, Darmstadt, Germany). Viability was expressed as % viable cells of totals with the Guava® software ($n = 3$, each done in duplicates). Further, 480 µL of the cell solution were used for glutamate detection in cell lysates (see Section 4.4).

4.4 Detection of neurotransmitters (BDNF, GABA, and glutamate)

The secretion of the neurotransmitter BDNF and GABA were measured in the supernatant of 24 h- stimulated SH-SY5Y$_{ATRA}$ cells using BDNF Quantikine™ ELISA Kit (R&D, Heidelberg, Germany) and ELISA kit for GABA (Abcam, Rozenburg, Germany). Glutamate as a precursor for GABA was measured in SH-SY5Y$_{ATRA}$ cell lysates according to the manufacturer's instructions with the Glutamate ELISA Kit (Abcam). Briefly, after incubation of the SH-SY5Y$_{ATRA}$, supernatants were collected and stored at −20°C until analysis for BDNF and GABA. For glutamate quantification, 480 µL of trypsinized SH-SY5Y$_{ATRA}$ cells (see Section 4.3) was used immediately and washed twice with PBS and lysed with lysis buffer for 20 min. Afterward, lysed cells were centrifugated (500 × *g*, 10 min) and supernatant was collected and used according to the manufacturer's instructions (Abcam). BDNF and GABA concentrations were measured at 450 nm and glutamate concentration were measured at 405 nm using the DigiScan microplate reader (Asys, Eugendorf, Austria). The BDNF and GABA concentrations were expressed as pg./mL and glutamate concentrations were expressed as µg/mL with $n = 3$ (each done in duplicates).

4.5 Choline levels

The total choline levels (free choline and acetylcholine) in SH-SY5Y$_{ATRA}$ cells were measured using the fluorometric Choline/Acetylcholine Assay Kit (Abcam) in freshly prepared samples according to the manufacturer's instructions. Briefly, after direct or indirect incubation of SH-SY5Y$_{ATRA}$ cells, cells were harvested by trypsinization (0.5% (w/v) trypsin/0.25 mM EDTA solution) and were washed twice with ice-cold PBS (Invitrogen GmbH). The cell pellet was resuspended in 500 µL choline assay buffer and homogenized by pipetting up and down ten times and leaving the cells for 10 min on

ice. After centrifugation (500 × *g*, 5 min, 4°C), the supernatant was collected and the assay was done according to the manufacturer's instructions. Acetylcholine was converted to choline by adding acetylcholinesterase and free choline was oxidized via the intermediate betaine aldehyde to betaine. The reaction generates products, which react with the choline probe to generate a fluorescence signal (Ex/Em 535/585 nm). Total choline concentrations were measured in black-clear microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) using the Ascent microplate fluorescence reader (Thermo Fisher Scientific, Germany). Fluorescence values (RFU) were finally expressed as % of controls with $n = 3$ (each done in duplicates).

4.6 Plasma membrane potential

Plasma membrane potential (PMP) was measured with the fluorogenic membrane Assay Kit (Abcam) according to the manufacturer's instructions. Briefly, after "direct" incubation of SH-SY5Y$_{ATRA}$ cells with 2′-FL and Fuc for 3 h or "indirect" incubation with basal media from transwell studies, the medium was replaced with 150 µL assay buffer (1:10) including 2 µL MP sensor dye. After 30 min of incubation in a 5% CO_2 incubator, fluorescence intensity (Ex/Em 535/585 nm) was measured using the Ascent microplate fluorescence reader (Thermo Fisher Scientific). Fluorescence values (RFU) were finally expressed as % of controls with $n = 3$ (each done in duplicates).

4.7 Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was measured with the fluorogenic JC-1 Assay Kit (Abcam) according to Sakamuru et al. (91). Briefly, after `direct´ incubation of ATRA-differentiated SH-SY5Y cells with 2′-FL and Fuc over 3 h or `indirect´ incubation with basal media from transwell studies, the medium was replaced with 200 µL HBSS buffer including 5 µM JC-1 solution (5,5′,6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazol-carbocyanine iodide). After 30 min of incubation in a 5% CO_2 incubator, and after two washing steps with HBSS, fluorescence intensity (Ex/Em 485/535 nm) was measured in using the Ascent fluorescence reader (Thermo Fisher Scientific). Fluorescence values (RFU) were finally expressed as % of controls with n = 3 (each done in duplicates).

4.8 Determination of mRNA expression of BDNF by RT-qPCR

mRNA was isolated directly from SH-SY5Y$_{ATRA}$ derived from the incubation experiments using the Dynabeads mRNA DIRECT™ kit (Invitrogen GmbH) according to the manufacturer's instructions. After the isolation of mRNA, cDNA synthesis was carried out with the iScript cDNA synthesis kit using the C1000 Touch Thermal cycler (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) in a reaction volume of 10 µL containing 20 ng mRNA with iScript reaction mix (5×), iScript Reverse Transcriptase and nuclease-free water (Bio-Rad Laboratories GmbH). Samples were incubated at 25°C for 5 min, followed by an incubation at 46°C for 20 min, and inactivation at 95°C for 1 min. Amplification of target genes (BDNF and ß-Actin (ACTB)) was measured using the C1000 Touch Thermal cycler (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) with gene-specific primers/probe sets labeled with FAM (BDNF-PrimePCR™ Probe Assay) or HEX (ACTB-PrimePCR™ Probe Assay). Amplification were done with 2 µL cDNA in a reaction volume of 20 µL containing iTaq Universal Probes Supermix (2×), PrimePCR™ Probe Assay (Bio-Rad Laboratories GmbH), and water in a two-step amplification with 3 min of initial denaturation at 95°C, followed by 45 cycles of 5 s at 95°C and 30 s at 60°C. The relative expression level was measured using the ΔΔ C_T-method, in which ΔC_T was calculated by subtracting the C_T value of ACTB from the specific C_T value of the BDNF. ΔΔ C_T was obtained by subtracting the Δ C_T of each experimental sample by the Δ C_T of a positive control (92, 93). Expression levels were given as % of control with $n = 3$ (each done in duplicates).

4.9 Inhibition of BDNF-secretion by calcium channel blocker verapamil

Inhibition of BNDF secretion was measured using BDNF Quantikine™ ELISA Kit (R&D GmbH, Heidelberg, Germany) as described above (see Section 4.4). Briefly, before incubation of SH-SY5YATRA cells with supernatants from batch culture collection at stat-growth phase, cells were washed twice with pre-warmed HBSS and were then pre-incubated for 30 min with 7.5 µM or without verapamil (> 99%; Calbiochem®, Merck, Germany). Afterward, the cells were washed twice with medium and finally incubated with the metabolite enriched media. Briefly, after 24 h of incubation, supernatants were collected and stored at −20°C until analysis for BDNF. Data are given as pg./mL with n = 3 (each in duplicate).

4.10 Flow cytometry analysis for TrkB expression

For cytometry analysis, cells were treated with accutase solution (Promocell, Heidelberg, Germany) for 10 min to ensure surface protein integrity. For TrkA staining cells were centrifugated (500 × *g*, 10 min) and stained immediately with anti-human TrkA PE-conjugated REAfinity antibody (Miltenyi Biotec B.V. & Co. KG) for 10 min in the dark (Table 2). For TrkB staining, cells were incubated after centrifugation with F_C-blocking reagent (Miltenyi Biotec B.V. & Co. KG) for 15 min. Then, cells were incubated with mouse anti-human TrkB Alexa Fluor® 405-conjugated monoclonal antibody or Alexa Fluor® 405-conjugated isotype control (IgG_1) antibody (R&D GmbH) for 10 min in the dark at room temperature. Unbound antibodies were removed by washing the cells in 1 mL running buffer (Miltenyi Biotec B.V. & Co. KG) and after centrifugation (500 × *g*, 10 min), cells were resuspended in 200 µL of running buffer for final flow cytometry analysis. Cell gating strategy and quantification (see Figure 6) were performed using the MACSQuant 2.13.0 software (Miltenyi Biotec B.V. & Co. KG) by comparing the median fluorescence intensities (MFI) of unstained, isotype stained, and Trk-stained cells with n = 3 (each done in duplicates).

4.11 Statistical analysis

Statistical analyses were carried out using GraphPad Prism 10.0.2 (GraphPad Software, MA, United States) As indicated, data were

TABLE 2 Antibodies for Trk staining of SH-SY5Y and SH-SY5Y$_{ATRA}$ cell.

Target	Primary recombinant antibodies (Ab)	Fluorochrome	Dilution	Incubation time	MQ10 channel
TrkA	REAfinity®	PE	1:50	10 min	B2 (PE)
TrkB	Monoclonal anti-human	Alexa Flour® 405	1:50	10 min	V1 (Vioblue)
Isotype control	Monoclonal mouse IgG1	Alexa Flour® 405	1:50	10 min	V1 (Vioblue)

analyzed by one-way ANOVA and multicomparison test or *t*-test. Differences were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

SK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. CK: Conceptualization, Funding acquisition, Resources, Writing – review & editing. CB: Investigation, Writing – review & editing. DH: Writing – review & editing. SM: Writing – review & editing. RB: Conceptualization, Writing – review & editing. SR: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

DH, SM, and RB were employed by Abbott, Nutrition Division.

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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1351433/full#supplementary-material

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Trans isomeric fatty acids in human milk and their role in infant health and development

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It is well known that long chain polyunsaturated fatty acids (LCPUFAs) play an important role in neurodevelopment in the perinatal life. The most important source of these fatty acids is the diet, however, they can also be formed in the human body from their shorter chain precursors, the essential fatty acids. Since the WHO recommends exclusive breastfeeding for the first six months after birth, the exclusive source of these fatty acids for breastfed infants is human milk, which can be influenced by the mother's diet. Unsaturated fatty acids can have either cis or *trans* configuration double bond in their chain with distinct physiological effects. Cis isomeric unsaturated fatty acids have several beneficial effects, while *trans* isomers are mostly detrimental, because of their similar structure to saturated fatty acids. *Trans* fatty acids (TFAs) can be further subdivided into industrial (iTFA) and ruminant-derived *trans* fatty acids (rTFA). However, the physiological effects of these two TFA subgroups may differ. In adults, dietary intake of iTFA has been linked to atherosclerosis, insulin resistance, obesity, chronic inflammation, and increased development of certain cancers, among other diseases. However, iTFAs can have a negative impact on health not only in adulthood but in childhood too. Results from previous studies have shown that iTFAs have a significant negative effect on LCPUFA levels in the blood of newborns and infants. In addition, iTFAs can affect the growth and development of infants, and animal studies suggest that they might even have lasting negative effects later in life. Since the only source of TFAs in the human body is the diet, the TFA content of breast milk may determine the TFA supply of breastfed infants and thus affect the levels of LCPUFAs important for neurodevelopment and the health of infants. In this review, we aim to provide an overview of the TFA content in human milk available in the literature and their potential effects on infant health and development.

KEYWORDS

development, human milk, infant, newborn, nutrition, review, *trans* fatty acid

1 Introduction

The Food and Drug Administration (FDA) provides a crucial definition of *trans* fatty acids (TFA) as "the sum of all unsaturated fatty acids that contain one or more isolated (i.e., non-conjugated) double bonds in a *trans* configuration" (1). Almost all naturally occurring unsaturated fatty acids have double bonds in a cis configuration, while natural TFAs are

formed in the rumen of ruminants (rTFA). Other types of *trans* isomers are formed during industrial processes, such as the partial hydrogenation of vegetable oils or the heating of oils at high temperatures (iTFA). Consequently, the primary sources of TFAs in human diets encompass milk and meat from ruminant animals, products containing partially hydrogenated oils (PHOs), and various foods subjected to cooking methods such as deep-frying and baking (2).

Research over the last 30 years has highlighted the negative effects of TFAs, including the direct link between increased dietary TFA intake and a worrying increase in cardiovascular disease (CVD) (3). TFA consumption may also reduce insulin sensitivity, increase diabetes risk, can act proinflammatory, impair endothelial function (4), and also can be associated with an increased risk of breast cancer (5). These detrimental effects were mainly attributed to the widespread use of PHOs, which serve as a fundamental source of TFAs, specifically iTFAs (6). As a result of the detrimental effects of iTFAs, the World Health Organization (WHO) announced an action plan called REPLACE, which aims to eliminate iTFA from the global food supply by the end of 2023 (7). However, of more important significance for the development of breastfed infants is the fact that several studies have suggested that *trans* isomers may interfere with the metabolism of long-chain polyunsaturated fatty acids (LCPUFAs), which are important for neurodevelopment (8–10). Since TFAs cannot be synthesized in the human body, diet is the only way to obtain these fatty acids. Hence, for breastfed children, human milk (HM) is the only source of TFAs and their potential adverse effects (11).

Breastfeeding is the optimal nutritional method for infants, offering a plethora of health benefits, not only for the infant but also for the mother. This natural feeding approach promotes healthy development and long-term benefits for breastfed infants (12). Public health organizations, including the WHO and the American Academy of Pediatrics (AAP), recommend exclusive breastfeeding for infants up to the age of 6 months (13–15). Consequently, HM is the only source of nutrients for breastfed infants during their first months of life to support their growth and development. However, the composition of HM is not constant, but it adapts to the different needs of the infant throughout lactation, including colostrum (C), transitional milk (TM), and mature milk (MM) (16). Moreover, it can differ depending on demographics (17) and genetic factors (18), as well as maternal diet and lifestyle (19). Maternal dietary changes seem to have a greater impact on the fat content of HM than on its protein and carbohydrate levels (20). The fatty acid (FA) composition of HM reflects the maternal dietary fat alterations over a period of 1–3 days (21).

In the perinatal period, LCPUFAs, mainly arachidonic acid (C20:4n-6, AA) and docosahexaenoic acid (C22:6n-3, DHA), play an important role in the neurodevelopment and visual acuity (22), because they serve as important components of the membranes of the retinal photoreceptors and brain cells (23). The only sources of these FAs during this period are maternal stores and the mother's diet. During the intrauterine period, these FAs are transferred to the fetus via the placenta and after birth through HM. Previous animal studies suggest that TFAs may decrease the activity of hepatic delta-6 desaturase, thereby interfering with the metabolism of physiologically important LCPUFAs (24, 25). The human body is not able to synthesize TFAs, so these fatty acids are ingested through our diet. As the disturbed metabolism of LCPUFAs by TFAs in early life may have a longer-term effect, we aim to provide an overview of the TFA content of HM worldwide based on previous publications and to summarize the results of animal models and human studies on its potential effects on infants and children.

2 Fatty acids in general

2.1 Nomenclature and metabolism

FAs are carboxylic acids with a carboxyl group at one (alpha end) and a methyl group (omega end) at the opposite end. Almost all naturally occurring FAs hold the double bonds in the cis configuration, where the hydrogen atoms on the double bond are on the same side of the molecule, causing a bend in the chain, while in *trans* isomers the hydrogen atoms are on opposite sides of the molecule. This results in a more stable and rigid chain, making it comparable to saturated fatty acids (Figure 1) (3, 26).

TFAs can be formed through industrial processes or naturally. iTFAs are created during food preparation at high temperatures or through partial hydrogenation of vegetable oils, which involves high temperatures, high pressures, and a catalyst. In this process, polyunsaturated fatty acids (PUFAs) undergo partial saturation of their double bonds to decrease melting point. Additionally, some double bonds in cis configuration are converted to a *trans* configuration. Several different mono-and polyunsaturated *trans* isomers can be formed as a result, with the double bond positioned at delta-6 or higher. However, the most prominent ones are the monounsaturated *trans* isomers of C18. Elaidic acid (EA, C18:1n-9 *t*), which is the geometrical isomer of oleic acid (C18:1n-9), has the highest prevalence in iTFAs. The most prevalent polyunsaturated TFA is linoelaidic acid (C18:2n-6*tt*), which is the *trans* isomer of the essential n-6 linoleic acid (LA, C18:2n-6) and the *trans* double bonds are in the delta-9 and delta-12 positions (3, 27).

In contrast, rTFAs are formed in the rumen of ruminants through microbial fermentation and biohydrogenation. Although the *trans* double bonds in rTFAs may be in a similar position to those in iTFAs, the probability of formation and frequency of occurrence differ. The most prominent rTFA is *trans* vaccenic acid (VA, C18:1n-7), which has a double bond in the delta-11 position. Moreover, ruminants also produce a group of conjugated linoleic acid (CLA), in which the double bonds are conjugated. This means that there is only one single bond between the two double bonds. The most prevalent CLA is rumenic acid (RA, C18:2n-9c7*t*) (28). Not only the position of the *trans* double bond can differ between the iTFAs and rTFAs, but also their concentration in foods. The concentration of iTFAs in products can reach up to 50% of the total fatty acids, whereas rTFAs in ruminant milk are typically found in much lower concentrations, usually around 4–6% of the total fatty acids. The most common TFA isomers are listed in Table 1 with their names and origin.

Dietary TFAs can be absorbed from the small bowel, incorporated into serum triacylglycerols (TGs) and structural lipids, and stored in adipose tissue (29, 30). However, TFAs are preferentially incorporated into TGs and phospholipids (PLs), and to a lesser extent into cholesterol esters (31). There may also be differences in tissue incorporation, for example, delta-9 and delta-11 C18:1 *t* TFAs are incorporated into tissues to a greater extent than other positional *trans* isomers (32). However, this incorporation is not irreversible, but

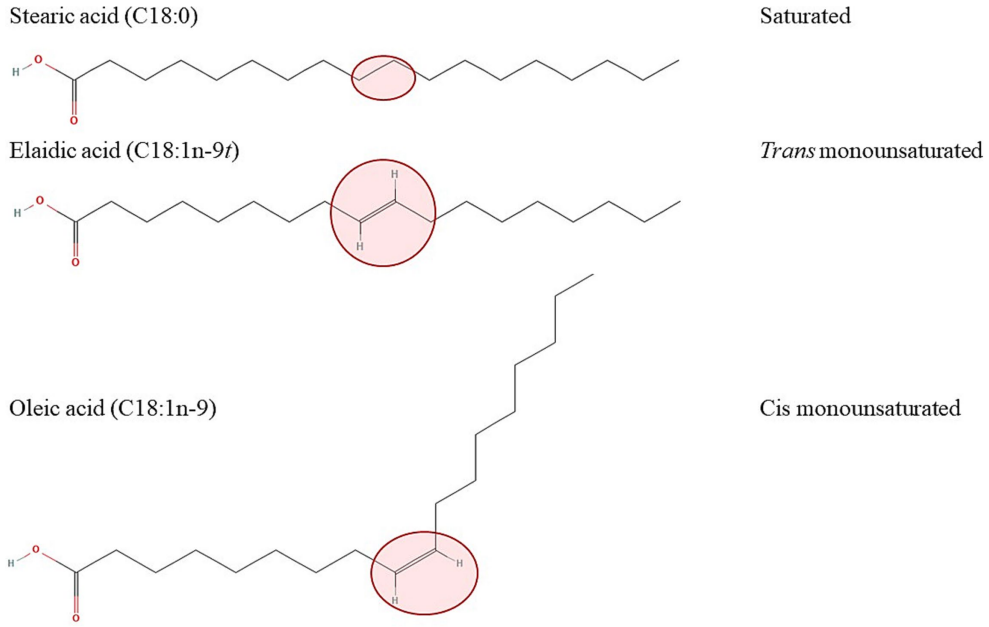

FIGURE 1
Effect of a configuration of the double bond on the spatial arrangement of the molecule with the example of 18-carbon saturated (stearic acid, top) *trans* (elaidic acid, middle) and cis (oleic acid, bottom) monounsaturated fatty acids. The structures of the fatty acids were downloaded from: https://pubchem.ncbi.nlm.nih.gov/compound/5281#section=2D-Structure (stearic acid), https://pubchem.ncbi.nlm.nih.gov/compound/637517#section=2D-Structure (elaidic acid), https://pubchem.ncbi.nlm.nih.gov/compound/oleic%20acid#section=2D-Structure (oleic acid).

TABLE 1 Most common *trans* isomers with name, origin, and formula.

Origin of TFA	Name	Structure	Systematic name
rTFA	Palmitelaidic acid	C16:1n-7 *t*	9E C16:1
rTFA	*trans* Vaccenic acid	C18:1n-7 *t*	11E C18:1
iTFA	Elaidic acid	C18:1n-9 *t*	9E C18:1
iTFA	Linoelaidic acid	C18:2n-6*tt*	9E,12E C18:2
rTFA	Ricinenic acid	C18:2n-9 *t*,7 *t*	9E,11E C18:2
rTFA	Rumenic acid	C18:2n-9c,7 *t*	9Z,11E C18:2

iTFA, industrial *trans* fatty acid; rTFA, ruminant *trans* fatty acid; TFA, *trans* fatty acid.

rather dynamic, so that when dietary intake of TFAs decreases, they are released from membranes. The *trans* double bond is recognized by enzymes in a similar way to the saturated bond, so EA can be incorporated into phospholipids at the expense of saturated fatty acids (e.g., stearic acid), or cis-9, cis-12, *trans*-15 C18:3 can be incorporated into cardiolipins instead of LA (33). The effect of TFAs in membranes is similar to that of saturated FAs, because they both have a more rigid structure than cis unsaturated fatty acids, so they both decrease membrane fluidity and therefore the affinity of molecules for receptors.

TFAs have been associated with a number of adult diseases and consequent death cases, such as atherosclerosis, cardiovascular disease and mortality (34, 35), and cancer (36). Animal studies have indicated that TFAs may inhibit the activity of the Δ-6 desaturase enzyme, disrupting the production of omega-3 and omega-6 LCPUFAs (Figure 2) (37). This interference may have significant effects on newborns as the activity of these enzymes is required for the synthesis of LCPUFAs (38, 39). As a matter of fact, the presence of TFAs in milk may cause an insufficient supply of essential fatty acids (EFAs) in both HM (38, 40) and infant blood (39). Some TFAs can also be metabolized in the body, for example, the human body can synthesize RA from VA in healthy adults (41), and also in lactating women (42).

The *de novo* synthesis of LCPUFAs is rather limited and therefore, infant levels of these FAs depend primarily on maternal dietary intake (43). In contrast, TFAs cannot be synthesized within the body and are entirely dependent on maternal diet. Therefore, a high concentration of TFAs could potentially have a negative impact on the supply of LCPUFAs due to direct competition with metabolic enzymes. It is important to note that there is a positive correlation between the concentrations of most FAs, including TFAs, EFAs, and LCPUFAs, in HM, and their corresponding levels in the infant's plasma, highlighting the significance of HM in the dietary intake of these FAs (9). On the other hand, the significant inverse relationship between the concentrations of TFAs and EFAs (LA and α-linolenic acid [C18:3n-3, ALA]) in both preterm (44) and term HM (9, 45) suggests a restrictive effect of TFAs on the EFA supply. These results support the need to ensure adequate EFA supply to infants by increasing EFA intake in the maternal diet on the one hand and by limiting TFA consumption on the other.

2.2 Fatty acids in the diet

HM is the best option for infants in the first months of life (46) and therefore exclusive breastfeeding is recommended until the 6th month of life (15). Fat in HM is the primary source of energy (47), while selected FAs, as well as crucial fat-soluble vitamins are essential for the development of infants (48, 49). The FAs of HM are either

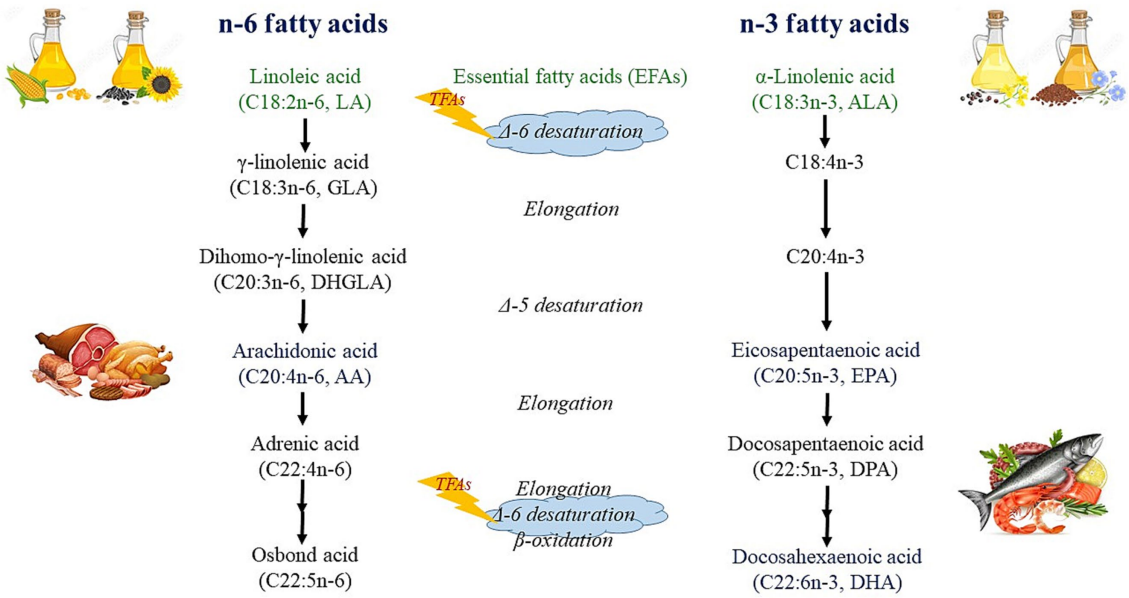

FIGURE 2
Metabolism of n-6 and n-3 fatty acids.

synthesized in the mammary gland (MG) or derived from plasma FAs that originate from lipid reserves, non-mammary tissues, or diet (47). Depending on the mother's intake, the concentration of FAs in HM is influenced by the type of diet prevalent in the area where the mother lives (11, 50–53). Therefore, a well-balanced diet is beneficial to both the mother and the breastfed child throughout the period of breastfeeding (54).

Maternal reserve capacity and the metabolic utilization of FAs (transport, synthesis, and oxidation) are directly related to FA levels during lactation (55). It has been estimated that between 60 and 70% of the fat in HM is derived from tissue synthesis and maternal fat deposits, while approximately 30% is derived from the diet of the mother (40, 50). This suggests that maternal diet, as well as the metabolism of FAs during lactation appear to be the most important factors that influence DHA concentration in HM (56). Additionally, the composition of fats in HM undergoes continuous change due to the rapid transport of dietary fats from chylomicrons. This process results in a peak occurring within 6 to 12 h after dietary DHA intake (55). Isotopic studies show, that about 30% of dietary LA intake is directly transferred to HM, but only a maximum 3% is converted and transferred as AA (57, 58). Although approximately 65% of HM ALA is derived from maternal dietary ALA intake, the low efficiency of conversion to its longer-chain metabolites and transfer to HM means that dietary ALA plays a negligible role in the eicosapentaenoic acid (C20:5n-3, EPA) and DHA content of HM (59).

TFAs cannot be synthesized *de novo* by the human body (60), so their exclusive sources are dietary intake. Ruminant animals naturally produce rTFAs through a process known as biohydrogenation in the rumen. Therefore, meat, milk and dairy products from ruminant animals (cattle, sheep, and goats) are the primary sources of rTFAs in the human diet. Conversely, iTFAs are primarily formed during the hydrogenation of vegetable oils during industrial processes of hardening and deodorization, so iTFAs are primarily consumed through PHOs and food products that contain them, including confectionery and food concentrates, but a small amount of iTFAs are also produced during the thermal processing of food, such as baking or frying (61, 62). The main iTFA present in the food supply is EA (27), which has been detected in larger proportions in HM (38, 40, 63, 64), but other iTFAs, like linoelaidic acid, have also been reported (40, 50, 63, 64). The most prevalent rTFA, found in milk, butter, and beef fat, is VA (26, 27), which has also been detected in HM by several research groups (40).

Based on reported data worldwide, rTFA intake from ruminant products, including meat, milk, and butter, constitutes a small, but constant portion of total TFAs, while the percentage of iTFAs derived from processed foods, bakeries, snacks, and fast food is continuously declining in most countries (65, 66). Bakery products, confectionery, and snack foods accounted for the majority of TFAs in the diets of breastfeeding women on a typical Western diet (11, 67) as well as on a Mediterranean diet (38). Moreover, maternal diet influences the HM TFA concentration with a significantly lower value (0.44%) in vegan compared to vegetarian (0.66%) and omnivorous (1.09%) lactating women (52). Previous studies have analyzed the fatty acid composition of various food categories containing TFAs. In general, older studies reported higher iTFA values, resulting in higher total TFA content in these foods. In a study conducted in Sweden (68), the total TFA content in certain foods was as high as 3.83 w/w% (digestive biscuits) during the period of 1995–1997. In contrast, Germany had even higher total TFA values in 2007–2009 (27): the highest levels were found in certain chocolate products (0.05–40.46 w/w%), doughnuts (0.14–35.11 w/w%), French fries and chips (0.12–16.30 w/w%), and biscuits (0.04–10.66 w/w%).

Considering the detrimental impact of iTFAs on human health, the European Food Safety Authority (EFSA) advises that TFA intake should be restricted to the lowest achievable level in a nutritionally balanced diet (69, 70). Accordingly, the World Health Organization (WHO) recommends limiting consumption of iTFA to less than 1% of daily energy intake to prevent chronic diseases, due to scientific

evidence demonstrating its harmful effects (71, 72). Several studies have also examined the impact of regulatory or voluntary restrictions on the total TFA content in various food groups. In Spain, certain products, such as chocolate-stuffed Swiss rolls (1.47–11.85 w/w%), doughnuts (8.47–10.89 w/w%), and chocolate-filled sponge cake (0.6–9.12 w/w%), had high TFA content due to their high iTFA content in 1999 (73). However, the values greatly decreased after regulation by 2010 and further decreased by 2015 (74), e.g., in confectionary and pastry products (2010: 0.657 w/w%; 2015: 0.034 w/w%), chocolate products (2010: 0.721 w/w%; 2015: 0.14 w/w%), and biscuits (2010: 0.311 w/w%; 2015: 0.008 w/w%). By contrast, the TFA content of mainly rTFA-containing products, such as dairy products, remained quite stable. For example, butter had a TFA content of 2.356 w/w% in 2010 and 2.452 w/w% in 2015, while spreadable cheese contained 2.646 w/w% and 2.524 w/w% TFA, respectively. Similar trends were observed in Sweden with the highest total TFA values in 1995–1997 and really low values in 2007 (68) as well as in Argentina (75) and Korea (76). Table 2 summarizes the total TFA values of various food groups based on recent measurements in Thailand (77), Egypt (78), Argentina (75), Spain (74), Jamaica (79) and Malaysia (80). Previous research has shown that both voluntary and regulatory measures to reduce iTFA content in foods have resulted in substantial reductions in global intakes of TFAs over the past two decades (61, 81, 82), including Canada (83) and the European Union (EU) countries (84). The estimated intake of TFAs in 2010 varied widely from 0.2 E% (Barbados) to 6.5 E% (Egypt) (82), while seven years later, intake was lower, ranging from 0.3 E% (China) to 4.2 E% (Iran). In addition, the average intake was below the maximum recommended intake of 1 E% in 22 out of the 29 countries (61). According to the most recent study, TFA intake was below 0.8 E% in most regions across the world, with the highest reported intake in North-, Central-, and Latin America, with levels ranging from 0.8 E% to 1.2 E% (85).

3 *Trans* isomeric fatty acids in human milk

3.1 *Trans* fatty acid content of human milk worldwide and its influencing factors

Maternal diet-derived TFAs can be transferred directly to HM (86). Based on the dietary sources and intake of TFAs, HM contains varying levels of TFAs across different populations and regions of the world (87). This systematic literature review found the highest TFA values in North America (6.99 [5.01–8.98]; mean [95% confidence interval for mean]), followed by South America (2.36 [1.14–3.58]), Europe (1.82 [1.61–2.03]) and Oceania (1.21 [1.19–1.23]), and the lowest values in Asia (0.71 [0.65–0.78]) and Africa (0.61 [0.55–0.67]). However, it is also important to consider that the TFA of HM is also dependent on the time of analysis. Higher values were reported in the literature prior to the implementation of the iTFA regulation. For instance, a Canadian study (83) investigated the effect of regulation on the TFA content of HM samples and found that the high values reported before regulation (7.2 ± 0.3% in 1992) were significantly reduced (1.9 ± 0.5% in 2011).

In the literature we can find a wide variety of TFA levels in HM among different populations (Supplementary Tables S1, S2), which range from the highest level in Iranian (88), Canadian (45, 89), and American (90) to the lowest level in Greek (91), Chinese (92) and Latvian lactating mothers (93). However, when dividing the literature into two groups based on the time of the study, it becomes apparent that the studies reporting very high levels of total TFAs are older, probably prior to the TFA regulation (Figure 3A). Total TFA values of 3% or more are exclusively found in studies published in 2010 or before (86, 88–90, 94–96), while very low TFA values of less than 0.5% are found in articles published both before (92, 97) and after 2010 (52,

TABLE 2 Total TFA content (w/w%) of some food groups based on recent studies worldwide.

Food category	Country (year of TFA determination)					
	Thailand (2017) (77)	Egypt (2019) (78)	Argentina (2015) (75)	Spain (2015) (74)	Jamaica (2019) (79)	Malaysia (n.d.) (80)
rTFA-containing products						
Butter	2.04–4.64	1.2		2.452		0.11–3.85
Cheese	0.68–1.08	1.5				0.33–2.57
Milk	0.08–0.11	2.1				
Whole milk powder	1.32	1.4				
iTFA-containing products						
Biscuits		2.0		0.008		
Chocolate and cocoa products		2.6		0.14		
Crackers		1.3		0.011	0.91	
Potato chips		1.5	0.4	0.088	0.87	0.05–0.50
French fries		0.5				0.09–0.51
Margarine			1.1	0.274	0.09	0.05–0.54
Confectionary, doughnut, pastries			3.7	0.721	0.06	

n.d. denote no exact data is given; TFA, *trans* fatty acid.

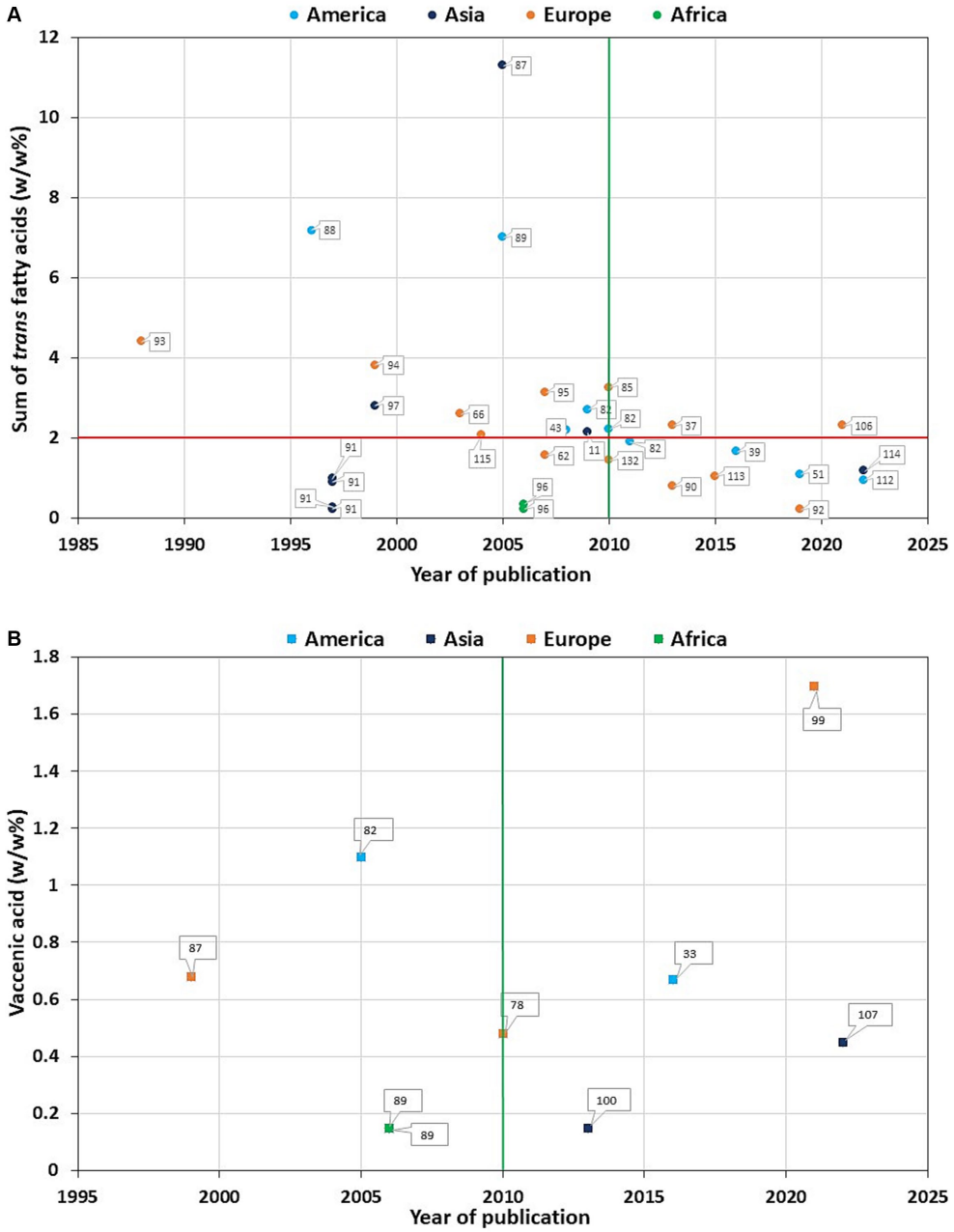

FIGURE 3
Total *trans* fatty acid **(A)** and *trans* vaccenic acid **(B)** values in mature human milk as a function of year of publication. The numbers assigned to each data item correspond to the article number in the bibliography. The vertical green line indicates the year 2010, the horizontal red line indicates the total *trans* fatty acid value of 2 w/w%.

91, 93, 97). However, this 2010 cut-off date is somewhat arbitrary, as the TFA legislation was adopted at different times around the world. It is worth noting that prior to 2010, neither Iran nor Kuwait had regulations on the TFA content of food, although Kuwait (98) had significantly lower levels of TFA in HM compared to Iran (88). According to WHO, only seven countries had best-practice TFA policy before 2017, including Denmark (since 2004), Austria (since 2009), Chile, Iceland, South Africa (all three since 2011), Hungary and Norway (both since 2014). In 2018 this best-practice TFA policy was implemented in Canada, Latvia, Slovenia, and USA, in 2019 in Lithuania and Thailand, and 2020 in Saudi Arabia also (99). Considering the increasing number of countries applying the regulation, it is expected that the amount of TFAs in HM will decrease further. However, the intake of rTFA remains constant, resulting in no change in the VA content of HM between before and after 2010 (Figure 3B).

The FA composition of HM, including the TFA content, can be influenced by several factors. One of these may be ethnicity, which probably leads to differences in HM TFA content due to distinct dietary habits. For example, in a Czech study, Roma women had significantly

higher mean C18:1 t levels in their HM than the average Czech population (2.73 ± 1.88 vs. 2.09 ± 1.24; w/w%, mean ± SD). The difference was explained by the higher consumption of foods with higher TFA content among Roma women, including buns, biscuits, butter, hamburgers, potato chips, and other fried food (96). This was also corroborated in an Asian study, where Chinese women on a Western-style diet had significantly higher TFA levels in their HM compared to lactating women on a traditional Chinese diet (92, 100). Similarly, different C18:1n-9 *t* values were found in an African country, Nigeria, when comparing the HM of urban and nomadic people (0.08 ± 0.07 vs. 0.03 ± 0.02, w/w%, mean ± SD), although no significant difference was found for most of the measured TFA values (97). However, if there is no difference in dietary habits, then ethnicity alone has no detectable effect on the amount of TFA in HM (63, 101). A Polish study found that EA values in HM was not significantly influenced by body fat, maternal BMI, health problems or n-3 LCPUFA supplementation (102).

When examining groups with differing ethnicities and geographic locations, even larger differences can occur. In HM samples from German mothers on a Western-style diet, significantly higher TFA levels were found in almost all measured TFA isomers compared to Nigerian mothers still on a traditional diet [C14:1 *t* 0.19 vs. 0.04, $p = 0.0009$; C16:1 *t*: 0.46 vs. 0.27, $p = 0.001$; C18:1 *t* 3.12 vs. 0.86, $p = 0.0017$; sum of TFAs: 4.40 vs. 1.20, $p = 0.0012$; w/w%, median (103)]. Similarly, large differences were measured in the TFA content of HM between US women on a Western-style diet (high in iTFAs) and Bolivian forager-horticulturalists (iTFA-free, but containing rTFAs); C18:1 *t* was about six times higher than in Bolivia [1.23 ± 1.05 vs. 0.21 ± 0.30; w/w%, mean ± SD (104)]. Although TFA regulation in recent years has resulted in much lower TFA intakes worldwide, it seems that Western-style diets still result in higher TFA intakes compared to traditional diets (sum of TFAs: Australia: 1.21 vs. Cambodia: 0.30; w/w%, median, $p < 0.001$) (105). Moreover, the different dietary intakes of mothers living in different countries can significantly affect not only relative but also absolute TFA concentrations in HM, as demonstrated in an Asian study (C18:1 *t*: China: 84.0 ± 468.3, South Korea: 5.9 ± 21.6; Pakistan: 433.6 ± 438.5, Vietnam: 31.4 ± 44.0; mg/l, mean ± SD) (106).

The exclusive source of TFAs in HM is the dietary TFA intake, as discussed previously, therefore, dietary intake of both rTFAs and iTFAs can affect the total TFA content of HM. Mothers who follow a vegan diet, excluding meat, eggs, and dairy products (including milk) have significantly lower levels of TFAs in their HM samples compared to those who follow a vegetarian diet (including dairy products and eggs) or an omnivorous diet (52). There was a positive correlation between higher consumption of eggs or meat and higher levels of TFAs in HM (93). In contrast, diets high in grains, cereals, vegetables, legumes, nuts, and seeds resulted in a low TFA intake, and therefore, in lower TFA values in HM. Not only VA but EA and total TFA were directly associated with maternal milk and dairy intake (107). In Croatian mothers, the consumption of bakery, confectioners, fried foods, dairy products, margarine, and sausages was significantly directly related to the TFA content of HM (38). In Polish mothers, only a high intake of bakery products and snacks (rich in iTFAs, as EA) increased HM total TFA content significantly (67). High dairy fat intake (rich in rTFAs, as VA), on the other hand, can lead to a significant increase in VA content in HM (86). However, in Malaysian mothers with low TFA intakes (1.27 g/day), neither a healthy dietary pattern rich in vegetables and fruits nor frequent consumption of unhealthy deep-fried foods or eating out affected the EA and total TFA content of HM (108). In Brazilian adolescents with somewhat higher daily TFA intake (3.07 g/day), also no correlation was found between dietary intake and HM TFA contents (40). In an experimental study, a high-TFA diet given to lactating women significantly increased C18:1 *t* in HM samples collected as early as day two compared to the control group, demonstrating that dietary TFA is excreted into HM very rapidly, within 24 h of consumption (109).

It is a well-known fact that fat content, and the concentration of FAs in HM varies over time, depending on the lactation stage and several factors can influence its values, like gestational age at delivery, maternal diet and time of sampling (46, 110). Not only the physiologically important PUFAs, but TFAs can also change depending on the lactation stage (Table 3). The total TFA values in the literature range from 0.19 [mean w/w% (92)] to 3.81 [mean w/w% (95)] in C, with the lowest value in mothers with a traditional Chinese diet and highest value in German mothers with Western-type diet. Previous studies have reported varying results regarding the levels of TFAs in HM during lactation. Some studies have shown a significant decrease in TFA levels over time, with the highest values observed in the C (40, 111, 112, 114, 115), while others have reported a similar trend, but without statistical significance (44, 91). In contrast, some research groups have found opposite results, with the lowest TFA values measured in C increasing over time (67, 92, 116), or showing an initial decrease followed by a non-significant increase (113). Interestingly, in a Spanish study, the highest C18:1 *t* values were only found in very-preterm and preterm HM that was significantly decreasing over lactation, but they failed to show this trend in the term group (117). A decrease in TFA content may also be associated with the duration of lactation, as the maternal diet becomes more balanced and diverse throughout breastfeeding (107). Since there is no clear change among TFA levels in C, TM, and MM, this raises the possibility of other factors that could also influence TFA levels in HM (47, 89).

Due to the different nutritional needs and metabolic capacities of preterm and term infants, the TFA content in HM may differ. It is essential that preterm infants receive higher amounts of some LCPUFAs, specifically DHA, in order to grow and develop at their optimal level (118). Consequently, preterm HM contains a higher concentration of DHA and other n-3 fatty acids compared to term HM (119–121) although other studies show no difference (43, 122, 123). However, preterm HM, may also contain higher levels of TFAs than term HM, which could potentially interfere with the metabolism and absorption of n-3 fatty acid (46). Although there have been few studies investigating the differences between TFA levels in preterm and term milk, the results are inconclusive. Some papers report no difference between the two groups (122, 124), while others have found higher levels in C in preterm infants (119), or even lower values during the first month of lactation (117) or only in MM (123). A systematic review summarizing EA values in preterm and term milk during lactation found lower values in the preterm group (preterm – C: 0.39 ± 0.02, TM: 0.26 ± 0.03; MM: 0.35 ± 0.04 vs. term – C: 1.95 ± 0.02, TM: 1.13 ± 0.02, MM: 1.16 ± 0.27; weighted least squares mean ± SEM), however, it should be noted that preterm data were based on a much smaller group of studies and included participants, so further studies are needed to demonstrate whether there is a difference between these two groups (125).

TABLE 3 Change in the values of *trans* isomers in human milk over lactation.

First author, Year	Place of study	Subgroups	Nr of mothers	Lactation stage	C18:1n9*t* (EA)	C18:1n7*t* (VA)	C18:2*tt* (linoelaidic acid)	Total TFA
Bousset-Alferes et al., 2022* (111)	Mexico	—	*n* = 33	C (1-5d)	1.334 (1.699)	—	0.196 (0.182)	1.529 (1.648)
				TM (5-15d)	0.503 (0.846)	—	0.246 (0.232)	0.748 (1.033)
				MM (>15d)	0.585 (1.042)	—	0.361 (0.535)	0.945 (1.368)
Chen et al., 1997* (92)	China	Hong Kong	*n* = 51	C (1-3d)	n.d.	n.d.	0.15 (0.012)	0.81 (0.30)
				TM (2w)	n.d.	n.d.	0.20 (0.16)	0.91 (0.79)
				MM (4w)	n.d.	n.d.	0.20 (0.16)	0.89 (0.67)
				MM (6w)	n.d.	n.d.	0.21 (0.12)	0.97 (0.49)
		Chongxing (Si Chuan)	*n* = 33	C (1-3d)	n.d.	n.d.	0.01 (0.02)	0.19 (0.25)
				TM (2w)	n.d.	n.d.	0.02 (0.02)	0.21 (0.04)
				MM (4w)	n.d.	n.d.	0.02 (0.03)	0.22 (0.04)
				MM (6w)	n.d.	n.d.	0.02 (0.03)	0.26 (0.05)
De Souza Santos da Costa et al., 2016* (40)	Brazil	—	*n* = 54	C (3d)	0.39 (0.08)	0.76 (0.08)	0.11 (0.04)	2.16 (0.19)
				MM (3 m)	0.26 (0.06)	0.67 (0.05)	0.10 (0.02)	1.65 (0.013)
Mihályi et al., 2015# (112)	Pécs, Hungary	—	*n* = 87	C (1d)	0.55 (0.40)	n.d.	0.06 (0.03)	1.18 (0.51)
			n = 61	MM (6w)	0.51 (0.48)	n.d.	0.06 (0.03)	1.04 (0.47)
Minda et al., 2004# (113)	Pécs, Hungary	—	*n* = 18	C (1d)	n.d.	n.d.	n.d.	1.69 (1.48)
				TM (14d)	n.d.	n.d.	n.d.	1.50 (0.65)
				MM (28d)	n.d.	n.d.	n.d.	2.06 (1.27)
Mojska et al., 2003& (67)	Poland	Spring	*n* = 50	C (3-4d)	n.d.	n.d.	n.d.	1.37 (1.00–2.00)
			n = 38	MM (5-6w)	n.d.	n.d.	n.d.	2.59 (1.49–3.34)
			n = 34	MM (9-10w)	n.d.	n.d.	n.d.	2.36 (1.55–3.92)
		Autumn	*n* = 50	C (3-4d)	n.d.	n.d.	n.d.	1.80 (1.42–2.48)
			n = 40	MM (5-6w)	n.d.	n.d.	n.d.	2.41 (1.79–4.31)
			n = 35	MM (9-10w)	n.d.	n.d.	n.d.	2.77 (1.53–4.18)
Tinoco et al., 2008* (44)	Brazil	—	*n* = 37	C (1-5d)	n.d.	n.d.	n.d.	2.34 (0.75)
				MM (35-42d)	n.d.	n.d.	n.d.	2.19 (0.47)
Urwin et al., 2013& (114)	China	River/lake	*n* = 42	C (3-5d)	3.98 (3.77–4.15)	n.d.	n.d.	n.d.
				TM (14d)	3.54 (3.27–3.66)	n.d.	n.d.	n.d.
				MM (25d)	3.37 (3.29–3.69)	n.d.	n.d.	n.d.
		Coastal	*n* = 42	C (3-5d)	3.50 (3.23–3.69)	n.d.	n.d.	n.d.
				TM (14d)	2.99 (2.53–3.56)	n.d.	n.d.	n.d.
				MM (28d)	2.80 (2.47–3.74)	n.d.	n.d.	n.d.
		Inland	*n* = 41	C (3-5d)	3.27 (3.03–3.57)	n.d.	n.d.	n.d.
				TM (14d)	2.96 (2.60–3.24)	n.d.	n.d.	n.d.
				MM (28d)	2.87 (2.56–3.14)	n.d.	n.d.	n.d.

*mean (SD), #median (IQR), &median (27–75 percentile); C, colostrum; d, day; EA, elaidic acid; m, month; MM, mature milk; n.d., no data; TFA, *trans* fatty acid; TM, transitional milk; VA, vaccenic acid; w, week.

3.2 Relationship between *trans* isomers and polyunsaturated fatty acids in human milk

LA and ALA cannot be synthesized in the human body, and therefore, they are called EFAs. They can be further metabolized into their longer-chain metabolites, where AA is formed from LA, while EPA, and DHA are formed from ALA (Figure 2) (126). This metabolic process takes place within the hepatic endoplasmic reticulum through the action of desaturase and elongase enzymes, with LA and ALA competing for these enzymes (127). Although humans can convert ALA to its longer-chain metabolites (including EPA and DHA), this conversion efficiency is rather limited, being highest in the perinatal period and decreasing to about 1% by adulthood (128). Due to this fact, DHA in the maternal diet provides a much more efficient source of DHA for the developing fetal and neonatal neural tissue than equivalent amounts of ALA (129).

Animal studies have shown that TFAs can inhibit the activity of the Δ-6 desaturase enzyme, which is involved in the metabolism of PUFAs, and thus TFAs may affect the availability of longer-chain n-3 and n-6 metabolites. As n-3 and n-6 LCPUFAs play an important role in perinatal life, including the maturation of the nervous system and the development of visual acuity, TFAs may negatively affect this process. An indirect sign of this disturbed metabolism can be the relationship between TFA and LCPUFA values. Previous studies found inverse associations between TFAs and the most important n-3 and n-6 LCPUFAs in blood lipids of pregnant women during pregnancy (130) and in term newborns at delivery (130–132). This suggests that there may also be a correlation between TFAs and PUFAs in HM. Although only a relatively small number of studies have examined this relationship, most of them have found inverse associations between these FAs.

EA values correlated inversely to LA in C ($r = -0.670$, $p < 0.001$), and to AA in MM ($r = -0.364$, $p < 0.05$), while there was a strong positive correlation with ALA values in C ($r = +0.844$, $p < 0.001$) and TM samples ($r = +0.844$, $p < 0.001$), but not in MM. In contrast, DHA values were significantly inversely correlated to EA in C ($r = -0.422$, $p < 0.05$), but directly in TM ($r = +0.615$, $p < 0.001$) samples, while in MM no significant correlation was found (111). An early study found an inverse association between C18:1 *t* values and both LA ($r = -0.29$, $p < 0.0001$) and ALA values ($r = -0.25$, $p < 0.001$), but not with their longer chain metabolites, AA, EPA, or DHA in Canadian mothers (89). In contrast, a decade later, no association was found between C18:1 *t* and EFAs (LA, ALA) in American HM (90). However, studies investigating European lactating women found significant negative correlations between C18:1 *t* and LA, AA, ALA, and EPA values at the 6th week (63), 3rd month (38) and 6th month of lactation (133), but DHA values were only inversely correlated to C18:1 *t* values at the 6th week (63), 3rd month of lactation (38).

Some studies also investigated the relationship between the total TFA content of HM samples and the PUFA values. In C, significant negative correlation was found between total TFA content on the one hand and essential n-6 LA values on the other hand (44, 111), while no clear relationship was found with the other n-3 and n-6 PUFA values (Table 4). One study found no significant correlations with AA, ALA, EPA, and DHA values (44), while the other study found significant positive correlations were found with AA, ALA, n-6 LCPUFA and n-3 PUFA values and negative correlations with DHA (111). In contrast, only one study investigated the relationship in TM (111) and found a significantly positive relationship between on the one hand TFA and on the other hand ALA, and DHA values. MM showed the clearest correlations: total TFA was significantly negatively correlated with LA in all studies (9, 38, 44, 63, 111, 133), while AA (38, 63, 111, 133), ALA (9, 38, 63, 133), EPA (38, 63), and DHA (38, 63) were significantly negatively correlated with TFA values in most studies, and only one study found a significant positive correlation with ALA, EPA, and DHA (111). In contrast, in American mothers no correlations were found between total or individual TFA values and the LA, ALA, n-3, and n-6 PUFA values (90).

Thus, the results so far suggest that TFAs may interfere with the metabolism of n-3 and n-6 PUFAs in HM during lactation, but the paucity of studies and conflicting results for some FAs suggest that further large-scale studies are needed to establish the actual relationship.

4 Relationship between human milk TFA content and infant health

The TFA content of HM can be a major concern, as newborns and infants are particularly vulnerable to the health effects associated with high intakes (134). The adverse effect of iTFAs in the perinatal period is mainly related to the disrupted metabolism of LCPUFAs and thus to the lower availability of these FAs crucial for the nervous system and somatic development (10). Human studies have demonstrated a negative correlation between TFA consumption and LCPUFA levels in both full-term infants (135) and healthy children aged 1–5 years (136). Additionally, an inverse association has been observed between infant plasma TFA concentrations and birth weight (137) indicating a possible early growth impairment linked to TFAs. There is a significant direct association between the proportion of TFAs in HM and their presence in plasma TGs and PLs of breast-fed infants (9). In this regard, the TFA content in HM should be reduced by modifying the diet of breastfeeding mothers, as dietary FAs are the major determinant of the composition of lipids in HM under sufficient energy supply (95). Since the largest part of the total TFA content of HM is EA, the total TFA content is closely related to its iTFA content.

During the prenatal stage, the fetus is exposed to TFAs through placental transfer, with the exposure relying on the concentration of the FAs in the mother's bloodstream, which, in turn, is influenced by her dietary intake (132, 138, 139). TFA levels in the umbilical cord blood at birth reflect the intrauterine supply and the dietary TFA intake of the mother during pregnancy. Previous studies have found a positive association between maternal and neonatal blood TFA levels (132, 138), supporting a direct effect of maternal nutrition, including TFA intake, during pregnancy on neonatal FA supply. TFAs are able to cross the placenta and thus appear in the umbilical cord plasma (131, 132, 138, 140, 141) and red blood cell (RBC) samples at birth (130, 132) and are also incorporated into the umbilical cord wall (135, 142). Higher maternal TFA intake during pregnancy may therefore interfere with the LCPUFA supply in the newborn infant at birth, an indirect sign of which may be the negative correlation between TFAs and the levels of LCPUFAs (AA, EPA, and DHA) important for the development of the nervous system (130–132, 135, 137, 138). Similarly, TFA intake during breastfeeding through HM may affect the n-3 and n-6 LCPUFA supply of breastfed infants and thus may have a longer-term adverse effect on development. Some previous studies

TABLE 4 Relationship between total TFA content and polyunsaturated fatty acids in human milk in different timepoints presented as correlation coefficients.

	C		TM	MM				
Fatty acids	Tinoco et al., 2008 (44)	Bousset-Alferes et al., 2022 (111)	Bousset-Alferes et al., 2022 (111)	Szabó É et al., 2007 (63)	Tinoco et al., 2008 (44)	Szabó et al., 2010 (133)	Krešic et al., 2013 (38)	Bousset-Alferes et al., 2022 (111)
LA (C18:2n-6)	−0.49*	−0.661***	−0.328	−0.34***	−0.35*	−0.230***	−0.275*	−0.377*
GLA (C18:3n-6)	n.d.	+0.621***	−0.196	n.d.	n.d.	+0.288***	n.d.	+0.379*
DHGLA (C20:3n-6)	n.d.	n.d.	n.d.	−0.53***	n.d.	−0.198***	n.d.	n.d.
AA (C20:4n-6)	−0.04	+0.758***	−0.106	−0.59***	+0.28	−0.356***	−0.440*	−0.521**
ALA (C18:3n-3)	+0.04	+0.837***	+0.867***	−0.35***	−0.12	−0.155***	−0.213*	+0.583***
EPA (C20:5n-3)	−0.26	+0.204	−0.236	−0.42***	+0.04	−0.046	−0.065*	+0.468**
DPA (C22:5n-3)	n.d.	n.d.	n.d.	n.d.	n.d.	−0.106*	n.d.	n.d.
DHA (C22:6n-3)	+0.16	−0.382*	+0.693***	−0.50***	+0.20	−0.006	−0.032***	+0.161
n-6 PUFA	−0.45*	−0.271	−0.315	−0.39***	−0.34*	n.d.	−0.122*	−0.313
n-6 LCPUFA	n.d.	+0.734***	−0.203	−0.61***	n.d.	n.d.	n.d.	+0.340
n-3 PUFA	n.d.	+0.735***	+0.710***	n.d.	n.d.	n.d.	−0.264*	+0.628***

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; AA, arachidonic acid; ALA, alpha-linolenic acid; C, colostrum; DHA, docosahexaenoic acid; DHGLA, dihomo-gamma-linolenic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acid; MM, mature milk; n.d., no published data; PUFA, polyunsaturated fatty acid; TM, transitional milk.

have reported possible negative effects of TFA levels during pregnancy or delivery on gestational age (141), placental weight (143), fetal head circumference (144) or birth weight (142, 143), raising the question of whether breast milk TFA levels also affect infants' somatomental development (Figure 4).

4.1 Animal studies

Ethical considerations have played a decisive role in the preference for animal studies over human studies when investigating the effects of TFAs in milk on infant health. The use of animal models, such as rats, mice or pigs allows researchers to investigate the effects of TFAs on infant health without exposing human infants to potential risks.

Animal models can also be used to study the effects of dietary TFA intake during pregnancy or lactation and its incorporation into maternal tissues such as the MG. In a rat experiment, high TFA intake during the early stages of pregnancy (until day 12), resulted in TFAs accumulating in maternal adipose tissue and MG and mobilizing from these stores despite a subsequent (after day 12) TFA-free diet and appearing in maternal, fetal and neonatal plasma samples. Moreover, these previously stored TFAs were distributed to the MG, making them available to the suckling offspring (145).

Dietary TFA intake during lactation is mainly accumulated in maternal liver tissues and MG and is also excreted in milk, where it limits the availability of EFAs to suckling offspring. Maternal TFA supply mainly increased MG lipoprotein lipase activity in a dose-dependent manner, with a less pronounced effect in the liver and no effect on adipose tissue suggesting that these two factors (TFA storage and increased lipoprotein lipase activity in the MG due to TFA) may underlie the higher TFA content in milk samples (146). A study conducted on animals reported that a diet rich in TFAs during lactation and after weaning had an impact on the FA supply of newborn piglets. At 3 days of age, their plasma levels of AA and docosapentaenoic acid (C22:5n-3, DPA) were significantly lower. Furthermore, the consumption of a TFA diet affected the composition of aortic PL FAs. At 3 days of age, it increased LA values and at 48 days of age, it increased C18:1 *t* and LA values, but decreased AA, EPA, and DHA values (39). Therefore, high maternal TFA intake during lactation can not only influence the plasma fatty acid concentration of their newborns, but also incorporate TFAs into their cells, potentially disrupting the metabolism of EFAs into their longer chain metabolites, AA, EPA, and DHA.

4.1.1 Brain development and behavior

It appears that higher maternal TFA intake during pregnancy and lactation may have a significant effect on the FA composition of the brain of the offspring. In an experimental study in which rats were fed a high-TFA diet or a control diet during pregnancy and lactation, the brains of 21-day-old offspring contained detectable amounts of TFAs. Moreover, high maternal TFA intake interfered with the incorporation of n-3 and n-6 LCPUFAs into the brain, leading to significantly lower AA and DHA levels in brain samples from the TFA group. However, when dams fed a high-TFA diet during pregnancy were switched to a

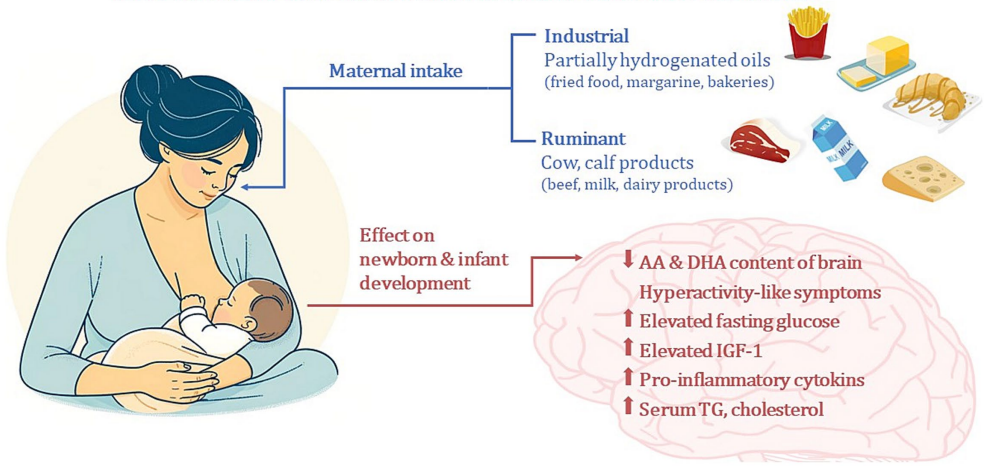

FIGURE 4
Possible effects of maternal industrial *trans* fatty acid intake during lactation on newborn and infant health.

normal (TFA-free) diet during lactation, EA levels in their adult offspring decreased, but AA and DPA levels increased significantly compared to the offspring of dams fed a high-TFA diet during both pregnancy and lactation. In fact, there was also a trend toward increased levels of EPA and DHA in this group, raising the possibility that the adverse effects of high TFA intake during pregnancy may be reduced by an adequate, low-TFA diet during lactation (147). This result was confirmed by another research group, where dietary TFA given to dams during lactation resulted in higher brain TFA levels in their adult offspring than in those who received a TFA diet only during the fetal period (during pregnancy). Moreover, the effect of TFA given only during lactation resulted in much lower AA and DHA content in the brains of adult offspring compared to the group that received TFA only during pregnancy (148). Similarly, a maternal TFA-rich diet during pregnancy and lactation increased TFA and decreased LA and ALA content in the offspring's brain at 21 days of life (after weaning) and LA and EPA content in adulthood (149) suggesting that maternal diet during pregnancy and lactation through HM may have a lasting effect on the FA composition of the infants' and children brain. Furthermore, the consumption of TFAs by mothers during pregnancy and lactation in two consecutive generations resulted in increased locomotor activity, impulsiveness, and agitation behavior in their offspring This suggests a correlation between TFA intake and hyperactivity-like symptoms (150). The FA composition of maternal diet during pregnancy and lactation, as well as the timing of brain development, can affect the incorporation of FAs into brain neural membranes. This can initiate early stages of inflammatory pathways in the brains of their offspring, ultimately impairing both short and long-term memory function (148, 149), increasing anxiety-like symptoms (151), and facilitating the development of hyperactivity-like symptoms (150) (Figure 4).

4.1.2 Glucose metabolism, insulin sensitivity

Animal studies have also focused on the impact of TFAs in breast milk on offspring's glucose metabolism and insulin parameters, providing valuable insights into their potential effects on early-life metabolic health. Maternal exposure to TFAs during lactation resulted in elevated fasting glucose levels in their offspring at weaning. However, HbA1c and insulin sensitivity remained unchanged, indicating a TFA-induced hepatic insulin resistance with adequate peripheral insulin sensitivity (152). In contrast, TFA intake via breastmilk did not affect serum glucose or insulin levels in rat pups, but insulin receptor and insulin receptor substrate-1 protein levels were significantly reduced in these rats (147). Additionally, adult offspring rats did not show any changes in blood glucose and insulin levels due to TFA intake during lactation (153). A study found that when mothers consumed TFAs during lactation, their adult offspring had significantly reduced levels of cardiac glucose transporter-4 levels, decreased hepatic glycogen content, and impaired insulin sensitivity. These results support the hypothesis, that early-life exposure to TFA-rich diets can lead to cardiac insulin resistance and have adverse consequences later in life (154).

Elevated plasma insulin-like growth factor-1 (IGF-1) in mouse pups with a high maternal TFA diet during lactation seemed to contribute to lower body fat, but this difference disappeared by adulthood (152). In pups of dams on a TFA diet during both pregnancy and lactation, there was no effect of this diet on appetite regulation, but when the newborn pups were switched from an intrauterine TFA diet to a postnatal control (TFA-free) diet, insulin-induced hypophagia disappeared, indicating that early TFA exposure may have long-term consequences on appetite regulation (147). In adult male rats on pre-and postnatal TFA-diet (including pregnancy and lactation) intracerebroventricular injection of insulin did not decrease 24-h feeding, although there was no difference in body weight compared to the control group (155). These studies underscore that early perinatal consumption of TFAs can affect food intake regulation in response to centrally administered insulin in young adult offspring, while the precise underlying mechanisms require further exploration, highlighting potential programming effects of early-life

exposure to TFAs on hypothalamic feeding control mechanisms, which could result in adverse outcomes later in life.

4.1.3 Body weight, adiposity

High maternal TFA intake during lactation delayed body weight gain only during the first week of life, but no difference was found later, although male offspring tended to be slightly smaller in adulthood ($p = 0.06$) compared to the group not receiving TFA during lactation. TFA-diet during lactation also affected the body composition in the offspring both at weaning and in adulthood, as measured by less abdominal fat (152). By contrast, in a rat study, maternal TFA-diet during pregnancy significantly decreased birth weight compared to n-6 PUFA of saturated fatty acid-rich diet, but at 21 days of age, these pups had significantly higher body weight, body weight gain, and relative weight of retroperitoneal fat compared to the n-6 PUFA group (156). However, others found no effect of perinatal TFA-intake on body weight in suckled pups (145, 153, 157) or adult offspring (147, 153, 155).

Maternal TFA-diet during pregnancy and lactation did not affect the weight of inguinal, retroperitoneal, epididymal, or mesenteric fat pads in their three-month-old male offspring. However, the mean areas of epididymal and inguinal adipocytes increased by 1.5 and 1.8-fold respectively, compared to the control diet-fed group. This suggests that maternal TFA-intake can enhance the deposition of visceral fat in their offspring, and therefore, may play a role in the development of later obesity (157).

4.1.4 Blood lipids

The most well-known adverse effect of iTFAs is the increased risk of cardiovascular morbidity and mortality, which is due, among other things, to the increased levels of atherogenic blood lipids. Animal studies may also help to investigate the effects of a high maternal TFA diet during lactation on the blood lipids of their offspring in adolescence or even adulthood. Only prenatal maternal TFA diet in mice pups increased the circulating free fatty acid (FFA) concentration, but this difference disappeared by adulthood. In mice pups exposed to TFAs only during lactation, however, no significant alteration was measured (152). After weaning, in 48-day-old piglets, no difference in total cholesterol and high-density lipoprotein (HDL) cholesterol values were found between the TFA and control groups (39). By contrast, TFA-diet during pregnancy and lactation resulted in significantly higher TG, cholesterol and lower HDL cholesterol levels in 21-day old rat pups. Similarly, pre-, and postnatal TFA diets significantly increased FFA and cholesterol levels, while TG values were decreased in adult offspring. A postnatal TFA-free diet could decrease FFA and cholesterol values, but they remained non-significantly higher compared to controls (153). Although there are animal studies about the effect of maternal TFA intake on the blood lipids of their offspring at different ages, most studies supplemented dams during both pregnancy and lactation, so we cannot conclude that they show the direct effect of TFA in breast milk. Another disturbing effect is that many studies continue the same diet in the pups even after weaning, so it also cannot show a direct effect of early postnatal TFA intake via breast milk.

4.1.5 Inflammation

Several studies have investigated the impact of maternal TFA consumption during pregnancy or lactation on the inflammatory status of offspring. In rat pups, maternal TFA intake during pregnancy and lactation increased the expression of pro-inflammatory adipokines such as plasminogen activator inhibitor-1 (PAI-1) and tumor necrosis factor-alpha (TNF-α) in white adipose tissue, but decreased the values and expression of anti-inflammatory adipokines, such as adiponectin or leptin (158). The changes observed were comparable to the low-grade inflammation status observed in obesity and metabolic syndrome. Therefore, it is possible that dietary TFA during the perinatal period may contribute to the perinatal programming of metabolic diseases. Similarly, maternal TFA diet in the pre-and postnatal period in 21-day-old rat offspring increased the expression of toll-like receptor 4 (TLR4), IL10Rα, and phosphorylated IκB kinase (p-IKKα+β) in the liver, although, there were no differences in the hepatic protein content of TNF-α, IL-6, and IL-10 (156). Based on a rat supplementation study, the long-term effects of pre-and postnatal exposure to TFAs might be different. Maternal TFA-supplementation during pregnancy or lactation significantly increased the pro-inflammatory cytokine levels (IL-1, IL-6, INF-γ, TNF-α) in both groups of adult offspring compared to controls, and this effect was more pronounced in the postnatal (TFA during suckling) group. By contrast, the anti-inflammatory IL-10 was decreased in both TFA groups, with significantly lower levels in the postnatal supplementation group. Moreover, increased reactive species generation, protein carbonyl levels, and catalase activity in the TFA-fed group also suggested increased oxidative stress in the adult offspring, that was even more pronounced when TFAs were consumed in the postnatal period via breastmilk (151).

The maternal TFA-diet during pregnancy and lactation may also impact the inflammatory state in the brain of the adult offspring. In an experimental study, the group fed with TFAs showed an increase in leucocyte rolling on the walls of brain venules, as well as an increase in leucocyte adhesion. These findings suggest leucocyte involvement in inflammation in the rat pups at 21 days of age (149). TFAs in the maternal diet during pregnancy and lactation may increase pro-inflammatory cytokine levels and decrease anti-inflammatory cytokine levels in the young and adult offspring. This may result in low-grade inflammation, but further research is needed to confirm these findings.

4.2 Human studies

Due to ethical considerations, human studies, contrary to animal studies, are not supplementation studies, but rather determine maternal TFA intake and/or HM TFA content and investigate their effects in early or late childhood. However, compared to animal studies, far fewer studies have examined these effects. As discussed above, the total TFA content of HM has been studied in several countries over lactation, in different ethnic groups, and in HM of preterm and term newborns. As previously stated, the largest portion of HM's total TFA content is attributed to EA. Therefore, the adverse effects of HM's total TFA content on infant development are primarily due to its iTFA content. Any potential beneficial effects of rTFA are discussed separately in the text.

Several studies have examined the relationship between maternal plasma TFA levels during pregnancy or at delivery and key pregnancy outcomes, including gestational age at birth, birth weight, and fetal head circumference. Higher maternal TFA levels in plasma may be associated with a shorter gestation period (138, 141), reduced birth weight, and small for gestational age at birth (159). A systematic

review and meta-analysis (160) found three studies about the inverse correlation between maternal TFAs during pregnancy and birth weight, while one study did not find any association. Moreover, the cord erythrocyte C18:1 *t* value, which is a better indicator of the newborn's supply, may also be inversely associated with birth weight (142). Higher maternal TFA levels during pregnancy may be related to lower fetal head circumference, but prenatal TFA exposure did not seem to affect global brain volume or nonverbal IQ in children (144). By contrast, neither cord plasma TFA (138) nor cord erythrocyte membrane C18:1 *t* (142) influenced head circumference at birth. From these studies, we can conclude that there might be a disturbing effect of maternal and neonatal TFA supply during pregnancy or at delivery on the infant development, but the effect of TFA intake through HM is a less researched topic and only a few studies can be found about short-and longer-term effects.

Maternal consumption of TFAs greater than 4.5 g/day during lactation appears to increase the odds ratio of higher percent body fat in both mothers (odds ratio: 5.81) and their 3-month-old infants (odds ratio: 2.13) for body fat percentages of 30% or greater in mothers and 24% or greater in infants (161). This study suggests that consuming TFAs during lactation may affect the body composition both mother and infant in the early postpartum period, potentially contributing to childhood and later obesity. However, no such relationship was found in Nigerian mothers regarding HM TFAs and maternal anthropometric values (97).

In a large birth cohort study ($n = 1{,}369$) maternal TFA intake during the second trimester was associated with higher fetal growth (162). In contrast, in a Norwegian birth cohort study ($n = 789$) no association was found between TFA content of HM samples and infant growth or rapid infant growth (163).

Although there were some correlations between maternal TFA supply during pregnancy and neonatal birth dimensions as discussed above, no relationship was found between TFAs in HM and head circumference, gestational age, and birth weight in term (40) as well as weight and height of preterm infants (44).

Animal studies, as discussed above, suggested an increased low-grade inflammation in those offspring that had higher TFA exposure during weaning (mainly iTFA, like EA), which might lead to an increased risk of developing allergies in infancy or childhood. However, rTFAs, like RA, and VA might be preventive. Increased concentration of both RA and VA in HM was correlated with a lower risk of eczema, atopic dermatitis, and allergic sensitization at one year of age, which was largely independent of HM n-3 LCPUFA values (164). Increased maternal VA-levels during pregnancy decreased the risk of atopic eczema in one-year-old children, but no other associations were found with other TFA values (165). Similarly, in children of mothers with allergy HM TFA content was associated with a significantly decreased odds ratio (OR: 0.57 95%CI: 0.33–1.00) of eczema at the age of four years, although no information is available, on whether these TFAs were iTFAs of rTFAs (C18:1 *t*, C16:1n-7 *t*, C18:2n-6 *t*) (166). By contrast, no relationship between maternal TFA intake during pregnancy and cow's milk allergy in their three-years-old children was found (167).

5 Conclusion

Thanks to recent regulations, the general population, including breastfeeding women, is now exposed to very low levels of dietary iTFA in most parts of the world. The effectiveness of iTFA regulation in food can be tracked by monitoring the iTFA content of HM samples in the literature. While early articles reported higher values, more recent studies have indicated much lower values. Although the TFA content of HM may change during breastfeeding, the data on the direction of this change are conflicting. There are contradictory data on the relationship between TFAs and n-3 and n-6 PUFAs, but most articles report a negative correlation between iTFAs and LA, AA, and DHA. The effects of iTFA supplementation during lactation have only been investigated in animal studies due to ethical considerations. These studies have shown that higher iTFA intake through breastmilk can lead to negative effects in the offspring, including altered brain fatty acid content, anxiety-like symptoms, increased visceral fat deposition, higher blood triglyceride and cholesterol levels, and pro-inflammatory status. However, few human studies have investigated the effects of breast milk iTFA content on infant and toddler health. While some data suggest that maternal iTFA intake may interfere with infant fatty acid supply, body composition, and the development of allergic diseases, further studies are needed to explore this relationship.

Author contributions

OH: Data curation, Writing – original draft. ÖFK: Data curation, Writing – original draft. HKK: Data curation, Writing – original draft. JLS: Visualization, Writing – review & editing. TM: Data curation, Software, Writing – review & editing. ÉS: Conceptualization, Data curation, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

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Supplementary material

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Human milk microbiome: associations with maternal diet and infant growth

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Introduction: Ingestion of human milk (HM) is identified as a significant factor associated with early infant gut microbial colonization, which has been associated with infant health and development. Maternal diet has been associated with the HM microbiome (HMM). However, a few studies have explored the associations among maternal diet, HMM, and infant growth during the first 6 months of lactation.

Methods: For this cross-sectional study, *Mam*-Mayan mother-infant dyads ($n = 64$) were recruited from 8 rural communities in the Western Highlands of Guatemala at two stages of lactation: early (6–46 days postpartum, $n = 29$) or late (109–184 days postpartum, $n = 35$). Recruited mothers had vaginally delivered singleton births, had no subclinical mastitis or antibiotic treatments, and breastfed their infants. Data collected at both stages of lactation included two 24-h recalls, milk samples, and infant growth status indicators: head-circumference-for-age-z-score (HCAZ), length-for-age-z-score (LAZ), and weight-for-age-z-score (WAZ). Infants were divided into subgroups: normal weight (WAZ ≥ −1SD) and mildly underweight (WAZ < −1SD), non-stunted (LAZ ≥ −1.5SD) and mildly stunted (LAZ < −1.5SD), and normal head-circumference (HCAZ ≥ −1SD) and smaller head-circumference (HCAZ < −1SD). HMM was identified using 16S rRNA gene sequencing; amplicon analysis was performed with the high-resolution ANCHOR pipeline, and DESeq2 identified the differentially abundant (DA) HMM at the species-level between infant growth groups (FDR < 0.05) in both early and late lactation.

Results: Using both cluster and univariate analyses, we identified (a) positive correlations between infant growth clusters and maternal dietary clusters, (b) both positive and negative associations among maternal macronutrient and micronutrient intakes with the HMM at the species level and (c) distinct correlations between HMM DA taxa with maternal nutrient intakes and infant z-scores that differed between breast-fed infants experiencing growth faltering and normal growth in early and late lactation.

Conclusion: Collectively, these findings provide important evidence of the potential influence of maternal diet on the early-life growth of breastfed infants via modulation of the HMM.

KEYWORDS

human breast milk microbiome, breastfeeding, maternal diet, infant growth z-scores, 16S rRNA gene, metagenomics 16S, lactation stage, Guatemala

Introduction

The human milk microbiota (HMM) is of growing interest in relation to infant microbial colonization and health outcomes (1, 2). Ingestion of human milk (HM) is considered one of the most significant factors associated with the composition and development of the infant gut microbiome (3–8). More recently, the maternal gut microbiome has emerged as an important source of the HMM (9–13), and although maternal gut microbiome has been shown to be highly influenced by maternal diet (14), only a few studies have explored the association of maternal diet with either the gut or the HMM.

Emerging evidence shows that the maternal diet via the HMM may influence infant growth by shaping the infant gut microbiota (14, 15). The HMM contributes to the establishment of an infant intestinal microbial community (16), and there is evidence that this infant intestinal microbial community is associated with infant growth during early life (17–19). There is also evidence that gut microbiota are associated with postnatal growth in animal models (20). These studies have shown that gut microbiota are involved in metabolic pathways that contribute to postnatal growth directly through energy harvesting (21), synthesizing vitamins (22), and affecting growth hormone and somatotropic axis sensitivity (20), and indirectly by regulating the immune system and preparing infants to face environmental challenges to their health (22).

Several studies have explored the association of the maternal diet/nutrients with the HMM, revealing multiple positive and negative associations between maternal micronutrient intakes and the HMM of lactating mothers (23–26). However, associations of HMM with infant growth mediated by maternal diet have not been explored. Maternal diet is known to affect the maternal gut microbiome community (27–32), which is a major source of the HMM (9, 10, 33). This is supported by evidence of the vertical transmission of the maternal milk microbiome and by shared bacterial species between paired maternal fecal and/or milk and infant fecal samples (3–5, 9, 34).

Guatemala is a developing country, with high exclusive breastfeeding rates approaching 77% (35). Moreover 90% of Indigenous mothers continue to breastfeed beyond 6 months for 17–21 months compared with 41.7% in the general population (36). The most recent available UNICEF 2015 Children Statistics for Guatemala reported that approximately 47% of Guatemalan children younger than 5 years of age were stunted (37). Although the Indigenous population of Guatemala complies with the WHO recommendations to exclusively breastfeed during the first 6 months of the infant's life (38, 39), earlier reports showed that the growth faltering occurs soon after birth in rural Guatemala (40) and is present for 3–6 months among breastfed Guatemalan infants (41). However, the association of the HMM with infant growth has been investigated in one study only (42). To date, no studies have explored the association between maternal diet and HMM in early and late lactation, both of which have been shown to have different HMM community composition (43), or examined potential associations of HMM with infant growth that may be mediated by maternal diet.

Recently, we reported differences in the HMM between infants with normal growth and infants with mild growth faltering (44, 45). We identified 30 differentially abundant (DA) taxa between the LAZ groups [mildly stunted (LAZ < −1.5SD) versus the non-stunted (LAZ ≥ −1.5SD)], 23 DA taxa between the WAZ groups [normal weight (WAZ ≥ −1SD) versus the mildly underweight (WAZ < −1SD)], and 26 DA taxa were identified between HCAZ groups [normal HC (HCAZ ≥ −1 SD) versus the smaller HC (HCAZ < −1 SD)] (44, 45). Our present study aimed to assess the association between maternal nutrient intakes and the DA HMM taxa identified at early and late lactation between infants with normal growth compared with infants with mild growth faltering among exclusively breastfed mother–infant dyads.

Materials and methods

Study setting, recruitment, and ethics

This cross-sectional study was conducted in eight rural *Mam*-Mayan communities of the Western Highland departments of Quetzaltenango between June 2012 and January 2013 (46). The *Mam*-Mayan community constitutes the fourth-largest Mayan population in Guatemala (47). The study began as collaboration between McGill University and the Center for Studies of Sensory Impairment, Aging, and Metabolism (CeSSIAM), a research organization based in Guatemala. Ethical approvals were obtained from ethics boards at McGill University and CeSSIAM. Further approvals were obtained from community leaders and the local authorities at the Ministry of Health in Guatemala. Community health workers used a participatory action research framework (48) to recruit lactating mothers through home visits, loudspeaker announcements, and word-of-mouth invitations (46). Recruited mothers provided fully informed written consent (thumbprint if unable to sign) if they wished to participate, and all mothers were informed of their rights to withdraw at any time from the study.

Study design

This cross-sectional study included healthy mother–infant dyads from two stages of lactation: "early" (6–46 days postpartum) or "late" (109–184 days postpartum). Inclusion criteria were healthy mother–infant dyads aged 6–46 days and 109–184 days postpartum, mothers who delivered vaginally, breastfed their infants exclusively or predominantly (used *agüitas*, a ritual fluid) for 6 months, and provided two 24-h dietary recalls at each stage of lactation. Exclusion criteria were non-singleton births, mother–infant dyads younger than 4 days, due to the possibility of still feeding colostrum, or did not have sufficient milk volume for the analysis, mothers treated with antibiotics, and mothers with sub-clinical mastitis (Na: K > 0.6) due to the possible effect of sub-clinical inflammation on the milk microbiome community and infant growth (49). Data included infant growth parameters, maternal anthropometries, maternal nutrient intakes (energy, macronutrients, and vitamins), and the DA species associated with infant z-scores.

Abbreviations: DA, Differentially abundant; HC, Head-circumference; HCAZ, Head-circumference-for-age-z-score; HMM, Human milk microbiome; LAZ, Length-for-age-z-score; WAZ, Weight-for-age-z-score.

Infant anthropometry

Infant age and infant anthropometric measurements (height, weight, and head circumference) were recorded by two trained Guatemalan nutritionists according to the standardized procedures. The detailed methodology was previously published (46). In brief, infant age was either calculated from the date of birth recorded on the maternal health card or was obtained from the mother in the absence of the health card. Infant anthropometric measurements were measured thrice, and the final value was the calculated mean of the three measurements. Infant recumbent supine length (cm) was measured thrice using an infant meter, a mobile baby measuring mat (SECA 210), and the calculated mean was rounded to the nearest 0.5 cm. Infant weight (kg) was measured using a digital infant scale (SECA 354) and rounded to the nearest 100 g. Finally, infant head-circumference (cm) was measured thrice using a head-circumference baby band (SECA 212). All infant anthropometric measures were completed on the same day of the milk sample collection.

Infant growth status indicators were calculated using the World Health Organization Anthro software (3.1) (50) for length-for-age z-score (LAZ), weight-for-age z-score (WAZ), and head circumference-for-age z-score (HCAZ) at early and late lactation. In brief, infants were divided into comparison groups: 'non-stunted' [LAZ ≥ -1.5SD (early: $n=11$; late: $n=16$)] versus 'mildly stunted' [LAZ < -1.5SD (early: $n=18$; late: $n=19$)], 'normal weight' [WAZ ≥ -1SD (early: $n=20$ late: $n=20$)] versus 'mildly underweight' [WAZ < -1SD (early: $n=9$; late: $n=15$)], and 'normal head-circumference' [HCAZ ≥ -1SD (early: $n=19$ late: $n=16$)] versus 'smaller head-circumference' [HCAZ < -1SD (early: $n=10$; late: $n=18$)]. The threshold of 1SD (i.e., more than 1SD below the WHO standard median) is used to define mild growth faltering. However, we used LAZ < -1.5SD to define mild stunting instead of LAZ < -1SD, to avoid over-estimation of both stunting prevalence and the association between the HMM and stunting, as stunting at birth and during the first month of life (4–33 days, median: 19 days) has been reported among Guatemalan infants (51).

Maternal diet records

Staff nutritionists conducted 2 comprehensive quantitative non-consecutive days of 24-h recalls in Spanish or Mam in both early and late lactation, as previously described (46). All foods and beverages were recorded and included in the analysis, and mothers were not taking food or vitamin supplements. National food composition tables for Central America from the Institute of Nutrition of Central America and Panama (INCAP) (52) were used to establish maternal intakes for energy, percent of energy from carbohydrates, protein, and total fat, macronutrient intakes for saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, cholesterol, fiber, sugar, and micronutrient intakes for vitamins including vitamin C, thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, folate, cobalamin, choline, vitamin A, retinol, alpha-carotene, beta-carotene, beta-cryptoxanthin, lutein + zeaxanthin, vitamin E, Vitamin D, and vitamin K. To estimate usual maternal intakes of energy, macronutrient intakes, and vitamins, average intakes of the 2 non-consecutive days of 24-h recalls for each nutrient were calculated at each stage of lactation. Maternal nutrient intakes were transformed using the nutrient density method where maternal macronutrient intakes were calculated as the proportion of total energy intake from carbohydrates, protein, and fat (e.g., % kcal from total fat), and micronutrient intakes were calculated in typical units per 1,000 kcal. This nutrient density method has been used among women in low-income, urban settings across three countries and has high probability of identifying inadequate intakes for several micronutrients (53). Due to a previously published report identifying true low intakes among our study population related to poverty (46), underreporting was not applied, and no dietary record was removed. Maternal dietary intakes were also compared with the Acceptable Macronutrient Distribution Range (AMDR).

Human milk sample collection

Milk samples were collected on the same day as the second 24-h recall to increase the accuracy of assessing the correlation between maternal diet and the human milk microbiome (HMM). To minimize the possibility of exchanging microbes, mothers were recruited from eight distinct remote communities. Mothers in the *Mam*-Mayan communities are known to comply with WHO recommendations to exclusively or predominantly breastfeed for the first 6 months of the infant's life (38, 39). Milk samples were collected from all mothers unilaterally from the breast that was not last used to feed the infant by full manual expression in a 3-h time window between 9 am and 12 pm (38). All milk samples were collected following an aseptic technique by a trained midwife, who used hand sanitizer before and after collection. The nipple and areola of the breast were cleaned with 70% ethanol prior to milk sample collection. Only manual expression was used for milk collection, without the use of a breast pump to exclude its potential influences on milk microbiome diversity (54). Milk samples were collected in acid-washed, sterile 60-ml plastic vials and stored on ice immediately and subsequently partitioned into four 15 mL vials and stored at −30°C in the field laboratory. Samples were shipped to McGill University, where they were stored at −80C (55), which is an optimum temperature for microbiome preservation prior to DNA extraction (56).

16S rRNA gene amplification and sequencing

A DNeasy Blood and Tissue mini kit from QIAGEN was used with 1 mL of milk to extract DNA, according to the manufacturer's protocol by Génome Québec laboratories. PCR amplification was conducted with the universal eubacteria primers 27F/533R (27F: AGAGTTTGATCCTGGCTCAG, 533R: TTACCGCGGCTGCTG GCAC) of the variable regions V1–V3 consisting of ~526 bp based on the *Escherichia coli* 16S rRNA gene (57–59). These primers have a high coverage of most genera currently considered "core" in human milk, including the genus *Cutibacterium* (60, 61). Sequencing was performed using the Illumina MiSeq platform. Reagent controls were below the detection limit. The amplification conditions have been previously described (43).

Contamination control steps were performed at multiple steps in this analysis. At the milk sample collection stage, a trained midwife followed an aseptic sampling protocol which included cleaning hands with hand sanitizers and using 70% ethanol prior to cleaning the

nipple and areola of the breast prior to milk sample collection. At the PCR step, the Genome Quebec Centre followed an aseptic technique.

Microbial data processing and bioinformatics

Analysis of the amplicon data was performed using the ANCHOR pipeline. ANCHOR is a method designed for improved species-level microbial identification through the utilization of direct paired-end sequences, which substantially improves the sequence resolution of 16 s rRNA amplification data. Furthermore, it uses multiple samples and integrated multiple-reference databases to annotate bacteria with criteria of >99% for identity and coverage to provide high confidence and resolution (62).

In brief, Mothur was used to align DE replicate sequences (63) before high-count ESV selection at a count threshold of 36 across all samples. The repository databases such as NCBI 16S rRNA RefSeq, NCBI non-redundant nucleotide, SILVA, and the Ribosomal Database Project (RDP) were used to annotate ESVs using BLASTn with criteria of >99% for identity and coverage. Priority was given to NCBI 16S rRNA RefSeq when BLASTn, 100% identity, and coverage hits were returned across multiple databases due to the high standard of curation. Amplicons with low counts (<36) were binned to high-count ESVs at a threshold of >98% identity/coverage. Multiple, equally good (highest identity/coverage), annotation was retained and reported as a multiple species (*_MS*). Taxonomy annotation, particularly species calls, should be considered putative even when sharing 100% sequence identity to a single species due to database errors.

Several contamination control steps via sample pre-processing were performed by the Canadian Centre for Computational Genomics (C3G) of McGill University at the bioinformatics stage, including controlling for prevalence and sparsity, ordination analysis, and identifying putative contamination, which is flagged by *Decontam* (*Decontam*, R package) (Supplementary file 1). *Decontam* flags putative contamination. Out of 1,505 ESVs, only one ESV was flagged as potential contamination, although it was not selected by DESeq2 as a differentially abundant species in our samples.

Statistical analyses

Initially, we assessed if maternal age and anthropometries (weight, height, and BMI) were associated with infant z-scores (WAZ, LAZ, WHZ, BMIZ, and HCAZ) in both stages of lactation. Thereafter, infant anthropometry and maternal nutrient intakes were compared for LAZ, WAZ, and HCAZ between infants with HCAZ ≥ −1 SD, LAZ ≥ −1.5SD, and WAZ ≥ −1SD compared with those infants with smaller head circumferences (HCAZ < −1 SD) and those with mild linear growth deficits (LAZ < −1.5SD) and those classified as mildly underweight (WAZ < −1SD); comparisons used *t*-tests or non-parametric Wilcoxon tests and chi-square for continuous and categorical variables, respectively. To describe population characteristics, anthropometric (Table 1) and dietary (Table 2) data

TABLE 1 Population characteristics of Guatemalan mother-infant dyads.

Characteristic	HCAZ ≥ −1 SD *n* = 34[1]	HCAZ < −1 SD *n* = 30[1]	*p*-value[2]	LAZ ≥ −1.5 SD *n* = 27[1]	LAZ < −1.5 SD *n* = 37[1]	*p*-value[2]	WAZ ≥ −1 SD *n* = 40[1]	WAZ < −1 SD *n* = 24[1]	*p*-value[2]
Lactation stage			0.2			0.5			0.3
Early	18 (53%)	11 (37%)		11 (41%)	18 (49%)		20 (50%)	9 (38%)	
Late	16 (47%)	19 (63%)		16 (59%)	19 (51%)		20 (50%)	15 (62%)	
Maternal characteristics									
Maternal age, years	24 (6.4)	23.4 (5.38)	0.8	24.1 (6)	23.4 (5.9)	0.6	23.3 (5.4)	24.3 (6.8)	0.8
Maternal height	147.6 (5.5)	146.6 (4.4)	0.4	148.2 (5.1)	146.3 (4.9)	0.15	148 (4.8)	145.7 (5.7)	**0.038**
Maternal weight	52.4 (8.3)	50.4 (7.1)	0.4	53.3 (7.7)	50.2 (7.7)	0.13	52.2 (7.3)	50.2 (8.4)	0.4
Maternal BMI	24 (3.1)	23.5 (3.6)	0.7	24.2 (3.2)	23.4 (3.4)	0.3	24 (3.3)	23.6 (3.5)	0.8
Infant characteristics									
Infant sex			0.8			0.14			0.2
Female	17 (50%)	16 (53%)		11 (41%)	22 (59%)		18 (45%)	15 (62%)	
Male	17 (50%)	14 (47%)		16 (59%)	15 (41%)		22 (55%)	9 (38%)	
Infant age, days	81 (64)	97 (61)	0.6	93 (60)	85 (65)	0.6	83 (66)	97 (58)	0.4
Infant WAZ	−0.46 (0.7)	−1.17 (0.95)	**0.001**	−0.28 (0.75)	−1.17 (0.79)	**<0.001**	−0.29 (0.56)	−1.63 (0.68)	**<0.001**
Infant LAZ	−1.4 (1.03)	−2.27 (1.17)	**0.008**	−0.8 (0.68)	−2.54 (0.87)	**<0.001**	−1.39 (1)	−2.5 (1.12)	**<0.001**
Infant WHZ	0.87 (0.9)	0.71 (1.32)	>0.9	0.43 (0.97)	1.06 (1.14)	**0.013**	1 (1.02)	0.47 (1.2)	0.15
Infant HCAZ	−0.09 (0.57)	−1.9 (1.03)	**<0.001**	−0.53 (1.2)	−1.2 (1.17)	**0.019**	−0.50 (1)	−1.62 (1.23)	**<0.001**
Infant BMIZ	0.46 (0.78)	0.18 (1.08)	0.4	0.22 (0.96)	0.41 (0.93)	0.5	0.7 (0.76)	−0.27 (0.91)	**<0.001**
Mild Underweight	7 (21%)	17 (57%)	**0.003**	5 (19%)	19 (51%)	**0.007**	-	-	-
Mild Stunting	15 (44%)	22 (73%)	**0.018**	-	-	-	18 (45%)	19 (79%)	**0.007**
Smaller head circumference	-	-	-	8 (30%)	22 (59%)	**0.018**	13 (32%)	17 (71%)	**0.003**

[1]n/N (%); Mean (SD). [2]Pearson's Chi-squared tests; Wilcoxon rank sum test; Wilcoxon rank sum exact test. HCAZ: head-circumference-for-age-z-score, LAZ: length-for-age-z-score, WAZ: weight-for-age-z-score, BMI: body mass index, WHZ: weight-for-height-z-score, BMIZ: body mass index-z-score. The bold values are statistically significant $p < 0.05$.

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TABLE 2 Maternal nutrient intakes by infant growth z-scores.

Nutrient intakes	HCAZ ≥ −1 SD n = 34[1]	HCAZ < −1 SD n = 30[1]	p-value[2]	LAZ ≥ −1.5 SD n = 27[1]	LAZ < −1.5 SD n = 37[1]	p-value[2]	WAZ ≥ −1 SD n = 40[1]	WAZ < −1 SD n = 24[1]	p-value[2]
Energy kcal	1,508 (243)	1,395 (275)	0.2	1,435 (329)	1,470 (205)	0.7	1,517 (247)	1,353 (260)	**0.018**
Protein g	46 (11)	43 (14)	0.4	44 (14)	45 (12)	0.6	46 (12)	43 (14)	0.4
Carbohydrates g	295 (55)	278 (54)	0.4	281 (73)	292 (36)	0.5	298 (55)	269 (51)	**0.049**
Fat g	24 (7)	20 (6)	0.081	23 (8)	22 (6)	0.4	24 (7)	20 (6)	**0.016**
Saturated fat g	5 (3)	4 (2)	0.3	5 (3)	4 (2)	0.4	5 (3)	4 (2)	0.069
Monounsaturated fat g	7 (3)	6 (2)	**0.049**	7 (3)	7 (2)	0.5	7 (2.6)	6 (2)	**0.016**
Polyunsaturated fat g	9 (2)	7 (2)	0.061	8 (2)	8 (2)	0.7	8 (2)	7 (2)	**0.026**
Cholesterol mg	97 (95)	85 (87)	0.6	94 (105)	90 (81)	0.6	105 (95)	70 (81)	0.2
Fibers g	30 (7)	28 (6)	0.9	28 (8)	30 (5)	0.3	30 (6)	28 (7)	0.4
Sugar g	77 (30)	64 (25)	0.2	70 (33)	71 (24)	0.4	75 (31)	64 (22)	0.2
Vitamin C mg	57 (35)	45 (27)	0.2	48 (31)	54 (33)	0.6	49 (30)	55 (36)	0.7
Thiamin mg	0.89 (0.24)	0.82 (0.2)	0.4	0.83 (0.26)	0.88 (0.2)	0.3	0.87 (0.22)	0.84 (0.23)	0.7
Riboflavin mg	0.71 (0.23)	0.66 (0.22)	0.6	0.68 (0.25)	0.69 (0.21)	0.7	0.73 (0.22)	0.62 (0.21)	0.058
Niacin mg	15.5 (3.7)	15.5 (6.1)	0.5	15.1 (6.1)	15.8 (4)	0.6	15.5 (4)	15.5 (6.3)	0.4
Pantothenic acid mg	2.6 (0.85)	2.5 (0.74)	0.5	2.5 (0.92)	2.63 (0.71)	0.8	2.69 (0.76)	2.4 (0.85)	0.2
Vitamin B6 mg	1.2 (0.32)	1.1 (0.41)	0.3	1.1 (0.36)	1.2 (0.38)	0.9	1.2 (0.37)	1.1 (0.37)	0.5
Folate DFE µg	528 (138)	490 (116)	0.4	503 (158)	516 (104)	0.6	527 (127)	483 (129)	0.4
Choline mg	183 (85)	177 (87)	0.9	181 (97)	179 (78)	>0.9	189 (81)	164 (91)	0.2
Vitamin B12 µg	0.74 (0.62)	0.71 (0.60)	>0.9	0.83 (0.72)	0.65 (0.50)	0.4	0.77 (0.60)	0.66 (0.63)	0.4
Vitamin A RAE	983 (326)	841 (410)	0.084	865 (377)	954 (368)	0.4	962 (371)	840 (366)	0.2
Retinol µg	567 (266)	433 (252)	0.055	488 (293)	515 (248)	0.3	548 (272)	430 (243)	0.10
Alpha-carotene µg	792 (771)	704 (964)	0.2	653 (743)	821 (941)	>0.9	742 (828)	765 (932)	>0.9
Beta-carotene µg	2,675 (2,231)	2,632 (2,563)	0.8	2,349 (2,164)	2,878 (2,520)	0.5	2,592 (2,300)	2,760 (2,536)	0.9
Beta-cryptoxanthin µg	171 (541)	203 (601)	0.6	70 (163)	271 (723)	0.2	245 (690)	88 (226)	0.7
Lycopene µg	338 (560)	427 (1,084)	0.3	247 (383)	477 (1,053)	0.9	286 (501)	537 (1,212)	0.4
Lutein + zeaxanthin µg	1,663 (1,828)	1,979 (2,054)	0.2	1,821 (1,998)	1,804 (1,903)	0.6	1,695 (1,657)	2,006 (2,337)	0.8
Vitamin E mg	3.4 (1.8)	2.3 (1)	**0.003**	2.8 (1.4)	2.9 (1.7)	0.9	3.2 (1.7)	2.34 (1)	**0.029**
Vitamin D µg	0.6 (0.9)	0.5 (0.7)	0.8	0.6 (0.9)	0.5 (0.7)	0.6	0.7 (0.9)	0.28 (0.5)	0.060
Vitamin K µg	86 (97)	84 (90)	0.7	92 (105)	80 (84)	0.8	81 (89)	92 (101)	0.6

[1]n/N (%); Mean (SD). [2]Pearson's Chi-squared tests; Wilcoxon rank sum test; Wilcoxon rank sum exact test. HCAZ: head-circumference-for-age-z-score, LAZ: length-for-age-z-score, WAZ: weight-for-age-z-score, BMI: body mass index, WHZ: weight-for-height-z-score, BMIZ: body mass index-z-score. The bold values are statistically significant $p < 0.05$.

were presented as means ± standard deviations (SD) for continuous variables. A p-value <0.05 was considered significant for these analyses.

Correlation analyses were performed to assess the relationships among maternal anthropometry and infant growth parameters, the dependency among maternal nutrient intakes, and among maternal anthropometry and infant growth parameters with maternal dietary intake per nutrient in early and late lactation (FDR < 0.05).

Spearman's rank-order correlation coefficients were calculated using normalized ESV abundance and the infant growth parameters from mildly stunted (LAZ < −1.5SD), non-stunted (LAZ ≥ −1.5SD), mildly underweight (WAZ < −1SD), normal weight (WAZ ≥ −1SD), normal head-circumference (HCAZ ≥ −1SD), and smaller head-circumference (HCAZ < −1SD) groups with the average of two 24-h recalls of maternal nutrient intakes in early and late lactation. Correlation coefficients were represented by heatmaps using the *corrplot* v0.92 R package. To control for multiple comparisons, a false discovery rate (FDR) was applied to p-values (FDR < 0.1).

Mantel correlations were obtained by performing Mantel test between distance matrices of clustered phenotypic values (maternal nutrients, infant growth parameters) and ESVs. Clusters were selected manually from heatmaps by applying a hierarchical clustering order (hclust option in *corrplot*) on each group correlation. Distance matrices were calculated by using Euclidean distance measure for phenotypic value and Jaccard distance (*vegdist* function in the vegan R package) of rlog-transformed ESV abundance.

Results

Population characteristics and maternal nutrient intakes of Guatemalan mother–infant dyads

Overall assessment of quality of maternal diet

Maternal macronutrient intakes of the majority of the lactating mothers were imbalanced and fell outside the Acceptable Macronutrient Distribution Range (AMDR) (64). The AMDR for adults for carbohydrate ranges from 55 to 70%, for protein from 7 to 20%, and for fat from 15 to 25% of the daily energy intake. In our study, Guatemalan mothers' carbohydrate intakes constituted more than 70% of the daily energy intakes for 95.3% of mothers and most (91.7%) had intakes of fat below its AMDR. For protein,

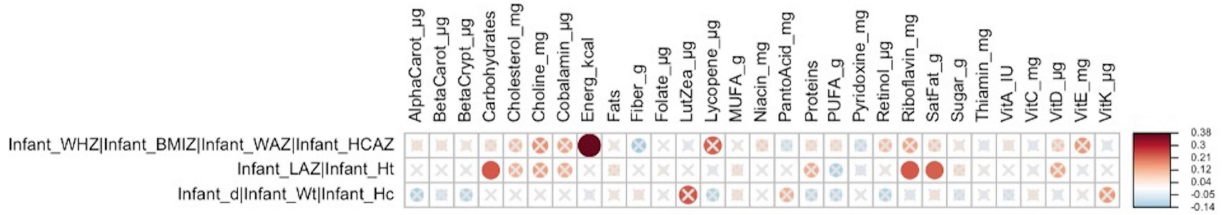

FIGURE 1
Heatmap of Mantel tests between maternal nutrient intakes (early lactation; Euclidean distance metric) and maternal and infant anthropometry clusters (based on Bray-Curtis distance metric). Red circles represent positive correlations and blue circles represent negative correlations. The intensity of the colors represents the degree of association. The solid circles represent significant correlations (FDR>0.1). Cluster 1: infant weight-for-height-z-score (WHZ), infant BMI-for-age-z-score (BMIZ), infant weight-for-age-z-score (WAZ), and infant head-circumference-for-age-z-score (HCAZ) was significantly correlated with maternal energy intakes (FDR=0.029; r=0.38), and Cluster 2: infant-length-for-age-z-score (LAZ), and infant height was significantly correlated with maternal intakes of carbohydrates (FDR=0.093; r=0.23), riboflavin (FDR=0.093; r=0.23), and saturated fat (FDR=0.093; r=0.22).

only 14.1% of mothers consumed proteins ≥15% of total energy intake. Mothers also had inadequate intakes of multiple vitamins when compared with two-thirds of the Recommended Dietary Allowances (RDA) or the national RDA known as *Recomendaciones Dietéticas Diarias* (RDD) (65, 66). The prevalence of inadequate intakes based on INCAP included thiamin (50%), riboflavin (94%), pyridoxine (70%), folate (97%), and cobalamin (90%). The mean energy intake among the Guatemalan mothers in our study was 1,455 calories/day which was uniformly lower than the estimated mean energy requirements of 2,065 calories per day based on estimated resting energy expenditure using the Dietary Reference Intake (DRI) equation of the Institute of Medicine (67, 68) and a moderate physical activity factor of 1.7 (69).

Association of anthropometry with maternal diet and nutrient intakes

Maternal–infant dyad anthropometric characteristics are shown in Table 1. Differences in maternal dietary intakes by infant z-scores: HCAZ ≥ −1SD versus smaller head-circumference (HCAZ < −1SD), LAZ ≥ −1.5SD versus mildly stunted (LAZ < −1.5SD), and WAZ ≥ −1SD versus mildly underweight (WAZ < −1SD) are shown in Table 2.

Maternal anthropometries were not associated with infant z-scores in either early (p=0.6) or late (p= 0.8) stages of lactation. On the other hand, comparisons showed that infants with HCAZ < −1 SD had a higher prevalence of mild stunting (p=0.018) and mild underweight (p=0.003) compared with infants with HCAZ ≥ −1 SD. These mothers also consumed less MUFA (p=0.049), vitamin E (p=0.003), and marginally less retinol (p=0.055) compared with the mothers of infants with HCAZ ≥ −1 SD. Mildly stunted (LAZ < −1.5SD) infants had a higher prevalence of mild underweight (p=0.007) and had smaller head-circumferences (p=0.018) compared with infants with LAZ ≥ −1.5SD. However, maternal intakes of the studied nutrients did not differ between infant LAZ groups. For the comparison of infants classified as having WAZ < −1SD, there was a higher prevalence of mild stunting (p=0.007) and smaller head-circumferences (p=0.003) compared with infants with WAZ ≥ −1SD. For these infants, maternal intakes of energy (p=0.018), carbohydrates (p=0.049), fat (p=0.016), and dietary fat components, including MUFA (p=0.016), PUFA (p=0.026), and vitamin E (p=0.029), were lower for mothers of mildly underweight (WAZ < −1SD) infants compared with maternal intakes for infants with WAZ ≥ −1SD (Tables 1, 2).

Cluster analyses in early and late lactation

Maternal and infant anthropometric correlations

In early lactation, the cluster of infant z-scores (WHZ, BMIZ, WAZ, and HCAZ) (Supplementary Figure S1) was correlated with maternal nutrient intakes including energy intake (FDR = 0.029; r = 0.4), and the infant linear growth cluster (LAZ and length/height) was correlated with maternal intakes of carbohydrates (FDR = 0.093; r = 0.23), riboflavin (FDR = 0.093; r = 0.23), and saturated fat (FDR = 0.093; r = 0.22) (Figure 1).

Of the six distinct nutrient clusters that were identified in early lactation (Figure 2), there was a significant correlation between maternal nutrient intakes of lutein + zeaxanthin and vitamin K as a cluster with the infant growth parameters (infant age, infant weight, and infant head-circumference) as a cluster (FDR = 0.08; r = 0.23) (Supplementary Figure S2).

In late lactation, no infant anthropometric cluster was correlated with any identified maternal nutrient intake cluster is shown in Figure 3.

Identification of nutrient patterns and clusters in early and late lactation

Correlation analyses of individual maternal nutrient intakes and nutrient clusters in early and late lactation are shown in Figure 2 for early lactation and in Figure 3 for late lactation. In both early and late lactation energy intakes were not correlated with intakes of other nutrients, but higher dietary intakes of carbohydrate as a percent of total kcal were inversely correlated with individual energy intakes from protein and total fat, also inversely correlated with gram intakes of saturated fats as MUFA or PUFA and cholesterol and finally with lower intakes of several micronutrients including fat soluble (E, D and/or A, K) and multiple water-soluble vitamins.

Cluster correlation analyses of maternal nutrient intakes in early (Figure 2) lactation and late (Figure 3) lactation represented inferred nutrient clusters with some differences between the clusters in early lactation and late lactation. First, an interesting cluster, possibly unique to the Guatemalan diet, emerged where sugar was clustered with retinol. In Guatemala, sugar is fortified with retinol, which was associated with lower intakes of fiber and pyridoxine in early and lower intakes of fiber and thiamine in late lactation. The second common cluster was of three vitamins—ascorbic acid, thiamine, and folate—with fiber. The third common cluster was lutein + zeaxanthin and vitamin

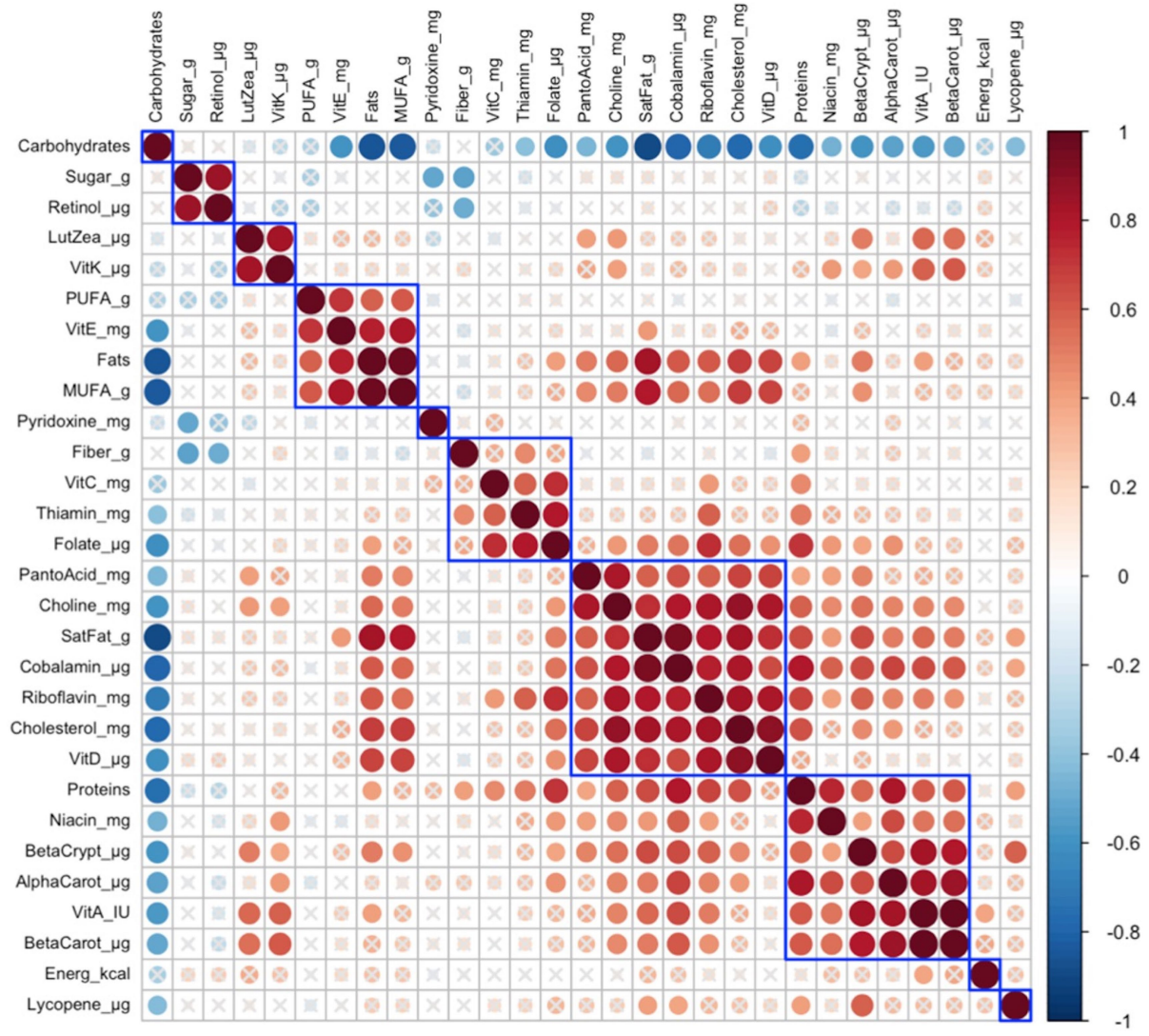

FIGURE 2
Hierarchical clustering of Spearman's rank correlation heatmap between maternal nutrient intakes in early lactation. Red circles represent positive correlations and blue circles represent negative correlations. The blue boxes represent inferred clusters. Cluster 1: maternal intakes of sugar and retinol, Cluster 2: maternal intakes of lutein + zeaxanthin and vitamin K, Cluster 3: maternal intakes of polyunsaturated fatty acids (PUFA), vitamin E, fats, and monounsaturated fatty acids (MUFA), Cluster 4: maternal intakes of fiber, vitamin C, thiamin, and folate, Cluster 5: maternal intakes of pantothenic acid, choline, saturated fatty acids, cobalamin, riboflavin, cholesterol, and vitamin D, and Cluster 6: maternal intakes of proteins, niacin, beta-cryptoxanthin, alpha-carotene, vitamin A, and beta-carotene.

K, which emerged as a cluster in early lactation; in late lactation, vitamin E was also emerged as positively correlated with this cluster. The fourth common cluster was vitamin A, its precursors were alpha-carotene, beta-carotene, and beta-cryptoxanthin with niacin. The last common cluster was choline, saturated fat, cobalamin, and cholesterol.

Despite similarities, differences occurred in the nutrient composition of clusters between early and late lactation. These clusters were associated with protein as a percentage of kcal, fat as a percentage of kcal, gram intakes of MUFA and PUFA, and mg intakes of vitamin D, vitamin E riboflavin, and pantothenic acid. A cluster with fat, MUFA, PUFA, and vitamin E in early lactation emerged, but a related cluster did not appear in late lactation, where fats and MUFA were positively clustered with a larger number of nutrients including higher intakes of protein as a percentage of kcal, saturated fats, cholesterol, choline, cobalamin, and nutrients often associated with intakes of animal source foods. In addition, in early lactation, protein was clustered with some nutrients that were related to plant intake including, beta-cryptoxanthin, alpha-carotene, and beta-carotene, and another two nutrients found in both plant and animal sources, vitamin A, and niacin. The opposite

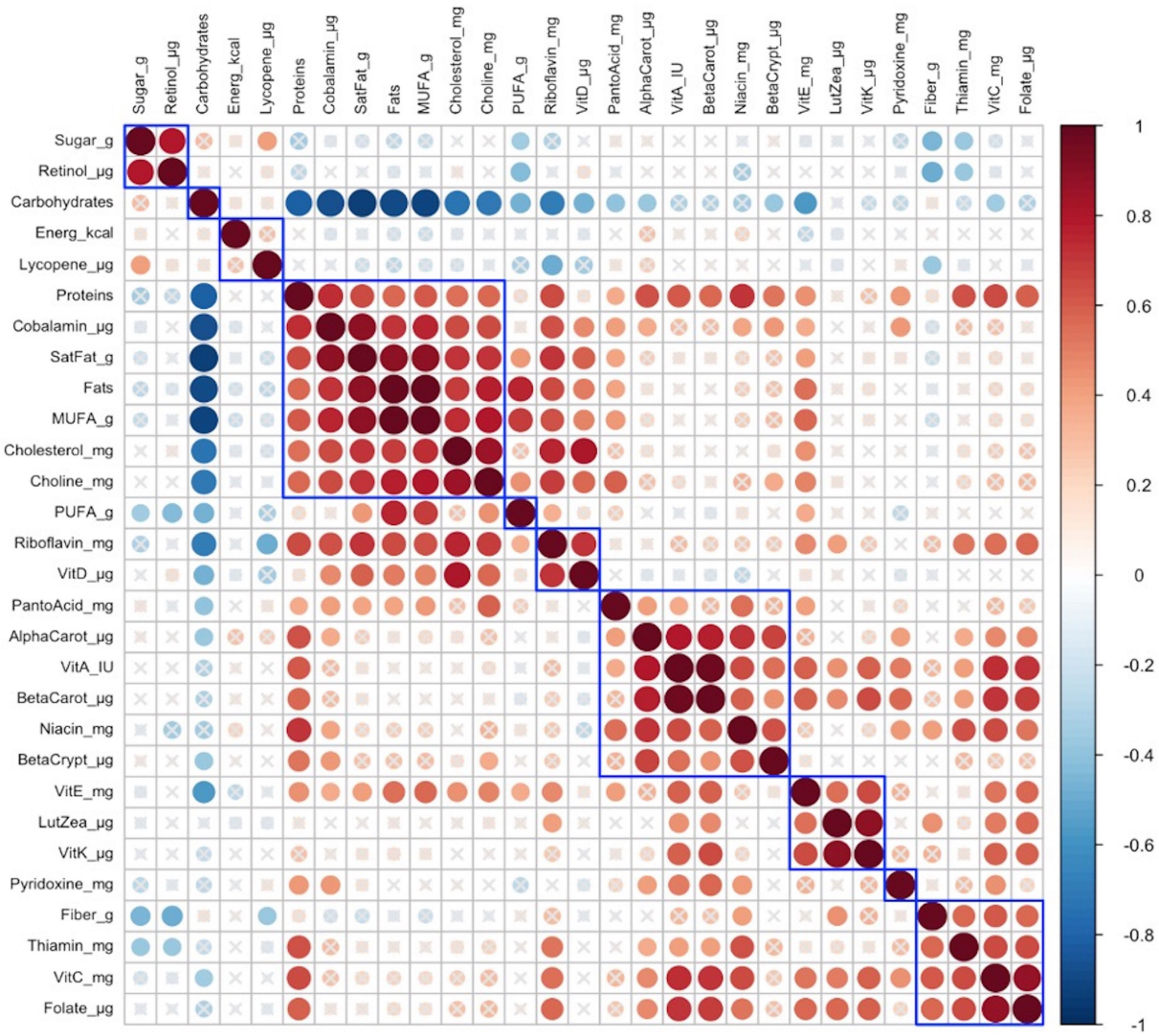

FIGURE 3
Hierarchical clustering of Spearman's rank correlation heatmap between maternal nutrient intakes in late lactation. Red circles represent positive correlations and blue circles represent negative correlations. The blue boxes represent inferred clusters. Cluster 1: maternal intakes of retinol, and sugar, Cluster 2: maternal intakes of protein, cobalamin, saturated fat, fats, monounsaturated fatty acids (MUFA), cholesterol, and choline, Cluster 3: maternal intakes of riboflavin and vitamin D, Cluster 4: maternal intakes of pantothenic acid, alpha-carotene, vitamin A, beta-carotene, niacin, and beta-cryptoxanthin, Cluster 5: maternal intakes of vitamin E, lutein + zeaxanthin, and vitamin K, and Cluster 6: maternal intakes of fiber, thiamin, vitamin C, and folate.

was true for pantothenic acid, which was clustered with animal-related nutrients including, choline, saturated fat, cobalamin, riboflavin, cholesterol, and vitamin D in early lactation, whereas it was clustered with more plant-related nutrients including alpha-carotene, vitamin A, beta-carotene, niacin, and beta-cryptoxanthin in late lactation.

Correlations of the human milk microbiome with maternal nutrient intakes

Human milk microbiome community

ANCHOR was able to identify 503 ESVs and captured 3,551,788 sequence reads across 64 human milk samples. Among the identified 503 ESVs, 256 were annotated at the species level, accounting for 81.2% of reads, 129 were annotated at the genera level, and 9 at the family-level or higher taxa in addition to 109 unidentified taxa that accounted for 6.5% of the total ESVs. These taxa were classified as Unknowns as they could not be identified at >99% similarity in both identity and coverage to any known taxa. There were also 67 ambiguous species that were given the suffix (*_MS*) 'Multiple Species'. The suffix was used for ESVs when multiple species are equally likely annotated.

Human milk microbiome and maternal nutrient intakes

Correlation analyses are illustrated in heatmaps and identified significant associations between maternal dietary intakes and HMM at the species level in early (Supplementary File 2) and late (Supplementary File 3) lactation.

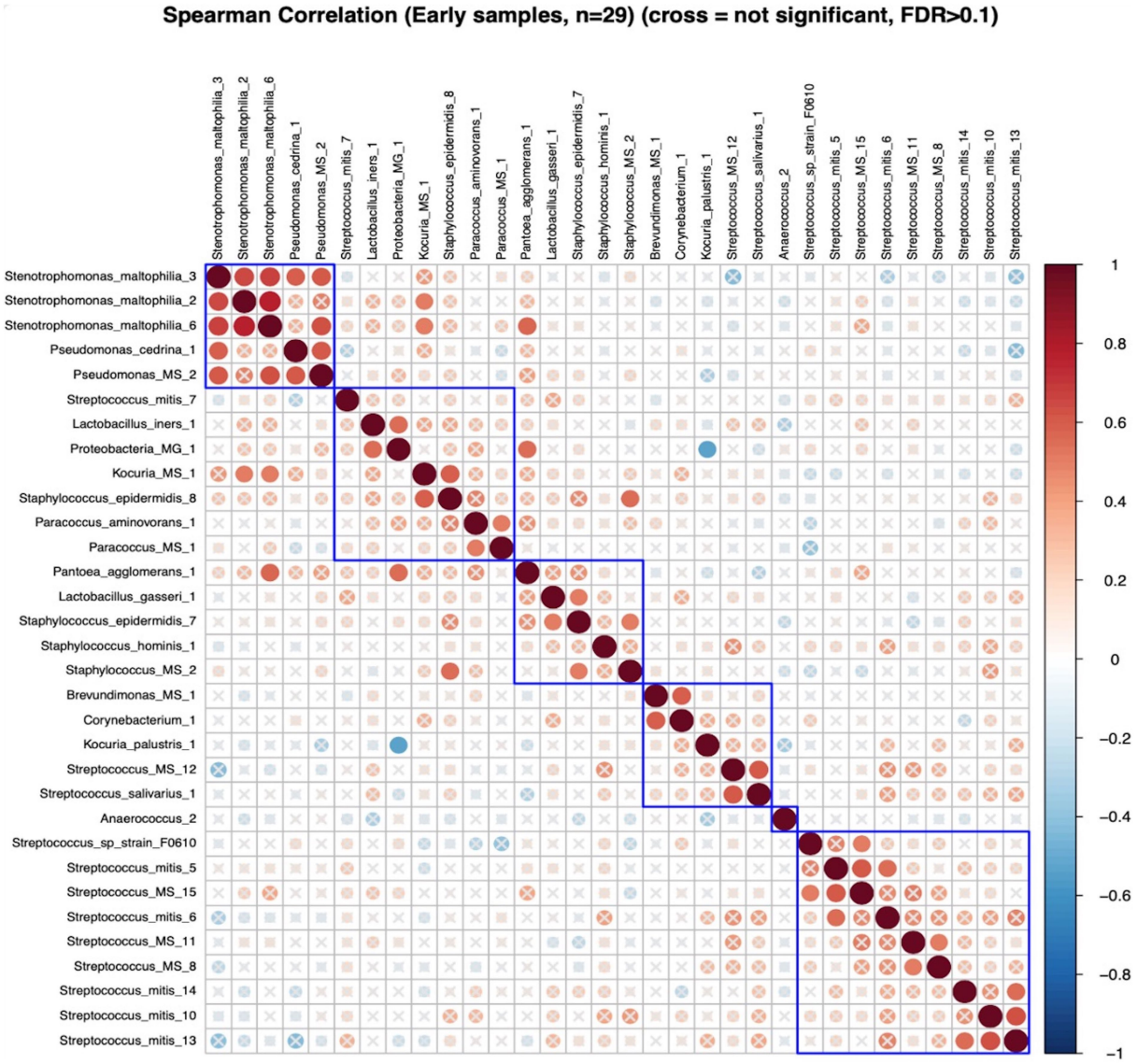

FIGURE 4
Hierarchical clustering of Spearman's rank correlation heatmap between differentially abundant ESVs in early lactation. Red circles represent positive correlations and blue circles represent negative correlations. The blue boxes represent the clusters. Cluster 1: *Stenotrophomonas_maltophilia_3, Stenotrophomonas_maltophilia_2, Stenotrophomonas_maltophilia_6, Pseudomonas_cedrina_1,* and *Pseudomonas_MS_2*, Cluster 2: *Streptococcus_mitis_7, Lactobacillus_iners_1,* and *Proteobacteria_MG_1, Kocuria_MS_1, Staphylococcus_epidermidis_8, Paracoccus_aminovorans_1,* and *Paracoccus_MS_1*, Cluster 3:*Pantoea_agglomerans_1, Lactobacillus_gasseri_1, Staphylococcus_epidermidis_7, Staphylococcus_hominis_1,* and *Staphylococcus_MS_2*, Cluster 4: *Brevundimonas_MS_1, Corynebacterium_1, Kocuria_palustris_1, Streptococcus_MS_12,* and *Streptococcus_salivarius_1*, Cluster 5: *Streptococcus_sp_strain_F0610, Streptococcus_mitis_5, Streptococcus_MS_15, Streptococcus_mitis_6, Streptococcus_MS_11, Streptococcus_MS_8, Streptococcus_mitis_14, Streptococcus_mitis_10,* and *Streptococcus_mitis_13*.

Cluster correlations of DA ESVs and maternal nutrient intakes

In early lactation, six differentially abundant species clusters were identified (Figure 4). One of the DA ESV clusters that included *Brevundimonas_MS_1, Corynebacterium_1, Kocuria_palustris_1, Streptococcus_MS_12, and Streptococcus_salivarius_1* was positively correlated with the maternal nutrient intake cluster that included pantothenic acid, choline, saturated fat, cobalamin, riboflavin, cholesterol, and vitamin D (FDR = 0.048; $r = 0.3$) (Figure 5). In late lactation, six differentially abundant species clusters were identified (Figure 6). However, none of the DA clusters were correlated with maternal nutrient intake clusters.

Univariate analyses of DA HMM by infant growth and maternal nutrient intakes

The DA species by infant growth groups [mildly stunted (LAZ < −1.5SD), non-stunted (LAZ ≥ −1.5SD), mild underweight (WAZ < −1SD), normal weight (WAZ ≥ −1SD), normal head-circumference (HCAZ ≥ −1SD), and smaller head-circumference (HCAZ < −1SD)] revealed multiple positive and negative correlations with maternal nutrient intakes in early lactation (Figures 7A–C) and late lactation (Figures 8A–C). Data are presented using heatmaps of Spearman rank-order correlation coefficient analysis (FDR < 0.1).

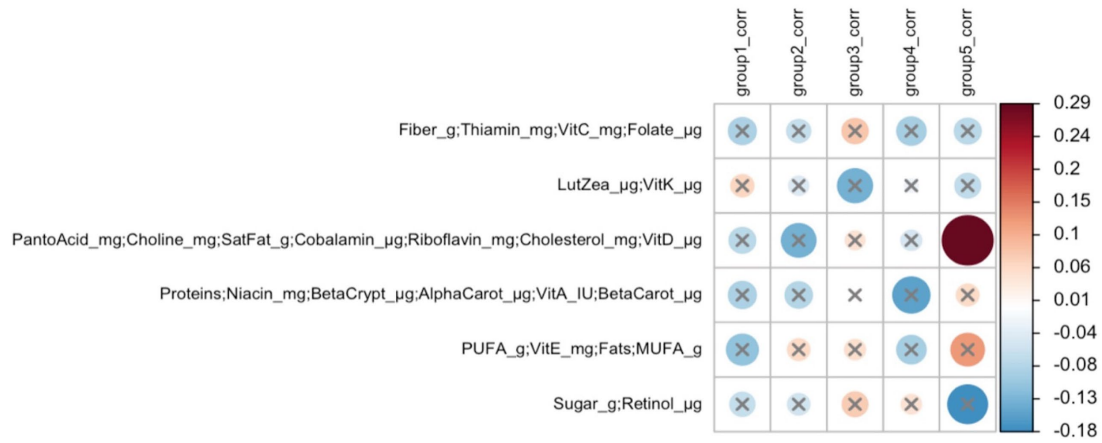

FIGURE 5
Heatmap of Mantel tests between maternal nutrient intake clusters (early lactation; Euclidean distance metric) and differentially abundant ESV clusters (based on Bray-Curtis distance metric). Red circles represent positive correlations and blue circles represent negative correlations. The intensity of the colors represents the degree of association. The solid circles represent significant correlations (FDR > 0.1). Cluster 5: *Brevundimonas_MS_1, Corynebacterium_1, Kocuria_palustris_1, Streptococcus_MS_12,* and *Streptococcus_salivarius_1* were significantly correlated with Cluster 3 of maternal nutrient intakes: pantothenic acid, choline, saturated fat, cobalamin, riboflavin, cholesterol, and vitamin D (FDR = 0.048; $r = 0.3$).

Correlations of DA ESVs and maternal nutrient intakes in early lactation with infant growth

LAZ

In total, 11 DA ESVs were identified; 9 were significantly more abundant in the mildly stunted (LAZ < −1.5SD) group compared with only 2 ESVs in the non-stunted (LAZ ≥ −1.5SD) group. Of the 9 DA species in the LAZ < −1.5SD, only 2, *Streptococcus_mitis, Streptococcus_mitis_14,* and *Streptococcus_mitis_5,* presented 10 distinct correlations with distinct maternal nutrient intakes. In terms of macronutrients, *Streptococcus mitis* was positively correlated with carbohydrates (FDR = 0.089; $r = 0.32$) but was negatively correlated with protein (FDR = 0.069; $r = -0.34$). In terms of micronutrients, both *Streptococcus_mitis_14* and *Streptococcus_mitis_5* were negatively correlated with alpha carotene (FDR = 0.073; $r = -0.34$) and (FDR = 0.058; $r = -0.35$) and beta carotene (FDR = 0.069; $r = -0.34$) and (FDR = 0.05; $r = -0.37$), respectively. *Streptococcus_mitis_14* was also negatively correlated with maternal intake of niacin (FDR = 0.009; $r = -0.47$), folate (FDR = 0.083; $r = -0.33$), and cobalamin (FDR = 0.087; $r = -0.32$), while *Streptococcus_mitis_5* was negatively correlated with maternal intake of vitamin A (FDR = 0.026; $r = -0.41$) (Figure 7A).

WAZ

Fifteen DA ESVs were identified as more abundant in the normal weight (WAZ ≥ −1SD) group; no DA species was identified in the mildly underweight (WAZ < −1SD) group. Of the 15 DA species, 4 DA species included two normal human microflora that were positively correlated with maternal nutrient intakes: *Streptococcus_salivarius_1* with lutein + zeaxanthin (FDR = 0.088; $r = 0.32$) and *Staphylococcus_epidermidis_8* with vitamin K (FDR = 0.094; $r = 0.32$). Two other correlated DAs were ambiguous species; these included *Kocuria_MS_1* which was positively correlated with energy intake (FDR = 0.038; $r = 0.39$) and *Pseudomonas_MS_2* which was negatively correlated with maternal intake of thiamin (FDR = 0.025; $r = -0.41$). The remaining DA species, *Streptococcus_mitis_14,* was also associated with the LAZ < −1.5SD group and presented similar correlations with maternal nutrient intakes that included negative nutrient correlations with both vitamin B and vitamin A precursors. These included negative correlations with niacin (FDR = 0.009; $r = -0.47$), folate (FDR = 0.083; $r = -0.33$), cobalamin (FDR = −0.087; $r = -0.32$), alpha carotene (FDR = 0.073; $r = -0.34$) and beta carotene (FDR = 0.069; $r = -0.34$), and protein (FDR = 0.069; $r = -0.34$) (Figure 7B).

HCAZ

In total, thirteen DA ESVs differed between the HCAZ groups; 12 were DA in the normal HC (HCAZ ≥ −1 SD) group compared with only 1 in the smaller HC (HCAZ < −1 SD) group. Three DA species of the normal HCAZ group were correlated with different maternal nutrient intakes. These correlations included two negative correlations: *Streptococcus_sp_strain_F0610* with pantothenic acid (FDR = 0.04; $r = -0.38$) and *Proteobacteria* at the genus-level and *Proteobacteria_MG_1* with vitamin K (FDR = 0.085; $r = -0.32$) and one positive correlation between *Pantoea_agglomerans_1* and retinol (FDR = 0.064; $r = 0.35$) (Figure 7C).

Correlations of DA ESVs and maternal nutrient intakes in late lactation with infant growth

LAZ

In total, 19 DA ESVs were identified between the LAZ groups; 15 were associated with the non-stunted (LAZ ≥ −1.5SD) group and 7 were correlated with maternal nutrient intakes. These DA species had both positive and negative correlations with diverse nutrients. Three were *Streptococcus* species that were correlated with maternal intakes of vitamin A and its precursors. These included *Streptococcus_4* that was negatively correlated with vitamin A (FDR = 0.011; $r = -0.42$), beta carotene (FDR = 0.021; $r = -0.4$), and alpha carotene (FDR = 0.067; $r = -0.31$), *Streptococcus_salivarius_5* was negatively correlated with retinol (FDR = 0.084; $r = -0.3$), and *Streptococcus_MS_16* was positively correlated with vitamin A (FDR = 0.097; $r = 0.3$), beta carotene (FDR = 0.091; $r = 0.3$), pyridoxine (FDR = 0.069; $r = 0.31$), and fiber (FDR = 0.093; $r = 0.3$). Other DA species were correlated with the fat component of the diet.

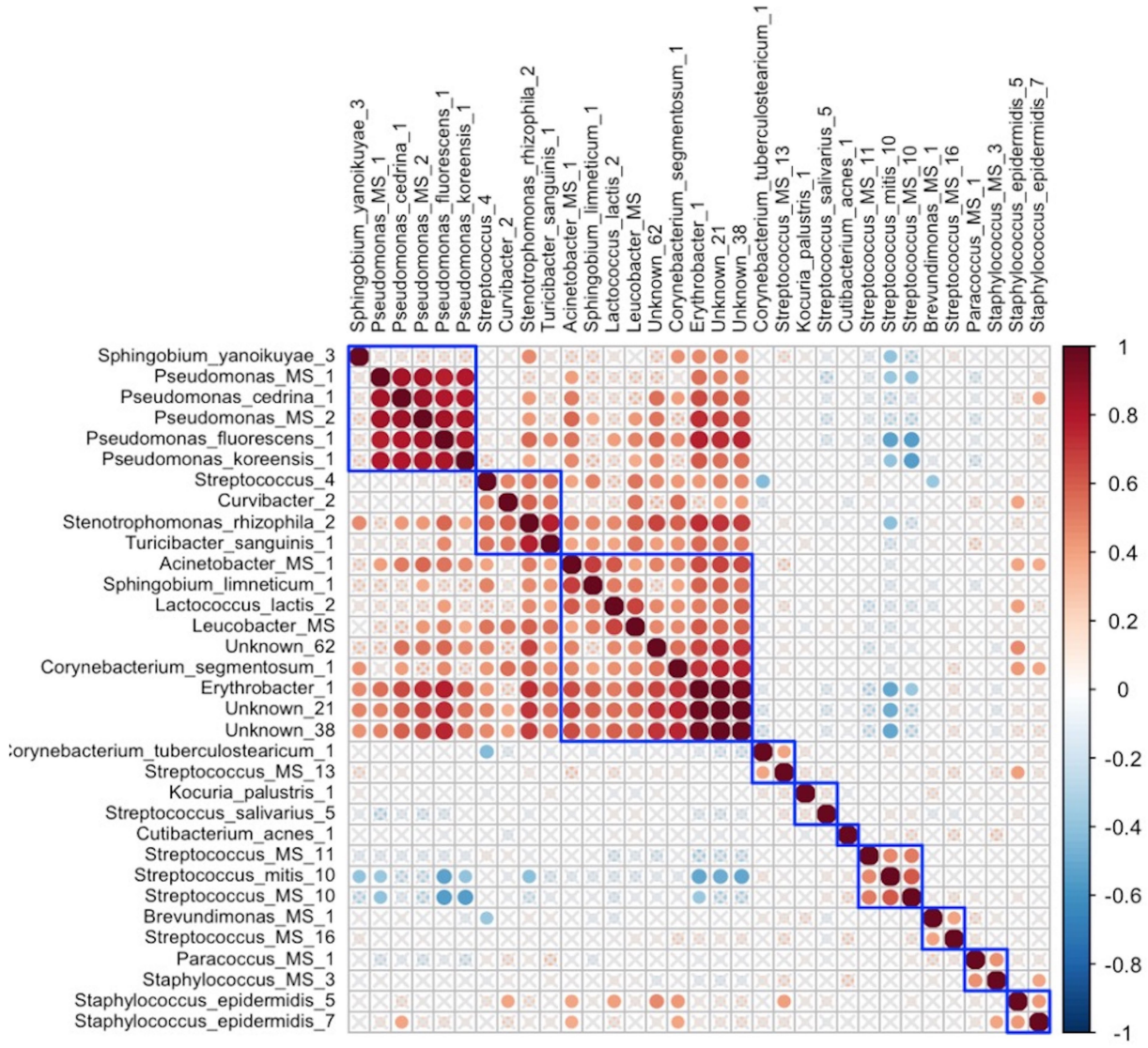

FIGURE 6
Hierarchical clustering of Spearman's rank correlation heatmap between differentially abundant ESVs in late lactation. Red circles represent positive correlations and blue circles represent negative correlations. The blue boxes represent inferred clusters. Cluster 1: *Pseudomonas_MS_1, Pseudomonas_cedrina_1, Pseudomonas_MS_2, Pseudomonas_fluorescens_1,* and *Pseudomonas_koreensis_1,* Cluster 2: *Streptococcus_4, Curvibacter_2, Stenotrophomonas_rhizophila_2,* and *Turicibacter_sanguinis_1,* Cluster 3: *Acinetobacter_MS_1, Sphingobium_limneticum_1, Lactococcus_lactis_2, Leucobacter_MS, Unknown_62, Corynebacterium_segmentosum_1, Erythrobacter_1, Unknown_21,* and *Unknown_38,* Cluster 4: *Streptococcus_MS_11, Streptococcus_mitis_10,* and *Streptococcus_MS_10,* Cluster 5: *Brevundimonas_MS_1* and *Streptococcus_MS_16,* Cluster 6: included *Paracoccus_MS_1* and *Staphylococcus_MS_3*, and Cluster 7: *Staphylococcus_epidermidis_5* and *Staphylococcus_epidermidis_7*.

These included *Acinetobacter_MS_1* that was positively correlated with fat (FDR = 0.065; $r = 0.32$) and choline (FDR = 0.088; $r = 0.3$), *Corynebacterium_segmentosum_1* was positively correlated with PUFA (FDR = 0.057; $r = 0.32$) and negatively correlated with retinol (FDR = 0.057; $r = -0.32$), and *Staphylococcus_epidermidis_5* was negatively correlated with vitamin D (FDR = 0.099; $r = -0.3$). Finally, one DA, *Sphingobium_yanoikuyae_3*, was positively correlated with protein (FDR = 0.066; $r = 0.31$) (Figure 8A).

Of the DA in the mildly stunted (LAZ < −1.5SD) group, we observed mainly correlations with maternal intakes of carotenes and vitamin B. These included positive correlations between *Brevundimonas_MS_1* and maternal intakes of beta-cryptoxanthin (FDR = 0.031; $r = 0.4$), beta carotene (FDR = 0.063; $r = 0.32$), and vitamin A (FDR = 0.025; $r = 0.4$) and between *Paracoccus_MS_1* and maternal intake of thiamin (FDR = 0.09; $r = 0.3$). Only *Streptococcus_MS_10* was negatively correlated with pantothenic acid (FDR = 0.08; $r = -0.3$) (Figure 8A).

WAZ

Eight DA ESVs were identified between the WAZ groups. Five were significantly more abundant in the normal weight group (WAZ ≥ −1SD) and three in the mildly underweight (WAZ < −1SD) group. Among these eight DA species, four species were correlated with maternal nutrient intakes, two DA species were associated with each group. The two DA species associated with the normal weight (WAZ ≥ −1SD) group were *Streptococcus*

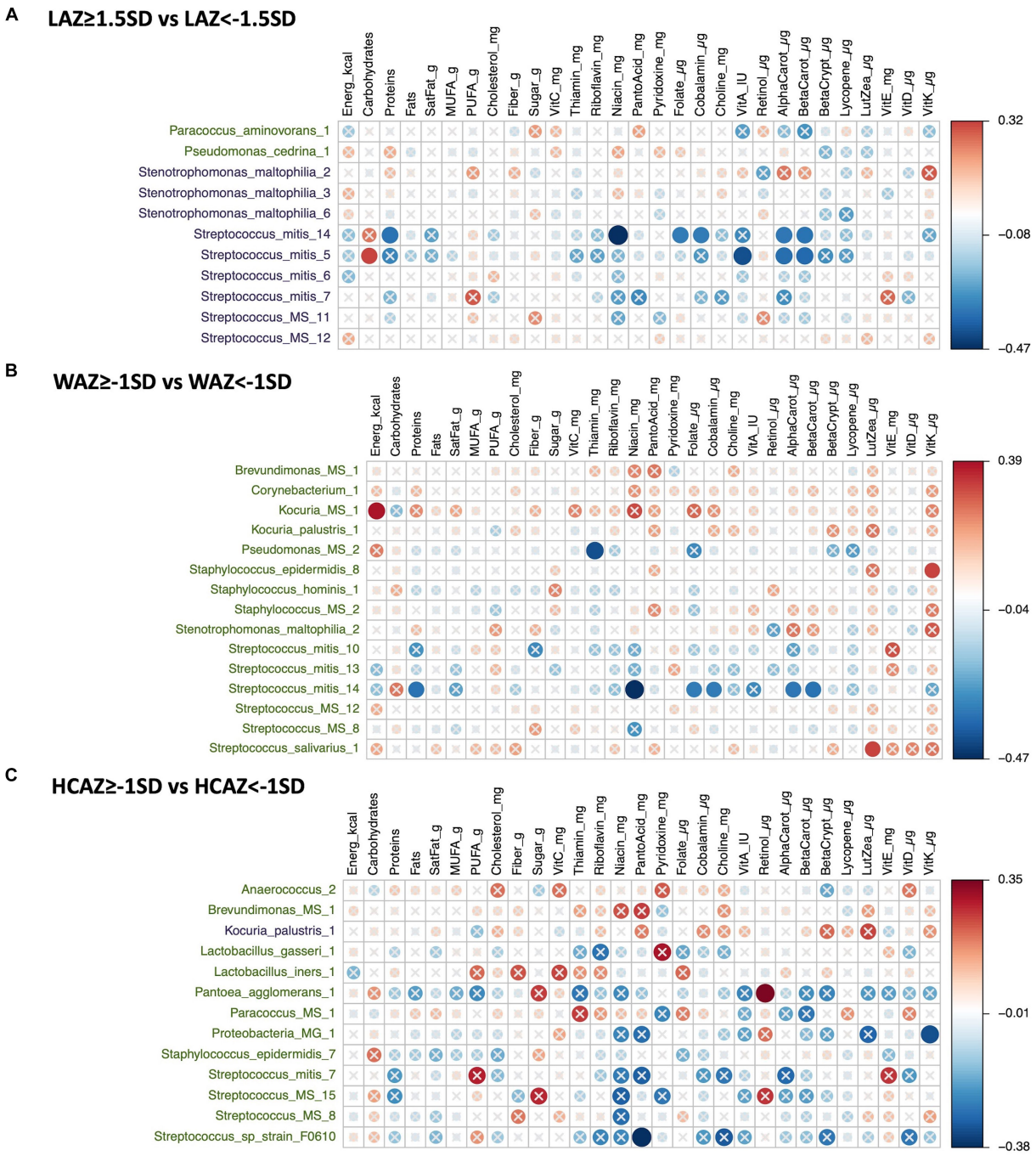

FIGURE 7
Heatmap of a univariate Spearman correlation matrix between the differentially abundant (DA) human milk microbiome (HMM) ESVs abundance (rlog; y-axis) *vs* maternal dietary information in early lactation (x-axis). **(A)** Shows the correlation between DA ESVs in the HMM of the mothers of non-stunted infant (LAZ ≥ −1.5SD) and the HMM of the mothers of mildly stunted infants (LAZ < −1.5SD) with maternal dietary information, **(B)** shows the correlation between DA ESVs in the HMM of the mothers of infants with normal weight (WAZ ≥ −1 SD) and the HMM of the mothers of infants with mild underweight (WAZ < −1SD) with maternal dietary information, and **(C)** shows the correlation between DA ESVs in the HMM of the mothers of infants with normal head-circumference (HCAZ ≥ −1 SD) and the HMM of the mothers of infants with mild smaller head-circumference (HCAZ < −1SD) with maternal dietary information. The black-colored taxa are the differentially abundant ESVs in the mild growth faltering groups: **(A)** LAZ < −1.5SD, **(B)** WAZ < −1SD, **(C)** HCAZ < −1SD. The green-colored taxa are the differentially abundant ESVs in the normal growth groups: **(A)** LAZ ≥ −1.5SD, **(B)** WAZ ≥ −1SD, **(C)** HCAZ ≥ −1SD. Red circles represent positive correlations and blue circles represent negative correlations. The intensity of the colors represents the degree of association between the HMM DA ESVs and nutrients as measured by Spearman's correlations. The solid circles represent significant correlations (FDR > 0.1).

that were mainly correlated with maternal intake of vitamin A-related nutrients. *Streptococcus_MS_16* was positively correlated with beta carotene (FDR = 0.091; $r = 0.3$), vitamin A (FDR = 0.097; $r = 0.3$), pyridoxine (FDR = 0.069; $r = 0.31$), and fiber (FDR = 0.093; $r = 0.3$). In contrast, *Streptococcus_salivarius_5* was negatively correlated with retinol (FDR = 0.084; $r = -0.3$) (Figure 8B).

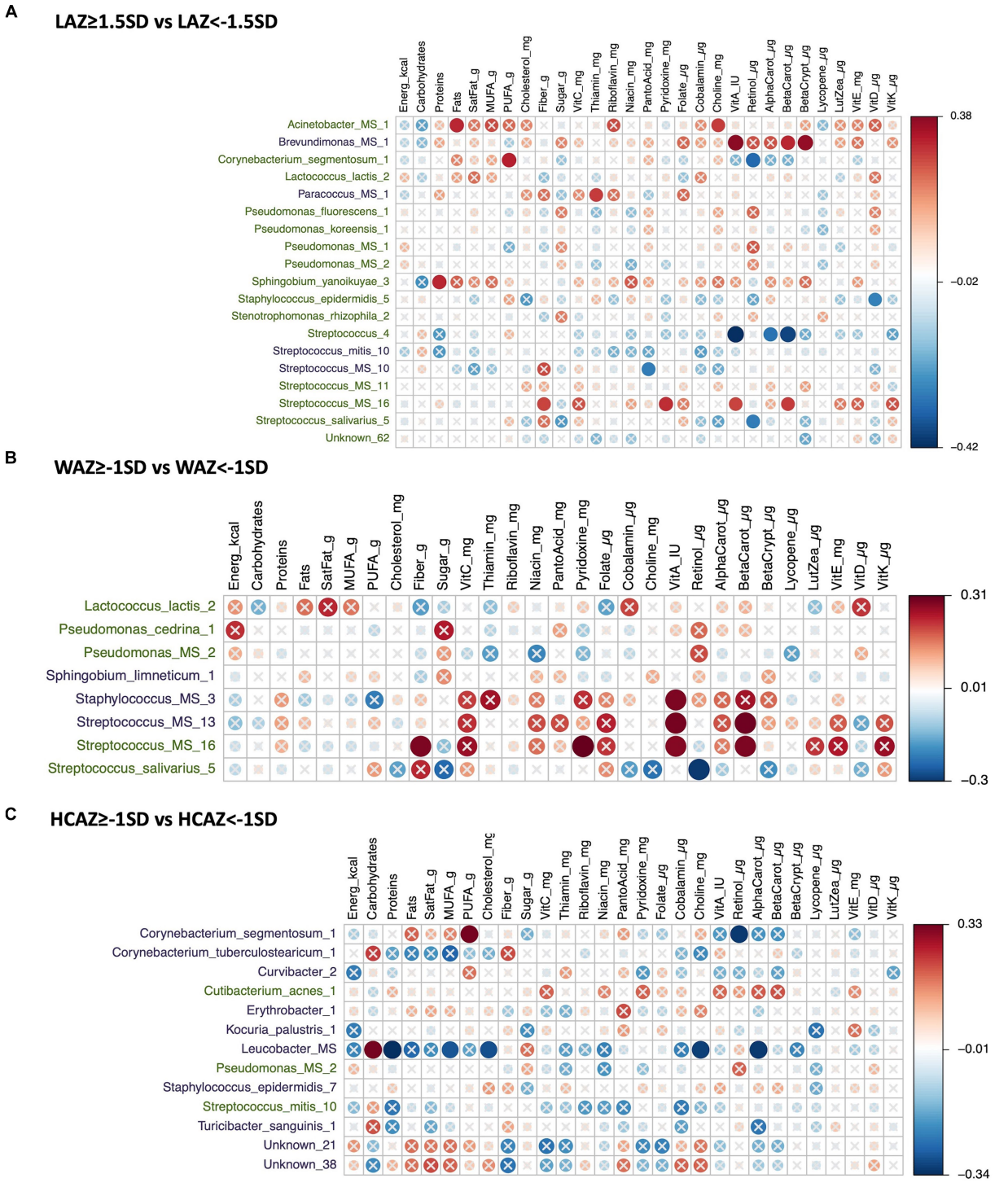

FIGURE 8
Heatmap of a univariate Spearman correlation matrix between the differentially abundant (DA) human milk microbiome (HMM) ESVs abundance (rlog; y-axis) *vs* maternal dietary information in late lactation (x-axis). **(A)** Shows the correlation between DA ESVs in the HMM of the mothers of non-stunted infant (LAZ ≥ −1.5SD) and the HMM of the mothers of midly-stunted infants (LAZ < −1.5SD) with maternal dietary information, **(B)** shows the correlation between DA ESVs in the HMM of the mothers of infants with normal weight (WAZ ≥ −1 SD) and the HMM of the mothers of infants with mild underweight (WAZ < −1SD) with maternal dietary information, and **(C)** shows the correlation between DA ESVs in the HMM of the mothers of infants with normal head-circumference (HCAZ ≥ −1 SD) and the HMM of the mothers of infants with mild smaller head-circumference (HCAZ < −1SD) with maternal dietary information. The black-colored taxa are the differentially abundant ESVs in the mild growth faltering groups: **(A)** LAZ < −1.5SD, **(B)** WAZ < −1SD, **(C)** HCAZ < −1SD. The green-colored taxa are the differentially abundant ESVs in the normal growth groups: **(A)** LAZ ≥ −1.5SD, **(B)** WAZ ≥ −1SD, **(C)** HCAZ≥ −1SD. Red circles represent positive correlations and blue circles represent negative correlations. The intensity of the colors represents the degree of association between the HMM DA ESVs and nutrients as measured by the Spearman's correlations. The solid circles represent significant correlations (FDR > 0.1).

The two DA species associated with the mildly underweight (WAZ < −1SD) group were two that were positively correlated with vitamin A: *Staphylococcus_MS_3* (FDR = 0.092; $r = 0.3$) and *Streptococcus_MS_13* (FDR = 0.088; $r = 0.3$) and beta carotene (FDR = 0.08; $r = 0.3$) (Figure 8B).

HCAZ

Thirteen DA ESVs were identified between the HCAZ groups, 3 in the normal HC (HCAZ ≥ −1 SD) group and 10 in the smaller HC (HCAZ < −1 SD) group. All the identified correlations between maternal nutrient intakes and DA species were identified with only two DA species, *Corynebacterium_segmentosum_1* and *Leucobacter_MS,* which were uniquely associated with the smaller HC (HCAZ < −1 SD) group. These mainly negative correlations included *Corynebacterium_segmentosum_1* with retinol (FDR = 0.057; $r = -0.32$) and *Leucobacter_MS* with protein (FDR = 0.044; $r = -0.34$), choline (FDR = 0.059; $r = -0.32$), alpha carotene (FDR = 0.054; $r = -0.33$), MUFA (FDR = 0.088; $r = -0.3$), and cholesterol (FDR = 0.079; $r = -0.3$). Other positive correlations with these two DAs were *Corynebacterium_segmentosum_1* with PUFA (FDR = 0.057; $r = 0.32$) and *Leucobacter_MS* with carbohydrates (FDR = 0.054; $r = 0.33$) (Figure 8C).

Discussion

A limited number of studies had previously reported associations between maternal nutrient intakes during lactation that had uncovered both multiple positive and negative associations with the HMM at the phylum and genus levels using correlation and cluster analyses (23–26). Since associations between the HMM and maternal nutrient intakes with infant growth parameters had not been explored, we also investigated associations of maternal nutrient intakes with previously established DA species that differed between infants with 'normal infant z-scores' defined as WAZ ≥ −1SD, LAZ ≥ 1.5SD, and HCAZ ≥ −1SD compared with infants experiencing growth faltering defined as mildly underweight (WAZ < −1SD), mildly stunted (LAZ < −1.5SD), and with smaller head circumferences (HCAZ < −1SD) by the stage of lactation (44, 45). Moreover, given that human milk continuously provides an infant with a dynamically changing community of commensal and potentially beneficial bacteria that differs between early and late lactation (43), we conducted our analyses at the species level during both early and late lactation.

Several novel findings emerged. First, in early lactation, cluster analyses revealed that infant z-scores were correlated with maternal energy intakes, and infant length parameters (height and LAZ) were correlated with maternal intakes of carbohydrates, riboflavin, and saturated fat. Second, with regard to maternal nutrient intake clusters, maternal intakes of lutein + zeaxanthin and vitamin K were correlated with infant growth parameters as a cluster that included infant weight and infant head circumference. However, in late lactation, infant anthropometric clusters were not correlated with maternal nutrient intakes or nutrient clusters. Third, univariate analyses in early and late lactation revealed multiple correlations between HMM DA taxa associated with infant growth and maternal nutrient intakes during lactation. Several of these were negative correlations of *Streptococcus* species with maternal intakes of vitamin B and vitamin A precursors during lactation. In late lactation, more environmental and ambiguous differentially abundant species were identified in the human milk microbial community compared with early lactation, and these taxa were correlated with several maternal nutrient intakes. Interestingly, the ambiguous environmental DA taxa uniquely identified in the smaller HC (HCAZ < −1 SD) group was *Leucobacter_MS*, which was negatively correlated with maternal nutrient intakes of choline that is required for the biosynthesis of the neurotransmitter acetylcholine.

Maternal nutrient intakes and infant growth parameters

Maternal intakes between early and late lactation showed comparable dietary intakes that only differed in vitamin E, which was higher in early lactation; however, maternal dietary intakes of macronutrients differed between infant z-scores. In our study, maternal intake of dietary fat and/or fat components and vitamin E were associated with both infant WAZ and HCAZ but not LAZ during the first 6 months of lactation. Mothers of infants with normal weight (WAZ ≥ −1SD) had higher intakes of total calories and carbohydrates and higher intakes of energy and fat including total fat, MUFA, PUFA, and vitamin E. Mothers of infants with normal head-circumference (HCAZ ≥ −1 SD) also consumed higher MUFA and vitamin E. Previous studies investigating maternal intakes of dietary fat and fat components have described associations of higher dietary fat and fat component intakes with higher infant adiposity during the first 6 months of lactation (70) and higher lipid components in breast milk with infant of length and weight up to 12 months postpartum (71).

There is also evidence that maternal concentrations of tocopherols also have been positively associated with better infant growth for at least one parameter (weight, length, head circumference (72), and percentile rankings for weight, length, and head circumference at birth (73)). There is also evidence in an older investigation that plasma concentrations of oxidative metabolites were higher, and levels of vitamins A, C, and E were lower in low-birth-weight infants compared with a normal birth-weight control group (74). More recent evidence continues to support the concept that oxidative stress during pregnancy may be associated with low birth weight, preterm delivery, and oxidative stress-related diseases (75). In our study, maternal dietary intakes of vitamin E were higher among mothers of infants with normal WAZ and HCAZ compared with underweight infants and infants with smaller HCAZ. Collectively, our findings highlight that the importance of adequate maternal energy, dietary fat, and vitamin E intakes in lactating mothers from marginalized communities that, if not adequate, do have consequences for early infant growth and can be associated with growth faltering and lower WAZ and HCAZ during the first 6 months of lactation.

Cluster analyses associating nutrients with anthropometry

In early lactation, cluster analyses revealed that the infant z-scores were correlated with maternal energy intakes, and infant length parameters (height and LAZ) were correlated with maternal intakes of carbohydrates, riboflavin, and saturated fat. Among the Guatemalan

population, higher energy intake is derived mainly from carbohydrates with lower intakes of animal proteins and dietary fat (76, 77). This was reflected in our study by the high carbohydrate intake (78% of the total energy) and the low intakes of saturated fat (2.8% of the total energy intake). When compared with the Acceptable Macronutrient Distribution Range (AMDR) for macronutrients, carbohydrate intakes in our population were above the upper AMDR limit of 65% and below the upper limit for saturated fat of <10%. Moreover, higher intakes of carbohydrates in our study population were negatively correlated not only with protein and fat intakes as a percentage of overall energy intakes but also with individual dietary lipids including saturated fat, MUFA, cholesterol, and micronutrients including two fat-soluble vitamins E and D and a range of vitamin B and vitamin C that slightly differed between early and late lactation.

Maternal nutrient intakes of lutein + zeaxanthin and vitamin K emerged as a cluster that was correlated with the infant growth cluster of infant head circumference, infant weight, and infant age in days. Lutein and zeaxanthin are fat-soluble dietary carotenoids found in dark green leafy vegetables and egg yolks and corn (78), which are principle components of the Mayan diet (76, 77). They have powerful anti-inflammatory properties that reduce oxidative stress and increase antioxidant capacities in newborns when supplemented during the first days of life (79, 80). Lutein and zeaxanthin are found with high concentrations in the brain tissues, and they were associated with cognitive function in young and older adults (81–84). In preterm infants, lutein was the prevalent carotenoid in the developing brain, and its concentration was lower in preterm neonates compared with term neonates (85), and when supplemented in healthy infants, it supported physical growth including HC growth (86). Our findings were associated with improved HC growth among infants of mothers with higher lutein and zeaxanthin intakes in early lactation.

Associations of maternal dietary intakes with the HM and the HMM

Studies exploring associations of maternal nutritional status with the vitamin composition of breastmilk have revealed that some vitamins might be inadequate for the growing infant, as a consequence of compromised maternal nutritional status (87–97) and poor maternal diet (97–100). However, the latest systematic review on HM components and infant anthropometry in the first 2 years of life among term infants uncovered no eligible studies for several vitamins including, riboflavin, C, D, E, and K and concluded that for others, the available evidence was largely inconclusive and further studies were needed (101). In our exploratory study in Guatemala where most women breastfeed but infants experience growth faltering by 3 months (40, 102), we were able to observe correlations between selected nutrient intakes, the HMM, and infant growth.

Researchers had previously associated several macronutrients including protein, fat, and fiber with HMM at the phylum and genera levels. Reportedly, maternal protein intakes have been associated with higher HM *Gemella* (25, 26), and maternal fiber intakes have been associated with one member of the Bacillota phylum and negatively with *Bifidobacterium*, *Ctibacterium, and Serratia.* At the genus level, total dietary fiber and insoluble dietary fiber intakes were negatively associated with *Finegoldia* and *Streptococcus* (23, 24), whereas *Veillonella* was positively associated with total fiber and soluble and insoluble fibers (26). Furthermore, these studies reported several associations between maternal intakes of the different dietary fat components (23–26) and vitamin B (23, 25) with the HMM. For example, maternal intakes of MUFA were negatively correlated with *Corynebacterium* (25) and *Sediminibacterium* at the genus level (26). In addition, maternal intakes of PUFA were positively correlated with *Gemella* (25), *Bifidobacterium,* and *Atopobium* (23) and negatively correlated with *Acinetobacter* (24), *Bifidobacterium, Serratia*, and *Ralstonia* (26). Furthermore, several B vitamins were reported to have negative correlations with HMM including, pantothenic acid with *Streptococcus* (25) and thiamin and folate with *Granulicatella* (23). In our study, we took the analysis a step further, and we uncovered maternal dietary intake associations at the species level.

Maternal nutrient intakes and DA human milk microbiome by infant growth

Our exploratory study uncovered previously unidentified correlations among maternal macronutrient and micronutrient intakes, infant growth in breast-fed infants, and the HMM in both early and late lactation. In both stages, univariate analyses of the DA HMM species by infant growth z-scores revealed multiple correlations with specific maternal nutrient intakes.

In our population, in early lactation, *Streptococcus_mitis* dominated the DA species in the mildly stunted (LAZ < −1.5SD) group with four distinct *Streptococcus_mitis species (Streptococcus_mitis_5, Streptococcus_mitis_6, Streptococcus_mitis_7, and Streptococcus_mitis_14)* and was positively correlated with maternal carbohydrate intakes, which are associated with plaque oral microbiome abundance and diversity (103–105). *Streptococcus_mitis* is identified as an oropharynx bacterium that has been found in the infant oral microbiome within a few days after birth (106). However, it is responsible for the development of dental caries (107, 108), and it is considered an opportunistic pathogen (109, 110), and it has demonstrated the highest level of penicillin resistance (111). Furthermore, there is existing evidence to support this negative association between oral bacteria involved in dental plaque with poor prenatal outcomes for infant birth-weight (112, 113). Our previous findings showed that the majority of women had periodontal disease in the forms of caries (78%) and/or gingivitis (66%) (46), suggesting potential impacts of the oral potentially pathogenic bacteria in the HMM on infant growth during lactation that might be exacerbated by a low-quality diet and higher carbohydrate intakes.

In addition, in early lactation, other DA *Streptococcus* species were negatively correlated with maternal intakes of B vitamins and vitamin A precursors regardless of the growth classification. *Streptococcus_sp_strain_F0610* in the normal head circumference (HCAZ ≥ −1SD) group was negatively correlated with maternal intake of pantothenic acid. Although this finding might be contradictory to what is known about *Streptococci* that require vitamin B5 for their growth *in vitro* (114), this finding is consistent with the study by Williams et al. (25) who reported that maternal pantothenic acid intake during lactation was negatively related to *Streptococcus* at the genus level (25). Pantothenate is involved in the Coenzyme A (CoA) synthesis, which functions as an acyl carrier and is a required cofactor for all living cells. Pantothenate analogs also have been shown to markedly suppress the growth of some opportunistic pathogens by inhibiting phosphorylation activity, which

catalyzes the first step of the CoA biosynthetic pathway in specific bacteria, such as *Staphylococcus* species that inhabit human skin including *Staphylococcus epidermidis, Staphylococcus saprophyticus, and Staphylococcus aureus* (115, 116), the most common microorganism identified in mastitis among lactating mothers (117, 118), suggesting pantothenate to be effective in preventing infections by opportunistic pathogens. In our study, *Staphylococcus aureus* was not identified as DA by infant growth and was not correlated with maternal intake of vitamin B5, possibly due to our exclusion criteria of mastitis and sub-clinical mastitis which might have yielded low abundance of *Staphylococcus aureus* in our study population.

In late lactation, most *Streptococcus species* exhibited similar patterns to those observed in early lactation; however, some exceptions were observed among the *Streptococcus ambiguous* species. In late lactation, a *Streptococcus species, Streptococcus_MS_10,* in the mildly stunted (LAZ < −1.5SD) group was negatively correlated with pantothenic acid, and *Streptococcus_salivarius_5* in the HMM of mothers of infants with WAZ ≥ −1SD was negatively correlated with maternal intake of retinol. However, there were two exceptions among the species of *Streptococcus ambiguous* that were positively correlated with maternal nutrient intake. These included the species *Streptococcus_MS_16,* which was DA in both the non-stunted (LAZ ≥ −1.5SD) group and the normal weight (WAZ ≥ −1SD) group, and was positively correlated with maternal intake of pyridoxine, beta carotene, fiber, and vitamin A, and the DA *Streptococcus_MS_13* in the latter group was positively correlated with maternal intakes of vitamin A and beta carotene.

In late lactation, one interesting correlation was observed with a non-Streptococcus ambiguous species. Interestingly, *Leucobacter_MS*, which were uniquely associated with a smaller head circumference (HCAZ < −1SD), was negatively correlated with maternal choline intakes, a nutrient required for the biosynthesis of the neurotransmitter acetylcholine. *Leucobacter_MS* was also negatively correlated with maternal intakes of protein, alpha-carotene, MUFA, and cholesterol but was positively correlated with carbohydrate intakes, possibly suggesting a higher abundance with a low-quality maternal diet.

Environmental bacteria in human milk linked to maternal intake and infant growth

In late lactation, HMM was more diverse and included ambiguous and environmental species. The latest systematic review of the HMM species origin reported that more than half of the studied species were first isolated from the environmental sources, suggesting their normal presence in human milk (119). In rural agricultural communities, which was the case for our study population, environmental bacteria contribute to the gut microbiome (120), which is one of the main sources of the HMM (9–13). In our study in late lactation, environmental bacteria were found in the HMM of both LAZ and WAZ infant subgroups (normal LAZ, mildly-stunted, normal WAZ, and mild underweight). Guatemalan mothers participate in fruit and vegetable harvesting (46), and they have a high interaction with the Guatemalan-rich soil (121), which would support the presence of environmental bacteria in the Guatemalan mother's milk and might be considered as integral components of breast milk in agricultural societies as previously reported (119).

In our population, we observed both non-pathogenic and potentially pathogenic environmental bacteria among infant z-score groups, which were also correlated with maternal nutrient intakes. In late lactation, the ambiguous species, *Leucobacter_MS,* was uniquely differentially abundant in the smaller HC (HCAZ < −1 SD) group. This ambiguous species could be either *Leucobacter_komagatae* or *Leucobacter_aridicollis,* and both have been isolated from contaminated plant and water environments (122, 123). Interestingly, this taxon was negatively correlated with the maternal intake of choline, which is a nutrient required to produce the neurotransmitter acetylcholine, and alpha-carotene, and its higher plasma levels were associated with higher cognitive scores in adults (124). *Leucobacter_MS* was also negatively correlated with protein, alpha-carotene, MUFA, and cholesterol, possibly suggesting a higher abundance with a low-quality maternal diet. This negative correlation is consistent with the finding of the lower intakes of MUFA among mothers of infants with smaller head circumferences (HCAZ < −1 SD). On the other hand, *Pantoea_agglomerans_1* was DA in the normal head circumference (HCAZ ≥ −1 SD) group and was positively correlated with maternal retinol intake. *Pantoea_agglomerans* is a plant non-pathogenic bacterium to humans that rarely causes opportunistic human infections (125–127).

Among the DA species between the LAZ and WAZ comparative groups, several ambiguous and environmental taxa were correlated with maternal nutrient intakes. Two ambiguous taxa in the non-stunted (LAZ ≥ −1.5SD) group were positively correlated with maternal nutrient intakes. The first one was *Acinetobacter_MS_1*, which could be either the commensal human skin and mucosal colonizer *Acinetobacter_lwoffii* (formerly known as *Acinetobacter calcoaceticus* var. *lwoffii*) (128, 129) or the non-pathogenic environmental species *Acinetobacter_guillouiae*, which was isolated from soil and water (Yoon et al., 2014). *Acinetobacter_MS_1* was positively correlated with higher maternal intakes of fat and choline. In contrast, among the DA in the mildly stunted (LAZ < −1.5SD) group, two ambiguous environmental taxa that were associated with soil and water were positively correlated with maternal dietary intake. *Brevundimonas_MS_1* was positively correlated with maternal intakes of vitamin A, beta-cryptoxanthin, and beta-carotene. This ambiguous taxon could be either *Brevundimonas_vesicularis or Brevundimonas_nasdae* that were isolated from the soil and aquatic environments, respectively (130–132). In addition, *Paracoccus_MS_1* was positively correlated with maternal intake of thiamin. This ambiguous taxon could be either *Paracoccus_carotinifaciens or Paracoccus_marcusii*. The latter has been shown to improve growth, elevate antioxidant properties, suppress the expression of some inflammatory genes in marine animals (133, 134), and increase the probiotic properties of whey proteins (135).

Strengths and limitations

Our study included major strengths. First, although our study had a cross-sectional design, milk samples and metadata were collected at two stages of lactation, allowing us to establish the association between the milk microbiome and maternal nutrient intakes. Second, the homogeneity of our cohort might have been an asset, as our population included healthy mothers, free from sub-clinical mastitis, did not take antibiotics, and complied with the

WHO recommendations to breastfeed for 6 months. These factors are known to affect the milk microbiome ecosystem but were absent in our study. Third, mothers were recruited from eight distinct remote communities to minimize the possibility of exchanging microbes among mothers. Fourth, milk samples yielded sufficient DNA extraction capturing six million sequence reads, which allowed proceeding with this secondary analysis. Fifth, we used the ANCHOR method (62), which uses multiple samples and multiple reference databases with the criteria of >99% for identity and coverage to annotate bacteria. These very high criteria provide high confidence and resolution for the annotation at the species level, which maximizes biological discovery. Sixth, we used the hypervariable regions V1–V3 for the sequencing. These regions have been shown to be highly informative and have produced comparable results to the full-length 16S rRNA V1–V9 in the human gut microbiome samples at species level when used in conjunction with an appropriate identity thresholds (136). In our study, we combined the ANCHOR pipeline, which provided high resolution at the species level microbial identification in conjunction with >99% identity and coverage threshold. However, we understand that species should be considered putative even when single species sequences share 100% 16S rRNA gene fragment similarity because many species remain poorly characterized and mistakes exist in major repositories. Seventh, for the first time, this study bridged maternal diet during lactation and the human milk microbiome with infant growth during the first 6 months of life, which could be mediated by the influence of the human milk microbiome on the infant gut microbiome.

We also recognize that our study had several limitations. First, our study was cross-sectional in design, thereby limiting us to correlation analyses and therefore we cannot infer causality. Second, this study might be under-powered, as originally, as it had been powered to detect only differences in infant growth in early and late lactation and not to detect associations with maternal nutrient intakes. Third, some of the nutrients might require more 24-h recalls to estimate the usual intake. Fourth, maternal diet may influence milk composition, providing a pathway through which maternal diet may directly influence offspring growth (137). However, limited evidence is available on the relationship between maternal diet during lactation on infant postnatal growth or adiposity. In our study, we did not assess the milk nutrient compositions. Fifth, in this study, we used the 27F/533R primer. This primer can amplify the core human milk genus *Cutibacterium* (60, 61, 138); however, it has limitations for amplifying *Bifidobacterium* genus (139). Sixth, due to the lack of studies that associate the maternal diet with human milk microbiome and infant growth, the exploratory nature of this study, and the potential significant biological role of some species, correlations with FDR < 0.1 and (rs) $\leq$0.3 or (rs) ≥ -0.3 were included in our study. We used FDR instead of value of p to limit false positive correlations in our study; however, our study might have included some.

Finally, our current study also addressed some concerns found in earlier studies including application of aseptic techniques such as not using breast pumps during milk sample collection and adding cross-contamination control measures performed during recruitment and at different stages of laboratory and bioinformatics analyses. We also collected the 24-h dietary recall data and the milk samples concurrently and controlled our inclusion criteria by excluding antibiotic use or mothers with diabetes. Finally, the milk samples in our study were collected within a time period to ensure that we did not collect colostrum, which might have different microbial characteristics and mothers who did not have elevated sodium:potassium ratios, indicative of breast inflammation and sub-clinical mastitis.

Conclusion

To the best of our knowledge, this study is the first to explore associations between maternal diet, the HMM, and infant z-scores and highlights an overlooked contribution of maternal diet and the HMM on early infant growth that can be mediated by the HMM during early and late lactation. In this exploratory study, we observed that maternal nutrient intakes during lactation were correlated with both infant growth parameters and milk microbiome. However, the presence of multiple positive and negative correlations suggests complex interactions between maternal nutrient intakes, the milk microbiota, and infant growth. Further research is required to understand how human milk micronutrients and microbiomes work independently and together to influence infant growth and identify new avenues for future maternal, newborn, and infant microbiome and nutritional interventions.

Data availability statement

The raw sequence data has been deposited at the European Genome-Phenome Archive (EGAD00001004160) and are available upon request to KK, kristine.koski@mcgill.ca.

Ethics statement

The study began as a collaboration between McGill University and the Center for Studies of Sensory Impairment, Aging, and Metabolism (CeSSIAM), a research organization based in Guatemala. Ethical approvals were obtained from ethics boards at McGill University and at CeSSIAM. Further approvals were obtained from community leaders and the local authorities at the Ministry of Health in Guatemala. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

TA: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. EG: Data curation, Formal analysis, Methodology, Software, Visualization, Writing – review & editing. NS: Funding acquisition, Methodology, Writing – review & editing. MV: Methodology, Writing – review & editing. KK: provided funding

for the 16s rRNA analysis, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1341777/full#supplementary-material

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The epidemiology and associated factors of non-exclusive breastfeeding: a comparative cross-sectional study of livelihood-secure and insecure areas

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Introduction: Regardless of national commitment, non-exclusive breastfeeding (NEBF) is a public health problem that worsens over time. It can be associated with sociodemographic, economic, and environmental factors and may vary depending on livelihood security. Hence, this study aimed to determine the magnitude of NEBF and identify its associated factors by considering two areas with varied degrees of livelihood security.

Methods: This study represented a comparative cross-sectional survey of 1,060 under 6 months (u6m) infant–mother pairs. Both descriptive and analytic statistics were evaluated using STATA version 17 packages. A binary logistic regression was used to identify associated factors of NEBF. The odds ratio (OR) with a 95% confidence interval (CI) was used to measure the significance of the association at a *p*-value of <0.05.

Results: The pooled magnitude of 51% of NEBF mothers (95% CI: 48.0, 54.0) was 53.1% (95% CI: 49.2, 57.0) and 48.1% (95% CI: 43.4, 52.8) in livelihood-secure and livelihood-insecure areas, respectively. The lack of recollecting the infant's birth date by mothers (AOR = 2.4; 95% CI = 1.15–4.40) had the highest odds of NEBF while household heads with tertiary education (AOR = 0.14; 95% CI = 0.01–0.54) and the poorest households (AOR = 0.43; 95%CI = 0.20–0.82) had the lowest odds of NEBF in livelihood-secure areas but not in livelihood-insecure areas. Moreover, mothers with male infants (AOR = 1.9; 95% CI = 1.18–2.92) had high odds of NEBF in livelihood-insecure areas but not in livelihood-secure areas. Infants of 2 to less than 4-month-old (AOR = 8.5; 95% CI = 3.47–18.63) and 4 to less than 6-month-old (AOR = 22.2; 95% CI = 8.02–51.97) in livelihood-secure areas and infants of 2 to less than 4-month-old (AOR = 4.3; 95% CI = 1.29–11.67) and 4 to less than 6-month-old (AOR = 8.3; 95% CI = 2.44–22.39) in livelihood-insecure areas had high odds of NEBF.

Conclusion: Over half of the mothers were practicing NEBF, which represents a failure to meet national and international targets. Area vulnerability to livelihood security modifies factors of NEBF. Male infants in insecure areas, infants of unknown age in secure areas, and infants aged 2 months or older, regardless of

setting, were more vulnerable to NEBF. However, households with the lowest wealth and higher household head educational status in livelihood-secure areas were less vulnerable to NEBF. Hence, livelihood-based interventions targeting mothers of 2 to less than 6-month-old infants, with emphasis on these factors, may help address and reduce NEBF.

KEYWORDS

breastfeeding, Deder, Jimma, Ethiopia, livelihood security

Introduction

The ideal diet for the optimal health and development of infants aged under 6 months (u6m) is exclusive breastfeeding. Non-exclusive breastfeeding (NEBF) is defined as the practice of introducing solid, semisolid, or liquid foods and/or anything else per mouth to breastfed infants before they reach the age of 6 months (1). Clinically indicated products are however allowed, e.g., oral rehydration solution (ORS) and vitamin and mineral drops or syrups; other medicines are given orally (1).

The World Health Organization (WHO) reported that more than two-thirds of mothers with u6m infants were found to practice NEBF in the years 2015–2020 (2). Other recent studies also show that the frequency of NEBF remains unacceptably high, with large geographic differences (3). It is particularly prevalent in high-income countries, where formula feeding promotion and insufficient maternity leave are among several factors undermining the recommended exclusive breastfeeding (EBF) practices (4, 5). Moreover, in high-income countries, there is also less societal consensus about the importance of promoting EBF practices than in most low- and middle-income countries (LMICs) (3, 6).

Overall, NEBF has slowly declined over the last decade, indicating that it remains a challenge and lagging behind in meeting both national and international targets of reducing by at least 50% and below (3, 7), as well as failing to achieve very good NEBF reduction rates of 0–10% (8).

Unless NEBF is tackled accordingly, it causes multiple problems including anemia due to iron-deficiency (9), infant growth faltering, and associated risk of early childhood mortality (2, 10). There are also large associated economic losses, particularly in sub-Saharan African countries (SSAs) (11). Recent evidence indicates that taking "4 months of age" as an optimal window for starting additional foods against the WHO recommendations at "6 months of age" (1), in both developed and developing countries (12, 13) along with other socioeconomic and demographic factors (14–18), is strongly believed to be associated with a higher magnitude of NEBF.

In Ethiopia, previous studies reported that NEBF prevalence was 28.3% in 2011 in Eastern Ethiopia (17), 47.5% in 2014 in north Ethiopia (19), 39.8% in 2019 in northwest Ethiopia (14), and 49.4% in 2015 in southern Ethiopia (18). Those studies, however, were conducted either in towns or only in rural areas, as well as over a quite longer period of time, which may raise issues of generalizability for the current population, as both the study setting and study period may have a significant effect on the magnitude and associated factors of NEBF (14, 17–19). More importantly, a thorough understanding of NEBF magnitude and its associated factors remains uncertain if studies fail to adequately account for the varying degrees of livelihood security across different population groups. This is because the associated factors of NEBF can be modified depending on the varying degree of livelihood security.

Mothers in relatively livelihood-secure areas with stable economies may have improved maternal nutrition, which is crucial for adequate breastmilk production and a reduced risk of NEBF. However, this may also increase accessibility to complementary foods, including formula (20, 21), and may increase the risk of NEBF (22). Conversely, mothers in livelihood-insecure areas may be unable to buy infant formula or other complementary foods, leaving EBF as their only alternative. These mothers, however, may experience poverty and labor-intensive jobs, resulting in maternal malnutrition and decreased milk production, potentially reinforcing NEBF.

Ethiopia is known among the resource-limited settings with a relatively varied degree of vulnerability to livelihood security across the different areas, for instance, in our study areas, Jimma (23–26) and Deder (27–29).

Jimma is a highland area with a tropical monsoon climate and is noted for its long wet with March to October rainy season, which makes the environment for surplus food crops and coffee production, making the area less vulnerable to livelihood insecurity in Oromia region, southwest Ethiopia (23–26, 30–33). In Jimma, studies reported that formula feeding is a common practice for u6m infants (21) and the level of care provided rather than wealth was reported as a significant predictor of malnutrition (26).

Deder in East Hararghe, a khat-dominant growing area from the Ethiopian lowlands in eastern Ethiopia, has long been known for its vulnerability in terms of climate shock with limited rainfall that exposed the area to repeated drought attacks, population migration, and water scarcity, making this an intervention target area for both the regional administration and other development partners (23–42). The presence of such internally displaced people (IDP) and frequent droughts in Deder have exacerbated livelihood insecurity, poor water, and sanitation practices that may affect infant feeding practices negatively or positively (29, 31–33).

Our recent qualitative study explored that mothers from Jimma areas had a strong habit of initiating complementary feeding at 4 months of age and were strongly defending "EBF until 6 months of infants age is a lie," which is against the 6 months of recommended practices of EBF

Abbreviations: CI, confidence interval; AIC, Akaike information criteria; EBF, exclusive breastfeeding; EDHS, Ethiopian Demographic Health Survey; ENN, emergency nutrition network; ICC, intra-cluster correlation; IYCF, infant and young child feeding; JUCAN, Jimma University Clinical and Nutrition Research Center; u6m, under-6-month; REDCap, research electronic data capture.

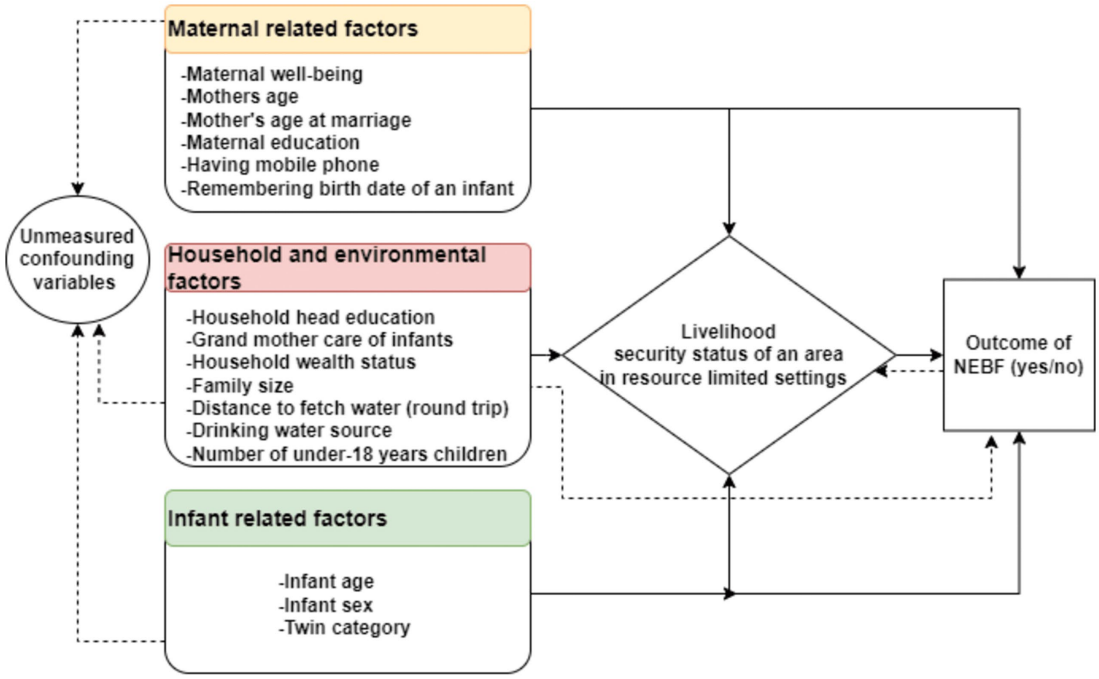

FIGURE 1
Conceptual framework of factors associated with non-exclusive breastfeeding practice in relatively livelihood-secure areas and insecure areas (adapted from different studies (3, 5, 17, 19, 22, 45)).

(1), whereas most of the mothers and community members from Deder areas discussed and explored that they had a good EBF culture (43).

Therefore, after analyzing differences between the two study sites in terms of sociodemographic, economic, maternal, infant, and other environmental characteristics using formative data from our clinical trial study (44), as well as gaps from our qualitative findings in the two study sites (43), we hypothesized that the epidemiology and factors of NEBF could differ across the aforementioned variables in those comparative areas. Hence, we aimed to conduct a separate and deeper analysis to quantify whether area vulnerability to livelihood insecurity status modifies the association between NEBF and aforementioned variables by examining our formative data preceding a clinical trial (44), which was set in the Jimma and Deder areas.[1]

By doing so, we hope to gain a better and more detailed understanding of the magnitude of NEBF across the different maternal, infant, sociodemographic, economic, and environmental characteristics, as well as its associated factors in two different settings of Ethiopia. This could help policymakers and programmers in contextualizing and strengthening breastfeeding guidelines and policies accordingly.

Below is a conceptual framework derived from various literature sources, illustrating the hypothetical association between maternal, infant, sociodemographic, economic, and environmental factors and NEBF outcomes with an effect modifier of "area vulnerability to livelihood-insecurity" (Figure 1).

1 https://www.isrctn.com/ISRCTN47300347

Methods and materials

Study design, areas, and period

We present a secondary analysis of previously reported comparative cross-sectional data from selected health centers both from Jimma and Deder, Oromia, Ethiopia (44). Jimma is located 360 km southwest of Addis Ababa, Ethiopia's capital city, whereas Deder is located 450 km southeast of Addis Ababa. Both Jimma and Deder are densely populated areas having diverse ethnicities, cultural traditions, and religious perspectives, with more than a fourth-fifth of the population being Oromo by ethnicity and Muslim religion followers (46–48).

Jimma zone is characterized by a highland with monsoon tropical climate features, a long annual wet, and almost year round rainy season in southwest Ethiopia (23–26, 30–33), whereas the Deder district is characterized by a tropical lowland climate with high temperature variability, limited rainfall, and known for its repeated drought attacks, a high rate of internally displaced peoples (IDP), and population migration in East Hararghe, eastern Ethiopia (23–27, 30–33, 38–42).

Such variation in our study sites help to gain representative formative data for our clinical trial study, which aims to provide comprehensive critical outcome and implementation evidence to inform national; specifically for Ethiopia regardless of area, cultural, religious, and ethnic diversity as well as for international policy and service development with a view to future sustainable scale (49).

In Jimma, Jimma University (JU) and Jimma University Clinical and Nutrition Research Center (JUCAN) are well known for performing an extensive track record of community-related activities

including delivering clinical services, teaching, and diverse research projects. In Deder, GOAL Ethiopia is a well-known partner working on at-risk u6m infant–mother pairs to improve their nutrition and health outcomes. Data were collected between 12 October 2020 and 29 January 2021 (44).

Population, sample size, and sampling procedure

The study contained the formative cross-sectional survey data from our trial study, which included 1,060 u6m infant–mother pairs (623 were from Jimma and 437 were from Deder) who visited health centers for delivery services, immunization, growth monitoring, and under-five clinics for the treatment of various acute diseases. As a result of their differences in terms of livelihood security and accessibility, we stratified and purposefully selected the Jimma and Deder areas of Ethiopia to obtain complete and representative population groups for our baseline and trial study. Our trial study is a new clinical care pathway (CP) designed for the management of small and nutritionally vulnerable infants and their mothers (MAMI) (50). This was created in order to translate high-level policy guidelines (such as the World Health Organization's 2013 guidelines on severe malnutrition) into effective front-line clinical and patient management practices (50).

Before using formative data from our trial study, we evaluated sample adequacy by reviewing NEBF and its associated factors reported in a previous study conducted in Ethiopia by Tadesse et al. (18). First, a single population formula was used for checking sample adequacy by considering 49.4% of NEBF practice (18), marginal error = 0.05, the standard normal deviate = 1.96 at 95% CI, design effect = 2, and 10% non-response rate, resulting in a total sample of 847 u6m infant–mother pairs. Second, the double population proportion formula for the sample size estimation technique was considered by undertaking the most commonly cited factor of NEBF practice, in general, and in this study, as "infant age" (18). With 80% power, 10.29 odds of NEBF among 4–5 months old infants compared with 0–1-month-old infants, 22.7% of mothers with 0–1-month-old infants practiced NEBF, a design effect of 2, and a non-response rate of 10%, the maximum sample size calculated using the Fleiss formula with the continuity correction was 75 u6m infant–mother pairs (51). However, the sample estimated using both methods was by far less than our formative data (44). As a result, this study used our MAMI health center survey formative data (44) to determine the magnitude and associated factors of NEBF using livelihood-security status as an effect modifier.

The original survey sampling procedure was carried out as follows: from the relatively livelihood-insecure areas, we included all eight available health centers and the catchment population in our study, but we used a systematic approach in relatively livelihood-secure areas to pick 10 health centers and their catchment population from a total of 124 alternatives. Then, the following steps were undertaken in our selection process. To begin, we did a thorough assessment of all 124 health center registers to obtain eligible information relative to the ease of access and patient load. As a result of the inadequate eligibility data, we excluded 60 health centers. In addition, we excluded seven health centers that had accessibility issues. Finally, we rated the remaining 57 health centers based on patient load and selected 10 health centers and their catchment population at random from the top 50% of the list to get a representative sample for our investigation. The overall sampling procedures are shown in the figure below (see Figure 2).

Study variables

The dependent variable was NEBF (yes vs. no), and the independent variables were as follows: infant and mother attributes (infant age, infant sex, maternal age at first marriage, maternal age, maternal educational level, twin category, number of children younger than 18 years within the household, maternal well-being, mobile phone use, grandmother care of infants, and ability to remember infant's birth date) and household attributes (wealth status, family size, household head educational level, household drinking water source, and distance to fetch water).

Operational definitions

Non-exclusive breastfeeding practice

To generate the NEBF variable, WHO and UNICEF Infant and Young Child Feeding (IYCF) practice assessment tools were used (52, 53). It was assessed by asking questions such as, "Does the infant ever breastfeed?" What was the infant given immediately after birth? Is the infant still breastfeeding? Was the infant on any medications, minerals, or liquids prescribed by a healthcare worker? Was the infant fed any liquids, semisolids, or solids in the last 24 h, day or night? An u6m infant was considered in "NEBF" for this study if he/she had ever breastfed but consumed any liquids, semisolids, or solid foods and/or anything else orally other than medicines, vitamins, or minerals.

Maternal wellbeing

It was measured by Patient Health Questionaire-9 (PHQ-9). The total PHQ-9 score ranges from 0 to 27. The severity of caregivers' mental health is classified as follows: 0 (no depressive disorder); 1–4 (minimum depression); 5–9 (mild depression); 10–14 (moderate depression); 15–19 (moderately severe depression); and 20–27 score (severe depression) (54). To meet the assumptions of Pearson chi-square, the study merged the data and treated them as binary variables, with a score of 0 indicating good maternal well-being and a score of 1–27 indicating poor maternal well-being.

Wealth index

It is the total value of a household's natural, physical, and financial assets minus its liabilities (55). Initially, the reliability test was carried out by the variables used in measuring wealth status. The variables were also entered into the principal component analysis, and the wealth index was calculated as a continuous scale of relative wealth. Wealth quintiles were created by assigning a score to each household; the wealth of each family was classified as follows: one as poorest (lowest), two as poorer (second), three as medium (third), four as rich (fourth), and five as richest (fifth).

Area vulnerability to livelihood insecurity

The Livelihood Vulnerable Index (LVI) tool is a latent variable commonly used in developing countries, such as Ethiopia, to aggregate and measure vulnerability to livelihood insecurity (27, 30, 38–43, 56, 57). This tool includes questionnaires on climate change and drought,

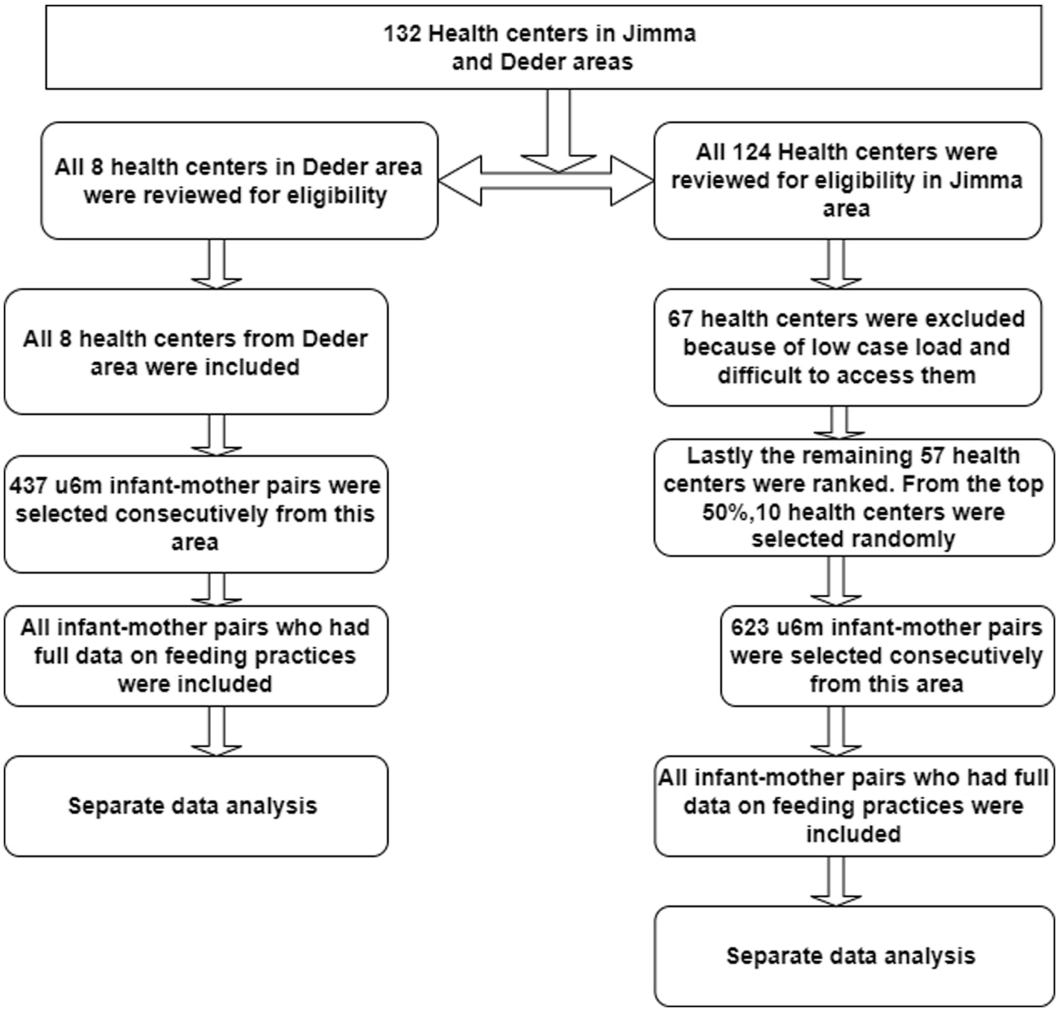

FIGURE 2
Schematic presentation of the sampling procedures to select the infant–mother pairs.

households' adaptive capacity or livelihood strategies, household assets, population demography including migration profile, shortage of water, and access to food in the area to be studied (56, 57). In a very recent study conducted in Jimma and Deder, the development partners in collaboration with the regional administration and local experts employed both this tool and historical metrological data to assess and clearly delineate local-scale vulnerability for effective area prioritization and project implementation with the goal of reversing and sustaining livelihoods (23–27, 30–33, 38–43, 58). Hence, based on this empirical information, we directly took the Deder area from a tropical lowland climate in East Hararghe, eastern Ethiopia, as more vulnerable to livelihood insecurity (27, 38, 39, 41, 58) and the Jimma area from a highland monsoon tropical climate in southwest Ethiopia as less vulnerable to livelihood insecurity (23–26, 30–33).

Data collection, quality management, and assurance methods

As published elsewhere (44), twelve nurses from JUCAN supervised by four individuals with master's degrees in health sciences collected the data. For 2 weeks, all data collectors and supervisors were trained in data collection tools and data entry techniques via the Research Electronic Data Capture (REDCap) tablet-based application. The data collection modules in REDCap were checked for accuracy. On the same day of the data upload, the collected data were meticulously assessed for completeness and clarity before and after being posted to the REDCap server.

Statistical analysis

Both the descriptive and analytic statistical analyses were performed using STATA version 17 packages (STATA 17, College Station, Texas 77,845 USA). Descriptive data were summarized using frequency tables and percentages. Before fitting into the final model, multicollinearity diagnosis was performed using the variable inflation factor (VIF) to check any significant intercorrelation issues across the independent factors. Then, a binary logistic regression model was used after checking model fitness via the Hosmer–Lemeshow goodness of fit test (59).

First, bivariate analysis was conducted to examine the association between NEBF and each independent variable. Then, variables with a

p-value of less than 0.25 in the bivariate analysis (60) and deemed important variables that can be associated with NEBF were included for the final different model building of multiple variable logistic regression. Finally, the Akaike Information Criterion (AIC) was used to evaluate the final model, and the best model with the lowest AIC value was chosen. The odds ratio (OR) with a 95% confidence interval (CI) was used to measure and identify the significant factors of NEBF at a p-value of <0.05.

Results

Sociodemographic and economic characteristics

This study included 1,060 u6m infant–mother pairs: 437 (41.2%) from relatively livelihood-insecure areas and 623 (58.8%) from relatively livelihood-secure areas. More than half of the infants (54.6% in livelihood-secure areas and 56.8% in livelihood-insecure areas) were male babies, while 91.5% of mothers in livelihood-secure areas and 90.6% of mothers in livelihood-insecure areas were at the age of 19 years and older. More than three-fourths of mothers in 75.4% of livelihood-secure areas and 89.7% in livelihood-insecure areas married for the first time before the age of 19 years (Table 1). According to the Pearson chi-square statistical tests, there were significant differences (a p-value of <0.05) in sociodemographic, economic, environmental, and infant-related characteristics between the livelihood-secure and livelihood-insecure areas (Table 1). This test implies that factors of infant feeding practice in each area are also different, emphasizing the need to do further separate analysis.

Magnitude of non-exclusive breastfeeding practice

The pooled magnitude of 51% of NEBF mothers (95% CI: 48.0, 54.0) was 53.1% (95% CI: 49.2, 57.0) and 48.1% (95% CI: 43.4, 52.8) in livelihood-secure and livelihood-insecure areas, respectively (*p*-value = 0.104). More than half of NEBF was practiced among mothers with male infants (62.9% in livelihood-secure and 51.1% in livelihood-insecure areas). A high magnitude of NEBF was reported among infants aged 2 to less than 4 months (49.8%) in livelihood-secure areas and infants aged 4 to 6 months (54.3%) in livelihood-insecure areas. More than three-quarters of early married women practiced NEBF (76.1% in livelihood-secure and 90% in livelihood-insecure areas). A high number of women who did not have mobile phone for communication practiced NEBF (58.6% in livelihood-secure and 76.2% in livelihood-secure areas) (Table 2).

Factors associated with non-exclusive breastfeeding practice

At first, we stratified the data based on areas, distinguishing between relatively livelihood-secure (Jimma) and insecure (Deder) areas. Subsequently, we conducted separate bivariate and multiple variable logistic regression analyses. Before fitting the data into the final model, we diagnosed multicollinearity among the confounding variables. Maternal education with household head education, household wealth index with family size, maternal and household education with recollection of the infant's birth date, and household wealth index with a mobile phone were assumed to be confounded. We have performed multicollinearity diagnostics across all the independent variables using VIF, and all of them had a VIF value of <10, indicating that no significant multicollinearity issue was found across the independent variables. However, the number of under 18-year-old children was strongly correlated with household family size, excluding them from the final model according to the settled criteria of data analysis in this study. A binary logistic regression model was used after checking model fitness via the Hosmer–Lemeshow goodness of fit test, which showed a *p*-value of 0.23, indicating good model fit (59).

From the final model, using livelihood security as an effect modifier, we have found different associated factors of NEBF in each area: from the relatively livelihood-insecure areas, mothers with male infants (AOR = 1.8; 95% CI = 1.15–4.61) had the highest odds of NEBF than mothers with u6m female infants. However, the sex of an infant was not statistically significant in relatively livelihood-secure areas (AOR = 0.9; 95% CI = 0.58–1.37). In relatively livelihood-secure areas, maternal lack of recollecting the infant's birth date (AOR = 2.1; 95% CI = 1.10–4.02) had the highest odds of NEBF, while it was not significant in comparatively livelihood-insecure areas (AOR = 0.9; 95% CI = 0.85–1.43). Furthermore, household heads with tertiary education had lower odds of NEBF than household heads with no education (AOR = 0.12; 95% CI = 0.02–0.76), but this was not significantly associated with relatively livelihood-insecure areas (AOR = 2.6; 95% CI = 0.58–11.68). Similarly, mothers from the poorest households in relatively secure areas had lower odds of NEBF than rich households (AOR = 0.41; 95% CI = 0.21–0.85) but not in generally insecure areas (AOR = 2.1; 95% CI = 0.89–4.75). Furthermore, the age of an infant was also associated with NEBF in both reasonably secure and insecure settings. In relatively secure areas, mothers with 2 to less than 4-month-old infants (AOR = 6.7; 95% CI = 2.95–15.25) and 4 to less than 6-month-old infants (AOR = 16.2; 95% CI = 6.62–39.52) and in insecure areas mothers with 2 to less than 4-month-old infants (AOR = 3.2; 95% CI = 1.15–9.11) and 4 to less than 6-month-old infants (AOR = 5.9; 95% CI = 2.10–16.83) had the highest odds of NEBF (Table 3).

Discussion

This study found that more than half (51%) of mothers practiced NEBF: 53.1% in livelihood-secure areas and 48.1% in a livelihood-insecure area. In line with the current findings, one study has also shown that three out of five mothers practiced NEBF, with minimal progress over the last 15 years (61). Similarly, NEBF was reported to be 49.4% in southern Ethiopia in 2016 (18) and 47.5% in northern Ethiopia in 2014 (19). However, the overall finding of NEBF in the current study was considerably higher than in a 2013 study in eastern Ethiopia, which was 28.3% (17) and in a 2019 study in northwest Ethiopia, which revealed that NEBF in the area was 39.8% (14). When compared with the last two NEBF reports, our NEBF data suggest that it is a continuing and even potentially an increasing issue of pressing public health concern, similar to other studies conducted on breastfeeding issues established thus far (5, 6, 62). It is possible that the dynamics of exclusive breastfeeding practice are

TABLE 1 Sociodemographic and economic characteristics.

Variables	Category	Relatively livelihood-secure area	Relatively livelihood-insecure area	*p*-value
		Number (%)	Number (%)	
Infant sex	Male	340(54.6)	248(56.8)	0.4830
	Female	283(45.4)	189(43.2)	
Infant age	0 to <1 month	63(10.1)	26(5.9)	<0.001*
	1 to <2 month	137(22.0)	49(11.2)	
	2 to <4 months	278(44.6)	176(40.3)	
	4 to <6-months	145(23.3)	186(42.6)	
Twin category	Singleton	611(98)	432(98.8)	0.3180
	Non-singleton	12(1.9)	5(1.1)	
Mother age	<19 years	53(8.5)	41(9.4)	0.6220
	≥19 years	570(91.5)	396(90.6)	
Mother's age at marriage	<19 years	470(75.4)	392(89.7)	<0.001*
	≥19 years	153(24.6)	45(10.3)	
Maternal education	No education	240(38.5)	194(44.4)	<0.001*
	Primary	275(44.1)	210(48.1)	
	Secondary	71(11.4)	25(5.7)	
	Tertiary	37(5.9)	8(1.8)	
Maternal wellbeing	Good	568(91.9)	373(85.9)	0.003*
	Poor	50(8.1)	61(14)	
Remembering the birth date of the infant	Yes	542(87)	336(76.9)	<0.001*
	No	81(13)	101(23.1)	
Household head education	No education	205(36.3)	135(31.3)	0.001*
	Primary	254(45.0)	198(45.8)	
	Secondary	60(10.6)	79(18.3)	
	Tertiary	45(8.0)	20(4.7)	
Family size	<5 family	388(62.3)	239(54.7)	0.013*
	≥5 family	235(37.7)	198(45.3)	
Telephone for communication	Yes	280(44.9)	105(24.0)	<0.001*
	No	343(55.1)	332(76.0)	
Household drinking water source	Improved	556(89.2)	196(44.9)	<0.001*
	Non-improved	67(10.8)	241(55.1)	
Household wealth	Poorest	156(25.0)	56(12.8)	<0.001*
	Poor	92(14.8)	121(27.7)	
	Middle	86(13.8)	126(28.8)	
	Rich	124(19.9)	88(20.1)	
	Richest	165(26.5)	46(10.5)	

*Indicates significant variables across the Jimma and Deder study areas.

changing as a result of societal changes, particularly in a market-driven environment (63), whereby u6m traditional breastfeeding-focused infant feeding practices are more challenging to maintain in today's culture (64). This cultural shift from human milk feeding to market driven foods for infants may explain the increased prevalence of NEBF in our study settings too, plus this may reflect wider challenges as cultural pressures elsewhere in Ethiopia also affect the feeding practices of u6m infants and not to be on track of NEBF rate reduction (43, 65, 66), which may be a barrier for Ethiopia not being on track to achieve the desired reduction in NEBF by 2025 (62).

Hence, the current study along with others mentioned here indicated that the NEBF rate remains high and has shown a slow reduction over the past decade, failing to meet the WHO's 2021 target for a very good NEBF practice reduction rate of 0–10% (8), failing to reach the Ethiopian Health Sector Transformation Plan (HSTP) 2016–2020 target of reducing NEBF to 28% (67), and also

TABLE 2 Magnitude of non-exclusive breastfeeding practice.

		Non-exclusive breastfeeding practice			
		Relatively livelihood-secure area		Relatively livelihood-insecure area	
Variables	Category	Yes (%)	No (%)	Yes (%)	No (%)
Infant sex	Male	132 (62.9)	182 (55)	116 (51.1)	158 (54.1)
	Female	78 (37.1)	149 (45)	111 (48.9)	134 (45.9)
Infant age	0 to <1 month	11(3.3)	52(17)	6(2.9)	20(8.8)
	1 to <2 month	46(13.9)	91(31.2)	13(6.2)	36(15.9)
	2 to <4 months	165(49.8)	113(38.7)	77(36.7)	99(43.6)
	4 to <6-months	109(32.9)	36(12.3)	114(54.3)	72(31.7)
Twin category	Singleton	322(97.3)	289(99.0)	208(99.0)	224(98.7)
	Non-singleton	9(2.7)	3(1.0)	2(1.0)	3(1.3)
Mother's age	<19 years	23(6.9)	30(10.3)	23(11.0)	18(7.9)
	≥ 19 years	308(93.1)	262(89.7)	187(89.0)	209(92.1)
Mother's age at marriage	<19 years	252(76.1)	218(74.7)	189(90.0)	203(89.4)
	≥ 19 years	79(23.9)	74(25.3)	21(10.0)	24(10.6)
Maternal education	No education	136(41.1)	104(35.6)	96(45.7)	98(43.2)
	Primary	147(44.4)	128(43.8)	94(44.8)	116(51.1)
	Secondary	32(9.7)	39(13.4)	16(7.6)	9(4.0)
	Tertiary	16(4.8)	21(7.2)	4(1.9)	4(1.8)
Maternal wellbeing	Good	179 (85.2)	300 (90.6)	194 (85.5)	268 (91.8)
	Poor	31 (14.8)	31(9.4)	33 (14.5)	24 (8.2)
Remembering the birth date of the infant	Yes	160 (76.2)	276 (83.4)	176 (77.5)	266 (91.1)
	No	50 (23.8)	55 (16.6)	51 (22.5)	26 (8.9)
Household head education	No education	116(39.5)	89(33.0)	60(29.1)	75(33.2)
	Primary	136(46.3)	118(43.7)	93(45.1)	105(46.5)
	Secondary	23(7.8)	37(13.7)	42(20.4)	37(16.4)
	Tertiary	19(6.5)	26(9.6)	11(5.3)	9(4.0)
Family size	<5 family	124 (59)	197 (59.5)	115 (50.7)	191 (65.4)
	≥5 family	86 (41)	134 (40.5)	112 (49.3)	101 (34.6)
Having mobile phone	Yes	137(41.4)	143(49.0)	50(23.8)	55(24.2)
	No	194(58.6)	149(51.0)	160(76.2)	172(75.8)
Grandmother care of infants	No	217(65.6)	206(70.5)	130(61.9)	128(55.5)
	Yes	214(34.4)	86(29.5)	80(38.1)	101(44.5)
Drinking water source	Improved	295(89.1)	261(89.4)	100(47.6)	98(42.3)
	Non-improved	38(10.9)	31(10.6)	110(52.4)	131(57.7)
Fetching water distance	< 30 min	218(85.2)	217(88.9)	81(41.5)	84(39.4)
	≥30 min	38(14.8)	27(11.1)	114(58.5)	129(60.6)
Household wealth status	Poorest	34 (16.2)	75 (22.7)	22 (9.7)	81 (27.7)
	Poor	58 (27.6)	53 (16)	63 (27.8)	39 (13.4)
	Middle	62 (29.5)	45 (13.6)	64 (28.2)	41 (14)
	Rich	35 (16.7)	72 (21.8)	53 (23.3)	52 (17.8)
	Richest	21 (10)	86 (26)	25 (11)	79 (27.1)

alarming one for not being on track toward progress of NEBF reduction rates in Ethiopia by 2025 (62). This implies that policies aimed at promoting EBF practice must be strengthened and critical gaps need to be addressed according to the local contexts for a successful EBF practice and effective health developmental gains (68).

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TABLE 3 Factors associated with non-exclusive breastfeeding practice.

Variables	Category	Relatively livelihood-secure area	Relatively livelihood-insecure area
		AOR (95% CI)	AOR (95% CI)
Intercept (β0)		0.4 (0.11, 1.4)	0.1 (0.01, 0.32
Sex of infant	Female	1	1
	Male	0.9 (0.58, 1.37)	1.8* (1.15, 4.61)
Infant age	0 to <1 month	1	1
	1 to <2 month	1.8 (0.75, 4.47)	1.4 (0.41, 4.61)
	2 to <4 months	6.7* (2.95, 15.25)	3.2* (1.15, 9.11)
	4 to <6 months	16.2* (6.62, 39.52)	5.9* (2.10, 16.83)
Twin category	Singleton	1	
	Non-singleton	2.0 (0.37, 10.66)	
Maternal age	<19 years	0.6 (0.25, 1.39)	1.7 (0.74, 3.66)
	≥ 19 years	1	1
Maternal age at marriage	<19 years	1.0 (0.60, 1.66)	0.8 (0.38, 1.60)
	≥19 years	1	1
Mother education	No education	1	1
	Primary	1.0 (0.59, 1.52)	0.8 (0.52, 1.36)
	Secondary	0.8 (0.34, 1.99)	4.1 (0.99, 16.97)
	Tertiary	3.4 (0.39, 30.00)	1.5 (0.17, 13.27)
Maternal wellbeing	Normal	1	1
	Abnormal	1.6 (0.75, 3.36)	1.2 (0.65, 2.29)
Remembering the birth date of the infant	Yes	1	1
	No	2.1* (1.10, 4.02)	0.9 (0.50, 1.43)
Having mobile phone	Yes	1	1
	No	1.0 (0.59, 1.55)	1.8 (0.95, 3.48)
Household head education	No education	1	1
	Primary	0.9 (0.56, 1.46)	1.3 (0.81, 2.18)
	Secondary	0.7 (0.32, 1.68)	1.5 (0.76, 2.89)
	Tertiary	0.1* (0.02, 0.76)	2.6 (0.58, 11.68)
Grandmother help in childcare	Yes	1	1
	No	0.8 (0.52, 1.36)	1.5 (0.97, 2.41)
Family size	< 5	1	1
	≥5	1.3 (0.93, 1.80)	0.8 (0.51, 1.28)
Drinking water source	Improved	1	1
	Non-improved	1.0 (0.53, 1.85)	1.2 (0.69, 1.91)
Fetching water distance	< 30 min	1	1
	≥30 min	1.6 (0.84, 3.21)	0.8 (0.51, 1.28)
Household wealth	Poorest	0.4* (0.21, 0.85)	2.1 (0.89, 4.75)
	Poor	0.9 (0.40, 1.80)	1.5 (0.78, 2.72)
	Middle	0.5 (0.25, 1.11)	1.6 (0.87, 2.97)
	Rich	1	1
	Richest	0.6 (0.32, 1.06)	1.4 (0.63, 3.20)

AOR, Adjusted Odds Ratio; CI, Confidence Interval. *Significant variables.

There is not enough evidence of the differences in the associated factors of NEBF across different degrees of livelihood security. This study and a few others indicated (22, 45) that livelihood security modifies the association between some of the sociodemographic and economic variables and NEBF outcomes. Our study found that, in relatively livelihood-secure areas, mothers who failed to remember

their infant's birth date had significantly higher odds of practicing NEBF than mothers who remembered their infant's birth date. However, this pattern was not found in livelihood-insecure areas. Though there is no supportive evidence in other similar settings, in reality, not knowing the infant's birth date is often a symptom of a larger issue—a sense of maternal disempowerment and vulnerability that can result in poor feeding choices, non-responsive feeding, and failure to follow guidelines (69–71). Additionally, mothers who fail to remember their infant's birth date may incorrectly assume that their infant has already reached the age of 6 months, leading to the introduction of additional foods before the recommended age. Another possible explanation is that educated mothers may use vaccination cards to track their infant's age and birth date, allowing them to feed infants EBF only until infants reach 6 months. However, mothers with the access and capacity to introduce infant feeds may be trying to justify their actions, based on pretending not to recall their infant's age, but rather by looking at other developmental cues associated with body language or behaviors of maturity to determine when an infant is ready to eat other foods (69). This is a difficult reality that must be addressed by underscoring the importance of empowering mothers with the knowledge and resources needed to make informed decisions about their infant's feeding options (72).

Mothers from the poorest households had lower odds of practicing NEBF than mothers from wealthier households in relatively livelihood-secure areas, but this difference was not statistically significant in livelihood-insecure areas.

Similarly, a previous study found that in wealthy countries, one out of every five infants did not exclusively breastfeed, whereas in poor countries, this ratio was one out of every 25 infants (73). Several studies, notably those conducted in Ethiopia and China, have revealed that the poorest mothers had the lowest NEBF rates (74, 75). Societal changes over the 21st century have led to substantial increases in NEBF rates in many nations, not only for those with high and increasing wealth (5) but also for some African countries (64), including Ethiopia (43, 65, 66). The lower NEBF rates among the poorest mothers can be because high-income households have better access to complementary foods than the most economically disadvantaged households (5, 63). This access, together with the means to the early introduction of infant feeds and an attempt to balance the challenges of working life for employed mothers, may further explain this finding.

Similar to high-income countries (3, 5, 6), our study found that NEBF is commonly practiced in wealthier households in Ethiopia. This supported that NEBF remains an unaddressed issue in low-income countries as well (64). Therefore, breastfeeding promotion via social mobilization, mass media, legislation, policy, enforcement, counseling, support, lactation management, and other behavior-change strategies that may reduce NEBF should also be included to target better-off households in low-income settings. However, attention should be paid to the fact that the association between high wealth index with NEBF could be an opportunity for providing adequate and diverse complementary feeds during 6–23 months of an infant age (76), which is crucial for infant growth and development (77).

In livelihood-secure areas, households with heads having tertiary education had lower odds of NEBF than non-educated household heads. Though specific evidence is lacking and further investigation is needed, similar studies conducted, regardless of settings, have consistently documented evidence supporting this finding (14, 74, 78–80). This is because household heads with tertiary education may have a better understanding of the benefits of EBF and the consequences of NEBF; thus, they can defend against interferences and pressures from traditional beliefs and misconceptions about NEBF.

The current study demonstrated that male infants had greater odds of NEBF in relatively livelihood-insecure areas, while this sex disparity was not observed in relatively livelihood-secure areas. Though the underlying factors need more investigation, consistent findings have been reported in Ethiopia (81), Angola (82), Malawi (83), and 29 sub-Saharan African countries (84). This difference is because scholars claimed that programs and policies are more focused on girls than boys to break the intergenerational cycle of malnutrition, so boys' vulnerability to NEBF and malnutrition (85, 86) may have received little attention in nutrition program design or nutrition policy formulation (87, 88). Hence, program staff and policymakers should be aware of boys' vulnerability both for NEBF and malnutrition. In livelihood-secure and insecure areas, mothers with infants of 2 months and older had higher odds of practicing NEBF which is in-line with reports from Ethiopia and across continents (18, 81, 89–94). As infants grow and approach 6 months of age, mothers tend to become less confident about EBF and are more likely to follow non-exclusive breastfeeding (NEBF). This may be due to a belief that breast milk alone is insufficient to meet their infants' nutritional and hydration needs (43, 95–98).

Additionally, while EBF is recommended for the first 6 months of an infant's life, working mothers may have difficulty continuing EBF as their infants grow older and require more frequent feedings (99). Because of these and others, many mothers may supplement breast milk with complementary foods and/or formula, which leads to a decrease in EBF rates, as infants approach 6 months. Furthermore, our previous qualitative findings also found that there is a cultural belief known as "there is no life without water," indicating cultural pressure to introduce complementary feeds before the age of 6 months (43). Therefore, it is imperative to underpin a comprehensive package of care that integrates breastfeeding guidelines with all the technical efforts that can break the aforementioned social and cultural norms to achieve a successful NEBF reduction.

To the best of the authors' knowledge, this is the first study that compares two different populations and examines the factors associated with NEBF practice while incorporating area vulnerability to livelihood security as an effect modifier. This is because Jimma from the central highlands in southwest Ethiopia and Deder from the eastern lowlands in eastern Ethiopia are comparatively dissimilar places in terms of topography, climate category, and vulnerability to livelihood insecurity, as their data are already documented elsewhere (23–27, 30–33, 38, 39, 41, 58). It also investigated the same research question for each independent variable and compared the NEBF practice across the two populations. The results showed that there is a marked difference in the associated factors between livelihood-secure and insecure areas and that livelihood security acts as an effect modifier of NEBF practice in resource-limited settings. Another strength of this study is that data were collected following strict procedures

along with daily monitoring and evaluation techniques via a Research Electronic Data Capture (REDCap) tablet-based application linked to its central server, both of which were critical for maintaining data quality and assurance throughout the data collection period.

However, the cross-sectional design of this study limits its ability to establish causality, more specifically, the longitudinal effect of livelihood security on NEBF outcomes, as it can only identify associations between variables rather than determine true cause-and-effect relationships (100, 101). Another limitation of our study could be the nature of feeding-related data collection, which could be influenced by social desirability and recall bias. However, the validated version of the WHO's and UNICEF's infant and young child feeding (IYCF) assessment tool (52, 53) was utilized to reduce information and recall biases associated with u6m infant feeding practices and maximize the accuracy of the data we have collected to date. There was also a paucity of data that may affect NEBF in this study, such as measures of women's empowerment, perceived social support, antenatal care, and the quality of EBF advice/support (81, 102, 103). This secondary analysis also missed religious, ethnic, and cultural data, which unfortunately limited us to further explore their association with NEBF in our study sites. In fact, our recent qualitative findings in the same study areas and a few other studies in low- and middle-income countries reported that religious and cultural narratives were explored as major attributes of NEBF (43, 63, 104).

There is a paucity of information on the association between Ethiopian ethnic diversity and u6m infant breastfeeding practices, especially among minority groups, instead, cultural pressure to introduce pre-lacteal feeds, other than breastmilk, within a few days after birth is observed across all ethnicities (105–107). Established literature studies from the United States, China, and Vietnam have shown marked differences in NEBF practices among different race/ethnic groups (108–110), highlighting the need for further investigation in the Ethiopian context.

Because this study focused on reporting only the epidemiology of NEBF and identifying associated factors in two areas with different climatic zones and vulnerability status, it does not definitively establish causal links between the effect of hot climatic zones and the provision of water and other fluids to u6m infants, as established elsewhere (111–113). As a result, we emphasize the careful interpretation of our findings and highlight the importance of further investigation to quantify the impact of climate change and seasonal variation on the population attributable risk (PAR) of NEBF practice in various climatic zones, particularly in resource-limited settings such as Ethiopia.

It is also possible that some infants older than 6 months were included in the study since mothers were not always sure of the exact age. There may also be selection bias since infants attending health centers do not fully reflect all infants in the catchment population.

Conclusion

Over half of mothers were practicing NEBF, which represents a failure to meet national and international targets. Livelihood security modifies associated factors of NEBF. Male infants in insecure areas, infants of unknown age in secure areas, and infants aged 2 months or older, regardless of setting, were more vulnerable to NEBF. However, households with the lowest wealth and higher household head educational status in livelihood-secure areas were less vulnerable to NEBF. Hence, livelihood-based interventions targeting mothers of 2 to less than 6-month-old infants, with emphasis on these factors, may help address and reduce NEBF.

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 humans were approved by Jimma University Institutional Review Board (JU-IRB) with a reference number (Ref.No) (IHRPGD/478/2020). Furthermore, the second ethical clearance was also taken from the London School of Hygiene and Tropical Medicine (LSHTM) of Observational/Interventions Research Ethics Committee with the Ref.No (LSHTM Ethics Ref: 18022). The third ethical approval was also obtained from the Hawassa University Institutional Review Board (IRB-HU) with the Ref.No. (IRB/033/14). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

AN: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – original draft, Writing – review & editing. TG: Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing. BD: Conceptualization, Data curation, Investigation, Methodology, Supervision, Validation, Writing – review & editing. MK: Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing. MB: Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

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Testing the reported long-term advantages of protein-fortified human milk in very low birth weight neonates

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Preterm infants are at-risk for extrauterine growth restriction and downward percentile-crossing between birth and discharge. Increased energy and protein intake through fortification of human milk during the first weeks of life has been associated with improved short-term growth and better developmental outcomes. The aim of this study was to evaluate whether these benefits persist up to children school age. The study was designed as an observational study. During hospitalization, 22 very low birth weight preterm infants were fed with increasing protein fortification of human milk (protein supplemented group, PSG). As a control group (CG), 11 preterm infants were fed with standard nutrition regimen. At children school age (9–11 years), we assessed anthropometric data (weight, height, BMI), global health (renal function), and specific psychological outcomes (Child Behavior Checklist 6–18). A global homogeneity between CG and PSG groups emerged: we found no significant differences in weight, height, and BMI, nor in internalizing symptom outcomes (all $ps > 0.05$). However, mothers reported significantly higher externalizing symptoms for the PSG infants compared to CG infants. Therefore, neonatal enteral protein supplementation in very low birth weight preterm infants leads to no positive nor adverse consequences in long-term assessment, suggesting that benefits are restricted to the neonatal term and first years of age.

KEYWORDS

very low birth weight infant, protein intake, human milk, long term neurological advantages, psychological outcomes

1 Introduction

Preterm infants frequently develop postnatal growth restriction during their hospitalization in Neonatal Intensive Care Units (1, 2). This could lead to negative consequences, like a higher risk of developing metabolic disorders such as obesity, diabetes and cardiovascular disease and increased motor, cognitive and socio-emotional impairment from school age to adulthood (3–5).

The European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) has recommended a high protein intake in preterm babies born between 26 and 30 weeks of gestational age (3.8–4.4 g/kg/d, energy ratio 3.4) given its positively association with lean body mass growth (6). Increased energy and protein intake through fortification of human milk during the first weeks of life have been associated

with improved short-term growth from infancy to adolescence in preterm and ELBW babies (7, 8). Furthermore, high-caloric nutrition has been associated with increased total brain and basal nuclei volumes as revealed by MR metric (9). Previous studies also described better developmental outcomes during the first 24 months of infant's corrected age, with a higher improvement especially for severe preterm infants, such as ELBW and SGA ones (8, 10–12).

Despite the rapid body mass index (BMI) gain and linear growth may improve cognitive development, they are accompanied by an increased risk of developing metabolic and cardiovascular disease in adulthood (13).

To our knowledge, up to now the literature mainly has focused on early years post-discharge, while the investigation of long-term impact of protein-fortification in preterm children is lacking. Therefore, the aim of this study was to further investigate the potential benefits of feeding preterm infants with human fortified milk; specifically, we hypothesized that this treatment would have been significantly associated to long term advantages in preterm auxological, and psychological outcomes evaluated at child's school age.

2 Method

This study is part of a wider research aimed to assess the effect of milk fortification on preterm infant outcomes in first two years of corrected age (8, 10–12). So, the present study represents a follow up with the objective to explore the state of children school age.

According to methodology explained on our previous published studies (8, 10–12), we recruited 61 preterm neonates born between January 2010 and March 2011 admitted at the level III Neonatal Intensive Care Unit of Bufalini Hospital, Cesena (Italy).

Inclusion criteria for the recruitment were: birth weight (BW) < 1,500 g (Very Low Birth Weight-VLBW), gestational age (GA) < 32 weeks, exclusive human milk feeding (own mother's milk or donor milk from the local human milk bank) during NICU stay, absence of sepsis, intraventricular hemorrhage grade 3 or 4, periventricular leukomalacia, retinopathy of prematurity grade 3 plus disease, necrotizing enterocolitis (NEC) Bell's stage 2 during hospitalization.

All newborns were fed with fresh maternal milk (OMM) and/or donor human milk (DHM), supplemented according to different protein fortification regimens, starting at post-natal day 1.

During hospitalization, neonates were randomly allocated to groups, according to a daily scheduled assignment: infants born at odd and even days were allocated in Protein Supplemented Group (PSG) and Control Group (CG), respectively.

PSG group included 34 neonates (19 ELBW, 15 VLBW), that were fed with fresh OMM or DM since their first day of life. Feeds started at volumes of 10–15 ml/kg/day, divided in 10 meals; when an adequate feeding tolerance was established, feeds were increased by 20–25 ml/kg/die.

Twenty-seven infants (13 ELBW, 14 VLBW) were included in the Control Group (CG): according to Standard Nutrition Protocol, administered by combined enteral and parenteral nutrition according to the European Society of Pediatric Gastroenterology and Nutrition (ESPGHAN) guidelines, they received 4.8 g of protein/kg/day and 3.5 g of protein/kg/day, respectively.

It was used "adjustment fortification" with level of fortification based on blood urea level. Assuming protein human milk content of 0.8–1.1 gr/dl and the diet volume intake of 160 ml/kg^{-1} per day, the max protein intake should have been about 3,5 g kg^{-1} per day in the control group whereas the protein supplemented group (PSG) would achieve a protein intake of 4.8 g/kg^{-1} per day receiving supplemented protein intake by graded amounts of protein. The end of the study was set at the time of discharge, transfer or when the baby was able to ingest >50% of his prescribed quantity directly from the breast of his/her mother.

In both groups, about 60% of milk was provided by the infants' own mother (OMM) and 40% was pasteurized donor milk from the hospital's milk bank (PDM). At discharge, 62.5% of all preterm infants included in the study were fed exclusively with OMM. More specific information on the kind of fortification and effects on brief term infant development were described in our previous works (11, 12).

The results of assessment during first two years were already published (8, 11, 12). The present study focused on a first assessment implemented when children were 6 years of age. Renal function of all children was evaluated by hospital pediatricians. The assessment regarded primarily PSG children in order to verify if the augmented protein in the neonatal period was adequate and if the possible transitional increase of glomerular filtration rate would have been a normal adaptative mechanism without any consequences for future kidney activity.

Subsequently, all children were assessed at about 10 years of age (mean 9.80 ± 0.59; range 8.9–10.8 years). We chose this age range to ensure that children data would not be influenced by adjustment to transition from pre-school to primary school, and also to prevent possible bias related to physiological changes occurring with the onset of puberty period.

All families were contacted through a phone call, to propose this follow-up research in February 2020, scheduling planned assessment in spring 2020. In order to facilitate families' participation, given the restriction measures related to Covid-19 pandemic, the assessment was organized sending the families by mail a booklet including a written informed consent, a form regarding on parental sociodemographic characteristics, anthropometric data (weight and height) and a questionnaire about parental perception of the child behavior. Parents were asked to fulfill and send back by mail in 15 days.

Among the 61 recruited families for previous studies (11, 12), only 33 agreed to maintain their participation to the study. Reasons of drop out were inability to accommodate into time schedule of the study or no interest into the study. The final samples included 22 (12 ELBW, 10 VLBW) and 11 (4 ELBW, 7 VLBW) families of children of PSG and CG groups (64.7% and 40% of the initial study group, respectively).

The study was conducted according to the guidelines of the Declaration of Helsinki and ethically reviewed and approved by the Head of the NICU at the beginning of original study in 2009.

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Anthropometric data on children height and weight given by parents were later correlated to Neonatal Anthropometric INeS Charts to detect percentiles and Z-scores for corrected age in the neonatal period (14). Data related to study group subjects, ages 2–10 years, were analyzed through the PediTools software, a clinical tool for Pediatricians based on CDC growth charts that enables determination of growth metric percentiles and Z-scores of children and adolescents from 2 to 20 years of age. Included in the metrics are calculation of Weight-for-age, Stature-for-age, and BMI-for-age (15). We used BMI-for-age-Z-score in childhood, according to the World Health Organization (WHO), as it represents the most widely available measure of adiposity and a predictor for overweight and obesity in adulthood. According to WHO definition, we considered obesity as BMI –for-age-Z score >3.0 and overweight by a BMI-for –age Z score >2.

Regarding child psychological outcomes through parental perception of the child, both parents fulfilled Child Behavior Checklist 6–18 (CBCL), a well-established parent-reported measure of children's emotional and behavioral functioning (16, 17). The CBCL includes 113 items describing the presence and the frequency of a specific behavior. Parents were asked to indicate how accurately each item applied to their child according to a three-point Likert scale (0, not true; 1, sometimes true; 2, very true). Scores of each item are allocated into 8 syndrome areas (anxious/depressed, withdrawn, somatic complaints, social problems, thought problems, attention problems, rule-breaking behaviour, aggressive behaviour) and summed into 2 main scales: internalizing and externalizing symptom scales. Cut off scores are considered clinically significant when the T-score is 65 or above (16, 17).

Statistical analyses were performed using the Statistical Package for Social Science software (SPSS version 21.0). Significant results were considered when p values were lower than 0.05.

At a preliminary level, homogeneity on clinical and sociodemographic variables between drop out and included participants groups, and between PSG and CG ones, were tested by Pearson Chi2 and Student t-test analyses for categorical and continuous variables, respectively. Given the small sample size, clinical and sociodemographic variables were included in analyses only in case of significant differences in their distribution in the two groups.

The significance of differences in anthropometric data (weight, height and BMI) reported for the two study groups (PSG and CG) was tested through unpaired t-tests performed between the two groups. Furthermore, we run unpaired t-tests to investigate possible differences between groups on CBCL scores, separately, for mothers and fathers.

3 Results

No statistical differences on clinical and sociodemographic variables among drop out infants and those included in the study emerged (all $ps > 0.05$). When we assessed clinical and sociodemographic variables between PSG and CG groups, a globally homogeneity emerged (all $ps > 0.05$) (Table 1).

TABLE 1 Child and parental characteristics according to group.

	PSG ($n = 22$)	CG ($n = 11$)	t/x^2	p
Neonatal Variables				
Birth weight, grams, mean (SD)	981.82 (181.56)	1,028.64 (158.48)	0.727	0.473
Gestational age, weeks, mean (SD)	28.08 (2.03)	29.07 (1.52)	1.435	0.161
CRIB score, mean (SD)	2.59 (2.11)	1.73 (1.27)	−1.244	0.233
SGA, n (%)	7 (31.8%)	3 (27.3)	0.072	0.789
Gender, n (%)			0.243	0.622
Male	12 (54.50)	5 (45.50)		
Female	10 (45.50)	6 (54.50)		
Parent variables				
Maternal age, years, mean (SD)	42.50 (4.27)	45.91 (6.14)	1.864	0.072
Maternal education, n (%)			0.330	0.566
Primary/Secondary school	6 (27.3)	2 (18.20)		
High school/University	16 (72.7)	9 (81.80)		
Paternal age, years, mean (SD)	47.68 (8.53)	48.55 (5.63)	0.303	0.764
Paternal education, n (%)			3.515	0.061
Primary/Secondary school	9 (40.90)	1 (9.10)		
High school/University	16 (59.10)	10 (90.90)		

PSG, protein supplemented group; CG, control group.
Parental variables regard the moment of the assessment.

Renal function was evaluated in 2 and 14 randomly selected CG and PSG children respectively (25% of each study group) at 6.3 years of age (SD 1.7) and resulted normal: mean creatinine value was 0.46 mg/dl (SD 0.06), mean urine pH was 5.9 (SD 0.8), mean urine specific molecular weight was 1018 (SD 10.5).

Assessment of anthropometric data showed no significant differences between PSG and CG weight mean Z-scores (Table 2). According to WHO definition, no children were classified as overweight nor obese.

The assessment of emotional and behavioral functioning showed specific profiles in children of the two groups. Specifically, PSG children reported a significantly higher score (worse) than CG ones when externalizing symptoms scales were measured by their mothers. Conversely, when externalizing scores were evaluated by fathers no significant differences in scores emerged between PSG and CG groups (Table 3).

Furthermore, a global homogeneity between groups was measured for internalizing scores, both when assessed by mothers and fathers (Table 3).

According to CBCL cut-off scores, no significant differences between PSG and CG emerged regarding the frequencies of

TABLE 2 Mean z-score values calculated with world health organization curves in PSG and CG groups.

	PSG ($n = 22$)	CG ($n = 11$)	t	p
Weight	−0.062 (1.06)	0.038 (0.95)	0.265	0.793
Height	−0.483 (0.84)	−0.211 (0.84)	0.877	0.387
BMI	0.185 (1.19)	0.217 (1.12)	0.076	0.940

PSG, protein supplemented group; CG, control group.
Data are expressed as mean (SD) values. p value was generated by t-test.

TABLE 3 CBCL mean scores in mothers and fathers.

Respondents	PSG ($n = 22$)	CG ($n = 11$)	t	p
Mothers				
Internalizing symptoms	49.00 (11.05)	41.67 (9.43)	−1.954	0.059
Externalizing symptoms	48.48 (8.33)	41.83 (6.12)	−2.435	**0.020**
Fathers				
Internalizing symptoms	46.14 (9.45)	44.14 (11.39)	−0.540	0.593
Externalizing symptoms	40.45 (8.00)	40.83 (7.18)	−1.305	0.201

PSG, protein supplemented group; CG, control group.
Data are expressed as mean (SD) values. p value was generated by t-test. Significant differences are highlighted in bold.

clinical risk considering maternal perception of internalizing nor externalizing scores or in paternal perception of internalizing nor externalizing scores (all $ps > 0.05$) (Table 4).

4 Discussion

This preliminary study was conducted with the aim to evaluate long-term outcomes in a NICU population fed with human fortified milk.

Our main hypothesis was that protein enriched human milk fed to preterm newborns during the critical period of first weeks of life would have led to long term advantages in auxological and psychological outcome without concomitant metabolic consequences, such as obesity, during childhood (18, 19).

While our previous studies underlined the benefits on patients' outcomes until 2 years of age (8, 10–12), in the present long-term evaluation the PSG children did not show any significant advantages compared to the standard protein intake group at neurodevelopmental level and psychological outcomes. Moreover, whereas some studies previously reported metabolic consequences, mainly obesity, in children born preterm and fed with high energy and protein intake, we did not find evidence of this in PSG group compared to CG sample.

The absence of long-term significant differences is consistent with a recent meta-analysis, which found no correlations between early nutritional supplementation—such as high protein intake—and increased incidence of later metabolic disease (20). The study of Lin et al. showed that supplemented groups had higher HDL concentration during childhood and a lower fasting blood glucose concentration than children feeding with lower protein intake: this difference seemed to vanish during adolescence. Childhood fasting blood glucose concentration is inversely related to pre-diabetes in adulthood. The meta-analysis substantially confirms that the early macronutrient supplementation in preterm and small for gestational age infants does not have adverse effect on metabolic outcome.

TABLE 4 CBCL cut-off scores in mothers and fathers.

Respondents	PSG ($n = 22$)	CG ($n = 11$)	X2	p
Mothers				
Internalizing symptoms	3 (13.0)	0 (0.0)	1.712	0.191
Externalizing symptoms	1 (13.0)	0 (0.0)	0.537	0.464
Fathers				
Internalizing symptoms	2 (9.1)	2 (16.7)	0.429	0.512
Externalizing symptoms	0 (0.0)	0.0 (0.0)	//	//

PSG, protein supplemented group; CG, control group.
Data are expressed as n (%) values. p value was generated by X2.

It is important to highlight that quick intrahospital growth rate may also be positively associated with the risk of future obesity and metabolic syndrome via epigenetic reprogramming (21). This is especially evident if the weight gain is accelerated; at age 8–11 the preterm children exhibit a greater risk of childhood obesity compared to those born at term. In contrast, the evidence linking rapid postnatal growth to later adiposity or cardiovascular disease risk factors in preterm children's results limited (22).

In the present study, children's BMI, corrected for gestational age at birth, was normal without significant statistical differences between the two CG and SPG groups. We did not find any causality between administration of an adequate protein content in human milk during the first periods of life and obesity in our preterm patients. According to parental report, all patients practiced at least one sport twice a week at the time of evaluation, excluding the possibility of cardiovascular fitness obfuscating interpretation of the results.

We further assessed the possible effects on neurobehavioral outcomes. From this perspective, aggressive nutritional intervention for "catch-up-growth" in preterm infants would appear largely justified. A systematic review, including observational studies, reported a positive association between postnatal weight or head growth and neurocognitive outcomes (22). Asuggesting that prenatal undernutrition turned out to be worse than postnatal rapid growth to determine neurodevelopmental outcomes.

Although Cochrane reports no benefits of fortification assessed beyond infancy based on limited available data (23), we have highlighted that the long-term benefits of extra-protein fortification were only present in fragile preterm infants (ELBW) aggressively fed during the short intrahospital period.

Premature infants weighing <1,000 g at birth may require more protein to grow compared to VLBW infants between 1,000 and 1,500 g. Enteral protein requirements likely need to be adjusted for growth as the infant develops, to accommodate for these differences, beginning with higher protein intake when the infant weighs <1,000 g with subsequent decreased amounts as weight and gestational age increase. Suitable growth, especially of lean body mass and particularly of the brain, is dependent on enrichment of human milk, well known for its beneficial effect, with adequate protein intake (24).

A final consideration regards the overall homogeneity in psychological outcomes. Our aim was driven by previous literature, which underlined that preterm children are at risk of emotional and behavioral problems, especially during the first years of life, that could also persist at school entry (25, 26). Regardless of nutrition, school-age could represent a sensitive time also for parents, induced by the expectations on children's performance, and by potential recollection of child's fragility. Consequent parental expectations could in turn represent a further pressure on children. We found no significant differences in internalizing or externalizing symptoms (in case of paternal

evaluation). Differently from our hypothesis, this result suggests a global homogeneity between the groups and the lack of significant advantages reported during earlier infancy periods. Unexpectedly, the only significant differences were found when evaluations were performed by mothers and suggested that PSG children had more externalizing difficulties than CG children. This result would seem to suggest that not only the advantages of protein supplementation disappear, but they might even represent a risk factor. However, it should be underlined that these differences disappear when categorical scores are used, suggesting an absence of pathology in the sample, as reported in previous studies (26). So, the differences in maternal perception could be not considered as an indicator of relevant difficulties.

Nevertheless, the presence of differences regarding maternal perceptions could suggest that these mothers might experience some difficulties with their children, with potential negative consequences on the mother-child relationship. Although the inclusion of both parents in the assessment of their children should enable less biased evaluation of children, it is not clear whether differences are due to a more accurate assessment by mothers, given their usually higher involvement into children-care, or the presence of specific characteristic in women that influence their answers. The small sample size did not allow to include possible influence of specific characteristics of parent or of the relationship between parent and child.

A final relevant question concerns methodological issues: while we previously assessed infant outcomes by developmental scales (27), in the present research we chose to investigate a larger definition of psychological states, considering child emotional and behavioral functioning. These measures have the advantage to give a realistic perspective of the emotional state inside the families, but it could be possible that changes into these variables are weaker to detect compared to other outcomes, such as cognitive development. Moreover, it could be possible that cognitive outcomes could play a significant role in mediating the relationship between early intervention and parental perception. Indeed, according to the study by Lowe and colleagues (28), the level of cognitive, language and motor development could influence parental perception of their children. So, further studies, including both developmental scales and parental-report questionnaires, are suggested.

Future studies could help to further characterize these factors by, for example, adding the evaluation of the effect of fortified nutrition on cognitive development of preterm children once they reach school age.

The present results should be considered as preliminary, and several limitations of the study should be noted. First, the results need to be confirmed on larger samples. The limited sample size could have reduced the power of analyses and have prevented the testing of more sophisticated hypotheses and analyses. Second, in the present study all the variables are assessed by parental evaluation, while objective height and weight measures, as a psychological profile, are lacking. Further studies, including also structured and standardized assessment, are required. Last, future studies are needed to confirm the results also controlling for the effect of other variables. Indeed, specific nutritional characteristics (frequencies of breastfeeding/bottle maternal milk/bottle formula milk, months of breastfeeding, specific diets,…), as well as variables regarding children (i.e., gender, severity of prematurity as defined by the presence of Sepsis, IVH, NEC, PVL, ROP, …), parents (i.e., parental age, level of education,…) and environment could play a relevant role in influencing child outcomes and parental affective reactions and perceptions of the child.

Therefore, all these variables need to be considered for their possible influences on the outcomes to help to generalize results.

Despite preliminary, these findings might contribute to deepen the understanding of the long-term effects of neonatal enteral protein supplementation in VLBW preterm infants, suggesting that benefits are restricted to the neonatal term and first two years of age.

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 humans were approved by Marcello Stella, Head of the Nicu where study was run. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

AB: Conceptualization, Methodology, Project administration, Writing – original draft, Writing – review & editing. EN: Conceptualization, Data curation, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. MS: Investigation, Project administration, Supervision, Writing – original draft. LM: Investigation, Writing – original draft. EM: Investigation, Writing – original draft. VR: Investigation, Writing – original draft. FA: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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An expert panel on the adequacy of safety data and physiological roles of dietary bovine osteopontin in infancy

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Human milk, due to its unique composition, is the optimal standard for infant nutrition. Osteopontin (OPN) is abundant in human milk but not bovine milk. The addition of bovine milk osteopontin (bmOPN) to formula may replicate OPN's concentration and function in human milk. To address safety concerns, we convened an expert panel to assess the adequacy of safety data and physiological roles of dietary bmOPN in infancy. The exposure of breastfed infants to human milk OPN (hmOPN) has been well-characterized and decreases markedly over the first 6 months of lactation. Dietary bmOPN is resistant to gastric and intestinal digestion, absorbed and cleared from circulation within 8–24 h, and represents a small portion (<5%) of total plasma OPN. Label studies on hmOPN suggest that after 3 h, intact or digested OPN is absorbed into carcass (62%), small intestine (23%), stomach (5%), and small intestinal perfusate (4%), with <2% each found in the cecum, liver, brain, heart, and spleen. Although the results are heterogenous with respect to bmOPN's physiologic impact, no adverse impacts have been reported across growth, gastrointestinal, immune, or brain-related outcomes. Recombinant bovine and human forms demonstrate similar absorption in plasma as bmOPN, as well as effects on cognition and immunity. The panel recommended prioritization of trials measuring a comprehensive set of clinically relevant outcomes on immunity and cognition to confirm the safety of bmOPN over that of further research on its absorption, distribution, metabolism, and excretion. This review offers expert consensus on the adequacy of data available to assess the safety of bmOPN for use in infant formula, aiding evidence-based decisions on the formulation of infant formula.

KEYWORDS

infant, milk, osteopontin, safety, immunity, neurodevelopment, gastrointestinal

Introduction

Optimal nutrition during the first year of life influences growth, susceptibility to infections, and the maturation of the gastrointestinal tract, immune system, and brain (1–4). Consistent evidence suggests that infants fed human milk (directly at the breast or by bottle) have more advantageous health outcomes than those fed formula (5–10). The benefits of human milk are attributed, in part, to its numerous biologically active components such as oligosaccharides, hormones, enzymes, cytokines, lactoferrin (LF), immunoglobulins, and milk fat globule membrane (1, 5, 11–16). It is believed the addition of such components to formula has potential to improve the health of formula-fed infants (17, 18). A joint workshop between the National Institutes of Health (NIH) and the Food and Drug Administration (FDA) was held to discuss an assessment framework surrounding the safe use of biologically active ingredients in infant formula (18). A post-meeting federal comment from the FDA noted that assessing the safety of such ingredients is complicated by the variability of human milk among mothers and across time, lack of consensus on follow-up periods in clinical trials, complexity of matrix effects, and lack of standardized approaches for evaluating the safety of bioactive ingredients (17).

Osteopontin (OPN) is one such protein that could be added to infant formulas to mimic the composition and functionality of human milk. OPN is particularly high in concentration in human milk compared to bovine milk (19), with growing evidence that its intake in early life supports immune, intestinal, and neural development (20–23). In 2022, the European Food Safety Authority (EFSA) published their scientific opinion that bovine milk osteopontin (bmOPN) is safe when added at a maximum concentration of 151 mg/L in infant formula (up to 6 months of age), follow-on formula (ages 6–12 months), and formula for young children (ages 1–3 years) (24). EFSA noted that while inconsistencies and limitations were present in the available science, they did not raise safety concerns (24). In the U.S., however, submission of the Generally Recognized as Safe (GRAS) Notice 716 for bmOPN was withdrawn after FDA concerns could not be alleviated in 2018 (25). The reasoning is stated in internal memos available at the CFSAN (Center for Food Safety and Applied Nutrition) FOIA (Freedom of Information Act) Electronic Reading Room on Bioactive Ingredients for Use in Infant Formula dated September 2020 (26). Since that submission and post-submission meetings thereafter (personal communication), the FDA noted concerns about bmOPN and its addition to infant formulas related to: high variability of dietary exposure to human milk OPN (hmOPN); relevance of the selected level of bmOPN in formula; lack of absorption, distribution, metabolism, and excretion (ADME) data; potential for crossing the blood–brain-barrier; its potential long-term immunomodulatory role and mechanism of action; its functional similarity to hmOPN; inadequacy of standard toxicological assessments for determining safety of bmOPN; and its potential relationship to various immune-related diseases.

Considering these concerns, a panel of experts convened to discuss the scientific evidence on the physiological roles and safety of orally ingested bovine milk OPN (bmOPN) for use in infant formulas for healthy term infants. The goal of the panel was to identify and interpret the research literature, identify what important gaps remain for determination of safe use of bmOPN in infant formula, and prioritize which gaps further research should address to inform future safety assessments. The efficacy of bmOPN was not of focus, however physiological effects that could potentially constitute or explain efficacy were in-scope. Here we report a narrative review on the physiological roles of milk OPN and evaluate existing data on bmOPN intake in early life, paying special attention to concerns raised previously by the FDA and at the FDA-NIH workshop.

Panel procedures

Authors SAF, SMR, and JCW were not considered expert panel members. Experts were selected by authors BL and JCW for their direct research experience on OPN or their expertise regarding gastrointestinal and immune development, or neuroimmunology. All experts declared direct and related conflicts of interest to the subject material in the preceding 36 months, which are detailed in the author disclosures. All authors (except SMR) attended a two-day, in-person workshop to discuss the concerns listed (described above) by the FDA and to review the available evidence. The workshop was organized by Building Block Nutritionals and Arla Foods Ingredients. Those with published research on OPN (BL, RJ, ESS, CEW, SMD, LS) were invited to present their research, those without presented research on gastrointestinal/immune development (OH, JN), and all were encouraged to provide their interpretation of the data. RK was chosen to lead the panel and solicit alternative scientific opinions on the interpretation of the data. After the panel was concluded, SAF and SMR independently conducted a narrative review to fact-check the evidence presented, corresponding with experts to clarify discrepancies or solicit further discussion. Two post-workshop remote meetings were held to review and interpret the evidence. A draft manuscript was written by SAF and SMR and reviewed over 3 rounds by all authors. As a final check against bias, SAF solicited alternative opinions on the interpretation of the data and conclusions of the panel on a 1:1 basis, such that responses remained anonymous to all authors except SAF. Experts were asked to provide their final opinion on the sufficiency of the data as it relates to safety, their interpretation of specific elements of the research, what gaps remain, and what future research to prioritize. Experts were not asked to provide a final conclusion on whether or not bmOPN is safe for use in infant formula. All authors were compensated by Arla Foods Ingredients for their efforts to attend the workshop and draft, revise, or edit the manuscript. Neither Arla Foods Ingredients nor Building Block Nutritionals were permitted to participate in the remote post-meeting workshops, nor to hold contributor or editorial roles in the writing of the manuscript.

Narrative review

The topics reviewed by the panel included dietary exposure to milk OPN, ADME of consumed milk OPN, OPN's role in gastrointestinal, immune, and neural development, the extent of the functional bioequivalence between forms of OPN (whether dietary intake of human, bovine, or recombinant forms of OPN demonstrate similar ADME profiles and physiological effects), and potential matrix effects with other components in infant formula and human milk. Although not a systematic review, study eligibility criteria (Table 1) were developed to guide the panel's assessment of data toward that of the appropriate developmental stage (infancy), type of exposure to

TABLE 1 Study eligibility criteria.[1]

Inclusion criteria	Exclusion criteria
Population:[1] *In vivo* trials in humans conducted in healthy pediatric populations or animal models of healthy early-life development.	Population: *In vivo* trials in adult humans or animals models of adulthood. Populations/models diagnosed with, at-risk for, or as a model of, any pre-existing disease, disorder, or injury, except for prematurity. *In vitro* trials in any model that examine the function of bmOPN, excepting those used to examine potential matrix effects.
Exposure:[1] Intake of any form of OPN (human, bovine, murine, recombinant, or otherwise) in any matrix (e.g., water, formula, milk), delivered using any dietary method (e.g., *ad libitum* feeding, oral gavage, or enteral feeding) that does not bypass the gastrointestinal tract.	Exposure: Studies in which delivery of OPN bypassed the gastrointestinal tract, such as through i.p. injection, parenteral nutrition, intranasal delivery, or other methods.
Outcomes: Outcomes related to exposure, ADME, anthropometric growth, immune function, brain development, cognition, or matrix effects with other milk components.	Outcomes: Studies assessing the function of endogenous OPN as an outcome in response to an intervention (e.g., OPN expression in response to injury or inflammation). Correlations between endogenous OPN and other physiological effects.
Study Design: *In vivo* research	Study Design: *in vitro* or *ex vivo* trials

[1]Exceptions were made for any criteria in order to describe endogenous concentrations of OPN in various fluids (e.g., milk, plasma) across the lifespan. ADME, absorption, distribution, metabolism, excretion; bmOPN, bovine milk osteopontin; OPN, osteopontin.

OPN (dietary), health status (no pre-existing diseases, disorders, or injuries, excepting prematurity), and study design (*in vivo* research).

Where appropriate, data that fit exclusion criteria were still used to describe exposure (e.g., endogenous levels of plasma OPN across the lifespan), and concepts related to its structure. It is well established that mechanisms of gastrointestinal, immune, and brain development are distinct from those supporting mature function in adulthood (27–30), thus the panel concluded that data generated in adult animals were not translatable to understanding the safety of bmOPN consumed by infants and were thus not reviewed. Then, the panel concluded that the inclusion of data generated on OPN in non-dietary contexts (including *in vitro* research) or in models of disease would not inform its function in a healthy, developmental, dietary context. For example, literature describing endogenously produced OPN or non-dietary OPN (e.g., administered by i.p. or i.v. injection) in adult models of inflammatory disease (31, 32) were not reviewed. Lastly, studies in preterm pig models were also included as they are relevant to exposure to OPN in early development.

Throughout, language denoting a "difference" or "change" between groups are only those that were statistically significant (as defined by the original authors, typically $p < 0.05$), unless otherwise stated. Likewise, the lack of a difference indicates a comparison made where no statistical significance was reached, unless otherwise stated.

Infant exposure to dietary osteopontin from milk and formula

OPN, found ubiquitously in nearly all body fluids, is specifically expressed at high levels from mammary tissue during lactation (33). Although hmOPN was originally estimated to be almost 10% of total protein in human milk (34), Schack et al. later reported term human milk to contain 138 mg/L hmOPN, about 2.1% (wt/wt) of the total protein in mature milk (19). Since 2009, several other studies have reported concentrations of hmOPN (23, 35–40). Collectively, these new data confirmed the observation that hmOPN concentrations decrease over the course of lactation, a trajectory also observed in murine and bovine milk (41, 42), suggesting a conserved biological pattern across mammals. The concentration of hmOPN is correlated with that of total protein, α-lactalbumin, LF, and casein (43). hmOPN is present at 250–350 mg/L in colostrum, declining to around 65–250 mg/L in mature, term human milk (Figure 1). For comparison, standard infant formulas (predominantly bovine milk-based) contain considerably less OPN (~9–15 mg/L of bmOPN) than is present in human milk (19).

Recent studies provide evidence that hmOPN concentration varies with many maternal factors including diet, parity, age, ethnicity and body composition (36, 40, 43). For example, higher levels of hmOPN were observed in women who delivered vaginally, had a non-obese postpartum BMI (<30 kg/m^2), and abstained from smoking (36). Additionally, among these women, low to moderate inverse associations were found between hmOPN and maternal intake of daily energy, and grams of fat, carbohydrate, and fiber intake (36). While variation in hmOPN appears high, most variation comes from between-study sources, with less variance within studies. Variation is even less pronounced when considering studies that use the same measurement method, such as ELISA versus HPLC (Figure 1). Notably, the human OPN (hOPN) ELISA from Immuno-Biological Laboratories (Gunma, Japan) has been reported to overestimate OPN concentrations compared to the hOPN ELISA from R&D Systems (Abingdon, UK) (19, 44). Given the heterogeneity in study design and assay method, it was not possible to ascertain true geographic differences in hmOPN concentration between mothers from Japan (45, 46), Denmark (19), the U.S. (23, 47, 48), Turkey (36), and China (38, 39, 43).

Panel conclusions
The panel concluded that the existing data enable quantification of hmOPN across the first six months of life.

Absorption, distribution, metabolism, and excretion of milk osteopontin

The structure and digestion of OPN from human and bovine milk have been extensively reviewed (21, 44). Briefly, OPN is a highly acidic protein that lacks a fixed tertiary structure (i.e., is intrinsically disordered) and undergoes extensive post-translational modifications including phosphorylation and glycosylation (49). There are 3 main splice variants of the protein: OPNa (the full-length form), OPNb (lack of exon 5), and OPNc (lack of exon 4), with OPNa being the only

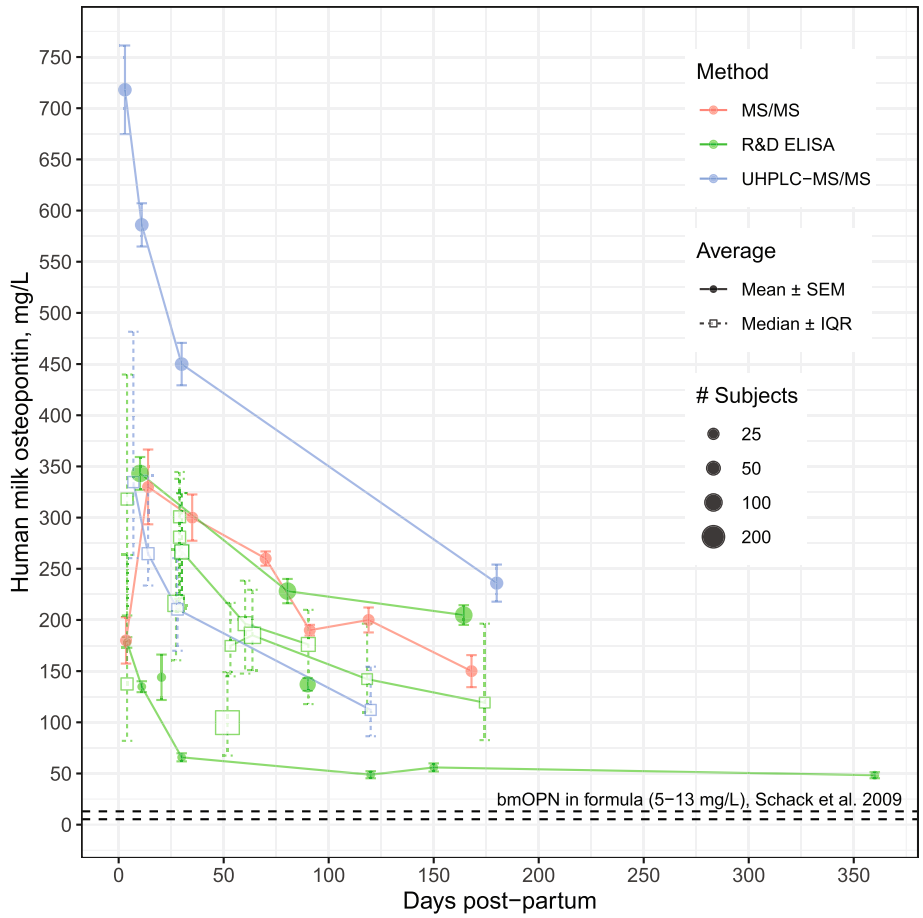

FIGURE 1
Osteopontin concentrations in human milk from mothers of healthy, term infants by analytical method. Modified from Sørensen and Christensen (44). Data used for visualization can be found at https://github.com/Traverse-Science/Osteopontin-Expert-Panel. For studies reporting samples collected across a range (e.g., 0–3months post-partum), the midpoint was used. Concentrations of hmOPN are high at birth and gradually decline over the first 6months of lactation. Points connected by a line represent longitudinal assessments from the same study. bmOPN, bovine milk osteopontin; IQR, Interquartile range; MS, mass spectrometry; R&D ELISA, enzyme-linked immunosorbent assay from R&D Systems (Abingdon, UK); SEM, Standard error of the mean; UHPLC, Ultra-High-Performance-Liquid Chromatography.

variant expressed in human milk (50). hmOPN and bmOPN are comprised of 298 and 262 amino acid residues, respectively, and are highly homologous with identical amino acids on 182 positions in addition to 44 structurally conserved amino acid substitutions (44). Full-length OPN undergoes cleavage by endogenous proteases in milk, resulting in several N-terminal-derived fragments (51, 52). C-terminal fragments are not detected in human and bovine milk, as they are likely further degraded to smaller peptides by proteases in milk (51, 52).

In vitro and *in vivo* studies have demonstrated that oral OPN is resistant to gastric and intestinal digestion. For example, human and bovine OPN have been detected intact after incubation with newborn gastric aspirates for 1 h at pH 3 (53). Additionally, intact bmOPN was found in both stomach and small intestinal contents of OPN knock-out (KO) mouse pups 30 min after oral gavage (54–56). Resistance to digestive proteases is primarily attributed to the glycosylated and conserved threonine residues close to the Arg-Gly-Asp integrin-binding sequence (44, 54, 57, 58). *In vitro* evidence suggests that intact OPN and/or fragments can cross the intestinal barrier via transcytosis (59), and dietary bmOPN has been detected in the plasma of rodents and humans (23, 55, 60), demonstrating absorption.

Assessing the distribution of dietary bmOPN is complicated by its ubiquitous endogenous presence in numerous tissues and fluids (21, 22, 31, 33, 44, 50, 61, 62). While human milk is particularly rich in OPN, plasma is not. The concentration of OPN in mature human milk ranges between 65–250 mg/L (Figure 1), term infant urine contains 6.6 mg/L (27 mg/L in adult urine) (63), adult cerebrospinal fluid contains 0.319 mg/L (64), term infant plasma contains between 0.075–0.170 mg/L (23), and adult plasma the lowest of these estimated at ≤0.080 mg/L (19, 65, 66). In infancy, the circulating concentration is a combination of dietary OPN and endogenously synthesized OPN. Jiang et al. reported ~75–170 µg/L hOPN in plasma of formula-fed infants compared to ~100–238 µg/L hOPN in plasma of breastfed (BF) infants (23) (Figure 2A). The presence of hOPN in plasma of formula-fed infants who consumed no hmOPN (at ~75% of the plasma concentration of hOPN in breastfed infants), suggests that the majority of circulating hOPN is endogenous in origin. Endogenous hOPN may be increased in response to dietary intake of bmOPN, as plasma concentrations of hOPN were higher in infants fed formula containing 65 or 130 mg/L bmOPN than those fed formula without supplementation of bmOPN (Figure 2A). This study also reported that bmOPN was detected in circulation but

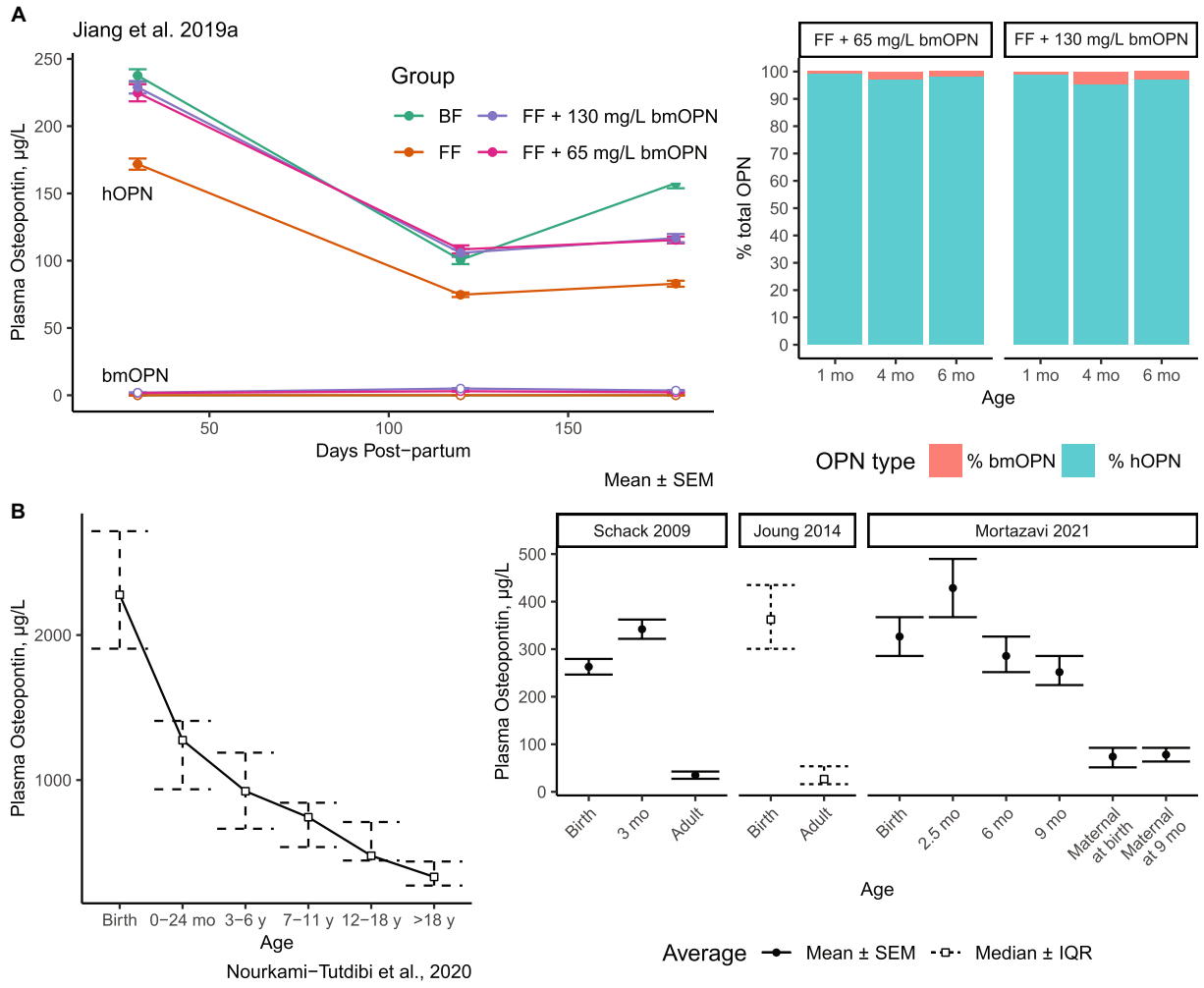

FIGURE 2
Concentrations of OPN in plasma in human infants and across the lifespan. **(A)** Concentration of bmOPN and hOPN among infants breastfed or fed formula supplemented with bmOPN Open points represent bmOPN, solid points represent hOPN. Stacked bars indicated the proportion of total OPN from bmOPN or hOPN. bmOPN represented less than 5% of total OPN at the greatest. **(A)** Modified from Jiang et al. (23). **(B)** Concentrations of plasma hOPN across the lifespan Modified from Nourkami-Tutdibi et al. (67); Schack et al. (19); Joung et al. (65); Mortazavi et al. (66). BF, breastfed; bmOPN, bovine milk osteopontin; FF, formula-fed; hOPN, human osteopontin; mo, Month.

represented 1.75–5% of total plasma OPN, even among infants receiving 130 mg/L bmOPN-enriched formula (Figure 2A).

Results from multiple studies demonstrate that the concentration of plasma hOPN is high at birth and gradually declines over the lifespan (Figure 2B) (67). By adulthood, plasma hOPN concentrations fall to between 26–80 µg/L (19, 65) (Figure 2B). Although absolute concentrations differ (representing the use of different ELISAs), the trend of decreased OPN from birth to adulthood is replicated (19, 65, 67). For comparison, circulating hOPN is about 1,000x lower in concentration than that found in human milk.

Insights into the distribution and metabolism of dietary OPN have emerged from studies conducted in mice. In an acute absorption study conducted by Rittling et al., OPN-deficient mice were fed milk enriched with bmOPN at 250 mg/mL, for a total one-time dose of 50 mg. Peak plasma levels of bmOPN (measured by an in-house competition ELISA; Rittling et al. suggest these were likely peptides) were observed at 1 and 4 h in 3-and 10-week-old mice, respectively (60) (Figure 3A). The levels of plasma bmOPN rapidly declined to an undetectable level between 4 and 8 h in 3-week-old mice, with low levels (compared to peak concentrations) detectable in 10-week-old mice. bmOPN appears to be rapidly (within 8 h) cleared from plasma, though whether that be in response to tissue uptake or metabolism is unclear. There were similar levels of biotinylated forms of bmOPN, recombinant human OPN, and recombinant bovine OPN in plasma 3 h after feeding in PND 12 mouse pups (55) (Figure 3B), which may represent peak levels (60).

After 1 to 3 h following oral gavage with radio-labeled hmOPN, label was detected in multiple organs and tissues of the mouse (41) (Figure 3C). After 3 h, >95% of the label was recovered, with ~62% found in the carcass, ~23% in the small intestine, 5.4% in the stomach, and ~3.8% in intestinal contents. The remaining ~6% was present at amounts of <2% in the cecum and liver, and <0.6% in the brain, spleen, and heart. Percentages reflect total label measured, unadjusted for tissue size. Over the course of 3 h, the labeled signal increased in carcass tissue, decreased in small intestinal perfusate and cecum colon, and remained relatively stable in other tissues (41). Further research is needed to characterize whether

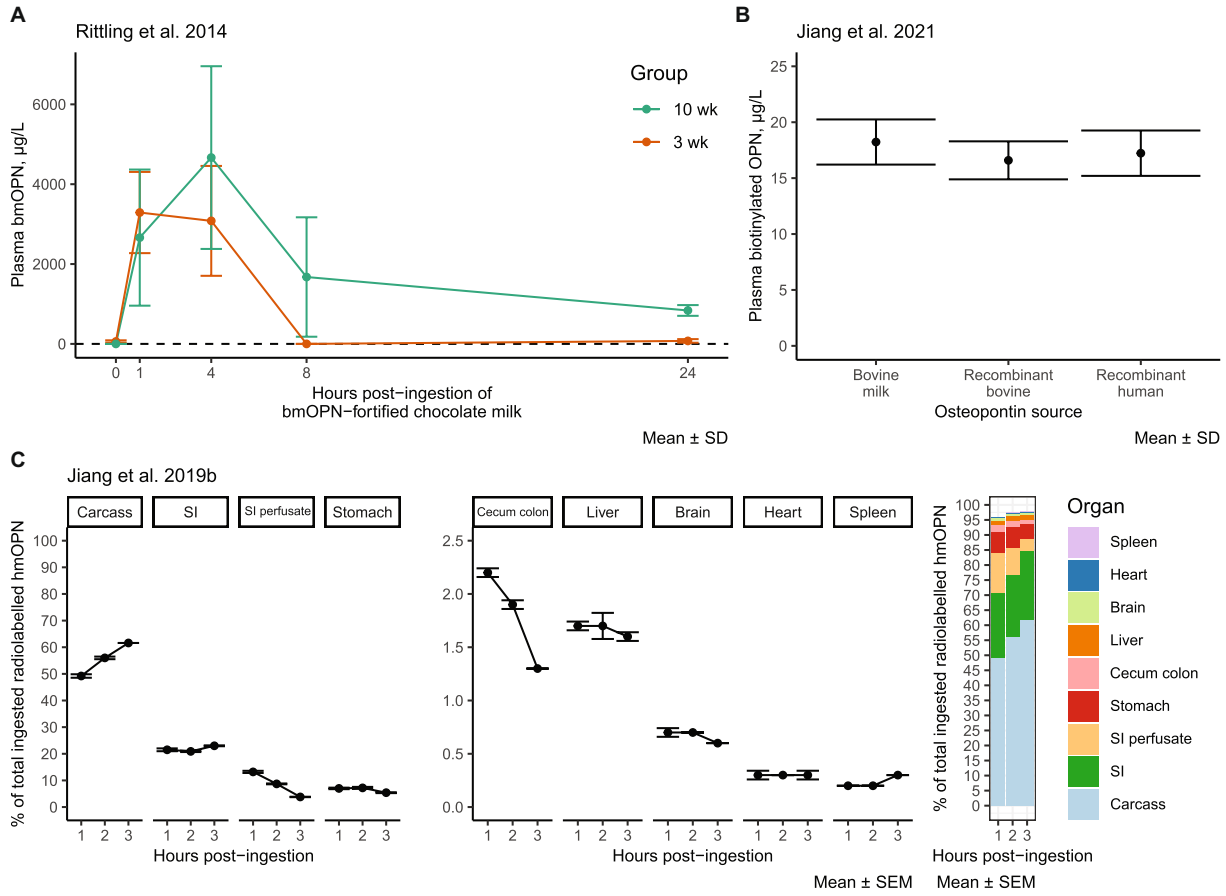

FIGURE 3
Experimental evidence of the distribution of dietary OPN: **(A)** bmOPN in plasma of OPN-deficient KO mice after oral gavage of 50 mg bmOPN in chocolate milk. Modified from Rittling et al. (60). **(B)** Plasma levels of biotinylated OPN 3 h after feeding (oral gavage) PND 12 WT mice with 12 mg OPN/kg bodyweight/day from bovine milk (Lacprodan OPN-10, Arla Foods Ingredients, Viby, Denmark), algal recombinant bovine OPN (Triton Algae Innovations, San Diego, CA), or algal recombinant human OPN (Triton Algae Innovations, San Diego, CA) in water. Modified from Jiang et al. (55). **(C)** Tissue distribution of [125I] radio-labeled hmOPN 1–3 h after oral gavage in WT mice. Modified from Jiang et al. (41). bmOPN, bovine milk osteopontin; hmOPN, human milk osteopontin; KO, knockout; PND, postnatal day; SD, standard deviation; SEM, standard error of the mean; SI, small intestine; wk., week; WT, wild-type.

orally ingested OPN has a distinct metabolic fate from that of endogenous OPN. It remains an open question if, to what extent, and how dietary OPN crosses the blood–brain barrier (BBB). Given that the hmOPN was radiolabeled with iodine-125 (41), which incorporates into tyrosine and histidine residues, it is not clear if the radiolabeled signal in these tissues is intact/partial hmOPN, smaller peptides and free amino acids thereof, or the same residues synthesized into new proteins. Osteopontin peptides have been detected in the blood of human subjects as early as 30 min or up to 7 h after consumption (68), suggesting the bmOPN signal detected by Jiang et al. within 3 h of bmOPN ingestion (41) could have represented absorption of OPN-related peptides in blood. It is also possible that the signal measured was indeed intact hmOPN, but the protein was located within blood or neurovascular tissue without crossing the BBB, as whole brain samples were not perfused prior to measurement, and thus contained blood. Regardless, whole brain lysates from OPN KO pups (devoid of tissue OPN) fed milk from wild-type (WT) dams demonstrate the presence of OPN (measured by Western blot) (41). The panel concluded that there is some evidence from the KO model that BBB transport of dietary OPN can occur, which may apply to endogenously produced OPN from serum as well. More research is required to definitively conclude that transport does occur and the physiological importance of such transport.

ADME summary

- In humans, OPN is 382-3333x higher in mature human milk, 39-88x higher in term infant urine, and 2-4x higher in adult cerebrospinal fluid as compared to term infant plasma (23, 63, 64). Plasma OPN is greatest in infancy and reduces across the lifespan to its lowest levels in adulthood (19, 65–67).
- bmOPN is resistant to gastric and small intestinal digestion, in mice (53–56).
- After acute intake of labeled hmOPN in mice, most of the label is found in carcass tissue (62%), small intestine (23%), stomach (5%), small intestinal perfusate (4%), with <2% each found in the cecum, liver, brain, heart, and spleen (41).
- It is unclear if, how, and to what extent OPN crosses the BBB.
- In 3-week-old mice, acute intake of bmOPN largely clears circulation within 8–24 h (60).

- After intake of bmOPN or algal recombinant human/bovine OPN, the forms of each are present in similar levels in the plasma of mice (55).
- In infants, bmOPN is present in circulation at 1.75–5% that of endogenous human OPN with daily consumption. Consumption of bmOPN may stimulate an increase in endogenous circulating OPN (23).
- Gaps in the ADME profile include clarification of transport across the BBB, quantification of dietary bmOPN's distribution in tissues and fluids relative to the concentration of endogenous OPN, quantification of its half-life, and replication of the effects shown thus far.

Panel conclusions
Although gaps in the understanding of the ADME profile of bmOPN exist, the panel questioned the utility of ADME as a paradigm to contextualize the physiological function or developmental impact of dietary bmOPN, given its low concentration in plasma relative to endogenous OPN. The panel suggested further research on clinically relevant outcomes be prioritized above ADME.

Orally ingested milk osteopontin and development

The FDA notes a need to standardize approaches for evaluating the safety of bioactive ingredients for use in infant formula (17). The FDA's state that, especially for potentially immunomodulatory substances, standard toxicological endpoints may not inform whether bmOPN is safe for use in infant formula or not (26). In an attempt to address this need, a previous expert panel separated safety endpoints related to immunity into those that are clinically relevant standard endpoints and those that are candidate markers of immune development (69). Clinically relevant endpoints included (but were not limited to) outcomes such as anthropometrics, incidence of infection, adverse events related to inflammation, vaccine response, hospitalizations, fevers, atopic dermatitis, and food allergy. Biomarkers included measurements of cytokines and immune cell populations. Callahan et al. recommended that the standard clinical endpoints are sufficient to demonstrate safety of novel bioactive ingredients for immune-related outcomes, and that the biomarkers can be used as indicators of stereotypical immune development (69). Using a range of clinically relevant outcomes and physiological biomarkers, 10 preclinical studies using either genetic knock outs or oral supplementation have investigated the role of dietary OPN and potentials mechanisms of action across gastrointestinal, immune, and neurodevelopmental outcomes. These include four KO studies in mice (41, 54–56); 1 rat trial (70); 1 term pig trial (71); 3 preterm pig trials (72–74); and 1 study in rhesus monkeys (75). Of those that measured bodyweight (BW) or anthropometrics, none reported an effect of OPN supplementation on growth (41, 55, 70–72, 75).

The role of dietary OPN examined in OPN knock-out mouse models

Jiang et al. report several cross-fostering experiments using WT and OPN KO mice to assess gastrointestinal development (54–56), neurodevelopment (41, 54, 55), and immunity (54, 55). In these experiments, WT or KO mouse pups (Pup^{WT}, Pup^{KO}) were cross-fostered to nurse WT or KO dams (Dam^{WT}, Dam^{KO}), creating up to 4 groups: Pup^{WT}/Dam^{WT} (a model replicating breastfed infants), Pup^{WT}/Dam^{KO} (a model replicating formula-fed infants), Pup^{KO}/Dam^{WT}, and Pup^{KO}/Dam^{KO} groups, respectively. The Pup^{WT}/Dam^{WT} group represents exposure to both endogenous and dietary OPN, and the Pup^{WT}/Dam^{KO} group represents exposure only to endogenous OPN. While not direct evidence of the safety of bmOPN, such models provide insight into the function of dietary OPN in early-life. Pups nursed to WT dams were exposed to an average of 12 mg OPN/kg BW/day (55) from milk until weaning on postnatal day (PND) 21 (41, 54–56). Milk yield was similar between Dam^{WT} and Dam^{KO} (41) mothers. Pup^{WT}/Dam^{KO} pups had lower jejunal cell proliferation (56), smaller inner surfaces of the jejunum on PND 10 and 20 (54–56), but not post-weaning on PND 30 (56) compared to Pup^{WT}/Dam^{WT} pups. Absolute length of the intestines was unchanged on PND 10 in 2 studies (54, 55), and shorter in Pup^{WT}/Dam^{KO} pups on a per BW basis from PND 4–6 and equivalent from PND 8–30 (56). Post-weaning at PND 30 Pup^{WT}/Dam^{KO} pups had lower alkaline phosphatase activity in the brush border of the duodenum and jejunum as well as fewer goblet cells, enteroendocrine cells, and Paneth cells compared to Pup^{WT}/Dam^{WT} pups (56). mRNA expression of the integrins α_V, β_3, and CD44 were generally lower in Pup^{WT}/Dam^{KO} compared to Pup^{WT}/Dam^{WT} between PND 4–20, but equivalent on PND 30. In contrast, protein expression of the same proteins was lower in Pup^{WT}/Dam^{KO} compared to Pup^{WT}/Dam^{WT} pups on PND 10, 20, and 30 (56). Moreover, compared to Pup^{WT}/Dam^{WT} pups, Pup^{WT}/Dam^{KO} pups had lower expression between PND 10 and PND 30 of several proteins related to signaling pathways important for intestinal development, namely extracellular signal-regulated kinase (ERK), phosphoinositide-3-kinase/protein kinase B (PI3K/Akt), Wnt, and focal adhesion kinase signaling (56, 76–78). Together, these data provided evidence that the absence of OPN in milk can alter some mechanisms of intestinal development, some of which remained altered after weaning and cessation of OPN intake.

Jiang et al. demonstrated that the absence of orally ingested milk OPN can lead to suboptimal myelination patterns and impaired performance on behavioral tasks measuring motor learning and memory (41). Although mRNA expression of OPN in whole-brain lysates of pups did not differ significantly between groups, compared to Pup^{WT}/Dam^{WT} pups, Pup^{WT}/Dam^{KO} pups had significantly lower concentrations of OPN protein in whole brain lysates on PND 6 and 8, a period considered critical for brain development (41). Immunohistochemistry revealed that Pup^{WT}/Dam^{KO} pups also had reduced proliferation and differentiation of glial cells into oligodendrocytes, fewer OPN+ cells, and fewer myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) positive cells detected in the hippocampus, corpus callosum, striatum, and cerebellum as compared to Pup^{WT}/Dam^{WT} pups between PND 6–20. These were concurrent with thinner myelin sheaths in the spinal cord on PND 8 in Pup^{WT}/Dam^{KO} pups compared to Pup^{WT}/Dam^{WT} (41). Of the outcomes measured after weaning, there were no differences in mRNA expression of MBP, MAG, proteolipid protein, 2′,3′- cyclic nucleotide 3′-phosphodiesterase, or myelin oligodendrocyte glycoprotein, or protein expression of MBP, MAG, or ERK-1/2 and PI3K/Akt signaling pathways. Protein expression of neural/glial antigen 2 (a glial cell marker) and anti-adenomatous polyposis coli clone 1 (an oligodendrocyte marker) remained reduced in $Pup^{WT}/$

Dam^{KO} pups compared to Pup^{WT}/Dam^{WT} after weaning, as well as behavioral performance on the passive avoidance and rotarod tasks, measures of memory and motor learning. Thus, lack of exposure to early-life OPN had transient impacts on markers of myelination and expression of OPN, and post-weaning impacts on markers of glial cell development and behavior.

Regarding immunity, two studies demonstrated that Pup^{WT}/Dam^{KO} weaned on PND 21 have increased plasma TNF-α in response to intraperitoneal injection of lipopolysaccharide (LPS) from *Escherichia coli* on PND 30 as compared to Pup^{WT}/Dam^{WT} (54, 55), indicating an increased response to immune challenges.

In summary, KO models confirmed that pups consuming dam's milk without OPN had altered gastrointestinal and neural physiology, impaired cognitive performance, and increased immune responses when compared to pups consuming dam's milk with OPN. Some of these changes remained after weaning and cessation of OPN intake. Such studies provided evidence that intake of milk OPN may play a role in development. Further research is needed to assess whether such effects extend to bmOPN supplementation, and not its absence.

Supplementation with bovine osteopontin

Throughout, references to the concentration of bmOPN refer to the concentration of the protein, and not the ingredient/source used. All studies used Lacprodan OPN-10 (Arla Foods Ingredients, Viby, Denmark) as the source of bmOPN, which was reported by the original publications or confirmed through personal communication with the manufacturer (70). Lacprodan OPN-10 is composed of ~80% protein, 9% ash, 4% moisture, and ~0.1% lactose. bmOPN represents over 88.5% of protein, with 25.4–26.5% of bmOPN in the product as full-length OPN, with 73.5–74.6% an N-terminal fragment, and no C-terminal fragments. A more detailed description of the composition and specifications is available elsewhere (24).

Using the same KO models and study design, Jiang et al. orally gavaged Pup^{WT}/Dam^{KO} pups with 12 mg bmOPN/kg BW/day in water (54). Supplementation of Pup^{WT}/Dam^{KO} with bmOPN compared to Pup^{WT}/Dam^{KO} without bmOPN increased whole-brain MBP and MAG, increased the villus height to crypt depth ratio, and post-weaning attenuated impairments in cognitive performance and lowered the level of plasma tumor necrosis factor α (TNF-α) in response to LPS, bringing the overall phenotype of bmOPN supplemented pups closer to that of Pup^{WT}/Dam^{WT} pups (54, 55). The same effects were found with supplemental algal recombinant bovine OPN (Triton Algae Innovations, San Diego, CA), or algal recombinant human OPN (Triton Algae Innovations, San Diego, CA), demonstrating functional similarity on these outcomes between forms of OPN despite their different post-translational modifications (the recombinant forms were not glycosylated and contained fewer phosphorylation sites than bmOPN) (55).

Chen et al. studied the effects of bmOPN supplementation on the adaptive immunity of Sprague Dawley rats by gavaging them with a standard (Biostime Beta-star, Biostime [Guangzhou] Health Product Company Ltd., China, containing 10 mg/L bmOPN) or bmOPN-enriched formula (Biostime Pi-star, Biostime [Guangzhou] Health Product Company Ltd., China, containing 65 mg/L bmOPN from Lacprodan OPN-10) in addition to nursing and compared them to an exclusively dam-fed group for 21 days (PND 7–28) (70). On PND 28 those exclusively dam-fed had higher concentrations of CD8+ T cells in lymph nodes compared to those fed standard formula, with no significant differences between bmOPN-enriched and dam-fed groups. Rats fed bmOPN-enriched formula had greater CD3+ cells in lymph nodes, but not in the spleen as compared to rats fed a standard formula. No significant differences were observed in CD4+, CD8+, or B220+ cell concentrations in lymph nodes or spleen between formula groups. After weaning, the immunoglobulin (IgG, IgA, and IgM) response to LPS was not different between formula groups, however anti-ovalbumin IgG (but not IgA or IgM) was increased in the bmOPN fed rats, shifting their response toward that of the dam-fed rats. Chen et al. interpreted these data as evidence that bmOPN-enriched formula promotes differentiation of $CD3^+$ T cells and the T-cell-dependent humoral immune response, with 65 mg/L bmOPN resulting in modest changes in immune markers and shifting the phenotype closer to that of nursed pups (70).

While the data on bmOPN supplementation in both mouse KO and WT models suggest numerous physiological functions of dietary bmOPN, large animal data have not reproduced such effects. Artificially reared term pigs were fed a soy-protein-isolate-based milk replacer (to eliminate residual bmOPN in bovine-milk-based replacers) supplemented with Lacprodan OPN-10 at 250 mg OPN/L (estimated 25–71.1 mg bmOPN/kg BW per day) in milk replacer from PND 3–32 (71). Magnetic resonance imaging revealed some differences in regional brain volumes, microstructure of the corpus callosum, and minor differences in behavior. While the authors initially hypothesized OPN supplementation would improve neurodevelopment, they concluded that the results were minimal and did not report any impairments to neurodevelopment (71).

Three studies used the preterm pig model, in which pigs were delivered via cesarean section between 89–92% of their full gestational length (115–117 days) (72–74). Pigs delivered preterm were reared individually in heated incubators and provided supplemental oxygen (72–74). They were fed via parenteral nutrition and gradually (72, 74) or entirely (73) transitioned to enteral nutrition. Given the lack of exposure to colostrum, pigs were provided maternal plasma to support passive immunity (72, 73).

Aasmul-Olsen et al. report that preterm pigs fed raw bovine milk supplemented with bmOPN (Lacprodan OPN-10, 46 mg bmOPN/kg BW per day, median 319 mg/L from PND 1–19) had higher villus height-to-crypt ratios on PND 19 compared to pigs fed raw bovine milk without supplementation (72). However, OPN-supplementation did not result in significant differences in the number of proliferative enterocytes on PND 19. For markers of immunity, OPN supplementation had low to modest effects. OPN-supplemented pigs had higher blood concentrations of monocytes and lymphocytes on PND 8, and a lower neutrophil phagocytic rate at PND 19 compared to pigs fed raw bovine milk without supplementation (72). However, there were no differences in T cell subsets including T-helper cells, cytotoxic T cells and regulatory T cells, and concentrations of interleukin-10 (IL-10) and TNF-α cytokines were similar in LPS-stimulated whole blood samples and undetectable in unstimulated samples (72). Among brain-related outcomes, there were no differences in whole and sub-region brain weights at euthanasia on PND 19, nor differences on open-field behavior, early motor development, or spatial learning on PND 12–18 compared to controls (72).

In another study, preterm pigs were exposed to prenatal inflammation via intra-amniotic LPS injection 3 days prior to birth and fed bmOPN (Lacprodan OPN-10, 2.22 g/L, 53.3 mg bmOPN/kg BW per day) for 5 days (74). Those fed bmOPN-enriched formula had no differences in clinically relevant endpoints of inflammatory intestinal injury or incidence of diarrhea compared to the control formula. Other biomarkers also did not differ, including: intestinal villus/crypt height/depth, lactase activity, distal/colon goblet cell density, colonic microbiota composition, blood chemistry, serum glucose, galactose, or iron, helper T cell concentrations, IL-1ß, lymphocytes, monocytes, neutrophils, or neutrophil phagocytic capacity, or time-to-stand (an indicator of motor development at PND 5) (74).

Additionally, Møller et al. enterally supplemented (in addition to parenteral nutrition) preterm pigs with 2 g bmOPN/L (Lacprodan OPN-10) in water at a rate of 5 mL/kg BW (10 mg bmOPN/kg BW) every 3 h from birth through PND 2, totaling 80 mg bmOPN/kg BW per day (73). Upon switching to enteral nutrition, preterm pigs were fed every 3 h with 15 mL formula/kg BW, with bmOPN supplemented pigs receiving 2.22 g bmOPN/L formula (266.4 mg bmOPN/kg BW per day) for 1.5 days. The high dose of bmOPN was used to replicate the concentration of OPN in colostrum, rather than mature milk. The authors reported reduced severity of inflammatory intestinal injury and greater absorption of mannitol in premature pigs fed bmOPN-enriched formula compared to those fed a standard formula (73). Otherwise compared to controls, bmOPN did not affect villus height; enzyme activity of lactase, maltase, sucrase, aminopeptidase, aminopeptidase N, or dipeptidyl-peptidase IV; or galactose absorption.

Donovan et al. found that the jejunal transcriptome of intestines from BF rhesus monkeys was distinct from those fed a commercially available infant formula (75). Monkeys fed the same formula supplemented with 125 mg/L bmOPN (Lacprodan OPN-10, intake on a BW basis not available) from birth to 3 months displayed an intermediate phenotype between BF monkeys and those fed un-supplemented formula in a primary cluster containing 50% of the genes. That module included genes related to functions including but not limited to: cell adhesion, cytoskeleton remodeling, neuronal development, protein modification, and the cell cycle (75). No differences in red blood cell concentrations, hemoglobin, hematocrit, white blood cells, or differential white blood cell counts were observed.

bmOPN supplementation in a soy-based formula to artificially-reared term pigs had minimal impacts on neurodevelopment (71). Studies in preterm pig models were heterogenous with respect to the dose and duration of exposure, with exposures to bmOPN 2-20x higher than OPN in mature human milk (72–74). No study reported an impact of bmOPN supplementation on brain development. bmOPN had a modest effect, if any, on markers of gastrointestinal and immune development. bmOPN supplementation in bovine-milk-based formulas to Rhesus monkeys altered the jejunal transcriptome with no impacts on hematology-related parameters (75).

Panel conclusions

- The heterogeneity in study designs and lack of consistent physiologic effects within and across species calls into question under what contexts dietary bmOPN has an immunomodulatory role for which a mode of action could be established.
  - o Some panel members concluded that the effects were modest and do not require further investigation.
  - o Some panelists suggested that further animal research should strive to reproduce the effects shown using longer study designs that replicate the first 12 months of human infant life. To further characterize potential immunomodulatory effects of bmOPN, using the response to vaccines or viral/bacterial pathogens may have greater utility than other markers of immune development (e.g., cytokines or T cell populations).
- All panelists agreed the data suggest OPN has no impact on BW growth in early life.
- All panelists agreed that the data on gastrointestinal development did not suggest adverse impacts on development, though an understanding of the mechanisms of some changes is nascent.
- All panelists agreed that despite potential crossing of the BBB, none of the behavioral or physiological outcomes suggested that dietary bmOPN has an adverse effect on neurodevelopment. Some panelists suggested no further research is needed to characterize the safety of bmOPN in this respect. Others suggested that further measures of cognitive and behavioral development would be the most clinically relevant outcomes to assess the effects of dietary bmOPN on brain development.
- The panel concluded that functional bioequivalence appeared high given the similarity in results comparing bmOPN, recombinant bovine/human, and murine OPN on response to LPS and behavioral performance.

Summary of preclinical evidence

Of the four KO mouse studies (41, 54–56), one rat study (70), one term pig study (71), three preterm pig studies (72–74), and one monkey study (75), none have reported any effect of bmOPN supplementation on body growth or anthropometrics, nor any adverse effects related to supplementation. Genetic KO studies in mice demonstrate that WT pups consuming milk with no OPN have impaired cognitive performance, increased immune responses to LPS, and altered gastrointestinal and neural physiology compared to WT pups consuming milk with OPN (41, 54–56). Supplementation with bmOPN, recombinant human, or recombinant bovine OPN can prevent/attenuate many of these effects, demonstrating functional similarity between forms (54, 55). Rats fed bmOPN-enriched formula in early life did not have an altered immunoglobulin response to LPS after weaning, but their response to ovalbumin shifted from those fed a standard formula toward that of dam-fed rats. bmOPN-feeding resulted in modest changes in T cell related immune markers (70).

Clinical evidence

One clinical trial has investigated the effects of orally ingested bmOPN and immune development in infants (79). In this double-blind randomized trial, 279 infants were BF or fed a standard formula with 15 (F0), 65 (F65), or 130 (F130) mg/L bmOPN (Lacprodan OPN-10) between 1 and 6 months of age (analyzed to contain 14, 72, and 150 mg OPN/L formula, respectively). Some infants in the F65 and F130 group started consumption of the experimental formulas prior to 1 month of age (personal communication with authors). Only small amounts of complementary foods were recommended to be introduced between 4 and 6 months of age. Of the clinically relevant endpoints identified by Callahan et al. (69), Lönnerdal et al. noted no differences between formula-fed groups in anthropometry/growth and adverse effects (79). At 6 months, the IgG response to tetanus (infants were vaccinated against diphtheria, pertussis, and tetanus at 4 months of age) was equivalent between F130, F0, and BF groups, with fewer antibodies in the F65 than the F0 group (79). Breastfed infants had fewer episodes of pyrexia than FF

infants. Among the FF infants, the F0 group had a significantly higher incidence and prevalence of pyrexia than the BF infants, whereas there was no significant difference between the F65 or F130 groups and the BF infants. These data are consistent with epidemiological findings that the concentration of mother's hmOPN is inversely associated with the number of infant hospital admissions due to fever in the first 3 months (N=85) (36). Ultimately, the clinically relevant immune endpoints indicated no or minimal differences between the F0 and F135 groups (79).

Of the immune markers (cytokines and immune cell populations) at 6 months of age, Lönnerdal et al. reported no difference in plasma levels of IL-6, IL-8, IL-12, IL-15, and transforming growth factor β2 between the F130 and F0 groups. By repeated measures analysis over all timepoints, the F65 and F130 groups were lower in concentrations of TNF-α (though not at 6 mo) and IL-10, and higher in IL-2, than in the F0 group (79). To better understand if such changes in cytokines corresponded with changes in T cell populations, West et al. performed a secondary analysis of the same subjects investigating peripheral blood immune cells via flow cytometry (80). West et al. report that the F130 and F0 groups appeared statistically equivalent for all outcomes measured except for greater T cells on average (averaged over all timepoints) in the F130 group (though not different from the BF group), in peripheral blood mononuclear cells (81). Although not statistically compared within each timepoint, the F130 group had higher T cells at 1 month of age than F0 and F165 groups, which decreased over 1 to 6 months and appeared more similar to all other groups by 6 months of age than at 1 month. This pattern echoed the concept of physiological "convergence" by 12 months of age between formula-fed and BF infants described by Callahan et al. (69). Greater T cell populations (and cytokines) in the F130 group at one month of age may have reflected consumption of formula by some infants prior to sampling. Otherwise, the F130 group did not differ from F0 in: concentration of circulating white blood cells, lymphocytes, monocytes, eosinophils, basophils, or neutrophils; immune cell composition of T helper or T cytotoxic cells; or proportion of naïve T cells (CD45T0+CD3+), memory/activated T cells (CD45R0+), HLA-DR T cells (HLA-DR+CD3+), double-positive T cells (CD3+CD4+CD8+), αβ T cells, γδ, T cells, B cells, or natural killer cells (81). Ultimately, this clinical trial demonstrated high similarity in clinically relevant immune endpoints between standard formula and OPN supplemented formula, with OPN-fed infants displaying modestly lower (but statistically insignificant) incidence of fever than those fed a standard formula. Although some markers of immune development (i.e., IL-2, IL-10, and T cell populations) differed, the overall phenotypes shifted toward that of the BF group by 6 months of age.

Panel conclusions
Although the trial did not extend beyond 6 months, given the similarity in clinical endpoints, the panel concluded that OPN-supplemented infants did not appear to have a different developmental trajectory than those fed standard formula.

Osteopontin and the infant formula matrix

The interactions between bioactive constituents within the infant formula matrix have been identified by the FDA as an important gap to address when assessing safety (17). For OPN, available preclinical research on this topic has focused primarily on the interaction between OPN and LF. Both OPN and LF are whey proteins present at up to 10-times higher in concentration in human milk compared to bovine milk (82), and LF is 10-times higher in milk than OPN (83). As oppositely charged ions, it has been hypothesized that OPN and LF may have a carrier-like relationship. Supporting this hypothesis, Yamniuk et al. demonstrated that LF and OPN form a complex in milk at a ratio of 3:1 (83). The LF-OPN complex has demonstrated greater resistance to digestion (84, 85), binding and uptake by human intestinal cells (84), promotion of proliferation and differentiation of intestinal cells (84, 85), anti-bacterial activity (84, 85), and stimulation of IL-18 compared to LF or OPN alone (84, 85). The LF-OPN complex consisting of iron-free LF (apo-LF) and calcium-bound OPN (holo-OPN), which are the predominant forms of LF and OPN in human milk, is resistant to digestion and has the strongest effect on cell proliferation compared with other forms (e.g., holo-LF and apo-OPN) (86). The LF-OPN complex may act by binding to cell surface receptors to activate the PI3K/Akt signaling pathway (86). While further research may elucidate the modes of action of the LF-OPN complex, it is clear that the complex does not impair the function of the individual proteins, and that they act in combination. Less is known regarding OPN's interaction with other components, though the combination of OPN, 2′-fucosyllactose, and docosahexaenoic acid was more effective at promoting maturation and differentiation of oligodendrocyte progenitor cells *in vitro* than either of the 3 individually (87).

Conclusion

The panel concluded that the existing data establish the following points: exposure to hmOPN is quantifiable through 6 months of age; dietary bmOPN comprises <5% of total circulating OPN and is cleared from plasma within 24 h; preclinical studies demonstrate no effect of dietary bmOPN on growth; dietary bmOPN does not appear to alter the trajectory of immune development; neither the gastrointestinal nor brain-related data demonstrate an adverse impact of bmOPN consumption; and multiple forms of OPN demonstrate high functional bioequivalence. For future research, the panel recommended prioritization of trials measuring a comprehensive set of clinically relevant outcomes on immunity and cognition to confirm the safety of bmOPN over that of further research on ADME. Such research would clarify the reproducibility of current findings, increase the confidence of these conclusions, and contribute to the body of evidence on safety.

Author contributions

SF: Conceptualization, Data curation, Formal analysis, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. SR: Writing – original draft, Writing – review & editing, Validation. SD: Writing – review & editing. OH: Writing – review & editing. RJ: Writing – review & editing. BL: Writing – review & editing, Conceptualization, Supervision. JN: Writing – review & editing. LS: Writing – review & editing. ES: Writing – review & editing. CW: Writing – review & editing. RK:

Writing – review & editing. JW: Writing – review & editing, Conceptualization, Supervision.

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Conflict of interest

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A combination of phospholipids and long chain polyunsaturated fatty acids supports neurodevelopmental outcomes in infants: a randomized, double-blind, controlled clinical trial

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Phospholipids (PLs) and long-chain polyunsaturated fatty acids (LCPUFAs) are naturally present in breast milk and play important roles in promoting the growth of the infant. Several studies have investigated the effects of the combination of PLs and LCPUFAs on neurodevelopment. However, data on the effectiveness of infant formula containing both PLs and LCPUFAs on the neurodevelopment of infants is still scarce. This randomized, double-blind, controlled clinical study was designed to evaluate the effect of an infant formula enriched with PLs and LCPUFAs on growth parameters and neurodevelopmental outcomes in term infants up to 365 days of age. Infants were enrolled within 30 days of birth who were then randomly assigned to either a control group ($n = 150$) or an investigational group ($n = 150$). Both groups consist of cow's milk-based formula which were generally identical in terms of composition, except that the investigational formula was additionally supplemented with PLs and LCPUFAs. The infants were followed for the first year of life. Breastfed infants were the reference ($n = 150$). Bayley Scales of Infant Development [3rd edition (Bayley-III)], Carey Toddler Temperament Scales (TTS), MacArthur-Bates Communicative Development Inventories (CDI), Single Object Attention and Free Play Tasks were used to evaluate neurodevelopmental outcomes of infant at 365 days of age. In addition, Ages and Stages Questionnaires (ASQ) were also conducted at 120, 180, and 275 days of age. Compared to breastfeeding, both infant formulas were well-tolerated and provided adequate growth, with no adverse events being reported throughout the study. Infants of the investigational group showed higher mean scores in Bayley-III cognitive performance (104.3 vs. 99.0, $p < 0.05$), language (106.9 vs. 104.5, $p < 0.05$), and motor skills (109.2 vs. 103.9, $p < 0.05$) compared the control group. Similar results were being reported for other developmental scales including TTS and ASQ. Notably, the test scores of infants fed the investigational formula were similar to those who were breastfed. Our results indicate that PL and LCPUFA supplementation may be beneficial for neurodevelopment of infants throughout the first year of life. Further studies are needed to investigation long-term effects PL and LCPUFA on neurodevelopment in early life.

KEYWORDS

brain development, anthropometry, early life nutrition, breastfeeding, bioactive substance

1 Introduction

Breast milk contains a wide range of components that are considered the optimal source of nutrients for feeding infants (1). It provides numerous advantages such as cognitive development, defense against pathogens, digestion and absorption of nutrients, and a lower risk of chronic diseases in later life (2). However, various circumstances can hinder breastfeeding, resulting in a significant number of infants worldwide receiving partial or exclusive formula feeding (3). Hence, it is crucial to develop an ideal infant formula that closely matches the composition, functional properties, and health-based outcomes of breast milk in order to meet the healthy growth needs of infants. In recent years, there has been growing recognition of previously overlooked functional components in breast milk, such as complex lipids, bioactive proteins, and oligosaccharides, which have been incorporated into infant formulas (4). Additionally, there is increasing focus on studying the potential biological functional effects of these components when added to infant formulas.

Approximately 60% of the energy consumed in an infant's first year of life is used for brain development, most of which is primarily allocated to the development of synthetic neuronal membranes and myelin. Lipids found in breast milk play a crucial role in providing the necessary energy and essential nutrients for the growth of the infant's brain and nerves (5). Approximately 98–99% of lipids in breast milk are glycerides, while phospholipids (PLs) account for around 0.2–2.0% of the total lipids (6). PLs have greater bioactivity compared to the main energy-supplying glycerides and offer various benefits in neurodevelopment, gut health, inflammation, and cardiovascular disease (7). PLs can be extracted from sources such as milk, soybeans, egg yolk or marine organisms (8). Approximately 60–65% of milk PLs are attached to milk fat globule membrane (MFGM). The MFGM not only contains PLs, but also a large number of bioactive compounds such as sphingolipids, glycolipids, and glycosylated proteins (9). Breast milk is also a source of long chain polyunsaturated fatty acids (LCPUFAs), specifically docosahexaenoic acid (DHA) and arachidonic acid (ARA). These fatty acids were found that are the most abundant in the structure of nervous tissue (10). The provision of LCPUFAs through breast milk has a positive effect on the development of retinal and brain cortical function in infants (11).

Dietary PLs phospholipids and gangliosides have been found to enhance spatial learning and impact brain growth and composition in neonatal piglets (12). Furthermore, early clinical studies have confirmed the safety of bovine MFGM (bMFGM) or its components in infant formula. An increasing number of clinical trials have associated the inclusion of dietary MFGM with beneficial effects on neurodevelopment (13–15) and child behavior (16). There have also been several early clinical trials investigating the beneficial effects of LCPUFAs -fortified formula on children's cognition (17, 18) and behavior (19). In addition, several clinical trials have investigated the effects of supplementing formula with bMFGM and LCPUFAs on infant neurodevelopment (14, 15, 20). However, data on the effectiveness of infant formula containing both PLs and LCPUFAs on infant neurodevelopment remain scarce and need to be supplemented in areas such as a longer feeding period up to 1 year of age for infants, a larger sample size, a more comprehensive neurodevelopmental assessment scale, and a more extensive control group design.

Consequently, further research is needed to fully understand and confirm the efficacy, mechanism of action, and long-term benefits of adding PLs from different sources, and LCPUFAs to infant formulas. The objective of the present study was to evaluate the neurodevelopmental outcomes at 12 months of age in healthy term infants who received formula supplemented with PLs and LCPUFAs as compared with infants receiving a routine cow's milk-based formula.

2 Methods

2.1 Design and participants

Eligible infants were enrolled in this single-center, randomized, double blind, controlled, parallel-designed, prospective study from August 2020 to June 2022 in Jinhua, Zhejiang Province, China. This research was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Shanghai Nutrition Society. This trial was registered at clinicaltrials.gov as NCT04508257. Informed consent was obtained from all subjects involved in the study. Acceptable participants were either exclusively formula-fed infants or breastfed infants. Participant inclusion criteria for participants were as follows: infants had to be at least 30 days old at the time of randomization, exclusively formula-fed for at least 3 days before randomization, born as singletons, with a gestational age between 37 and 42 weeks (36 weeks and 6 days was considered 36 weeks gestational age), a birth weight between 2,500 g to 4,000 g, and informed consent obtained from the parent or guardian for the infant's participation in the study. Additionally, the parent or guardian agreed not to enroll the infant in any other interventional clinical research study while participating in this study. The exclusion criteria included individuals with a history of underlying metabolic or chronic disease, congenital malformation, or any other condition that, in the investigator's opinion, could interfere with the infant's ability to ingest food, normal growth and development, or the evaluation of the infant. Additionally, infants with evidence of feeding difficulties or formula intolerance, such as vomiting or poor intake, at the time of randomization were excluded (at the discretion of the investigator). Infants with a weight at visit 1 less than 95% of their birth weight [(weight at Visit 1 ÷ birth weight) × 100 < 95%] were also excluded. Furthermore, infants who were immunocompromised (according to a doctor's diagnosis of immunodeficiency) or had known head/brain disease/injury, such as microcephaly or macrocephaly, were excluded.

The study period involved feeding and cognitive testing up to 365 days of age (Supplementary Table 1). The study consisted of 6 visits at specific time points: 30 (±7 days; enrollment), 90 (±7), 120

TABLE 1 Nutritional composition per 100 mL.

	Study formula (target values)			
	Stage 1		Stage 2	
	Control	Investigational	Control	Investigational
Energy, kcal	67	64	71	69
Protein, g [1]	1.40	1.35	2.18	2.26
Casein, g	0.52	0.50	1.09	1.13
Whey, g	0.88	0.85	1.09	1.13
Carbohydrate, g [2]	7.0	6.7	8.1	7.8
Lactose, g	6.8	6.5	7.8	7.5
GOS, g	0.45	0.45	0.64	0.68
Fat, g [3]	3.3	3.4	3.1	3.1
Linoleic acid, g	0.53	0.53	0.37	0.37
α-Linolenic acid, mg	53	53	37	37
DHA, mg	0.1	12.2	0.1	15.0
ARA, mg	1.3	20.8	2.0	25.0
PLs, mg	20	67	19	70
PC, mg	6	22	6	24
PE, mg	5	21	5	23
PI, mg	2	6	2	6
PS, mg	2	6	2	6
SM, mg	5	12	4	11

GOS, galactooligosaccharide; DHA, docosahexaenoic acid; ARA, Arachidonic acid; PLs, Phospholipids; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; PS, Phosphatidylserine; SM, Sphingomyelin.

[1]Sources of protein for control: skim milk and whey protein concentrate (WPC); and for investigational: skim milk, WPC, and whey protein-lipid concentrate (source of bMFGM protein; Lacprodan® MFGM-10, Arla Foods Ingredients P/S, Denmark).

[2]Sources of carbohydrate for control and for investigational: lactose and GOS.

[3]Sources of fat for control: base blend of sunflower, coconut, and flaxseed oils; and for investigational: base blend of sunflower, coconut, flaxseed oils, fungal-derived single cell oil (source of ARA), algal-derived single cell oil (source of DHA), whey protein-lipid concentrate (source of bMFGM phospholipids; Lacprodan® MFGM-10, Arla Foods Ingredients P/S, Denmark) and soybean phospholipids (source of phospholipids; soybean phospholipids ingredient, Cargill).

(±7), 180 (±7), 275 (±7), and 365 (±7) days of age. Participants in the formula feeding groups were given stage 1 formula until 180 days of age and then transitioned to stage 2 formula until 365 days of age. Cognitive tests were conducted by trained professionals at the study sites. All participant data was recorded in an electronic case report form (eCRF) by designated and trained study personnel. The study adhered to good clinical practices and was registered with ClinicalTrials.gov (NCT04508257).

2.2 Randomization and study group allocation

Eligible infants who were exclusively consuming their mother's breast milk with the intent to continue through at least 4 months of age and continue with supplement food without any marketed infant formula were registered in the breast milk reference group. Infants whose mothers had chosen to exclusively feed them infant formula until 365 days of age were randomly allocated to either a control formula (*CF*) group or an investigational formula (IF) group with a ratio of 1:1 stratified by gender. The composition of two formulas were identical except that the IF was fortified with whey protein-lipid concentrate (Stage 1&stage 2: source of bMFGM; Lacprodan MFGM-10, Arla Foods Ingredients P/S), soybean phospholipids (Stage 1& stage 2: source of PLs; soybean phospholipids ingredient, Cargill), fungal-derived single cell oil (Stage 1& stage 2: source of ARA), and algal-derived single cell oil (Stage 1& stage 2: source of DHA; Table 1). Both formulas were produced in the same factory in China Feihe, using identical production and processing equipment. In addition to the added PLs and LCPUFAs, both formulas also contain a certain baseline level of these components. This is because the main ingredients of both formulas are raw cow's milk, and PLs and LCPUFAs naturally exist in raw cow's milk. The randomization scheme was generated using Version 9.4 of SAS® statistical software. The identities of the specific products are blinded to participants, parents or legal guardians, support staff and investigators. Investigator will receive one sealed and numbered envelope for each participant containing the identification of the study product administration. The unblinding will occur to the statistical analyses team only after completion of statistical analyses. Upon database lock the treatment codes were transmitted to the statistical group generating the final analysis for incorporation into the study analysis datasets.

2.3 Study outcome measures

The Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III) evaluates infants and children from 1 to 42 months

of age. The cognitive, language (receptive and expressive communication), and motor (fine and gross motor) domains were assessed by a trained evaluator. The social–emotional and adaptive behavior scales were assessed by parent questionnaire (21). The Bayley-III has been previously translated into Chinese (Mandarin), adapted for the Chinese population. The study aimed to assess infant neurodevelopment at 365 days of age using the Bayley -III as the primary outcome. In addition to the Bayley-III, the following instruments used were previously translated, revised, and adapted for use in Chinese populations: TTS, CDI, Single Object Attention and Free Play Tasks, and ASQ. The study analyzed the output of the TTS on 9 domains of infant temperament, including activity level, regularity, approach/withdrawal, adaptability, intensity, mood, persistence, distractibility, and sensor threshold. The Chinese (Putonghua) Communicative Developmental Inventory (PCDI) scores were used to measure word production and other early language skills. The study measured overall fixation (attention) and looking away (inattention) in terms of duration and frequency for the Single Object Attention and Free Play Task. TTS, CDI, and Single Object Attention and Free Play Tasks were administered at 365 days of age. The ASQ assesses 5 domains: communication, gross motor, fine motor, problem solving, and personal/social, and was conducted at 120, 180, and 275 days of age. Measurements of body weight, length, and head circumference were taken at 30, 90, 120, 180, 275, and 365 days of age. Formula intake, stool characteristics (frequency and consistency) and formula tolerance (fussiness and gassiness) were assessed through a 24-h recall beginning at 90 days of age (Supplementary Table 2). To determine breast milk intake, we utilized data from a survey conducted by the Chinese Center for Disease Control and Prevention on the breast milk intake of Chinese infants aged 0–5 months from 2019 to 2021. The weighing method was employed to measure the 24-hourly breast milk intake of infants. The average breast milk intake of exclusively breastfed infants aged 0–5 months was 800.1 g/d, ranging from 696.4 to 937.7 g/d. Breast milk intake increased with age and remained stable at 5 months (22). Oral, intramuscular and intravenous (IV) antibiotic treatments throughout the study period, and medically-confirmed adverse events (AEs) including respiratory and gastrointestinal infections, were also monitored.

2.4 Statistical analysis

The primary outcome for this study is the cognitive scale of the Bayley-III at 365 days of age. Assuming a difference of 6 points and a SD of 15, a sample size of 105 per group is necessary to have a power of 80% when testing at an alpha level of 0.05, two-tailed test. We enrolled 150 participants per group to accommodate a potential attrition rate of 30%. All enrolled participants were included in the analysis. Missing data from participants who withdrew early from the study were not replaced or imputed. Descriptive statistics were used to summarize the continuous variables, including the number of observations, mean, median, standard deviation (SD), and quartiles. The discrete variables were summarized using counts, proportions, and/or percentages. These descriptive analyses were presented separately for each treatment group. A significance level of 0.05 was used for statistical testing, unless otherwise specified. Prior to testing, the distributional assumptions of the outcomes were assessed, and transformations or nonparametric tests were used if necessary. A 95% confidence level was used for confidence intervals. Pairwise group comparisons were conducted for outcome variables that showed significant overall group differences. Tukey adjustment was applied to these comparisons, and adjusted confidence intervals with adjusted p-values were reported. All analyses were performed using Version 9.4 of SAS® statistical software.

2.5 Intention to treat analysis

Sensitivity analysis was also performed for the primary and secondary outcomes in the intention-to-treat (ITT) population. For outcomes that only observed at post-baseline visits, missing data of early withdrew subjects were handled with multiple imputation with fully conditional specification regression method, based on sex, birth weight, family income, number of family members living in the household, father education, mother education, mother's age when participant was born, maternal DHA supplement use during last 12 weeks of pregnancy, and maternal prenatal vitamin use during last 12 weeks of pregnancy. Five imputations were conducted for all variables. Group differences were evaluated for each imputed dataset, and the results were combined using the SAS MIANALYZE procedure. For outcomes with longitudinal measurements starting from baseline, we used a mixed model for repeated measures with feeding group, age and their interactions as fixed effect, and an unstructured covariance matrix to compare the differences among feeding groups (23). All outcome data were used, regardless of whether an individual has complete data or not.

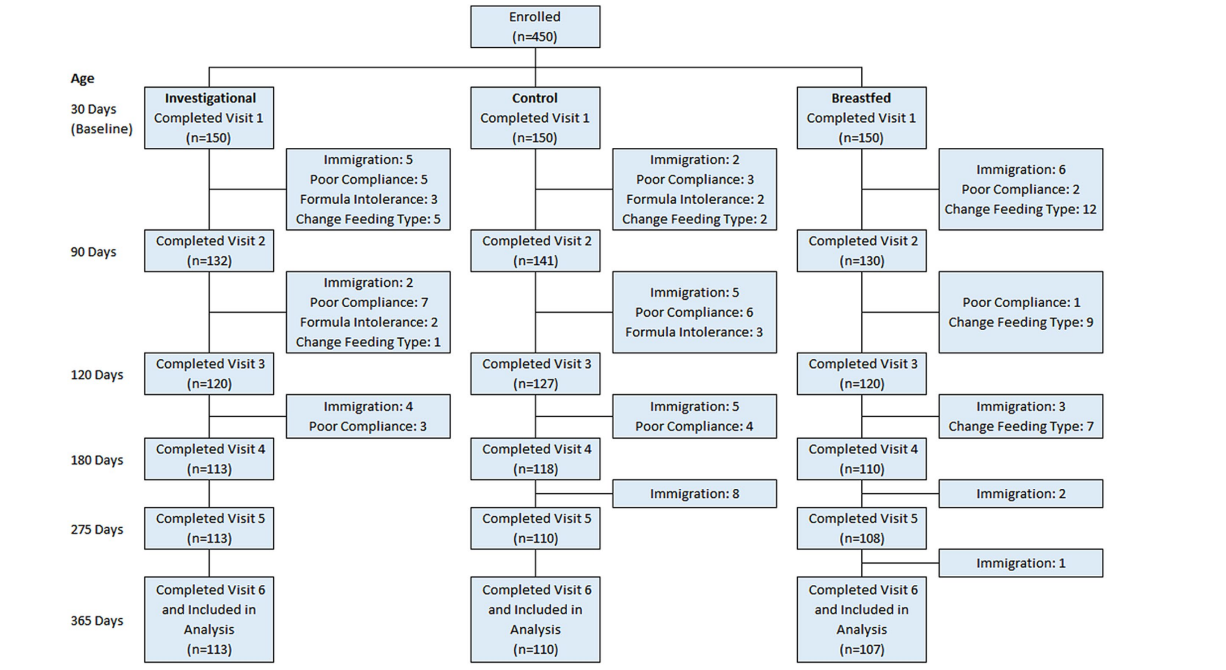
3 Results

3.1 Study population and baseline characteristics

Among the 450 participants recruited, 150 were assigned to the Breastfed group and 300 were randomized to either the Control group ($n = 150$) or the Investigational group ($n = 150$). A total of 330 participants completed the assigned treatment according to study instructions and were included in the analyses. The participant flow from recruitment to study completion is illustrated in Figure 1. There was no significant difference between the formula feeding groups in terms of total discontinuation rate ($p = 0.692$) or the reasons for discontinuation. Table 2 provides a summary of the baseline characteristics for each study group. There were no significant differences in baseline characteristics between the two formula groups. However, the breastfed group had mothers who were younger at the time of participant's birth, had fewer household members and lower monthly family income compared to the formula groups. Other baseline characteristics were comparable between the breastfed group and the formula groups.

3.2 Primary outcome Bayley-III

The Bayley-III assesses development in five areas: cognitive, language, motor, social–emotional, and adaptive behavior, which were administered at 365 days of age. Considering the baseline differences in characteristics between the breastfed group and the formula milk group. The five developmental scores were analyzed separately using



Overall dropout rate:				Difference between Formula Feeding Groups p-value	Difference among All Groups p-value
Immigration	11 (7.33%)	20 (13.33%)	12 (8.00%)	0.128	0.153
Poor compliance	15 (10.00%)	13 (8.67%)	3 (2.00%)	0.843	0.014
Formula intolerance	5 (13.51%)	5 (13.51%)	NA	1.000	NA
Change feeding type	Formula feeding to breastfeeding 6 (4.00%)	Formula feeding to breastfeeding 2 (1.33%)	Breastfeeding to formula feeding 28 (18.67%)	0.282	<0.0001
Total	**37 (24.67%)**	**40 (26.67%)**	**43 (28.67%)**	**0.692**	**0.736**

FIGURE 1
Flow chart of study. Data were presented frequency (%). Group difference was evaluated using chi-square/Fisher's exact test.

analysis of variance (ANOVA) and using analysis of covariance (ANCOVA) to adjust for potential confounding variables (gender, birth weight, household income, number of family members living in the household, father education, mother education, mother's age when participant was born, maternal DHA supplement use during last 12 weeks of pregnancy, maternal prenatal vitamin use during last 12 weeks of pregnancy, and exposure to smoking at study enrollment). At 365 days of age, the Bayley-III cognitive composite score was significantly higher for the investigational group compared to the control group (adjusted $p < 0.0001$ for pairwise group comparison, Table 3). The investigational group exhibited significantly higher language ($p < 0.05$) and motor ($p < 0.05$) composite scores compared to the control group. The investigational group and the breastfed group showed similar cognitive and language scores. No significant differences were found between the groups in terms of social–emotional or general adaptive mean scores. These findings remained consistent even after adjusting for factors such as gender, birth weight, family income, parental education, and other socio-environmental variables. In addition, the mean Bayley-III cognitive, language, and motor scores for each group fell within the standardized mean score (mean ± SD = 100 ± 15) of the 50th percentile, indicating mid-average functioning (24). Meanwhile, the results of the ITT analysis were basically consistent with the Per-protocol (PP). Therefore, the study suggests that the infant formula used provided adequate neurodevelopmental support.

3.3 Secondary outcomes through 12 months of age

The study analyzed the output of the TTS on 9 domains of infant temperament. A significant difference was found in the activity level domain ($p = 0.007$) for TTS scores at 365 days of age. Pairwise comparisons revealed significant differences between the investigational group ($p < 0.05$) and the control (Table 4). However, no statistically significant differences were observed in the other domains of TTS scores. The study measured overall fixation (attention) and looking away (inattention) in terms of duration and frequency for the Single Object Attention and Free Play Task. At 365 days of age, the investigational group had significantly longer total look duration compared to the control group ($p < 0.05$, Table 5). The investigational group and the breastfed group had similar total and mean look duration. There were no significant group differences observed for the longest look duration or the number of look episodes. The ASQ assesses 5 domains: communication, gross motor, fine motor, problem solving, and personal/social. ASQ scores in all five domains were significantly higher in the investigational group compared to the control group at 120 days of age (Supplementary Table 3). The number and percentage of subjects with ASQ scores above, close to or below the cut-off points for each domain are presented in Supplementary Table 4. No significant difference was observed between the investigational group and the

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TABLE 2 Baseline characteristics of participants.

Variable	Investigational ($n = 150$)	Control ($n = 150$)	Breastfed ($n = 150$)	Group difference p-value
Birth History				
Weight at birth, g	3402.4 ± 372.0	3327.6 ± 372.4	3356.9 ± 380.8	0.221
Length at birth, cm	50.0 ± 0.8	50.0 ± 0.8	49.9 ± 0.8	0.309
Demographics				
Infant age at enrollment, days	33.9 ± 2.2	34.1 ± 2.3	34.1 ± 2.2	0.653
Infant Han ethnic group, %	116 (77.3)	125 (83.3)	112 (74.7)	0.174
Infant boys, %	76 (50.7)	75 (50.0)	75 (50.0)	0.991
Vaginal delivery, %	85 (56.7)	99 (66.0)	97 (64.7)	0.196
Infant gestational age, weeks	39.1 ± 1.0	39.0 ± 1.0	38.9 ± 1.1	0.341
Mother's age when participant was born, years	30.7 ± 5.1	30.4 ± 4.3	28.6 ± 4.7	**0.0002**
Mother's marital status, %				0.367
Single	0 (0.0)	0 (0.0)	1 (0.7)	
Married	150 (100.0)	150 (100.0)	149 (99.3)	
Number of previous live births to the infant's mother				0.698
0	59 (39.3)	50 (33.3)	54 (36.0)	
1	74 (49.3)	86 (57.3)	83 (55.3)	
2	17 (11.3)	13 (8.7)	12 (8.0)	
3	0 (0.0)	1 (0.7)	1 (0.7)	
Infant insurance, %	144 (96.0)	139 (92.7)	141 (94.0)	0.460
Anthropometrics at baseline				
Weight, g	4658.6 ± 520.9	4573.6 ± 489.0	4591.0 ± 497.7	0.303
Length, cm	55.2 ± 1.7	55.0 ± 1.8	55.1 ± 1.8	0.517
Head circumference, cm	37.0 ± 1.0	36.9 ± 1.0	36.9 ± 1.0	0.784
Socioeconomic status				
Number of family members at household	3.5 ± 0.7	3.6 ± 0.6	3.3 ± 0.6	**0.0003**
Household size, %				0.555
<60 m^2	4 (2.7)	3 (2.0)	7 (4.7)	
60–90 m^2	83 (55.3)	81 (54.0)	89 (59.3)	
90–120 m^2	54 (36.0)	59 (39.3)	50 (33.3)	
>120 m^2	9 (6.0)	7 (4.7)	4 (2.7)	
Mother's educational level, %				0.872
Primary school	6 (4.0)	6 (4.0)	4 (2.7)	
Junior school	43 (28.7)	43 (28.7)	51 (34.0)	
High school/ Technology school	88 (58.7)	87 (58.0)	82 (54.7)	
Bachelor	13 (8.7)	14 (9.3)	12 (8.0)	
Master and above	0 (0.0)	0 (0.0)	1 (0.7)	
Father's educational level, %				0.318
Primary school	2 (1.3)	3 (2.0)	5 (3.3)	
Junior school	33 (22.0)	32 (21.3)	45 (30.0)	
High school/ Technology school	84 (56.0)	83 (55.3)	79 (52.7)	
Bachelor	31 (20.7)	32 (21.3)	21 (14.0)	
Master and above	0 (0.0)	0 (0.0)	0 (0.0)	
Mother currently employed, %	87 (58.0)	78 (52.0)	73 (48.7)	0.260
Father currently employed, %	149 (99.3)	147 (98.0)	149 (99.3)	0.626

(Continued)

TABLE 2 (Continued)

Variable	Investigational ($n = 150$)	Control ($n = 150$)	Breastfed ($n = 150$)	Group difference p-value
Monthly average household income, %				**0.001**
<3,000 RMB	0 (0.0)	0 (0.0)	0 (0.0)	
3,000–5,999 RMB	14 (9.3)	19 (12.7)	34 (22.7)	
6,000–8,000 RMB	86 (57.3)	82 (54.7)	94 (62.7)	
>8,000 RMB	50 (33.3)	49 (32.7)	22 (14.7)	
Family medical history				
Infant asthma or allergy history, %	0 (0.0)	0 (0.0)	0 (0.0)	NA
Father asthma or allergy history, %	0 (0.0)	0 (0.0)	0 (0.0)	NA
Mother asthma or allergy history, %	0 (0.0)	0 (0.0)	0 (0.0)	NA
Sibling asthma or allergy history, %	0 (0.0)	0 (0.0)	0 (0.0)	NA
Half-sibling asthma or allergy history, %	0 (0.0)	0 (0.0)	0 (0.0)	NA
Smoker among family members, %	140 (93.3)	140 (93.3)	131 (87.3)	0.103
Exposed in smoking environment, %	3 (2.0)	4 (2.7)	5 (3.3)	0.933
Mother supplement intake within 12 weeks before delivery				
DHA	7 (4.7)	12 (8.0)	11 (7.3)	0.472
Vitamins	33 (22.0)	26 (17.3)	21 (14.0)	0.191

Data presented are mean ± standard deviation for continuous variables and frequency (%) for categorical variables. Group difference was evaluated using one-way analysis of variance for continuous variables and chi-square/Fisher's exact test for categorical variables.

control group for the proportion of participants at risk developmentally in all ASQ domains from 120 to 275 days of age. Meanwhile, the ITT analysis results for secondary outcomes were basically consistent with the PP.

3.4 Infant growth

Growth rates were analyzed from 30 days to 90, 120, 180, 275 and 365 days of age. No statistically significant group differences by gender were observed in weight, length, or head circumference growth rate (Supplementary Table 5). According to guidance from the American Academy of Pediatrics, the rate of weight gain is a crucial factor to consider when evaluating infant formula clinically. Differences of more than 3 g/day over a 3- to 4-month period are deemed clinically significant (25). The mean weight growth rate of both the control group and the breastfed group did not exceed that of the IF by a clinically relevant amount (3 or more g/day for the 30 to 120-day interval), indicating that the IF provided adequate growth. No statistically significant group differences by gender were observed for mean achieved weight, length or head circumference at any measured time point up to 365 days of age (Supplementary Table 6). Similarly, the results of the ITT showed that both the group effect and the interaction of group and age was not significant, indicating similar patterns of change in achieved weight, length and head circumference over time among the feeding groups (Supplementary Table 7). In addition, the mean achieved weight, length and head circumference for participants on the WHO weight-for-age, length-for-age and head circumference-for-age standard growth chart for boys and girls in each group remained near 50th and 75th percentiles of growth through 365 days of age (Supplementary Figure 1).

3.5 Stool characteristics and tolerance

The breastfed group had a significantly higher mean daily stool frequency compared to the investigational group and the control group at 30 days of age. Additionally, the breastfed group had significantly softer stool texture compared to the two formula feeding groups. There were no significant differences in mean daily stool frequency or stool consistency detected between the two formula feeding groups at any visits (Supplementary Table 8). Similarly, the results of the ITT showed that the interaction of group and age was not significant for stool frequency and stool consistency, indicating similar patterns of change in both outcomes over time among the feeding groups. No overall differences were observed between the investigational group and the control group throughout the study period (Supplementary Table 9). Fussiness and amount of gas were similar among the three study groups at all study visits (Supplementary Table 10). Similarly, the results of the ITT showed that the interaction of group and age was not significant for all fussiness and gassiness outcomes, indicating similar patterns of change in tolerance over time among the feeding groups (Supplementary Table 11).

3.6 Formula intake and adverse events

The amount of study formula intake was similar between the two formula groups for both boy and girl participants (Supplementary Table 12). In this study, the intake of formula milk fell within the range of breast milk intake, indicating that intake did not affect the study results. The number of subjects AEs was summarized by study group, Medical Dictionary for Regulatory Activities (MedDRA) system organ class and preferred term. There was no

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TABLE 3 Bayley III composite scores at 365 days of age.

Domain	Investigational	Control	Breastfed	Overall group difference (*p*-value)
Per-Protocol	**n = 113**	**n = 110**	**n = 107**	
Unadjusted				
Cognitive	104.3 ± 7.7[a]	99.0 ± 5.9[b]	104.7 ± 7.7[a]	**<0.0001**
Language	106.9 ± 5.9[a]	104.5 ± 6.4[b]	107.8 ± 6.7[a]	**0.0002**
Motor	109.2 ± 8.6[a]	103.9 ± 9.5[b]	109.3 ± 12.6[a]	**<0.0001**
Social–emotional	107.7 ± 14.4	105.4 ± 12.9	109.3 ± 15.3	0.136
General adaptive	90.3 ± 11.1	89.7 ± 10.0	91.7 ± 11.3	0.365
Adjusted[1]				
Cognitive	104.3 ± 7.7[a]	99.0 ± 5.9[b]	104.7 ± 7.7[a]	**<0.0001**
Language	106.9 ± 5.9[a]	104.5 ± 6.4[b]	107.8 ± 6.7[a]	**0.0002**
Motor	109.2 ± 8.6[a]	103.9 ± 9.5[b]	109.3 ± 12.6[a]	**<0.0001**
Social–emotional	107.7 ± 14.4	105.4 ± 12.9	109.3 ± 15.3	0.082
General adaptive	90.3 ± 11.1	89.7 ± 10.0	91.7 ± 11.3	0.232
Intention-To-Treat[2]	**n = 150**	**n = 150**	**n = 150**	
Unadjusted				
Cognitive	103.9 ± 9.2[a]	99.8 ± 10.6[b]	104.4 ± 8.8[a]	**<0.0001**
Language	106.7 ± 10.0[a]	105.2 ± 8.8[b]	107.7 ± 6.8[a]	**0.012**
Motor	108.3 ± 13.6[a]	104.7 ± 14.8[b]	108.8 ± 13.5[a]	**0.0003**
Social–emotional	107.8 ± 17.4	105.4 ± 17.1	108.8 ± 16.3	0.240
General adaptive	90.2 ± 15.3	89.7 ± 12.2	91.4 ± 14.6	0.397
Adjusted[1]				
Cognitive	104.3 ± 7.7[a]	99.0 ± 5.9[b]	104.7 ± 7.7[a]	**<0.0001**
Language	106.9 ± 5.9[a]	104.5 ± 6.4[b]	107.8 ± 6.7[a]	**0.002**
Motor	109.2 ± 8.6[a]	103.9 ± 9.5[b]	109.3 ± 12.6[a]	**0.0004**
Social–emotional	107.7 ± 14.4	105.4 ± 12.9	109.3 ± 15.3	0.193
General adaptive	90.3 ± 11.1	89.7 ± 10.0	91.7 ± 11.3	0.244

Data are summarized by mean ± standard deviation. Means with different letters were significantly different ($p < 0.05$).
[1]Group differences were analyzed using analysis of covariance, with adjustment for gender, birth weight, family income, number of family members living in the household, father education, mother education, mother's age when participant was born, maternal DHA supplement use during last 12 weeks of pregnancy, and maternal prenatal vitamin use during last 12 weeks of pregnancy. Tukey adjusted p-values are presented for pair-wise group comparisons.
[2]Results are summarized from 5 imputations. Data are summarized by mean ± standard deviation. Group differences were analyzed using mixed model, with adjustment for sex, birth weight, family income, number of family members living in the household, father education, mother education, mother's age when participant was born, maternal DHA supplement use during last 12 weeks of pregnancy, and maternal prenatal vitamin use during last 12 weeks of pregnancy. Tukey adjusted *p*-values are presented for pair-wise group comparisons.

significant difference in the rate of each AE type among the study groups (Supplementary Table 13). All AEs reported were of mild intensity and not related to the study products. No serious AEs occurred throughout the study period.

4 Discussion

In this study, infants fed an PLs and LCPUFAs supplemented infant formula received significantly higher scores on cognitive testing (Bayley-III) at 12 months of age than did infants fed a control formula. The effect size between *CF* and IF group were reported to be 5.4 for cognitive (104.3 vs. 99.0), 2.4 for language (106.9 vs. 104.5) and 5.3 for motor (109.2 vs. 103.9), respectively.

Previously, RCTs have been carried out to explore cognitive function of either PLs or LCPUFAs for infants. In general, results of PLs (bMFGM) are inconsistent. For example, a Swedish study showed that infant formula supplemented with PLs (bMFGM) significantly increased cognitive level (105.8 vs. 101.8) based on Bayley-III at 1 year of age (14), however, there was no significant difference observed at 6.5 years, with the Breastfeeding Group, used as a reference, showing higher scores in full-scale IQ, verbal comprehension, perceptual reasoning, and working memory from WISC-IV compared to the supplemented group (13). Another study failed to show any effect of PLs (bMFGM) on cognitive level (Bayley-III) of Chinese infants, even though significant improvements were observed for Social emotional and General adaptive scores after adjustment for confounders (15). In terms of LCPUFA, a large number of systematic reviews of RCTs do not support its beneficial role on neurodevelopmental outcomes of infants (26–28). Our results clearly showed that combination of PLs and LCPUFAs support neurodevelopment of infants (three domains were improved), which are robust against a group of known confounders.

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TABLE 4 Toddler temperament scale domain scores at 365 days of age.

Domain	Investigational	Control	Breastfed	Overall group difference (*p*-value)
Per-Protocol[1]	**n = 113**	**n = 110**	**n = 107**	
Activity level	3.8 ± 0.9[a]	3.5 ± 1.0[b]	3.9 ± 0.9[a]	**0.007**
Regularity	3.6 ± 1.3	3.5 ± 1.1	3.6 ± 1.1	0.864
Approach/withdrawal	3.2 ± 1.1	3.2 ± 1.2	3.3 ± 1.2	0.932
Adaptability	3.6 ± 1.1	3.4 ± 0.9	3.6 ± 1.0	0.331
Intensity	3.8 ± 0.8	3.7 ± 0.8	3.8 ± 0.7	0.924
Mood	3.5 ± 1.2	3.4 ± 1.0	3.6 ± 0.9	0.467
Persistence	3.7 ± 1.2	3.6 ± 1.4	3.6 ± 1.2	0.714
Distractibility	4.4 ± 0.9	4.4 ± 1.0	4.3 ± 1.1	0.763
Sensor threshold	3.4 ± 1.3	3.4 ± 1.0	3.4 ± 1.1	0.973
Intention-To-Treat[2]	**n = 150**	**n = 150**	**n = 150**	
Activity level	3.8 ± 1.0[a]	3.6 ± 1.1[b]	3.8 ± 1.1[a]	**0.042**
Regularity	3.6 ± 1.4	3.5 ± 1.3	3.6 ± 1.4	0.561
Approach/withdrawal	3.2 ± 1.3	3.2 ± 1.3	3.3 ± 1.2	0.674
Adaptability	3.6 ± 1.3	3.4 ± 1.2	3.6 ± 1.1	0.407
Intensity	3.8 ± 0.9	3.8 ± 0.8	3.8 ± 1.0	0.460
Mood	3.5 ± 1.2	3.4 ± 1.1	3.6 ± 1.0	0.408
Persistence	3.7 ± 1.3	3.6 ± 1.7	3.6 ± 1.3	0.532
Distractibility	4.4 ± 1.0	4.4 ± 1.2	4.3 ± 1.3	0.540
Sensor threshold	3.4 ± 1.5	3.4 ± 1.3	3.5 ± 1.3	0.700

Data presented are mean ± standard deviation. Means with different letters were significantly different ($p < 0.05$).
[1]Group differences were analyzed using analysis of covariance, with adjustment for gender, birth weight, family income, number of family members living in the household, father education, mother education, mother's age when participant was born, maternal DHA supplement use during last 12 weeks of pregnancy, and maternal prenatal vitamin use during last 12 weeks of pregnancy. Tukey adjusted p-values are presented for pair-wise group comparisons.
[2]Results are summarized from 5 imputations. Data are summarized by mean ± standard deviation. Group differences were analyzed using mixed model, with adjustment for sex, birth weight, family income, number of family members living in the household, father education, mother education, mother's age when participant was born, maternal DHA supplement use during last 12 weeks of pregnancy, and maternal prenatal vitamin use during last 12 weeks of pregnancy. Tukey adjusted *p*-values are presented for pair-wise group comparisons.

TABLE 5 Five-minute single object free play at 365 days of age.

	Investigational	Control	Breastfed	Overall group difference (*p*-value)
Per-Protocol[1]	**n = 113**	**n = 110**	**n = 107**	
Look duration				
Longest	58.7 ± 31.9	51.3 ± 39.6	60.6 ± 39.3	0.197
Total	224.7 ± 38.6[a]	201.5 ± 54.7[b]	225.0 ± 43.6[a]	**<0.0001**
Mean	18.1 ± 8.8[ab]	15.8 ± 12.5[b]	21.0 ± 17.5[a]	**0.014**
Look episodes	14.2 ± 4.3	15.0 ± 5.2	14.0 ± 5.2	0.421
Intention-To-Treat[2]	**n = 150**	**n = 150**	**n = 150**	
Look duration				
Longest	60.6 ± 33.1	54.1 ± 41.4	61.1 ± 40.9	0.296
Total	224.1 ± 42.4[a]	206.0 ± 56.8[b]	222.2 ± 43.6[a]	**0.001**
Mean	18.5 ± 16.2[ab]	18.7 ± 27.7[b]	20.7 ± 18.0[a]	**0.044**
Look episodes	14.1 ± 4.8	14.8 ± 5.7	13.9 ± 5.7	0.384

Data presented are mean ± standard deviation. Means with different letters were significantly different ($p < 0.05$).
[1]Group differences were analyzed using analysis of covariance, with adjustment for gender, birth weight, family income, number of family members living in the household, father education, mother education, mother's age when participant was born, maternal DHA supplement use during last 12 weeks of pregnancy, and maternal prenatal vitamin use during last 12 weeks of pregnancy. Tukey adjusted p-values are presented for pair-wise group comparisons.
[2]Results are summarized from 5 imputations. Data are summarized by mean ± standard deviation. Group differences were analyzed using mixed model, with adjustment for sex, birth weight, family income, number of family members living in the household, father education, mother education, mother's age when participant was born, maternal DHA supplement use during last 12 weeks of pregnancy, and maternal prenatal vitamin use during last 12 weeks of pregnancy. Tukey adjusted *p*-values are presented for pair-wise group comparisons.

Notably, the Bayley-III profiles are similar between the IF group and breastfed group (Table 3). Even though direct comparisons are not possible as the infants of breastfed group were not randomized, our exploratory analysis showed that the differences were not significant using the method described in Table 3 (data not shown). Also, it is interesting to note that upon reviewing previous research on LCPUFAs (DHA and ARA) supplementation in infant formula, it was observed that limited studies have explored the specific ratio of ARA to DHA. Despite being often overlooked, this aspect holds significant importance. The balance between ARA and DHA could play a crucial role in influencing cognitive and developmental outcomes during infancy and early childhood. Research on the neurodevelopment of infants who were fed formulas with varying ARA to DHA ratios suggests that the specific ratio utilized may have a substantial impact on the benefits of LC-PUFAs in early childhood, with neurodevelopmental outcomes showing preference for an ARA/DHA ratio of 1:1 or 2:1 (29). Notably, during the initial stages of this clinical trial, researchers meticulously considered the ARA to DHA ratio in the formula design, ultimately settling on a ratio of approximately 1.7. Remarkably, this ratio closely mirrors that found in Chinese breast milk (30). Breast milk is generally considered to be optimal for infant aged 0 ~ 6 months of age, whereby causal relationship has been established between breastfeeding and cognition development (31, 32). It has been recognized in recent years that breast milk represents a complex biological system, such that numerous interacting parts of the system outperforms the sum of those individual parts (33, 34). Therefore, we argue that the neuro-supportive effect of IF in our study may possibly be due to the interplay between PLs and LCPUFA. Gázquez A et al. have demonstrated that incorporating bMFGM plus milk fat into infant formulas may increase the bioavailability of DHA in both plasma and tissues in suckling piglets. The structures of PLs and gangliosides found in bMFGM could potentially enhance the uptake of DHA from milk triacylglycerol and increase its uptake in tissues. The developing brain has the ability to synthesize and incorporate DHA from blood vessels into membrane PLs, which can result in improved neurite outgrowth, synaptogenesis, and neurogenesis (35–37).

To minimize the impact of confounding factors on the results of this study, several potential influencing factors were considered. One of these factors is dietary intake, as higher intake may lead to infants receiving more nutrients and bias the results. Our study specifically examined the intake of formula milk and found no significant difference between the two groups, which was comparable to breastfeeding. Another factor is the addition of dietary supplements for infants, such as supplements containing key components like PLs and LCPUFAs, which may affect the study results. We collected data on dietary supplement usage and found that infants only used supplements containing vitamin D and iron, without including the key components of PLs and LCPUFAs. Additionally, there is limited evidence suggesting that supplementing with omega-3 fatty acids during pregnancy may have a positive impact on cognitive development in children (38). Our study reviewed supplementation during the 12 weeks prior to delivery. Interestingly, we found no significant difference in DHA supplementation among all groups. In addition to diet-related factors, maternal factors may also influence infant cognition. Liu Xiaoning et al. reported that maternal obesity can lead to cognitive and social behavioral deficits in both human and mouse offspring. They also discovered that overweight and obese mothers tend to have lower educational attainment and family income compared to normal-weight mothers (39). Another study found a correlation between economic vulnerability during pregnancy and a higher risk of adverse neurodevelopmental outcomes in 2- and 5-year-old children (40). In our study, we examined the participants' family income and observed that the monthly income of the breastfeeding group was lower than that of the formula feeding group. However, it is important to note that the monthly income of all participants was above 3,000 yuan, indicating that they were not below the poverty level. Furthermore, we evaluated the education level of the infants' parents and found no significant difference between the two groups. Unfortunately, we did not track the mothers' weight during pregnancy. Data from the Children's Nutrition and Health System Survey in China conducted between 2019 to 2020 suggested that cesarean section delivery was associated with neurodevelopment outcomes. Specifically, it was found that cesarean section might decrease the developmental scores of gross motor, fine motor and language (41). In our study, we did not observe any significant difference in the proportion of mothers' modes of delivery between the groups. Notably, our research data at each visit were analyzed using analysis of covariance (ANCOVA) to adjust for gender, birth weight, household income, number of family members living in the household, father education, mother education, mother's age when participant was born, maternal DHA supplement use during last 12 weeks of pregnancy, maternal prenatal vitamin use during last 12 weeks of pregnancy, and exposure to smoking at study enrollment, thereby enhancing the accuracy of result evaluation.

Compared to several previously reported clinical studies (14, 15, 20), our research has the following advantages: a longer feeding period up to 1 year of age for infants, a larger sample size, a more comprehensive neurodevelopmental assessment scale, and a more extensive control group design. However, several limitations of the present study should be noted. Firstly, we did not specifically investigate the content of PLs and LCPUFAs in breast milk in the breastfeeding group. If we had collected such data and compared it to the formula group, there might have been more surprising findings. Secondly, the neurodevelopment of infants is substantially affected by maternal status during pregnancy. However, we only had complete statistics on the occurrence of adverse events during pregnancy, focusing solely on the presence of asthma or allergy history. Thirdly, we did not evaluate the neurodevelopmental aspects of the infants at the time of enrollment, making it impossible to determine whether the effects of innate genetic factors differed between the groups. Nonetheless, it is worth mentioning that our study had a sufficient and randomly distributed sample size, which helps to minimize the influence of confounding factors. Fourthly, human milk oligosaccharides (HMOs) seem to be linked to enhanced white matter development and increased gray matter development in a dose-dependent manner, subsequently leading to improved neurological outcomes during childhood. Various bioactive components influence brain development, particularly myelination, either directly, via the gut-brain axis, or through the immune system (42). HMOs are believed to significantly contribute to long-term cognitive improvement. However, the study did not yield any significant findings regarding the impact of HMOs on the microbiota in the two experimental groups. Fifthly, it is unfortunate that this clinical study did not incorporate relevant blood measurements, particularly the levels of DHA in red blood cell membrane PLs, which indicates the level of DHA in cerebral cell membranes. The inclusion of this data would have further strengthened the evidence supporting the hypothesis that LCPUFA and PLs work

together to produce neurodevelopmental effects. Lastly, it is important to highlight that our study only reported short-term results within the first year of birth, and further investigation is needed to assess long-term outcomes. It is worth noting that in two other studies, the investigational group (bMFGM + DHA + ARA) demonstrated better cognitive scores at 18 months, 30 months, and 4 years of age (43, 44).

In conclusion, the study found that the infant formula, which included PLs and LCPUFAs are safe, tolerated which support normal growth in infants. More importantly, infants fed the PLs and LCPUFAs supplemented infant formula performed significantly better (including cognitive, language, and motor skills, as well as longer sustained attention) on cognitive testing at 12 months of age than did infants fed a control formula. Further research is needed to understand any potential long-term effects on growth and neurodevelopment due to possible programming and interacting effects of PLs and LCPUFAs.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Institutional Review Board of Shanghai Nutrition Society. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

QR: Formal analysis, Investigation, Writing – original draft. XZ: Investigation, Writing – review & editing. JP: Investigation, Resources, Writing – review & editing. KL: Formal analysis, Writing – original draft. YZ: Resources, Writing – review & editing. YL: Formal analysis, Writing – review & editing. QX: Conceptualization, Writing – original draft, Writing – review & editing. YX: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

QR, JP, KL, and QX are employed by Feihe Dairy Co., Ltd., Tsitsihar, China. Feihe Dairy Co., Ltd., Tsitsihar, China supported the processing and production of clinical trial formula.

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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1358651/full#supplementary-material

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New alternatives to holder pasteurization in processing donor milk in human milk banks

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Infectious and toxicological risks are the main potential hazards that operators of Human Milk Banks (HMBs) encounter and must eliminate. HMBs are trying to implement procedures that allow to manage and sanitize human milk without altering significantly its nutritional and biologically protective components, obtaining a product characterized by a valid balance between safety and biological quality. The history of human milk processing is linked to the origins of HMBs themselves. And although other forms of sterilization were used originally, pasteurization soon became the recognized most effective means for sanitizing milk: all the milk that arrives at the HMB must be pasteurized. Holder pasteurization (HoP) is the most used methodology, and it is performed using low temperature and long time (+62.5°C for 30 min). With HoP some bioactive milk components are lost to varying degrees, but many other precious bioactive compounds are completely or partially preserved. To improve the quality of human milk processed by HMBs, maintaining in the meantime the same microbiological safety offered by HoP, new technologies are under evaluation. At present, High-Temperature Short-Time pasteurization (HTST) and High-Pressure Processing are the most studied methodologies. HTST is already utilized in some HMBs for daily practical activity and for research purposes. They seem to be superior to HoP for a better preservation of some nutritional and biologically protective components. Freeze-drying or lyophilization may have advantages for room temperature storage and transportation. The aim of this study is to evaluate the advancement regarding the processing of DHM with

a literature search from 2019 to 2022. The effects of the new technologies on safety and quality of human milk are presented and discussed. The new technologies should assure microbiological safety of the final product at least at the same level as optimized HoP, with an improved preservation of the nutritional and bioactive components of raw human milk.

KEYWORDS

human milk, donor human milk, human milk bank, HTST, HPP

1 Introduction

Holder pasteurization (HoP) is the processing method most often recommended in relevant guidelines around the world (1). For this paper, we are including some information about the historical origins of processing of human milk (HM) in donor human milk (DHM) banks. We can always learn from the past, although each of the specific processing methods we are going to consider (some of which we also discussed previously) have a history of their own. We begin this paper with a discussion of the original processing method conducted in Vienna in 1909.

It has been recognized that the origins of milk banking itself are derived from the scientific advancement of HM processing, and to the laboratories in Vienna in 1909 linked to the clinical biologist Theodor Escherich (1–4). The first DHM service in Vienna did not use pasteurization, but instead used the most popular European method for sterilization at the time, known as the "Budde" method, named after Carl Christian Leopold Getter (C.C.L.G) Budde from Denmark, and based on original experiments from 1906 which were first published in 1908. The Budde method became the state-of-the-art process and was increasingly popular across Europe (5). It had previously been reported on in *Scientific American* in 1903 (6). The Budde method includes heating at a low temperature, but it also involves the addition of hydric dioxide to sterilize the milk. This obsolete term "buddeization", meaning to sterilize milk, was widely used at least until World War II, as is evidenced by a 1937 American thesis which compared this method to milk "iodization" (milk treatment by iodine) and considered the effects of these two processes on enzymes, bacterial content, physical and chemical properties, and nutritional value of milk (7).

In light of limited refrigeration, preservation was a driving concern, and this was especially true during the summer months when spoilage occurred much more quickly, and these concerns were linked to the commercial pasteurization of bovine milk. The history of pasteurizing milk in bottles while heating dates to the 1890s (8, 9). The "in bottle" process of heating to 62.8°C (145°F) for 20–30 min, which also has been argued to date from the early 1900s, whereas the so-called commercial use of the "holder" or "in vat" method of pasteurization at a similar temperature for a similar length of time is argued to have become common, especially in large urban centers, by 1917 (9, 10), although used commercially earlier. It is around this time that we start to see this method become more common when pasteurizing HM as well, at least the in-bottle version, beginning it seems in US urban centers (11), in particular New York (12).

High-temperature-short-time (HTST) pasteurization, also known as "flash" pasteurization, was also conducted in the US in the early part of the twentieth century, but was not considered appropriate and abandoned until the 1920s (9, 10). High pressure processing or high hydrostatic pressure processing (HPP), also known as "pascalization" or "bridgmanization," also dates to the end of the nineteenth century (13, 14) but is not used more widely or with HM until much more recently (15). This is likewise the case with short wave ultraviolet (UV) irradiation (16). On the other hand, the history of lyophilization or freeze drying of HM for their conservation could be traced back to the 1920s (17, 18) and is tied to the service at the so-called Boston Floating hospital and Massachusetts Institute of Technology-MIT. Although, each of these processes has its own history, it becomes clear that even some of the most recent processes have been part of the scientific imagination for a very long time.

2 Aim of the study

In most human milk banks (HMBs) worldwide, milk processing includes HoP, which has a significant effect on nutrient content and biological properties of DHM. In a recently published paper on this topic by the European Association of Human Milk Banks (EMBA) (19), the conclusions were that in processing of DHM new technologies are developing rapidly, and EMBA recommendations were that "*…the final aim of these technologies should be an improved preservation of the nutritional and bioactive components of raw human milk, while assuring microbiological safety of the product, at least at the same level of optimized HoP.*"

This aspect is considered by EMBA as a scientific priority in the field of HM donation and processing in HMBs. Therefore, the EMBA Board of Directors set up a Working Group (WG) in the year 2018 with the purpose to perform research in this field. The components of this WG are relevant scientists, from different European countries with a great experience in the field of HMBs and HM treatment. The first step of this WG was to update the knowledge about the technologies most utilized in HMBs and to come out with recommendations based on the results of this search and on their personal experience (19). The second step is the present study, aimed to evaluate the state of the art today, with a literature search from 2019 (year of publication of the first EMBA paper on processing of DHM) to the end of 2022. The purpose is to evaluate the new most advanced technologies of HM treatment, and to compare, when possible, the results obtained from these technologies with HoP. Review articles were excluded as well as conference paper/abstract, book chapters, and position paper.

3 High-temperature short-time (HTST) pasteurization

HTST (generally 72°C for 15 s) is a thermal sanification technology whose application in dairy and beverage industry dates to the beginning of XX century. Since the publication of the last version of EMBA recommendations (19), the number of reports on the efficacy and effect of HTST treatment applied to HMBs environment has increased. Some of the improvement points previously listed by EMBA working group have been further addressed, concerning instrumental and processing conditions and validation in HMB environment. Also, studies on the inactivation of HM viruses by HTST pasteurizers have been carried out in recent years. It is important to underline that HTST efficacy against viruses was previously investigated at laboratory level by two research teams (20, 21), whose work contributed to the design of HTST devices that are still being used nowadays (22–24), also in the clinical practice (25, 26).

Briefly, a literature review has been conducted by searching separately the following terms in the Title/Abstract/Keywords domains in Scopus, and in Title/Abstract/MeSH terms in PubMed, limiting the search in the time range 2019–2022: ("human milk" OR "donor milk" OR "breastmilk") AND ("High – Temperature – Short – Time" OR "High Temperature Short Time" OR "HTST" OR "Flash pasteurization" OR "Flash pasteurization"). Results retrieved from single database were: 27 in Scopus and 106 in PubMed. After filtering by 2 independent authors (Ma.G. and C.Y.B.), a total of 17 papers were considered as relevant for the present review.

3.1 HTST results

Tables 1–3 summarize the reports that have been found from 2019 to 2022 on the use of HTST processing for treating donor HM. The most relevant point is that the number of patented and/or commercially available instruments has increased, with respect to the two prototypes already described in 2018 (Spanish patented prototype – PCT/ES2016/070594; Italian patented prototype –EP 15176792.8–1358). Nevertheless, literature search still reports experiments that simulated HTST processing by means of submerging bottles/tubes for few seconds in hot water (27, 29), or by using small volumes, like polymerase chain reaction (PCR) tubes (34). These processes do not meet the requirements of repeatability and monitoring of the temperatures that are needed for implementation in HMB environment. Another aspect is that different groups reported results achieved by different time/temperature combinations. Although these results can be interesting to optimize the processing parameters of HTST, and to limit the thermal damage of DHM components, the pre-requisite for testing different processing conditions should be that these combinations using the same equipment have been already proved as effective as HoP in eradicating bacterial and viral pathogens.

3.1.1 Effect on safety of human milk

The effects of HTST on safety of human milk are summarized on Table 1. Several reports on the eradication of bacteria, as well as viruses (mainly CMV), have been published (19, 20, 35). Evidence on the efficacy of HTST on HM microflora is now available for every commercial system (26, 28). The pathogens showing higher resistance to thermal treatments, such as *Enterococcus faecalis*, required temperatures above 72°C, and were more effectively reduced by HoP (26). *Bacillus cereus* was not eradicated even in the most challenging conditions tested, and its presence in DHM is not efficiently resolved by thermal pasteurization alone, requiring a multi-step integrated approach consisting in donor education, monitoring milk management at home, and the instauration of a routine analysis of pasteurized donor milk (36), and a specific detection system in HMBs.

Another aspect that was investigated is the residual bactericidal/bacteriostatic activity of DHM after pasteurization with either HoP or HTST. While the raw HM bacteriostatic activity against *Staphylococcus aureus* was preserved by HTST, as opposite to HoP (24), the bactericidal/bacteriostatic activity against *Escherichia coli* was more reduced over incubation time when HTST was simulated in a modified HoP pasteurizer (29), than by standard HoP. Although a reduction in *E. coli* bacteriostatic activity was reported also by performing HTST in a flow-type laboratory scale system (24), in this case, HTST proved to be more similar than HoP to the native HM bacteriostatic activity, thus demonstrating that the nature of the pasteurization device may have an influence on the HTST performances.

Endospore-forming spoilage bacteria (*Bacillus* spp. and *Paenibacillus* spp.) are the main biological barrier to extend the shelf life of HTST milk. After a storage at 6°C for 21 days, *Bacillus* and *Paenibacillus* counts (when present) were higher in cow's milk as the temperature of the HTST treatment increased from 72 to 85°C, remaining $<2\log_{10}$ CFU/mL for the first 7 weeks of cold storage (37, 38). So far, there are no specific works dealing with the effect of HTST treatments on the spore-forming bacterial populations of human milk stored at frozen temperatures, but work is in progress to fill this knowledge gap.

3.1.2 Effect on quality of HM

The amount of research data concerning the effect of HTST technology on nutritional and bioactive compounds in HM has increased significantly since the previous edition of EMBA paper (19) (Table 2). The most interesting aspect of those additional data is that they enforce the opinion that batch and continuous processes performed with commercial devices often result in different degrees of thermal damage, affecting the protein fraction. Overall, HTST performed by continuous devices is reported to not affect the total protein content (24, 28, 30), nor the total amino acid profile (39), although protein denaturation and aggregation have been reported (35). Denaturation and aggregation are highly dependent on the applied time/temperature conditions, and, as observed recently by Escuder-Vieco et al. (30), duration, rather than temperature, seems to affect the degradation process of nutrients. They observed that, as compared to raw HM, the lactose concentration was slightly higher in HTST-treated samples, except for longest tested processing time (25 s). Also, glucose concentration in HTST-treated samples was only affected by 25 s treatments. Myo-inositol was not affected by any HTST condition. The fat content after HTST treatments for 5–15 s was not affected, but longer treatments showed lower levels of fat. Compared to raw milk, the content in triglycerides was similar, while diglycerides increased after 5–10 s treatments, showing a concurrent decrease in monoglycerides (about −50%). The phospholipid fraction doubled in proportion (but no difference in the levels of the individual phospholipid was found). The cholesterol fraction and free fatty acids concentrations were both reduced after all HTST treatments. HTST treatment for 15–25 s resulted in lower saturated fatty acids

TABLE 1 Effects of HTST on safety of human milk.*

	References	HTST treatment parameters	Device	Milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HTST – HoP
Pathogens Microbicidal/ Antimicrobial activity	Capriati et al. (27)	Fixed time/temperature: 70°C for few min. (not specified) pasteurized volume: 30 mL each batch *Donors n = 2*	Submerged bottles in a HoP pasteuriser modified to reach the determined plateau temperature.	Microbiological counts of native milk microflora (Total aerobic, *Staphylococcus aureus*, *Bacillus cereus*)	Undetectable after treatment (except *B. cereus* – one sample)	Similar eradication in HoP
	Aceti et al. (28)	Fixed time/temperature: 72°C for 15 s pasteurized volume total: 100 mL *Donors n = 5*	Patented prototype (EP 15176792.8–1,358).	Microbiological counts of native milk microflora (Total aerobic, *Staphylococcus aureus*, Enterobacteriaceae)	Undetectable microbial counts after treatment	Similar eradication in HoP
	Manzardo et al. (26)	Different time/ temperature combinations: 62 and 81°C for 5 s. pasteurized volume 60 mL each batch *Donors n = 30*	Virex II (Lauf, Tübingen, Germany)	Efficacy against inoculated microorganisms	Combination 81°C/5 s. resulted in no bacterial growth, with the exception of *E. faecalis*	HoP: 5 log reduction for all microorganisms except *C. sakazakii* (RV00078)
				Enterococcus faecalis ATC 29212– 5.8×10^4 CFU/mL	62°C for 5 s: 0.76 log reduction; 81°C for 5 s: 3.3 log reduction	
				Cronobacter sakazakii RV00078 and RV5-I-92 – 1.4 and 2.1×10^5 CFU/mL	62°C for 5 s: 1.9 and 1.3 log reduction; 81°C for 5 s: 5 log reduction	
				Listeria monocytogenes 00218 and 0015–1.8 and 1.1×10^5 CFU/mL	62°C for 5 s: 1.2 and 1 log reduction; 81°C for 5 s: 5 log reduction	
				Cytomegalovirus (HCMV)	As effective as raw human milk	
				Respiratory syncytial virus (RSV)	As effective as raw human milk	
				Rotavirus (HRoV)	As effective as raw human milk	
				Rhinovirus (HRhV)	Reduced inhibitory dilution (HTST 0.06 vs. Raw 0.02)	
				Herpes simplex virus type 1 and 2 (HSV-1, HSV-2)	As effective as raw human milk against HSV-2; reduced inhibitory dilution against HSV-1 (HTST 0.08 vs. Raw 0.05)	
	Patil et al. (29)	Fixed time/temperature: 72°C for 15 s. pasteurized volume: 10 mL each batch *Donors n = 48, pooled*	Submerged bottles in a HoP pasteuriser modified to reach the determined plateau temperature:	Bactericidal activity against *E. coli*	*Reduction in bacterial growth:* - after 4 h incubation: 95% - after 8 h incubation: 94% - after 24 h incubation: 26%	HoP showed a higher *E. coli* bactericidal activity than HTST after 24 h incubation (46%)
	Kontopodi et al. (24)	Fixed time/temperature: 72°C for 15 s. pasteurized volume total: 50 mL *Donors n = 4, pooled*	Laboratory scale pasteuriser	Bacteriostatic activity against *E. coli* and *S. aureus*	*Growth rate per hour:* significant increase in *E. coli* growth rate (HTST 4.2 vs. Raw 3.6), but similar for *S. areus* (growth rate 2.0 -fold per hour)	HoP caused the highest reduction in bacteriostatic capacity.

*p-value are indicated only when significant and/or reported in the original article; NS, not significantly different.

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concentrations (−5%), higher proportion of polyunsaturated fatty acids (PUFA) (+7%) and similar monounsaturated fatty acids. HTST treatment did not affect the concentration of any water-or lipid-soluble vitamins (30). Neither HTST nor HoP treatments had a significant effect on the concentration of the Human Milk Oligosaccharide (HMO) Disialyllacto-N-tetraose (34), calcium and amino acid concentrations (27), while insulin content was better retained by HTST than by HoP method (23).

Many recent investigations focused on the possible modifications in the amount or bioactivity of single HM proteins following HTST pasteurization. Alkaline phosphatase activity was suppressed by both continuous (24, 39) and batch (26) processes, at temperatures above 72°C, while activity was partially retained by using mild conditions (62°C, 5 s) (31). Also, bile salt stimulated activity was highly affected by HTST (20% retention rate after 5 s), although to a lower extent than HoP (retention 7%) (24, 30). Lactoferrin concentration is also affected by HTST in a time/temperature correlation depending on manner by both continuous (24, 28) and batch processes (26, 31), as a consequence of aggregation phenomena (28).

Major difference in the performance between batch and continuous devices was observed in the retention of important immunological factors, namely immunoglobulins. Immunoglobulin A (IgA) retention rates with continuous devices were all above 80% (24, 28), better than standard HoP technique, while batch methods showed much lower retention rates (about 30%) at 72°C (26). Escuder-Vieco and colleagues also demonstrated a higher retention for other HM immunoglobulins after HTST pasteurization with respect to HoP, IgG showing the highest preservation rate (99%), followed by IgM (60%), at a combination of 72°C for 10 s (39).

3.1.3 Effect demonstrated *in vivo* and *in vitro* models

The effects of HTST *in vivo* and *in vitro* models are reported on Table 3. HTST was the first investigated HoP alternative to be addressed for possible differences in donor HM digestion with respect to raw DHM (32, 33). By performing simulated *in vitro* dynamic digestion using preterm infant conditions, HTST treatment resulted in a higher retention of immunoglobulins and lactoferrin content, and higher intestinal release of total and essential free amino acids, with respect to HoP (32). Both thermal pasteurization methods were recently found to affect the ability of HM to induce blood plasma clotting, probably because of protein denaturation over the surface of extracellular vesicles (22), although the *in vivo* role of this ability has not been clarified.

3.2 HTST discussion

Currently, two different HTST pasteurizers, specifically designed and validated for HM treatment, are available. One batch device is inspired by the pioneering work by Hamprecht and is used mainly as a cytomegalovirus (CMV) limiting tool in Germany (40), at lower time temperature combinations. Due to its low operating volumes (approximately 50–95 mL), it is suitable for the pasteurization of milk from single donation of single mothers in neonatal units. One continuous patented device has been used since 2020 in the Regional HMB, Madrid, Spain, in clinical routine (30, 39). The validation procedure of the device demonstrated that a processing treatment including a combination of 72°C for at least 10 s is efficient in killing any vegetative cell initially present in raw HM samples, including Gram negative and Gram-positive bacteria, yeasts, at microbial counts up to 5.77 $\log_{10}$, except for the spore forming *B. cereus*, similarly to HoP.

HTST treatment resulted in better preservation of the nutritional quality of DHM compared to HoP because relevant thermosensitive components (phospholipids, PUFAs, and BSSL – Bile Salt-Stimulated Lipase) were less affected. Furthermore, vitamins did not present a significant reduction after both HTST pasteurization and HoP (30, 39).

Recent evidence for continuous devices confirms that HTST pasteurization of HM allows a higher retention of bioactive factors, such as immunoglobulins, including sIgA, lactoferrin, and BSSL, when compared to HoP. This is very likely to be at the basis of the antiviral activity of HTST treated HM, that, unlike HoP, retained the original HM inhibitory activity against a set of viruses of clinical relevance in infants, as demonstrated for another commercially available continuous pasteurizer (41). Moreover, bacteriostatic activity against *E. coli* and *S. aureus* was less affected than by HoP (24). The degree of thermal damage for specific bioactive proteins, such as immunoglobulins, is more relevant when batch processes are used, and may thus affect the anti-infectious capacities (26, 29).

Last, the closer biochemical profile of HTST and raw HM resulted in a similar peptide digestion profile at gastric level and in higher release of total and essential amino acids at intestinal level from HTST treated HM, with respect to HoP, as reported by simulated preterm infant digestion (32, 33).

4 High pressure processing (HPP)

HPP is used since several years in the food industry to increase the safety and to improve the shelf-life of different products. HPP is usually performed, as a non-thermal pasteurization method, by applying high hydrostatic pressure, from 350 to 800 MPa, for a short period of time (5–10 min). The first review on HM processing by EMBA in 2019 (19) showed promising results when HPP was applied for the treatment of DHM. Scientific teams have demonstrated the destruction of multiple vegetative bacteria, as well as sporulating bacteria, and a better retention of immunoglobulins, lysozyme, lactoferrin, lipase, and cytokines in HPP-treated milk, compared to milk pasteurized by HoP. Given these data, HPP currently experience an increased interest in research on HM and other recent advancements have been made using different HPP settings.

Briefly, searches on Pubmed and Scopus databases have been conducted by using the following terms: ("human milk" OR "donor milk" OR "breastmilk") AND ("high-pressure processing" OR "high hydrostatic pressure"), limiting the search in the time range 2019–2022. After the screening of 37 publications for eligibility, a total of 23 papers were used for the present review.

4.1 HPP results

Tables 4–6 report the most relevant data that have been found in literature on the use of HPP processing for the treatment of DHM.

TABLE 2 Effects of HTST on quality of human milk: nutrients and bioactive factors retention.*

	Reference	HTST treatment parameters	Device	Milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HTST – HoP
Nutrients	Capriati et al. (27)	Fixed time/temperature: 70°C for few min. pasteurized volume: 30 mL each batch *Donors n = 2*	Submerged bottles in a HoP pasteuriser modified to reach the determined plateau temperature	Calcium (mg%)	Not affected	HoP showed similar results, with the exception of slightly higher triglycerides decrease (~21%, not significant)
				Amino acids profile (mcgmol/L)	Not affected	
				Triglycerides (mg%)	Decreased (~18%)	
	Aceti et al. (28)	Fixed time/temperature: 72°C for 15 s pasteurized volume total: 100 mL *Donors n = 5*	Patented prototype (EP 15176792.8–1,358)	Total protein content	Not affected	HoP showed similar results
	Escuder-Vieco et al. (30)	Different time/temperature combinations: 70, 72, 75°C for 5, 10, 15, 20, and 25 s. Pasteurized volume total: 9760 mL each batch *Donors n = 12, pooled in each batch*	Patented prototype – (PCT/ ES2016/070594)	Total protein content (g/L)	Not affected	HoP - preserved the fat and lactose content, myo -inositol and vitamins - decreased the total protein content (−1%); pyridoxal (−12%) - - increased the glucose (+8%); vitamin D3 (+47%).
				Total fat content (g/L)	Not affected at 5–15 s; −4% at 25 s.	
				Lactose content (g/L)	Slightly increased (<1%)	
				Glucose content (mg/L)	Not affected until 20 s.; −10% at 25 s.	
				Myo – inositol content (mg/L)	Not affected	
				Vitamin content	Not affected	
				Lipid profile	– Similar triglyceride content – Higher diglycerides at 5–10 s. – Lower (~50%) mono-glycerides – Doubled phospholipid fraction – 1/3 reduced cholesterol and free fatty acid – Minor significant reduction of Saturated Fatty Acids (−5%), and increase in Polyunsaturated Fatty Acids (+7%) Duration rather than temperature seemed to have an impact on nutrients	
Bioactive factors	Aceti et al. (28)	Fixed time/temperature: 72°C for 15 s pasteurized volume total: 100 mL *Donors n = 5*	Patented prototype (EP 15176792.8–1,358)	Lactoferrin content	Decreased (−83.5%)	HoP shows a similar retention of lactoferrin content and sIgAs content. The protein profile revealed aggregation phenomena for native lactoferrin that was significantly reduced by HoP (−35%)
				Secretory IgA content	Preserved (−20%, not significant)	
				Protein denaturation (PAGE)	Protein profile analysis revealed an induction of protein aggregation (>40%), and a decrease of native lactoferrin band (although not significant)	

(Continued)

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TABLE 2 (Continued)

	Reference	HTST treatment parameters	Device	Milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HTST – HoP
	Escuder-Vieco et al. (30)	Different time/temperature combinations: 70, 72, 75°C for 5, 10, 15, 20, and 25 s. pasteurized volume total: 9760 mL each batch *Donors n = 12, pooled in each batch*	Patented prototype – (PCT/ES2016/070594)	Bile salt stimulated lipase activity (%)	Mean retention <20%	Lower retention by HoP (7%).
	Kontopodi et al. (24)	Fixed time/temperature: 72°C for 15 s. pasteurized volume total: 50 mL *Donors n = 4, pooled*	Laboratory scale pasteuriser.	Native serum protein content (mg/ml)	Not affected	HoP caused a lower retention than HTST for all proteins studied (~50% serum proteins; 44% IgAs; 19% lactoferrin; 2% BSSL concentration and 4% activity), except lysozyme, and alkaline phosphatase (not significant)
				IgAs concentration	Not affected	
				Lysozyme concentration	Not affected	
				Lactoferrin concentration	Decreased (~50%)	
				Bile salt-stimulated lipase concentration and activity (%)	Decreased (−91% concentration and −81% activity)	
				Alkaline phosphatase activity (U/ml)	No detectable activity	
	Mank et al. (23)	Fixed time/temperature: 72°C for 15 s. pasteurized volume: 10 mL *Donors n* = 28	Laboratory scale pasteuriser	Insulin concentration (pmol/L)	Not affected (retention 78%)	HoP significantly reduced insulin concentration (retention 67%)
	Manzardo et al. (26)	Different time/temperature combinations: 62°C for 5 s. 72°C for 5 s. 81°C for 5 s. Pasteurized volume: 60 mL *Donors* n = 10	Virex II (Lauf, Tübingen, Germany)	Alkaline phosphatase activity (mU/ml)	Not detected at 72 and 81°C	HoP caused a lower retention than HTST at 62°C, but similar at higher temperatures, except for sIgAs, that were more preserved by HoP and HTST at 62°C.
				Lactoferrin concentration (g/L)	−50% at 62°C, −75 to 80% at 72–81°C	
				Secretory IgA concentration (g/L)	−20% at 62°C, −70% at 72°C, undetectable at 81°C	
	Müller et al. (31)	Fixed time/temperature: 62°C for 5 s. Pasteurized volume: 60 mL *Donors n = 15*	Virex II (Lauf, Tübingen, Germany)	Alkaline phosphatase activity (mU/ml)	Decreased (−50%)	HoP caused a lower retention than HTST for secretory IgAs, lactoferrin, and alkaline phosphatase, in particular when using dry tempering devices.
				Lactoferrin concentration (g/L)	Decreased (−20%)	
				Secretory IgA concentration (g/L)	Decreased (−30%)	

**p*-value are indicated only when significant and/or reported in the original article; NS, not significantly different.

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TABLE 3 *In vivo*/*In vitro* metabolic studies on HTST effect.*

	References	HTST treatment parameters	Device	Studied milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HTST – HoP
In vitro	Nebbia et al. (32)	Fixed time/ temperature: 72°C for 15 s Pasteurized volume total: 100 mL *Donors* n = 5	Patented prototype (EP 15176792.8–1,358)	Macronutrient composition, protein profiling, confocal microscopy, dynamic light scattering and aminoacid composition of human milk following *in vitro* simulated dynamic digestion with preterm infant model.	Formation of protein aggregates mainly composed by lactoferrin, xanthine dehydrogenase, and fatty acid synthase; 75% retention of native lactoferrin content. Formation of smaller particles during gastric digestion; increased rate of digestion for some protein fractions. Similar lipid composition, and release of free NH2 during digestion.	HoP increased the interaction between proteins and fat globules, decreased the retention of native lactoferrin (33%), and showed a lower abundance of native BSSL than in HTST (−66% vs. −39%). Digestion patterns were similar for proteins (a slightly higher retention of immunoglobulins and lactoferrin, and a higher intestinal release of total and essential free amino acids was seen after HTST).
	Giribaldi et al. (33)	Fixed time/ temperature: 72°C for 15 s pasteurized volume total: 100 mL *Donors n* = 5	Patented prototype (EP 15176792.8–1,358)	Peptidomic analysis of *in vitro* simulated dynamic digestion with preterm infant model.	A slightly more similar pattern in peptide release during gastric digestion was found between HTST and raw human milk, in particular with peptide release from beta casein digestion.	During gastric digestion, HOP-HM presented a greater number and more abundant specific peptides from beta casein.
	Hu et al. (22)	Fixed time/ temperature: 72°C for 15 s. pasteurized volume total: 10 mL *Donors* n = 30	Laboratory scale pasteuriser	Blood plasma clotting time	Significantly increased >10 fold	HoP significantly increase more than HTST plasma clotting time (>20 fold)

*p-value are indicated only when significant and/or reported in the original article; NS, not significantly different.

4.1.1 Effect on safety of HM

The last EMBA literature review (19) reported a destruction of *E. coli*, *S. aureus, L. monocytogenes,* and *Salmonella* sp. in HM within the 300–400 MPa pressure range.

Different HPP settings (400–600 MPa) have been tested to reduce bacterial load in DHM (24, 42–47) (Table 4). Total inactivation of *E. cloacae* and *S. aureus* (10^8 CFU/mL) was obtained by subjecting milk to various pressure and holding time (400 MPa for 5, 10, and 30 min, 500 MPa for 1.5, 2 × 1.5, 3, and 5 min, 600 MPa for 1.5, 2 × 1.5, 3, and 5 min) (23). By applying a pressure of 450 MPa for 15 min, Jarzynka et al. (45) reported a total inactivation of *E. coli* (1.2×10^7 CFU/mL), *L. monocytogenes* (8.2×10^7 CFU/mL), *S. aureus* (6.9×10^6 CFU/mL), and *C. sakazakii* (8×10^5 CFU/mL). Rocha-Pimienta et al. (58) proposed a model to predict the inactivation of microorganisms by HPP of HM. By using this model, a reduction of 10^6 CFU/mL of *S. aureus* and 10^7 CFU/mL of *B. cereus*, in its vegetative form, was achieved by applying a pressure of 600 MPa for almost 4 min.

Sporulating bacteria such as *Bacillus cereus* are a major problem in human milk banks, and optimizing the HPP process to destroy such bacteria is still a challenge. Dussault et al. observed that the use of a higher temperature, 37°C rather than 4°C, during an HPP treatment of 4-cycles of 6 min each at 425 MPa, is more efficient in eliminating bacterial load naturally present in raw milk (42). When the treatment was performed at 37°C, the researchers observed an unequal destruction of the initial bacterial load (between 10^2 and 10^3 CFU/mL) according to the pools, with *B. cereus* surviving, probably due to bacterial spores resistant to HPP treatment (42). Applying a pressure of 600 MPa for almost 4 min, Rocha-Pimienta et al. also failed to destroy *Bacillus cereus* spores inoculated at an initial concentration of 10^7 CFU/mL in milk (58).

TABLE 4 Effects of HPP on safety of human milk*.

	Reference	HPP treatment parameters	Device	Studied milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HoP – HPP
Pathogens	Kontopodi et al. (24)	Many conditions have been tested: −400 MPa 5, 10, 30 min −500 MPa 1.5, 2 × 1.5, 3, 5 min −600 MPa 1.5, 2 × 1.5, 3, 5 min (n = 2)	Pilot-scale equipment (Resato, Roden, Netherlands).	Inactivation of *E. cloacae* and *S. aureus*	>7.8 Log_{10} reduction (below detection limit) for all HPP-tested parameters	The same inactivation was obtained after HoP for all tested bacterial strains. (>7.8 Log_{10} reduction; below detection limit).
				Bacteriostatic capacity	NS	NS
	Dussault et al. (42)	4 cycles of 6 min; 425 MPa; 4°C and 37°C (n = 6)	Industrial-scale equipment (Model 135; Hiperbaric, Spain).	Diminution of initial milk bacterial flora	Preincubation of milk to 37°C seem to facilitate bacterial destruction by HPP treatment	No difference was observed in post-pasteurization bacterial loads between HPP and HoP treated milk (p > 0.16).
	Irazusta et al. (43)	Many conditions have been tested at 20°C: −400 MPa 5 min −450 MPa 5 min −500 MPa 5 min (n = 8)	S-IL-100-250-09 W (HP Food Processor, UK).	Microbiological Inactivation: - Coliforms - Total aerobic bacteria	Decreased bacterial concentration below detection limit Results for raw milk were not presented	NS (Median total counts obtained for HPP-treated samples at 400, 450, and 500 MPa were equal to median counts obtained for the HoP-treated samples)
	Pitino et al. (44)	500 MPa; 8 min T° not mentionned (n = 17)	Industrial-scale equipment (Model 135; Hiperbaric, Spain).	Diminution of initial milk bacterial flora	All methods of pasteurization increased the number of culture negative milk samples (<1 × 103 CFU/L) compared with pre pasteurization p (<0.05).	Higher proportion of samples with negative cultures after HPP than after Holder but NS (p = 0.06)
B. cereus	Jarzynka et al. (45)	450 MPa; 15 min; 21°C (n = 6)	Pilot-scale equipment (U 4000/65, Unipress Equipment, Poland).	Microbiological Inactivation: - *E. coli* (1.2×10^7 CFU/mL) - *L. monocytogenes* (8.2×10^7 CFU/mL) - *S. aureus* (6.9×10^6 CFU/mL) - *C. sakazakii* (8×10^5 CFU/mL) - Sporulating *B. cereus* (4×10^5 CFU/mL)	Complete inactivation	Total vegetative forms of the native microbiota were destroyed by HoP; no result for sporulating forms after HoP

(Continued)

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TABLE 4 (Continued)

	Reference	HPP treatment parameters	Device	Studied milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HoP – HPP
Microbicidal activity	Barbarska et al. (46)	Many conditions have been tested: −450 MPa; 15 min −200 MPa; 10 min + 400 MPa; 10 min Inoculation of 6×10^7 CFU/mL of *E coli*, post-HPP treatment; incubation for 2 h at 37°C. ($n = 6$)	Pilot-scale equipment (U 4000/65, Unipress Equipment, Poland).	Bactericidal activity against *E. coli*	NS (reduction of *E. coli* growth from 29.6 to 50.3%)	Not compared
	Kothari et al. (47)	1 cycle of 8 min; 500 MPa, 4°C Following HPP treatment, milk was spiked with CMV (~5 log PFU/mL) and incubated up to 4 h at room temperature. Post-incubation CMV titers were measured.($n = 10$)	Industrial-scale equipment (Model 135; Hiperbaric, Spain).	Anti-cytomegalovirus activity	HPP-treated milk maintains anti-CMV activity comparable to raw milk. After a 30-min incubation period, 3.4 and 3.7 log PFU/mL were measured in raw and HPP-treated milk, respectively.	HoP-treated milk maintains anti-CMV activity comparable to HPP-treated milk after a 30-min post-pasteurization period. Not significantly different.
Viruses	Pitino et al. (48)	Many conditions have been tested at <10°C: −350 MPa 8 and 10 min −500 MPa 8 and 10 min −600 MPa 8 and 10 min	1 L-pilot unit Dustec Hochdrunktechnik GmbH (Germany).	Cytomegalovirus (CMV) Hepatitis A virus (HAV)	Reduction of CMV (initial load: 5.1 log PFU/mL) and HAV (initial load: 5.7 log PFU/mL) below the detection limit for all HPP treatments;	NS for reduction of CMV HoP was less effective than HPP treatments in reducing HAV in human milk ($p < 0.05$).

*p-value are indicated only when significant and/or reported in the original article; NS, not significantly different.

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Bactericidal and bacteriostatic activity of DHM is its ability to reduce the growth of milk pathogens through the presence of bioactive compounds, such as lactoperoxidase.

Barbarska et al. (46) processed milk samples in two ways: (1) 450 MPa for 15 min; or (2) 200 MPa for 10 min + 10 min break + 450 MPa for 10 min. They used a 0.2 mL inoculum with a concentration of 3×10^8 CFU/mL of *E. coli* to inoculate 0.8 mL milk samples. After a 2 h-incubation at 37°C, the bactericidal capacity of HPP-treated milk against *E. coli* was preserved at 29.6% (450 MPa) and at 50.3% (200 MPa + 450 MPa). These results were not significantly different from those obtained with raw milk, for which a 46.6% reduction in *E. coli* growth was observed ($p \geq 0.34$). These results suggest that, at these conditions, HPP treatment did not negatively affect the bactericidal activity of human milk against *E. coli*.

It should be noted that the elimination of contaminating pathogens by HPP has led to the elimination of all bacteria present in raw milk and likely to colonize infant gut in early life (59).

Pitino et al. (48) reported the reduction of CMV (initial load of 10^5 PFU/mL) and hepatitis A virus (HAV) (initial load of 10^6 PFU/mL) below the detection limit (4 PFU/mL) for multiple HPP treatment tested (350–600 MPa; 8–10 min). Furthermore, Kothari and colleagues (47) reported a maintained anti-CMV activity in milk treated by one HPP-cycle of 500 MPa for 8 min. Therefore, HPP used in these conditions preserves antiviral activity.

In summary, most pathogens currently present in HM, including 4×10^5 CFU/mL of vegetative cells of *B. cereus*, and 10^5–10^6 PFU/mL of CMV and HAV are destroyed by HPP, and antimicrobial activity of HM is preserved (Table 4).

The HoP treatment shows a destruction of the milk bacterial load and the initial CMV load very similar to that of the HPP treatment (23, 41, 42, 46). HoP-treated milk maintains post–pasteurization anti-CMV activity comparable to HPP-treated milk (48). However, HoP is less efficient in destroying hepatitis A virus than HPP at a pressure equal or over 500 MPa (48).

4.1.2 Effect on quality of HM

The 2019 review (19) indicates that an HPP treatment from 100 to 500 MPa, up to 10 min, allows retention of macronutrients when compared to raw milk. The new evidences on the effects of HPP on quality of HM are summarized on Table 5.

Macronutrients' content is similar in raw milk and in HPP treated milk at 350, 500, or 600 MPa for 8 or 10 min (48). Moreover, in another study, the same research team detected no statistical difference in folate levels between raw milk and milk subjected to an HPP treatment at 500 MPa for 8 min, while vitamin C was destroyed by HPP treatment at 500 MPa for 5 min or more (44). In summary, HPP has no significant effect on nutrient content, except for vitamin C.

As emphasized in the past review (19), bioactive components are generally well preserved after HPP treatment with a few exceptions. Wesolowska and colleagues demonstrated that HPP treatment, especially at 450 MPa for 15 min, lead to almost complete retention of lipase activity (87.3%) (51). Other studies have also reported no loss of BSSL activity after HPP treatments of DHM at 400 MPa for 5 min (50), or 500 MPa for 8 min (46). However, lipase activity is decreased by HPP treatment above 500 MPa: a 62% retention of lipase activity in milk treated at 550 MPa for 5 min when compared to raw HM ($p < 0.05$) was reported (53). Wesolowska and colleagues (48) detected retention of only 16.5% of the lipase activity present in raw milk after a treatment at 600 MPa for 10 min.

Regarding protein, no change in lipoprotein lipase abundance in the milk fat globule membrane fraction was reported following HPP at 400 MPa for 5 min (60). In the same study, protein profiles were less affected in the whey fraction, compared to the milk fat globule membrane or casein fraction. Given these results from studies on HPP impact on lipase, this treatment has no or limited effect on lipase activity when used at pressure below 500 MPa.

Lysozyme is a major component of breastmilk and has been widely studied with respect to its retention in DHM after pasteurization processes and shown to be resistant to HPP treatment. By treating maternal milk at 400 MPa for 5 min, Zhang et al. (59) observed an increase of lysozyme activity to approximately 120% of the activity detected in raw milk. This result agrees with other studies, such as Pitino et al. (44) and Dussault et al. (42). Increase of lysozyme activity could be attributed to a partial unfolding of the lysozyme protein during the HPP treatment. This increases the surface area and lytic activity of the enzyme against the cell wall of *Micrococcus lysodeikticus* used for the turbimetric test. This results in an increased lysozyme activity (61).

Many discrepancies are observed for lactoferrin between the results obtained after different HPP protocols. Because of the high sensitivity of lactoferrin to HPP and to different heat treatments of milk, its measurement is often considered as a reference for evaluation of the impact of milk processing on its quality. From recent studies a trend has emerged: the higher the pressure, the lower the milk lactoferrin content. From 500 MPa for 8 or 10 min, Pitino et al. (48) observed a significant decrease in lactoferrin levels compared to raw milk. Lactoferrin analysis was performed by HPLC. At 600 MPa for 10 min, Kontopodi et al. suggest an aggregation of lactoferrin that could be associated to the protein aggregation observed by proteomics after treatment at 600 MPa for 3 or 5 min (24). Aceti et al. (28) (600 MPa, 3 min), Zang et al. (50) (400 MPa, 5 min), and Dussault et al. (42) (4 cycles of 425 MPa, 6 min) used ELISA to assess lactoferrin levels and all reported a non-significant decrease in lactoferrin after treatment. Kontopodi et al. also obtained non-significant decrease in lactoferrin after HPP treatment, proteins were analyzed by LC–MS/MS (24). However, the temperature of the treatment may have an impact on lactoferrin levels: Dussault et al. (42) observed a significant decrease (37%) when HPP is performed at lower temperature (at 4°C) compared to untreated milk.

Application of HPP at 400 MPa for 5 min did not affect IgA content of treated milk (50). Similarly, when applying 4-cycles at 425 MPa for 6 min each, there was no significant difference in IgA and IgG levels between raw milk and HPP-treated milk (42). Contrary to IgG and IgA, IgM is significantly reduced by a 4-cycles HPP treatment at 425 MPa and 37°C for 6 min (42). After a treatment of 5 min to 500 MPa, Irazusta et al. (43) reported a halved (−49%) IgM content when compared to raw milk.

Lactoperoxidase and xanthine oxidase are two natural enzymes found in breast milk that prevent the multiplication of bacteria. Zhang et al. (50) showed preservation of activity up to 110 and 90%, respectively, after HPP at 400 MPa for 5 min.

There are more than 160 different human milk oligosaccharides (HMOs), some of which exert beneficial effects on newborn

TABLE 5 Effects of HPP on quality of human milk: nutrients and bioactive factors retention.*

	Reference	HPP treatment parameters	Device	Milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HoP -HPP
Nutrients (Protein, Fat, Carbohydrates)	Marousez et al. (49)	4 cycles of 5 min; 350 MPa; 38°C Compression rate 1 MPa/s ($n = 8$)	Not precised	HMO Maillard reaction products: -Furosine -CML -CEL	NS NS NS NS NS	NS All components were statistically increased compared to HPP ($p \leq 0.01$)
	Aceti et al. (28)	600 MPa; 3 min; 4°C ($n = 5$)	Industrial device AV-30 (Avure Technologies, Inc., OH).	Total amount of proteins	NS	Not compared
				Secretory IgA	Decreased (−39%) (significant reduction)	
				Lactoferrin content	Decreased (−24%) (*not significant*)	
	Zhang et al. (50)	400 MPa for 5 min, 20°C ($n = 2$)	Hippo 20 food isostatic press (Ilshin Autoclave, Daejeon, Korea)	casein proteins whey proteins MFGM (Milk Fat Globule Membrane) proteins	Relative abundance of some MFGM Proteins significantly altered Increased abundance of bile salt-dependent lipase and no change in the abundance of lipoprotein lipase in the MFGM fraction Lactoferrin unchanged (whey) Protein profiles less affected in the whey fraction, compared with the MFGM or casein fraction	MFG were aggregated by HoP. MFGM and casein proteins were affected by HoP Better preservation of the protein profile for HPP compared to HoP
	Wesolowska et al. (51)	Many conditions have been tested at ~20°C: −600 MPa 10 min −100 MPa 10 min + 600 MPa 10 min −200 MPa 10 min + 400 MPa 10 min −200 MPa 10 min + 600 MPa 10 min −450 MPa 15 min ($n = 6$)	pilot-scale equipment (U 4000/65, Unipress Equipment, Poland)	Lipase activity	A slight decrease was observed for all tested parameters. The decrease was less important for 200 + 400 MPa (17.8%) and for 450 MPa (−12.7%);	Better preservation in HPP-treated milk than HoP for these treatments ($p < 0.05$): −600 MPa 10 min −200 MPa 10 min + 400 MPa 10 min −450 MPa 15 min
				Free fatty acids (FAA) content	Decreased	Decreased ($p < 0.05$)
				Oxidative stability	Longer induction time (IT) for 600 MPa and 200 + 600 MPa (~ + 5%); decreased IT for other treatments (≥ − 3 min) (Longer IT is associated with better stability)	
				Fatty acid composition	Acids in milk after HoP at 450 MPa ($p < 0.05$). Significant decrease in the percentages of oleic acid and fatty	Decreased induction time (IT) ($p < 0.05$)

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TABLE 5 (Continued)

	Reference	HPP treatment parameters	Device	Milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HoP -HPP
				Fatty acid distribution	Increase in polyunsaturated fatty acids and monounsaturated fatty acid and decrease in saturated fatty acid esterified at sn-2 position for all treatments except for fatty acid esterified at sn-2 position in milk treated at 200 + 600 MPa (NS)	NS between HoP and HPP below 450 MPa
				Carotenoids	Destructive effect on lutein+zeaxanthin; no change for β-carotene and a slight increase for lycopene	Destructive effect on lutein+zeaxanthin of HPP compared to HoP ($p < 0.05$)
	Pitino et al. (52)	500 MPa; 8 min ($n = 8$)	Industrial-scale equipment (Model 135; Hiperbaric, Spain).	Fatty acid composition Oxylipins (ng/ml)	NS Higher levels of some arachidonic-derived oxylipins following HPP can be a consequence of pressure-mediated quaternary structure stabilization of cyclo-oxygenase and lipoxygenase.	NS Lower concentration of a-linolenic acid oxylipin ($p < 0.04$) and higher concentration of EPA oxylipin ($p < 0.05$) in HoP-treated HM compared to HPP-treated HM
	Pitino et al. (44)	Many conditions have been tested at <10°C: −350 MPa 8 and 10 min −500 MPa 8 and 10 min −600 MPa 8 and 10 min	1 L-pilot unit Dustec Hochdrunktechnik GmbH (Germany).	Macronutrients content (g/L) Lactoferrin (g/L)	NS In absolute values, there was no statistical difference between raw milk and HPP treatments; once corrected for true protein, only the 600 MPa treatment of milk for 10 min shows a significant decrease in lactoferrin content compared to raw milk.	NS In absolute values, no statistical difference between HPP and HoP; once corrected for true protein, HoP shows a significant decrease in lactoferrin content compared to HPP treatments at 500 MPa or less ($p < 0.05$).
Bioactives factors	Zhang et al. (50)	400 MPa; 5 min; 25°C	Hippo 20 device (Ilshin Autoclave, Daejeon, Korea).	Lysozyme Xanthine oxidase Lactoperoxidase IgA Lactoferrin Lipoprotein lipase BSSL	NS NS NS NS NS NS NS	All statistically different from HPP (decreased) ($p < 0.05$)
	Mank et al. (23)	500 MPa; 5 min ($n = 28$)	pilot-scale equipment (Resato, Roden, Netherlands).	Insulin concentration (pmol/L)	NS	Not compared

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TABLE 5 (Continued)

	Reference	HPP treatment parameters	Device	Milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HoP -HPP
	Jarzynka et al. (45)	450 MPa; 15 min; 21°C ($n = 6$)	pilot-scale equipment (U 4000/65, Unipress Equipment, Poland).	Lipase Leptin Adiponectin Insulin Hepatic growth factor	NS NS Decreased (−85%) NS Decreased (−21%)	Not compared
	Dussault et al. (42)	4 cycles of 6 min; 425 MPa; 4°C and 37°C ($n = 6$)	Industrial-scale equipment (Model 135; Hiperbaric)	IgA (μg/mL) IgG (μg/mL) IgM (μg/mL) Lysozyme (U/ml) Lactoferrin (mg/ml) Lipase (pg/mL) Cytokines (pg/mL)	NS NS NS for 4°C-HPP treatment NS NS for 37°C-HPP treatment NS NS	NS Significant decrease ($p < 0.01$) Significant decrease ($p < 0.05$) NS Significant decrease ($p < 0.01$) NS NS
	Koh et al. (53)	550 MPa; 5 min ($n = 3$)	Not precised	BSSL activity (U/mL)	Decreased (−38.4%)	Not compared
	Wemelle et al. (54)	4 cycles of 5 min; 350 MPa; 38°C Compression rate 1 MPa/s ($n = 8$)	Not precised	H_2O_2 (nM)	Significantly decreased	HPP treatment significantly decreased H_2O_2 level compared to HoP ($p < 0.01$)
				Vitamin A (mg/L)	NS	NS
				Vitamin E (mg/L)	NS	Not compared
				Total antioxidant capacity	NS	NS
	Wemelle et al. (55)	4 cycles of 5 min; 350 MPa; 38°C Compression rate 1 MPa/s ($n = 8$)	Not precised	Apelin (ng/ml) GLP-1 (ng/ml)	NS NS	Decreased in HoP ($p < 0.05$) Decreased in HoP ($p < 0.0001$)
	Irazusta et al. (43)	Many conditions have been tested at 20°C: −400 MPa 5 min −450 MPa 5 min −500 MPa 5 min ($n = 8$)	S-IL-100-250-09 W (HP Food Processor, UK).	IgA (μg/mL) IgM (μg/mL)	For statistical analysis, authors did not compare HPP with raw milk but with HoP-treated milk 86–101.5% of raw milk content 49–97.5% of raw milk content	Retention of IgA, IgM, and IgG in HM samples was at least 35.5, 23, and 22.5% higher in HPP-treated HM than in HoP-treated HM samples ($p < 0.05$)
				IgG (μg/mL)	98–104.5% of raw milk content	
				Lysozyme ($\times 10^3$LU/mL)	125–128% of raw milk content	Significantly higher in HPP-treated HM (at least 52.5% higher) ($p < 0.05$)
				TGF-β2 (ng/mL)	69.9–88.5% of raw milk content	Significantly higher after the HPP treatment of more than 450 MPa (at least 15.6% higher) ($p < 0.05$)
				sCD14 (μg/mL)	28.3–111.2% of raw milk content	Significantly higher after HPP treatment (at least 28.2% higher) ($p < 0.05$)
				Nutritional components (lipids, proteins, lactose) (mg/mL) (energy) (Cal/mL)	Results for raw milk were not presented	NS

(Continued)

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TABLE 5 (Continued)

	Reference	HPP treatment parameters	Device	Milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HoP -HPP
	Smyczynska et al. (56)	450 MPa; 15 min ($n = 3$)	Pilot-scale equipment (U 4000/65, Unipress Equipment, Poland).	microRNA content	Some miRNAs are degraded under high pressure. The diminution of miRNAs could decline functions of human milk.	Not compared
	Marousez et al. (57)	4 cycles of 5 min; 350 MPa; 38°C Compression rate 1 MPa/s ($n = 8$)	Not precised	Leptin	NS	Compared to HoP-treated HM, HPP treatment significantly increased concentration of insulin, nesfatin-1, cortisol, leptin, apelin and GLP-1 ($p < 0.05$) and reduced adiponectin by 52% ($p < 0.01$).
				Insulin (mU/L)	NS	
				Apelin (ng/ml)	Reduced (−20%)	
				Adiponectin (ng/ml)	Reduced (−50%)	
				Nesfatin-1	NS	
				Cortisol (ng/ml)	NS	
				GPL-1	Increased (+64%) → *maybe due to a release of GLP-1 from fat globules to the aqueous phase following HPP treatment*	
	Kontopodi et al. (24)	Many conditions have been tested: −400 MPa 5, 10, 30 min −500 MPa 1.5, 2 × 1.5, 3, 5 min −600 MPa 1.5, 2 × 1.5, 3, 5 min ($n = 2$)	Pilot-scale equipment (Resato, Roden, Netherlands).	Total protein concentration (mg/ml)	NS	Not compared
				Proteome	Most intense HPP treatment (600 MPa; 3 and 5 min) have a larger effect than other treatments	
				IgA	NS for 400 and 500 MPa treatments	
				Lactoferrin	NS	
				Lysozyme	NS	
				BSSL activity (%)	NS	
	Pitino et al. (44)	500 MPa; 8 min ($n = 17$)	Industrial-scale equipment (Model 135; Hiperbaric, Spain).	Macronutrients content (g/L)	NS (significant reduction in mean total carbohydrate content after HPP ($p = 0.04$))	Significant reduction in mean total carbohydrate content after HPP ($p < 0.05$).
				Folate (nmol/L)	NS	NS if compared to HPP
				Vitamin C (mg/L)	Decreased (−11 mg/L) (*76%*)	NS if compared to HPP
				BSSL (U/ml)	NS	Significant decrease ($p < 0.05$)
				Lactoferrin (g/L)	Decreased (−0.44 g/L)	Significant decrease ($p < 0.05$)
				Lysozyme (U/L)	NS	Lysozyme activity after HPP was higher ($p < 0.05$) than with HoP

*p-value are indicated only when significant and/or reported in the original article; NS, not significantly different.

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TABLE 6 *In vivo*/*in vitro* metabolic studies on HPP effect.*

	Reference	HPP treatment parameters	Device	Milk components/ properties	Impact of treatment (compared to raw milk)	Comparison HoP – HPP
In vivo/ metabolic studies	Wemelle et al. (54)	4 cycles of 5 min; 350 MPa; 38°C Compression rate 1 MPa/s ($n = 8$)	Not precised	Level of antioxidant gene expression in the liver of mice treated with HPP-donor milk Inflammatory markers in liver and ileum of mice treated with HPP-DM	Increase of catalase and Superoxide dismutase mRNA expression → stimulation of antioxidant defense NS for other genes (Sod1, Gpx1, Gpx2, Nox1/2) Decrease in F4/80 mRNA expression (ileum) Decreased expression of Tnfα et F4/80 mRNA (liver) → reduced inflammation	Catalase and Sod2 mRNA expression were significantly decreased in HoP-treated HM ($p < 0.05$) After supplementation of mice with HPP-treated milk compared to HoP-treated milk: - Decrease in F4/80 mRNA expression in both the ileum and ($p < 0.05$). - Better stimulation of antioxidant defense and reduction of inflammation in both the ileum and liver
	Wemelle et al. (55)	4 cycles of 5 min; 350 MPa; 38°C Compression rate 1 MPa/s ($n = 8$)	Not precised	Glucose tolerance in mice Isotonic intestinal contraction in mice	Not compared	In mice supplemented with HPP-treated milk compared to HoP-treated milk: - Improvement of glucose tolerance ($p < 0.05$) - Decrease of the amplitude of duodenum contraction ($p < 0.05$)
In vitro	Zhang et al. (50)	400 MPa; 5 min; 25°C	Hippo 20 device (Ilshin Autoclave, Daejeon, Korea).	*In vitro* static digestion assays	HPP increased proportion of larger fat globule but HPP milk had a similar size distribution pattern as raw milk following a 60-min of gastric digestion. As in raw milk, lactoferrin remain largely undigested in HPP milk following gastric digestion.	Not compared

*p-value are indicated only when significant and/or reported in the original article.
NS, not significantly different.

development and protection (62, 63). Very little information on the impact of HPP on HMOs levels is known. Marousez et al. (49) quantified 22 major HMOs in raw milk and in milk sample that underwent 4 cycles of 5 min each of HPP treatment at 350 MPa. HMOs remained unaffected by this HPP treatment. The authors also studied the formation of Maillard reaction products (MRPs) as a result of chemical reactions between reduced sugars and amino groups in proteins. Marousez et al. (49) quantified 3 MRPs (furosine, N-epsilon-carboxymethyllysine and N-epsilon-carboxyethyllysine) in maternal milk exposed to the previously described HPP and concluded that this protocol avoids MRPs formation.

Of the 26 cytokines analyzed by Dussault et al. (42), only IL-8, IP-10, MCP-1, MIG, and TGF-β_2 were detected at measurable levels, even in raw milk. No significant difference was observed between milk

samples treated with 4 cycles of HPP treatment (425 MPa for 6 min) and untreated milk samples, suggesting that HPP do not affect these factors.

The sCD14 is a protein that detects bacteria by binding to lipopolysaccharides. While this protein is well preserved in milk after HPP treatment at 400 MPa for 5 min at 20°C (111% of the amount found in raw milk), increasing the pressure seems to have a deleterious effect on sCD14 since 51% and only 28% are retrieved after treatment at 450 MPa and 500 MPa, respectively (43). These results suggest that milk treated at more than 400 MPa may not provide preterm infants with an adequate response to inflammation in comparison to milk treated to low pressure.

Hormones are active factors present in human milk that contribute to the control of energy intake and gut maturation in newborns, both directly and indirectly. Thus, Marousez and colleagues (57) studied the impact of HPP treatment on 6 peptide hormones (leptin, insulin, adiponectin, apelin, GLP-1, and nesfastin-1) and a steroid hormone (cortisol). No significant loss of leptin, insulin, nesfastin-1, or cortisol was observed after 4 cycles of 5 min HPP treatment at 350 MPa compared with raw milk. Similarly, Jarzynka et al. (45) investigated hormone retention in human milk and reported comparable levels of leptin and insulin in milk samples treated with HPP at 450 MPa for 15 min and in untreated samples. Preservation of insulin levels is also reported by Mank et al. (23) following a different HPP protocol (500 MPa for 5 min). However, in a few studies, HPP of human milk seems to have a deleterious effect on the retention of some hormones present in milk since both Marousez et al. (57) and Jarzynka et al. (45) observed a reduction of adiponectin by 50 and 85%, respectively, after a treatment of 4 cycles of 5 min at 350 MPa or at 450 MPa for 15 min (holding time). Marousez et al. (57) also reported a significant decrease in apelin level (20%) after HPP treatment.

HPP can affect the quality of HM with respect to certain nutrients and bioactive factors. For example, oxylipin which derived from PUFAs by oxidation and may be involved in resolving inflammation (52), have been shown to be destroyed by HPP treatment at 500 MPa for 5 min or more. For lutein, which has been shown to have antioxidant and anti-inflammatory properties, Wesolowska and colleagues (51) detected a retention of only 39.8% of lutein present in raw milk, after a treatment at 600 MPa for 10 min. Some miRNAs are also degraded under high pressure, mainly miRNAs related to pathways involved in extracellular matrix -receptor interactions, cell proliferation and metabolism regulation (56). Below 500 MPa, HPP treatment has limited effect on the quality of human milk except for vitamin C, IgM, and apelin.

Compared to HoP, HPP has no effect on the macronutrient composition of milk (48) except for a significant decrease in total carbohydrate content in HPP-treated HM (41). Zhang and colleagues, however, showed that MFG tended to aggregate in HoP-treated milk and that milk fat globule membrane proteins as well as casein were affected (50). Polyunsaturated fatty acids and monounsaturated fatty acids were increased in milk after HPP at 450 MPa ($p < 0.05$), while saturated fatty acids decreased (51). Many authors have shown significant decrease in bioactive factors in milk following HoP compared with HPP. Thus, immunoglobulins (42, 43, 50), lactoferrin (42, 44, 48, 50), lysozyme (43, 44, 50), lipase activity (24, 44, 45, 50, 51, 53) and many hormones (54, 57) are largely affected by HoP.

4.1.3 Effect demonstrated *in vivo* and *in vitro* models

A growing number of experiments are being conducted to investigate the effect of HPP-treated HM on metabolism and physiology (Table 6). An increase in the expression of antioxidant genes (catalase and super dismutase) and in glucose tolerance was observed in mice fed with HPP-donor milk, as demonstrated by Wemelle et al. (55). Authors also showed a decrease in inflammation markers (F4/80, TNF-α) in these animals. After a 60-min *in vitro* gastric digestion assay, Zhang et al. (50) reported a particle size distribution of HPP-milk similar to that of digested raw milk despite the initial observation of an increased proportion of larger fat globules in HPP-treated milk (400 MPa for 5 min) compared with raw milk. Possible aggregation of MFG may occur during HPP treatment since Ye et al. (64) also observed that α-lactalbumin, κ-casein, as well as β-lactoglobulin, associate with milk fat globule membrane proteins through disulfide bonds after HPP treatment.

Importantly, as in raw milk, Zhang and colleagues observed that lactoferrin in HPP-treated milk (400 MPa, 5 min) remained largely undigested after the digestion test (50). Given the antimicrobial properties of lactoferrin, this resistance to digestion is important for regulating the gut microflora of preterm infants.

Studies comparing mice fed HoP- and HPP-treated milk showed a loss of antioxidant defenses and an increase of inflammatory markers in animals fed HoP-treated milk (54).

4.2 HPP discussion

The lack of consensus regarding a "gold standard" HPP protocol makes that each research team develops its own protocol, making it very complex to navigate through the different results. Moreover, all technical details and HPP parameters are not always efficiently mentioned by authors, thus compromising repeatability of an HPP protocol. In order to completely destroy bacterial spores, several parameters must be considered and monitored during HPP treatment of milk: pressure level, temperature, compression and decompression rates, number of cycles and duration of each cycle. All these parameters should be presented by authors. Studies reported bacterial spores resisting to HPP treatment using pressure higher than 400 MPa (42, 58, 65). This phenomenon was studied in more detail by Wuytack et al. using *B. subtilis* spores. This group observed incomplete spore germination after one 600-MPa treatment. At this pressure, the small acid-soluble spore proteins (SASPs) would not be degraded, thus interrupting the germination process (66). In contrast, when pressure between 50 and 300 MPa is applied at 40°C for about 30 min to sporulated bacteria, germination is activated by the stimulation of nutrient receptor, leading the bacteria to act as if the environment is no longer hostile and to germinate into vegetative cells, which would then be destroyed by subsequent HPP cycles (67) underlying the importance of the number of cycles. It is also crucial to present the compression time leading to a progressive increase in pressure, allowing bacterial spores to germinate and bacteria to return to their vegetative form as previously demonstrated for another *Bacillus* sp. (68). Lastly, the initial milk temperature is also important since treatment above 30°C seems to facilitate the germination of bacterial spores which are eliminated by subsequent treatments (69). Dussault

et al. (42) have shown that a pressure of 425 MPa tend to be more effective in destructing bacterial spores when milk samples are previously heated and treated at 37°C, than when they are treated at 4°C. This synergy between pressure, number of cycles and temperature is also observed and well described by Van Opstal and colleagues (69). Authors demonstrated that germination of bacterial spores (6 log) is efficient when milk temperature is above 30°C even at low pressure (200 MPa for 30 min). The bacteria are then eliminated by subsequent treatment. All these parameters have to be considered when establishing an HPP protocol in order to ensure safety and quality of DHM. The first review of the EMBA working group (19) reported the pioneering work of Demazeau et al. (70) based on an original protocol for using HPP: 4 cycles at 350 MPa, during a 5-min holding time, at a temperature of 38°C, with a compression/decompression rate of 1 MPa/s and a latency time of 5 min (at normal pressure) between each cycle. In these specific conditions, HPP treatment was able (i) to destroy at least 10^6 CFU/mL bacteria, which meets the definition of a sterilization/ pasteurization process (ISO14937) (71), including multidrug-resistant *S. aureus*, and (ii) preserve lipase, lactoferrin, and lysozyme (70). These results still need to be verified. Only a comparison between such a multi-cycle process and such a single-cycle treatment, for the destruction of *B. cereus and B. subtilis* spores at a similar level, optimally at 10^6 CFU/mL, would allow to conclude on the effectiveness of the treatment.

At present, to our knowledge, no HMB uses HPP as a pasteurization method. All the devices on the market are used for research and development purposes only. Clinical studies demonstrating all the benefits of using HPP rather than HoP are still needed before the widespread implementation of HPP into milk bank operations. Research so far, however, has been able to show that HPP would ensure comparable or even improved safety of DHM compared to HoP, in addition to allowing better preservation of nutrients and bioactive components (24, 42, 43, 45, 48, 50, 53, 55). As previously shown, preterms' growth rate during their hospital stay depends on overall quality of lipidome (72). Better preservation of human milk lipids would therefore be an advantageous asset for human milk banks. As the cost of the device is high, a full medico-economic evaluation is needed before its routine use in HMBs (73).

5 Other new advanced technologies of HM treatment

A literature review has been conducted by searching separately the following terms in the Title/Abstract/Keywords domains in Scopus, and in Title/Abstract/MeSH terms in PubMed, limiting the search in the time range 2019–2022: ("human milk" OR "donor milk" OR "breastmilk") AND search n°1 ("Ultraviolet C radiation" OR "UV-C"); search n°2 ("Microwave Treatment"); search n°5 ("Gamma-Irradiation"); search n°6 ("Thermoultrasonication" OR "TUS"); search n°7 ("Freeze-drying" OR "FD" OR "lyophilization").

5.1 Ultraviolet C (UV-C) radiation

Short-wavelength ultraviolet C (UV-C) radiation is a treatment commonly used in the food industry (range of waves: 200–280 nm). Despite penetrating the microorganism, ultraviolet light has no effect on the organoleptic characteristics of the food being treated. It works by damaging the nucleic acids in these organisms and prevent them from carrying out essential physiological processes. However, depending on the product's optical characteristics, UV light can only penetrate food materials a few millimeters. The liquid's turbidity and/or color affect the optical absorption coefficient. Milk and other cloudy foods cannot be penetrated by UV radiation. This poses a problem when treating routinely significant quantities of donor HM in HMBs making necessary to expose the milk to the UV-C in a thin layer.

Since our last revision, some papers have increased the knowledge on the effects of UV-C on HM. Thus, Kontopodi et al. (24) demonstrated that UV-C may be considered as a suitable alternative to HoP, since it was able to ensure sufficient microbiological inactivation while at the same time better preserving the bioactive components of DHM. The milk was treated in a sterile beaker glass filled with 140 mL of milk with a UV-C lamp placed diagonally, while magnetically stirred. A UV-C dosage of 4,863 J/L was the UV-C dosage effective in causing a >5 log reduction of *E. cloacae*, *E. coli* and *S. epidermidis* counts. This dosage did not cause a significant reduction on the levels of IgA, lactoferrin, lysozyme or BSSL, and the changes in the DHM proteome after UV-C treatment were minimal. By subjecting DHM to the same treatment, Mank et al. (23) showed that UV-C treatment preserves insulin in DHM. In addition, Almutawif et al. (74) showed that treatment with UV-C at a dosage of 2,259 J/L reduces *S. aureus* growth with similar kinetics to raw milk, thus confirming that the immune protein bioactivity present in HM is not affected by the treatment with UV-C irradiation. Moreover, in parallel with this reduced growth, no enterotoxin production was detected in UV-C treated DHM. Also in this case, the DHM was treated in a glass beaker with a germicidal UV-C lamp introduced diagonally.

The absence of a proper equipment in the setting of a HMB presents the biggest obstacle to evaluating this technology. However, some promising equipment have been analyzed in the treatment of other products than human milk, like donkey milk or swine plasma. This equipment, a pilot-scale SurePure Turbulator UV-C reactor system, creates a turbulent flow and is designed for continuous flow inactivation of turbid fluids. Considering that it is operating at a flow rate of 4,000 L/h, depending on the UV dose applied, the time to treat 1 L would be around 1 min. Papademas et al. (75) tested this device with 7 L of artificially inoculated (*L. innocua*, *S. aureus*, *B. cereus*, *Cronobacter sakazakii*, *E. coli*, *Salmonella enteriditis*; 1×10^5 CFU/mL) donkey milk and observed a complete inactivation at doses in the range of 200–600 J/L, except for *L. innocua* who need a dose of 1,100 J/L. By using the same prototype, a series of papers (76, 77) of the same group confirmed the capability of Sure Pure Turbulator UV-C treatment system to inactivate significant levels of swine enveloped and non-enveloped viruses as well as bacterial strains (*Salmonella typhimurium*, *Salmonella choleraesuis*, *Enterococcus faecium*) inoculated in commercially collected porcine or bovine plasma.

Regarding the effect of UV-C on the elimination of bacterial spores present in human milk, there are no published data. However, there are promising data on the ability of UVC irradiation to eliminate bacterial spores present in different liquids such as orange juice (78),

distilled water (79), coconut water (80), platelet concentrates containing Ringer's solution (81) or grape and apple juices (82).

5.2 Microwave treatment

Almost all international procedural guidelines forbid using household microwaves to defrost or heat-treat HM. This advice is mainly based on two studies that showed how using a household microwave to treat HM caused degradation of IgA and lysozyme and a quicker development of *E. coli* (83, 84). However, the methodology employed in these articles have been called into doubt (85) and a growing number of papers demonstrate that this technique is more harmless than previously believed (86–93).

To treat samples of DHM by microwave heating (MWH) and examine the impact on bioactive components of human milk, Malinowska-Panczyk et al. (93) evaluated a prototype microwave device where the temperature is maintained constant for a short period of time. While baseline lipase and TGF-B2 levels were lowered, like in milk that had been treated with HoP, lactoferrin and IgA were better conserved. Additionally, the treatment with MWH had a greater detrimental impact on the concentration and activity of lysozyme. Inoculated microbiota (1×10^5 CFU/mL) was also shown to be inactivated following microwave heating at 62.5°C or 66°C for 5 or 3 min, respectively. The same team reported that, compared to the HoP method, pasteurization of HM using MW heating under controlled circumstances resulted in decreased loss of Brain derived neurotrophic factor and Glial cell line-derived neurotrophic factor (91).

Two additional studies demonstrated that samples microwave-treated at 62.5°C for 5 min had enzyme activities that were comparable to or greater than those subjected to HoP (88), and that the concentration of macronutrients, fatty acids, lipid peroxides, and α-lactalbumin after using MWH at 62.5°C for 5 min was on a par with that of raw milk, and furosine was not formed. Malonaldehyde and protein carbonyls (PC) levels in DHM slightly increased after MWH treatment. Malonaldehyde concentration in DHM was, however, lower due to MWH than it was following the use of convection heating. Mikawa et al. (92) reported that human CMV was completely destroyed from HM by MWH treatment at 500 W for 40 s. After a treatment at 2450 MHz with a maximum power of 300 W, at 60°C for 30 s, Leite et al. (86) detected the same decrease of microbial contamination in experimentally inoculated human milk (*S. aureus* and *S. typhimurium*, 10^6 CFU/mL) as HoP. The same group reported in a different study (87), that immunoglobulins and lactoferrin did not significantly decrease in concentration following microwave treatment (60°C for 30 s, 65°C for 15 s), and oligosaccharides and fatty acids were not considerably altered. IgA, IgG, IgM, and lactoferrin, on the other hand, fall by 47, 45, 68, and 85%, respectively, following HoP.

5.3 Combination of freeze-drying (FD) or lyophilization and gamma irradiation

Gamma-irradiation is another non-thermal treatment that has been tested. Blackshaw et al. (94, 95) used 2 kGy gamma irradiation to treat freeze-dried DHM samples (moisture 2.2%), and they found no significant change in the protein profile or end-products of lipid oxidation. Additionally, this method lowered the amount of bacteria inoculant like HoP, without decreasing the DHM's antibacterial effectiveness. There is no published data on the effect of this technique on the elimination of bacterial spores present in human milk. However, gamma irradiation has destructive capacity on the bacterial spores present in apple and orange juices (96) and deionized water (97).

Freeze-drying (FD), when utilized in HMBs, may have advantages for room temperature storage and transportation. FD is a low temperature dehydration process that involves freezing the product and lowering pressure, removing the ice by sublimation, contrary to conventional dehydration methods that evaporate water using heat (98). Sublimation allows the passage from the solid state to the gaseous state without passing through the liquid state. Freeze-dryer should be a high-quality device compatible with aseptic clean room operations allowing the production of sterile products. FD is the technique of choice for drying high-value products as the low operating temperatures allow preservation of product quality. Freeze-dried products can be easily re-hydrated. FD enables in-packaging pasteurization of dried DHM powder with a longer shelf-life, stored at ambient temperature, and easier to transport, which reduces storage and transportation costs. It can help to increase availability for newborn babies in hospitals or other places in demand, such as in crisis conditions for humanitarian aids. Main limitation for FD use is the very high cost of high-quality freeze-dryer. It requires high technical expertise and freeze-dryers usable routinely in HMBs. Therefore, it can be used only for large amounts of high-value products. Freeze-drying should not be confused with spray-drying, which is a process used to produce powdered formula or fortifiers, but uses very high temperatures, and have more deleterious effects than FD on milk components (87). Effects of freeze-drying on HM properties were reported in 8 studies between 1983 and 2020, only 3 after 2019. Most studies were performed using a laboratory bench freeze-dryer that can only process a limited volume of milk and requires prolonged (72 h) exposure to pressure, both being not compatible with the requirement of routine HMB operation (99–101). Milk properties of freeze-dried were preserved, even after few months of storage. Oliveira et al. demonstrated preservation of osmolality and stability of nutritional content in freeze-dried human milk samples that were subsequently stored for 3 and 6 months (99). Lactoferrin, lysozyme, total anti-antioxidant capacity and fat remained stable regardless of time (6 weeks) and temperature (5°C and 25°C) of storage (100). Only the activity of superoxide dismutase was significantly reduced in the stored freeze-dried samples, mainly during the first week of storage. However, that decrease was significantly less than the decrease reported during freezer storage of liquid HM (100).

As freeze-drying has no effects on microorganisms, it needs to be associated with a process ensuring microbiological safety (102). Recently, techniques have been tested in association with FD. Combination of high positive pressure (HPP) and FD preserved better the nutritional value than HoP and offered microbiological safety of DHM (45). Combination of FD and then low-dose (2 kGy) gamma irradiation shows no post-treatment difference in lipid oxidation end-products and variation in protein profile (94). This hybrid method also preserves antimicrobial properties and enables bulk pasteurization within sealed packaging of powdered DHM (93).

5.4 Thermoultrasonication (TUS)

Thermoultrasonication (TUS) is a process that combines ultrasonication (microbubble formation and their rapid collapse though inertial cavitation) with mild heating. By using a laboratory configuration of a sonifier and a circulating bath, Mank et al. (23) described that the treatment of DHM (60 W, 6 min: 1080 kJ/L) did not affected the concentration of insulin.

By testing two different conditions, 40 W, 9 min (1,081 kJ/L) and 60 W, 9 min (1,620 kJ/L), Kontopodi et al. (24) observed that all tested conditions achieved a >5-log10 reduction for all bacterial strains analyzed. In addition, the retention of sIgA, lysozyme and lactoferrin showed a decreasing tendency with increasing TUS intensity and exposure time, whereas BSSL retention decreased significantly, that is a pattern of protein damage similar to that of HoP-treated DHM. By using LC–MS/MS-based proteomics analysis, the authors described a pattern of protein damage like the one observed after HoP treatment of DHM.

Regarding the effect of TUS on the elimination of bacterial spores present in human milk, there are no published data. In addition, TUS have also demonstrated their harmful effect on bacterial spores present in water or cow's milk (103).

6 Discussion and conclusion

This paper presents the new most advanced technologies of HM treatment realizing a literature search from 2019 to the end of 2022 as a continuation to the first EMBA paper on processing of DHM in 2019. In this sense, two of the technologies described in our previous paper, HTST pasteurization and HPP, are available on the market today. The only technique among those described in this paper that is regularly used in some HMB is HTST. A first clinical trial to compare the incidence of microbiological proven late onset sepsis, NEC, ROP, mortality, oxygen supplementation, and infant growth in newborns <1,000 g that in the first 28 days of life need to be supplemented with donor milk pasteurized by HTST method versus the Holder method has been recently completed (NCT04424667) in two hospitals, the Neonatology Services of the Hospital 12 de Octubre and the Hospital de La Paz in Madrid, Spain, and the results will be available soon. Recently, a randomized clinical trial funded by Polish Medical Agency Research has just begun, on the prevention of cow's milk allergy through DHM supplementation. In this trial one arm of the intervention is HPP treated HM (Wesolowska, personal communication).

Recent evidence confirms that HTST pasteurization of HM allows a higher retention of bioactive factors, such as immunoglobulins, including sIgA, and lipase, when compared to HoP. It has also been observed that HTST processing resulted in improved preservation of the nutritional quality of HM than HoP, since relevant thermosensitive components, such as phospholipids, PUFAs and BSSL, were less affected. At present no other technique has been implemented in HMB practice, demonstrating a Technology Readiness Level of TRL9, and clinically demonstrated as alternative to HoP in a clinical trial. Concerning HPP, research so far has been able to show that this methodology would ensure comparable or even improved safety of human milk compared to HoP: most bacteria currently present in human milk, including the vegetative forms of *Bacillus cereus*, are destroyed by HPP (provided the results will be confirmed in further studies), and antimicrobial activity of HM is preserved. In addition, HPP allows better preservation of nutrients and bioactive components in comparison to HoP (24, 42, 43, 48, 50, 53, 55). However, the fact that several methodological approaches are used makes extremely difficult to compare studies. There is no doubt that determining the winning conditions for HPP should be a focus for future research. However, HPP as well as HTST seem to be excellent alternatives to HoP for ensuring safety, and quality of human milk for preterm babies is quite comparable to that of raw milk. Regarding other technologies for processing of HM, particular attention should be paid to the determination of parameters to avoid the loss of major components and/or biological activities while ensuring the destruction of both pathogenic bacteria and viruses. On the other hand, FD has no major effects on milk properties and offers advantages such as extended shelf-life, reduced storage and transport costs, larger capacity for storage at room temperature. It has to be associated with a process enabling microbiology safety of milk, such as HoP, HPP or Gamma irradiation. The main limitations are the costs and the required technical expertise to produce a safely dehydrated DHM.

The aim of this study is to evaluate what is new in the processing of DHM from a literature search of the period 2019–2022, and to compare the results derived from the new technologies with the results derived from traditional HoP. It is not our intention to make a classification of the new technologies based on the results obtained, because there are very few studies comparing the new methodologies and the results are scarce and not significant. The effects of the new technologies on safety and quality of human milk have been presented and discussed in the previous sections of this paper. Another thing that may be interesting for the reader is to evaluate the feasibility of the new technologies within HMBs with a practical approach, considering financial, human and technical aspects.

Regarding HTST, there are at present two devices available on the market (SIVE Fluid Systems, Alcalá de Henares, Madrid, Spain, and Labor Baby, Tribiano, Milan, Italy). They can be easily allocated in a HMB and both can be utilized by bank staff after a training course. The cost is higher than the cost of a traditional pasteurizer, and it can be addressed by many banks in high-income countries but not in low- and medium-income countries. HPP is a more sophisticated technology that seems able to inactivate *B. cereus* spores (even if this aspect has to be evaluated more deeply). The utilization of the device needs a high specialized and well-trained staff, a situation not easy to realize in the majority of HMBs. The dimensions of the instrument, and its allocation in HMBs is not compatible with the space available in the vast majority of HMBs. The cost of DHM processed with HPP is estimated to be 7 times higher than that of the milk treated with HoP. Even if we consider that the inactivation of *B. cereus* could reduce the amount of DHM discarded, it is very unlikely that the saving of money deriving from a better utilization of DHM is able to compensate the very high cost of the processing. Future studies should be carried out to assess the actual cost/effectiveness of HPP methodology. At present, no HMB uses HPP as a pasteurization method. All the devices on the market are used for research and development, waiting for a miniaturization of the machinery in order to be easily placed and

routinely utilized for human milk processing in HMBs. FD is an emerging technology that is becoming more and more important in this period due to the possibility to increase the utilization of human milk in emergencies (war, epidemics, and natural disasters). Main limitations for FD use is the very high cost of high-quality freeze-dryer. UV-C radiation, TUS, microwave treatment and gamma-irradiation are all promising technologies but at the moment they are under evaluation in a lab phase and many practical problems have still to be solved before their utilization in a human milk bank environment.

HM processed by HMBs is destined to very vulnerable babies. Since HM may contain potential pathogenic microorganisms and viruses for these babies, their effective removal by pasteurization is a major concern for HMBs. A predominance of *Staphylococcus* species has been reported by several studies, but other miscellaneous bacteria have been identified in HM samples, including *Klebsiella* sp., *Streptococcus* sp., *Pseudomonas* sp., and *Enterobacter* sp. (104). Contamination of breastmilk may occur from a variety of sources including skin, breast pump and environment, and cases of severe sepsis caused by these microorganisms, transmitted by unpasteurised HM to premature babies, have been reported (105–108). *Bacillus cereus*, an environmental organism, can cause fatal infections in vulnerable patients such as premature babies. *B. cereus*, in its sporulating form, can survive in adverse conditions, making it very difficult to eliminate, even by pasteurization. *B. cereus* is an opportunistic microorganism that can cause nosocomial infections, including sepsis and digestive infections in immunocompromised newborns. Moreover *B. cereus* produces toxins, that may intoxicate infants even in the absence of vegetative forms and are heat resistant (109). While unprocessed HM has been pointed out as a potential source of *B. cereus* (110), no causal link between ingestion of banked milk and *B. cereus* infection in preterm babies has been confirmed according to Lewin et al. (111). Contamination could rather be attributed to immediate environment contamination, such as ventilation or invasive care equipment. Nonetheless, several publications show great interest in the presence of *B. cereus* in HM, whether it is in its vegetative or sporulating form.

Development of any sterilization/pasteurization process should not allow the growth of any pathogenic bacteria in treated milk and ensure the destruction of at least 10^6 CFU/mL of reference bacteria and viruses (70). This should be considered when evaluating a new technology and developing an HM treatment protocol. Moreover, the establishment of an effective HM processing method in an HMB should ensure that maximum components and biological activity are retained in processed milk compared to raw milk. Consensus about the best techniques for analyzing these components will allow for consistency in results, considering that different factors may influence the results: i.e., the detection method (ELISA kit, HPLC, mass spectrometry) and the value assessed (concentration, activity). The consequences of partial elimination of pathogens and/or a loss of essential nutrients and bioactive components in milk for vulnerable premature babies could be devastating. These newborns need to grow rapidly and to develop their digestive and immune systems effectively, among other things, to ensure their survival. As previously mentioned, infection can cause fatal sepsis and severe injury, while a lack of essential components can compromise proper development. Moreover, it is also important to keep in mind the programming effect of a deleterious perinatal nutrition on later life metabolic and intestinal heath. For these reasons, it is relevant to assess the health benefits of donor's milk after treatment, which are currently being evaluated *in vitro* and through animal studies, but not actually analyzed in a human population. Milk banks must be aware of these issues when choosing a method of human milk processing.

Author contributions

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Conflict of interest

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

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The association between maternal factors and milk hormone concentrations: a systematic review

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Background: Breast milk is the gold standard for infant feeding. It is a dynamic biological fluid rich in numerous bioactive components. Emerging research suggests that these components, including hormones, may serve as signals between mother and offspring. From an evolutionary perspective, maternal hormonal signals could allow co-adaptation of maternal and offspring phenotype, with implications for their Darwinian fitness. However, a series of steps need to be considered to establish the role of a component as a signal and this systematic review focuses on one step: 'Do maternal factors influence the concentration of milk hormones?'

Objective: To systematically review human studies which analyze the association between maternal factors and the concentration of hormones in breast milk.

Methods: Three databases were searched for studies reporting the association of maternal factors including body mass index (BMI), weight, fat mass, age, ethnicity, smoking with hormones such as adiponectin, leptin, insulin, ghrelin, and cortisol in breast milk.

Results: Thirty-three studies were eligible for inclusion. Maternal BMI was positively associated with milk leptin (20/21 studies) and with milk insulin (4/6 studies). Maternal weight also displayed a positive correlation with milk leptin levels, and maternal diabetes status was positively associated with milk insulin concentrations. Conversely, evidence for associations between maternal fat mass, smoking, ethnicity and other maternal factors and hormone levels in breast milk was inconclusive or lacking.

Conclusion: Current evidence is consistent with a signaling role for leptin and insulin in breast milk, however other steps need to be investigated to understand the role of these components as definitive signals. This review represents a first step in establishing the role of signaling components in human milk and highlights other issues that need to be considered going forward.

KEYWORDS

breast milk, maternal factors, breast milk hormones, parent-offspring signaling, breast milk composition

Introduction

The World Health Organisation (WHO) recognizes breast milk as the gold standard for infant feeding. It offers evident short-term advantages for infants, including a decrease in the incidence of mortality and morbidity related to infectious diseases (1). However, breast-feeding is also a mode of nutrition that exhibits substantial variability among mothers, for example in the volume and composition of the milk produced (2).

In this context, breast milk can be considered a medium through which the mother can communicate through different biological pathways with her offspring, potentially regulating the offspring's growth and development. From an evolutionary perspective, a mother could optimize her Darwinian fitness if her investment in lactation can adapt in response to ongoing environmental factors, whether these relate directly to the mother (e.g., her energy reserves, which support lactation), or to external factors such as the supply of food (e.g., famine), or to psychosocial stress, which could divert maternal metabolic resources to the stress response, and thus reduce nutritional supply to the offspring. Beyond transferring macronutrients and micronutrients to the offspring, other biological molecules could influence how the offspring utilizes its nutritional supply, and hence its developmental trajectory. However, the volume and composition (including hormone content) of breast milk that maximizes maternal Darwinian fitness is not the same that maximizes the offspring's fitness (3). The offspring may also influence maternal biology, for example through the strength of the suckling response.

Therefore, breastfeeding can be viewed as a dynamic process which involves complex physiological and psychosocial signaling or communication between the mother and the offspring (4).

Human milk contains numerous components that could act as signals between the mother and offspring including hormones, bacteria, nutrients, and growth factors. These components may interact with the infant's cells, tissues, and organs, triggering various signaling pathways and physiological responses. Among the underlying mechanisms could be epigenetic modifications, including DNA methylation, histone modification, and microRNA effects, and impacts on the establishment of the infant microbiome (5). There are various steps that apply to any component being considered as a signal in milk such as the origin of milk components, whether they come from the mother's circulation or are synthesized in the breast (or both), if the milk components reach the infant intestine or if they have specific gut receptors. Additional steps include if the milk components are absorbed and influence infant outcomes and whether maternal or environmental factors influence the concentration of milk components. This systematic review focuses on whether maternal and environmental factors influence the concentration of one group of milk components – milk hormones.

Previously, Andreas et al. (6) conducted a systematic review which indicated that there was an association between the concentration of leptin in breast milk and maternal BMI in ten out of fifteen studies (6). Furthermore, a narrative review undertaken in 2016 highlighted reported evidence to support the role of specific bioactive components and explained that several maternal factors such as BMI have been proposed to influence levels of these bioactive components in breast milk (7). In this review, leptin provided the clearest indication that maternal BMI was positively associated with leptin concentrations in breast milk.

The purpose of this review was to systematically search the literature for evidence on maternal and environmental factors that influence the concentration of hormones in human milk.

Methods

Eligibility criteria

Types of studies

Observational studies and randomized controlled trials (RCTs) that reported on the association between maternal factors and breast milk hormones were eligible for inclusion. Data from RCTs were included only if they reported associations between an exposure (maternal factors) and subsequent measures of milk hormones. Full text studies published in English were included. Studies reported only in abstract form were omitted.

Types of participants

Eligible participants included human mother-infant pairs. Studies in which most (>50%) of the infants were exclusively or predominantly breast-fed at the time of sampling were eligible in order to isolate the potential association of maternal factors on breast milk composition. There were no restrictions on participant health status; this was due to the research interest being in how mothers signal their condition and experiences to their offspring, including markers of living conditions, lifestyle, ill health, and good health. Studies in animals were excluded.

Maternal factors (exposure)

The exposure was any maternal factor related to the mother's condition, health, lifestyle, living condition or environment at any time period. It was expected that this would include factors such as maternal anthropometry, adiposity, gestational diabetes, age, ethnicity, socioeconomic status, stress, smoking or climate. Any time period was chosen because of the potential of the factors influencing outcomes via various mechanisms such as epigenetic programming and regulation of gene expression which can potentially affect human milk composition.

Breast milk hormones (outcomes)

Studies were considered eligible if they reported on at least one breast milk hormone. It was expected that this would include cortisol, leptin, insulin, ghrelin, adiponectin, prolactin, oxytocin or resistin. Studies analyzing these hormones in colostrum, transitional or mature milk at any time-point were considered eligible.

Information sources

Studies were identified by searching electronic databases with no limits on date of publication. The electronic databases searched were MEDLINE Ovid (from 1946), EMBASE Ovid (from 1974) and Cumulative Index of Nursing and Allied Health Literature (CINAHL).

Search strategy

The search strategy included database-specific search terms and medical subject headings (MeSH) terms with Boolean operators (NOT, AND, OR) were used on synonyms and variations of the terms relating to human milk, hormones, and maternal factors. The search terms were: "breast milk" OR "breastmilk" OR "human milk" OR "breastfeeding" OR "lactation" OR "breastfed" OR "breastfeed" OR "breast fed" OR "breast feed" OR "milk, human" OR "breast feeding" AND "hormones" OR "hormone concentrations" OR "hormonal concentrations" OR "hormone profile" OR "hormones profile" OR "hormone" OR "leptin" OR "insulin" OR "ghrelin" OR "cortisol" OR "prolactin" OR "oxytocin" OR "resistin" OR "adiponectin" OR "thyroid" OR "interleukin-6" OR "tum?r necrosis factor-a" OR "hydrocortisone" AND "parental factors" OR "maternal factors" OR "environmental factors" OR "stress" OR "inflammation" OR "BMI" OR "body mass index" OR "maternal BMI" OR "maternal body mass index" OR "obesity" OR "type 2 diabetes" OR "type 2 diabetes mellitus" OR "T2D" OR "diabetes" OR "diabetes mellitus" OR "university education" OR "weight" OR "height" OR "maternal weight" OR "maternal height." Searches were limited to human studies. The search was run on 19th June 2023. An example search of MEDLINE can be found in Appendix A.

Selection strategy

Duplications were removed and references were imported to Covidence for screening according to the eligibility criteria. Titles were screened by RQ, then abstracts and full-texts were screened by two authors independently (RQ and SD). Any discrepancies between the reviewers were discussed to reach consensus.

Data extraction

Data to be extracted was agreed by the research team. One reviewer (RQ) independently extracted the data from each study. Extracted data included author name, date of publication, sample size, location of study, study design, feeding type and duration, human milk hormones assessed, stage and type of milk analyzed, how the milk sample was processed for analysis, how the samples were obtained, the maternal factors assessed, results of associations, correlation coefficients and confounders adjusted.

Quality assessment

One independent reviewer (RQ) used the revised Downs and Black Quality Index score system (Appendix B), known to be a reliable and valid tool for assessing bias in observational and randomized studies to assess the quality of individual publications (8). The quality assessment tool provided an overall score based on four assessed domains; reporting, external validity, internal validity bias and internal validity confounding. Each domain had an overall total score out of 10, 3, 7 and 6, respectively. Item 27 relating to the statistical power was given a score of 1when a power analysis had been conducted. Thus, the highest possible score for the checklist was 28 (9). The quality assessment can be found in Appendix C.

Data synthesis

It was not possible to conduct a meta-analysis on the relationship between maternal factors and human milk composition due to highly heterogenous data for both maternal factors and the hormonal composition of human milk. As a result, the data was synthesized narratively and presented in tables.

Results

The database searches identified 6,493 studies, of which 4,994 titles were screened following the removal of duplicates. Four hundred ninety abstracts were screened for appropriateness; 398 studies were excluded based on inappropriate title. The full text of 92 studies were reviewed; 59 studies were excluded after the review. This left 33 studies suitable for inclusion in the systematic review. A Preferred Reporting for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram is presented in Figure 1.

Study characteristics are summarized in Table 1. All included studies were conducted in high-income countries with the exception of two conducted in Turkey (11, 35) and one in Iran (20), both classified as upper-middle-income economies. The majority of studies were published in the last 8 years ($n = 29$), except for 5 studies published in 2002, 2006, 2007, 2011 and 2014 (10, 25, 34–36). The main type of study design was observational ($n = 29$), with 4 studies consisting of data from randomized controlled trials (10, 13, 18, 24). Most of the studies had a sample size of 100 or less ($n = 26$) with the largest study including 767 participants (36).

Human milk components analyzed included concentrations of adiponectin ($n = 15$), leptin ($n = 26$), insulin ($n = 12$), cortisol ($n = 4$) and ghrelin ($n = 2$). Maternal factors assessed included BMI ($n = 23$), fat mass ($n = 7$), weight ($n = 6$), maternal age ($n = 3$), gestational diabetes ($n = 4$), smoking ($n = 2$) and 'other' including hip, waist and mid-upper arm circumference, ethnicity, carbohydrate intake, energy intake, psychological stress, and mode of delivery ($n = 7$). Data for all eligible studies including the methods and analysis of sample collection are summarized in Table 1. Mature milk was the main type of milk sample analyzed ($n = 33$), with three studies also analyzing colostrum and one study analyzing transitional milk.

Synthesis of results

Twenty-three studies examined the relationship between maternal body mass index (BMI) and human milk composition in exclusively or predominantly breastfed infants. The studies investigated associations between maternal BMI pre-, during- and post-pregnancy and adiponectin, leptin, insulin, cortisol, and ghrelin concentrations in milk as shown in Table 2. Among the ten studies focusing on adiponectin, two demonstrated a positive association at 2 weeks and 1–3 months (38, 39), while one reported a negative association stronger at 1 month than 3 months (39). In 20 studies, leptin concentrations, analyzed in twenty-one studies, generally showed a

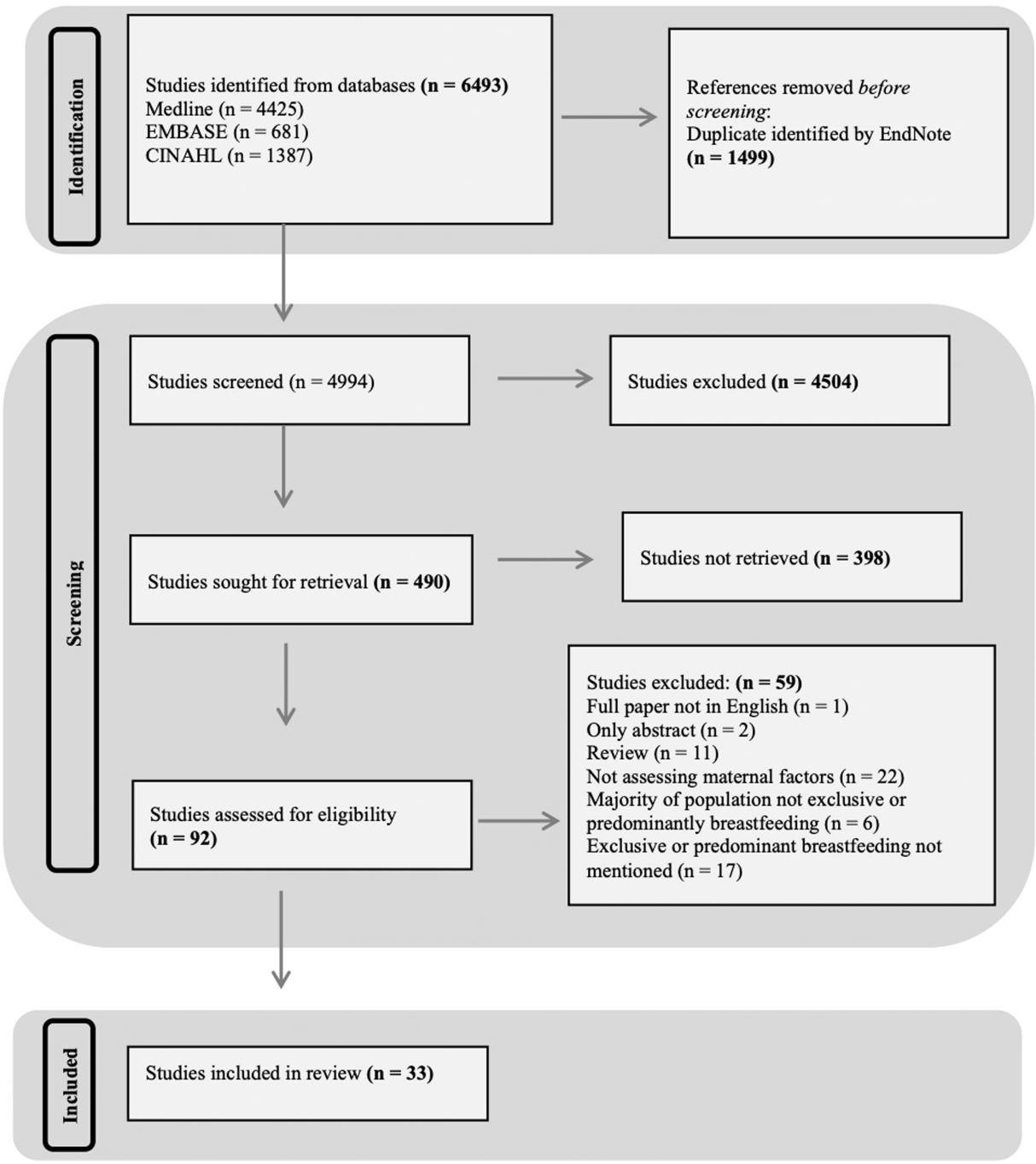

FIGURE 1
Flow diagram summarizing the process of study screening.

positive correlation with maternal BMI post-pregnancy with higher levels in overweight and obese mothers. For insulin, six studies indicated a positive correlation with maternal BMI post-pregnancy, particularly strong at 3 months, with higher levels in overweight and obese mothers. Cortisol, analyzed in two studies, exhibited a positive correlation with maternal BMI. Ghrelin concentrations, studied in two investigations, displayed an inverse association in one study, while the other found no significant correlation with maternal BMI post-pregnancy. Only three studies among the twenty-three adjusted for potential confounding factors (12, 14, 32).

Seven studies examined the relationship between maternal fat mass and human milk composition, focusing on adiponectin ($n = 5$), leptin ($n = 6$), insulin ($n = 2$), cortisol ($n = 1$), and ghrelin ($n = 1$) as summarized in Table 3. Notably, none of the studies adjusted for potential confounding factors. Regarding adiponectin, only one study reported a significant negative association with fat mass at 6 months (20), while the 4 others found no significant correlations. In the case of leptin, all six studies identified significant associations, with three indicating a positive link between maternal fat mass and leptin concentrations (20, 21, 33). For insulin, one study reported a positive

TABLE 1 Summary of the characteristics of included studies.

Paper	Size	Location	Design	HM hormone	Milk type	Analysis of milk	Collection time	Collection method	Maternal factors assessed
Brunner et al. (10)	208	UK	RCT	Adiponectin, Leptin	Mature	Whole milk	After overnight fast	Full breast expression (EP)	BMI, Fat Mass
Cagiran-Yilmaz et al. (11)	65	Turkey	Observational	Leptin	Mature	NS	2 h after feed	Single breast expression (EP)	Weight, BMI, Hip, Waist & Mid-Upper arm circumference
Chan et al. (12)	430	Canada	Observational	Adiponectin, Leptin, Insulin	Mature (foremilk & hindmilk)	Skim Milk	Multiple feeds during 24 h	HE, if unable to do so EP	BMI, Ethnicity, Age, Smoking
Choi et al. (13)	189	USA	RCT	Adiponectin, Leptin, Insulin	Mature	Skim Milk	10 am-12 pm, 2 h post-feed	Single breast expression (EP) post-feed	GDM
Christensen et al. (14)	223	Denmark	Observational	Adiponectin, Leptin, Insulin	Colostrum, Mature	Whole milk	Colostrum – 24-72 h after birth, Human milk – 9 am or 2 pm	Colostrum – HE, Human Milk – Full breast expression (EP)	BMI
Cortes-Macias et al. (15)	100	Spain	Observational	Adiponectin, Leptin	Mature	NS	NS	NS	BMI
De-Luca et al. (16)	100	France	Observational	Leptin	Mature	NS	9 am-11 am during feed	EP	BMI
Ellsworth et al. (17)	55	USA	Observational	Insulin	Mature	Whole milk	8 am-10 am 2 h post-feed	Single breast expression (HE/EP) post-feed	Weight
Enstad et al. (18)	40	USA	RCT	Leptin	Mature	Whole milk	NS	NS	BMI
Fields et al. (7)	37	USA	Observational	Leptin, Insulin	Mature	NS	NS	Single breast expression (EP)	BMI
Gridneva et al. (19)	20	Australia	Obersvational	Adiponectin, Leptin	Mature	Whole milk	NS	NS	Maternal Body Composition
Khodabakshi et al. (20)	80	Iran	Observational	Adiponectin, Leptin, Ghrelin	Mature	NS	8 am-10 am after an overnight fast 2 h post-feed	Full breast expression (EP) post-feed	% Maternal Fat, BMI
Kunganathan et al. (21)	59	Australia	Observational	Adiponectin, Leptin	Mature	Whole & Skim milk	9.30–11.30 am	HE/EP	BMI, % fat mass
Larson-Meyer et al. (22)	22	USA	Observational	Leptin	Mature (foremilk, hindmilk)	NS	7 am-10 am after an overnight fast	Own pump/ Mechanical pump	Body mass, fat mass, BMI
Larsson et al. (23)	30	Denmark	Observational	Leptin	Mature (foremilk, hindmilk)	NS	NS	Manual breast pump	BMI
Leghi et al. (24)	15	Australia	RCT	Adiponectin, Leptin, Insulin	Mature	Whole milk	NS	Full breast expression (HE/EP) pre- and post-feed	Body weight, fat mass, carbohydrate and energy intake
Miralles et al. (25)	28	Spain	Observational	Leptin	Mature	NS	9 am-10 am after a 12 h fast	HE	BMI

(Continued)

TABLE 1 (Continued)

Paper	Size	Location	Design	HM hormone	Milk type	Analysis of milk	Collection time	Collection method	Maternal factors assessed
Nuss et al. (26)	33	USA	Observational	Leptin	Mature	NS	In the morning after a 2 h fast	Single breast expression (EP)	BMI
Pundir et al. (27)	18	Australia	Observational	Cortisol	Mature	NS	9.30 am-10.30 am	Pre- and post-feed	Height, BMI, % fat mass
Pundir et al. (28)	650	Finland	Observational	Cortisol	Mature	NS	Morning	Single breast expression (HE)	Weight, age, GDM, mode of delivery
Rodel et al. (29)	36	USA	Observational	Insulin	Mature	NS	Fasting and mid-feed	HE/EP	Type 2 Diabetes, GDM, Normal glucose tolerance
Romijn et al. (30)	63	Netherlands	Observational	Cortisol	Mature	NS	NS	HE/EP pre-feed	Psychological stress
SadrDadres et al. (31)	135	USA	Observational	Adiponectin, Leptin, Insulin	Mature	Skim milk	8 am-10 am	Single breast expression (EP) post-feed	BMI, gestational weight gain, postpartum weight loss
Savino et al. (32)	58	Italy	Observational	Leptin	Mature (foremilk)	Skim milk	7 am-9 am	HE	BMI
Schneider-Worthington et al. (33)	25	USA	Observational	Adiponectin, Leptin, Insulin	Mature	Skim milk	8 am-10 am following an overnight fast	Personal breast pump	Fat mass
Schuster et al. (34)	23	Germany	Observational	Leptin	Colostrum, Transitional, Mature	Skim milk	NS	NS	BMI
Uysal et al. (35)	50	Turkey	Observational	Leptin	Mature	NS	8 am-11 am	EP post-feed	BMI
Weyermann et al. (36)	767	Germany	Observational	Adiponectin, Leptin	Mature	Whole milk	NS	Full breast expression (HE/EP) pre-feed	Smoking
Young et al. (37)	41	USA	Observational	Insulin	Mature	Whole and skim milk	Fasted mid-feed	HE	Weight
Young et al. (38)	48	USA	Observational	Adiponectin, Leptin, Insulin	Mature	Skim milk	Mid feed	HE	BMI
Yu et al. (39)	96	China	Observational	Adiponectin, Leptin, Insulin, Ghrelin	Colostrum, Mature (foremilk, hindmilk)	NS	Colostrum – 8 am pre-feed, Mature – 2 pm & 4 pm pre-feed	Single breast expression (EP)	GDM, BMI
Zamanillo et al. (40)	59	Spain	Observational	Adiponectin, Leptin	Mature	NS	9 am-2 pm	Full breast expression, (HE, manual breast pump)	BMI
Zielinkska-Pukos et al. (41)	38	Poland	Observational	Cortisol	Mature	NS	4 time periods: 6 am-12 pm, 12 pm-6 pm, 6 pm-12 am, 12 am-6 am	Pre- and post-feeding (HE/EP)	Age, BMI, postpartum depression score, percieved stress scale

HM, human milk; RCT, randomized controlled trial; EP, electronic pump; HE, hand expressed; BMI, body mass index; GDM, gestational diabetes mellitus; NS, not stated.

association, while another found no correlation (17). The sole study on cortisol showed no association with maternal fat mass, and the only study on ghrelin also found no correlation.

Six studies explored the relationship between maternal weight and human milk composition in predominantly breastfed infants, focusing on adiponectin ($n = 2$), leptin ($n = 3$), insulin ($n = 4$), and cortisol ($n = 1$), as detailed in Table 4. Only three studies accounted for potential confounding factors (17, 28, 33). Concerning adiponectin, the two available studies found no correlation with maternal weight. Regarding leptin, all three studies identified significant associations. For insulin, two out of four studies reported a significant association between maternal weight and insulin levels, with one study noting elevated insulin levels in overweight mothers (17). The lone study on cortisol found that normal-weight mothers had higher levels of milk cortisol than mothers with overweight/obesity.

Three studies investigated the relationship between maternal age and human milk composition, specifically examining adiponectin ($n = 1$), leptin ($n = 2$), insulin ($n = 1$), and cortisol ($n = 2$), as shown in Table 5. Only two studies accounted for potential confounding factors (12, 28). Regarding adiponectin, a single study found no correlation between maternal age and adiponectin concentrations in human milk (12). In the case of leptin, one study revealed that the concentration of leptin was lower in older women. For insulin, a lone study found no correlation between maternal age and insulin levels in human milk. Two studies explored the association between maternal age and cortisol, with both studies observing no correlation (28, 41).

Four studies investigated the link between pre-gestational and gestational maternal diabetes and human milk composition, examining adiponectin ($n = 2$), leptin ($n = 2$), insulin ($n = 3$), cortisol ($n = 1$), and ghrelin ($n = 1$), as detailed in Table 6. Notably, only two studies adjusted for potential confounding factors (13, 28). Regarding adiponectin, one study found a significant negative association with gestational diabetes mellitus in both colostrum and mature milk, while another study found no association (13, 39). For leptin, both studies observed no correlation with maternal diabetes (13, 39). All three studies exploring insulin concentrations reported significant associations with maternal diabetes, with one study revealing lower insulin levels in mothers with gestational diabetes (13) and another indicating that women with type 2 diabetes mellitus had twice the milk insulin levels compared to those with gestational diabetes and normal glucose tolerance (29). The sole study on cortisol found no correlation between maternal diabetes and cortisol in human milk (28) while the single study on ghrelin reported a significant negative correlation with gestational diabetes in both colostrum and mature milk (39).

Two studies investigated the relationship between maternal smoking and human milk composition, focusing on adiponectin ($n = 2$), leptin ($n = 2$), and insulin ($n = 1$), as summarized in Table 7. Both studies adjusted for potential confounding factors. However, no significant associations were observed between maternal smoking and adiponectin, leptin, or insulin. Notably, one of the studies found that levels of adiponectin were higher in the milk of non-smoking mothers, suggesting a potential impact of smoking on this specific milk component (36).

Table 8 presents a summary of findings on the associations between the hormonal composition of human milk and other maternal factors. Seven studies explored the relationships between these factors and adiponectin ($n = 3$), leptin ($n = 4$), insulin ($n = 2$), and cortisol ($n = 3$), with two studies adjusting for potential confounding factors. Regarding adiponectin, a study found lower levels in Asian mothers compared to Caucasian mothers (12), while another reported a positive association with changes in carbohydrate and total energy intake (24). However, a study observed no significant association with maternal body composition (19). For leptin, previous research identified positive correlations with hip, waist, and mid-upper arm circumferences at different postpartum time points (11). No associations were observed with ethnicity, maternal body composition, and carbohydrate and energy intake in other studies. In terms of insulin, Chan et al. (12) reported higher levels in Asian mothers compared to Caucasians, while another study found no association with maternal carbohydrate and energy intake (12). For cortisol, Romijn et al. (30) noted lower levels in mothers seeking psychiatric consultation compared to healthy controls, and another study found no association with postpartum depression score or perceived stress (41). Pundir et al. (41) observed no association between mode of delivery and cortisol levels at 3 months.

Discussion

This systematic review explored the relationship between different maternal factors and hormones in breast milk, as a first step to establishing their role in signaling mechanisms between mother and infant. The review of 33 papers suggests a positive association between maternal adiposity (BMI and weight either pre-pregnancy and during lactation) and breast milk leptin concentrations (Figure 2). However, the evidence regarding maternal fat mass, age, smoking, and other factors was inconclusive. The review underscores the need for more research in this area, emphasizing the inconsistency in findings, likely due to variations in data collection and sampling methods across studies.

Maternal adiposity was assessed using a variety of measures in different studies. There was a positive association between maternal BMI and breast milk leptin and insulin in the majority of studies included with only 2/10 studies showing a positive association between maternal BMI and adiponectin. This aligns with a previous systematic review (6) by Andreas et al. (6) which also found an association of maternal BMI with breast milk leptin but not adiponectin (6). A 2016 narrative review further highlighted the positive association between maternal BMI and leptin and insulin levels in human breast milk (7). We also found that maternal weight was positively associated with milk leptin in all included studies, but its relationship with milk insulin was less clear. Inconsistent associations were also observed between maternal fat mass and milk leptin. It is possible that the associations of milk hormones with BMI were stronger than with fat mass due to the ease with which BMI could be assessed and thus the larger sample sizes in studies using this outcome.

Leptin in breast milk could serve as a signaling mechanism for the infant, affecting aspects such as metabolism, appetite, and fat storage. The leptin gene (LEP), which is responsible for the production of leptin, is expressed in mammary epithelial cells and can be influenced by many factors including maternal diet, nutritional status and hormone regulation (42). Since leptin signals the level of fat reserves to the brain, maternal leptin transfer to the infant could act to inflate

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TABLE 2 Summary of the association between human milk composition and maternal BMI.

Paper	Feeding type	HM measured time-point	HM component	BMI measure time-point	Reported associations and correlations	Confounders adjusted
Brunner et al. (10)	85% EBF and 15% PBF to 6 weeks; 83% EBF and 17% PBF to 4 months	6 weeks and 4 months	Adiponectin (ng mL^{-1})	6 weeks and 4 months	No significant correlations found with BMI	None
			Leptin (ng/mL)	NS	Strong correlation with pre-pregnancy BMI **($p < 0.001$, $r = 0.55$)**	
Cagiran-Yilmaz et al. (11)	EBF for 3+ months	1, 3 and 6 months	Leptin (ng/mL)	1, 3 and 6 months	Positive correlation with BMI **($r < 0.73$ across all months and $p < 0.05$)**	None
Chan et al. (12)	EBF for 3–4 months	3 months	Adiponectin (ng mL^{-1})	Pre-pregnancy	No linear correlation observed with pre-pregnancy BMI ($p = 0.68$), however concentrations lower from obese mothers **($p = 0.02$)**	Maternal BMI, pre-pregnancy weight, gestational weight gain, mode of infant feeding (exclusively or partially breastfed without formula at 3 months postpartum)
			Leptin (ng mL^{-1})		Strong correlation with pre-pregnancy BMI **($p < 0.001$, $r = 0.71$)**	
			Insulin (ng mL^{-1})		Strong correlation with pre-pregnancy BMI **($p < 0.00$, $r = 0.4$)**	
Christensen et al. (14)	EBF for 3+ months	Colostrum – 24-72 h after birth, Mature milk – NS	Adiponectin (ng mL^{-1})	NS	No significant associations with maternal BMI	Infant age and mean-center age
			Leptin (ng/mL)		Positive correlation with BMI **($p < 0.01$)**	
			Insulin (ng mL^{-1})		Positive correlation with BMI **($p < 0.01$)**	
Cortes-Macias et al. (15)	EBF for 3+ months	15 days post birth	Adiponectin (ng mL^{-1})	NS	No correlation observed ($p = 0.28$)	None
			Leptin (ng/mL)		Positive correlation with BMI **($p < 0.01$, $r = 0.388$)**	
De-Luca et al. (16)	EBF for 1 month	1 month	Leptin (ng mL^{-1})	1 month	Positive correlation with BMI **($p < 0.01$, $r = 0.33$)**, concentration higher in obese mothers	None
Enstad et al. (18)	EBF but duration not stated	1 and 4 months	Leptin (ng/mL)	1 and 4 months	Positive correlation with BMI at both time-points	None
Fields et al. (7)	EBF for 3+ months	1 and 6 months	Leptin (ng/mL)	1 and 6 months	Significant association with maternal BMI **($p < 0.0001$)**, levels 96.5% higher in overweight mothers vs. normal-weight	None
			Insulin (ng mL^{-1})		225% higher in obese mothers with female infants	

(Continued)

TABLE 2 (Continued)

Paper	Feeding type	HM measured time-point	HM component	BMI measure time-point	Reported associations and correlations	Confounders adjusted
Khodabakshi et al. (20)	EBF for 3+ months	6 months	Adiponectin (ng/mL)	6 months	No significant associations with maternal BMI	None
			Leptin (ng/mL)		Significant association with maternal BMI **($p < 0.001$, $r = 0.39$)**	
			Ghrelin (ng/mL)		No significant associations with maternal BMI	
Kunganathan et al. (21)	EBF for 2–5 months	One of 4 timepoints: 2, 5, 9 and 12 months	Adiponectin (ng/mL)	One of 4 timepoints: 2, 5, 9 and 12 months	No significant associations with maternal BMI ($p = 0.17$)	None
			Leptin (ng/mL)		Significant association with maternal BMI **($p < 0.001$)**	
Larson-Meyer et al. (22)	EBF for 3+ months	1, 6 and 12 months	Leptin (ng/mL)	1 and 6 months	Significant association with maternal BMI **($p < 0.001$, $r = 0.79$)**	None
Larsson et al. (23)	EBF for 3+ months	6 weeks	Leptin (ng/mL)	5 and 9 months	Positive correlation with BMI **($p < 0.01$)** at 5 months and 9 months **($p = 0.0057$)**	None
Miralles et al. (25)	EBF for 3+ months	1, 3, 6 and 9 months	Leptin (ng/mL)	1, 3, 6 and 9 months	Positive correlation with BMI **($p < 0.01$, $r = 0.33$)** at each time-point	None
Nuss et al. (26)	EBF for 2 months	NS	Leptin (ng/mL)	NS	Positive correlation with BMI **($p < 0.01$)**, levels higher in normal BMI	None
Pundir et al. (27)	EBF for 2 months	2 and/or 5, 9 and 12 months	Cortisol (ng/mL)	2 and/or 5, 9 and 12 months	Significant, positive correlation with maternal BMI **($p = 0.009$, $r = 0.33$)**	None
SadrDadres et al. (31)	EBF for 3+ months	1 and 3 months	Adiponectin (ng mL^{-1})	1 and 3 months	Negative association with BMI **($p = 0.02$, $\beta = -0.07$)**, weaker at 3 months	None
			Leptin (ng/mL)		Positive association with BMI **($p < 0.001$, $\beta = 0.49$)**	
			Insulin (ng mL^{-1})		Positive association with BMI **($p = 0.03$, $\beta = 0.14$)**, stronger at 3 months	
Savino et al. (32)	EBF for 3+ months	3 months	Leptin (ng/mL)	3 months	Positive correlation with BMI **($p = 0.004$, $r = 0.37$)**	Infant age and gender
Schuster et al. (34)	EBF for 3+ months	1, 2, 3, 4 weeks and 2, 3, 4, 5, and 6 months	Leptin (ng/mL)	1, 2, 3, 4 weeks and 2, 3, 4, 5, and 6 months	Positive correlation with BMI **($p < 0.0001$, $r = 0.3$)** over 6 months	None

(Continued)

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TABLE 2 (Continued)

Paper	Feeding type	HM measured time-point	HM component	BMI measure time-point	Reported associations and correlations	Confounders adjusted
Uysal et al. (35)	EBF for 3+ months	NS	Leptin (ng/mL)	NS	Significant correlation with maternal BMI	None
Young et al. (38)	EBF for 3+ months	2 weeks and 1, 2, 3 and 4 months	Adiponectin (ng/mL)	2 weeks and 1, 2, 3 and 4 months	Positive association at 2 weeks **($p = 0.04$, $r = 0.09$)**	None
			Leptin (ng/mL)		Increased levels in overweight mothers **($p < 0.001$)**	
			Insulin (ng/mL)		Increased levels in overweight mothers **($p < 0.03$)**	
Yu et al. (39)	EBF for 3+ months	Colostrum – 72 h after birth, Mature – 1 (42 days) and 3 months (90 days)	Adiponectin (ng/mL)	Postnatal days 3, 42 and 90	Positive association with BMI **($p = 0.001$, $\beta = 0.06$)**	None
			Leptin (ng/mL)		Positive association with BMI **($p < 0.001$, $\beta = 0.16$)**	
			Insulin (ng/mL)		Positive association with BMI **($p < 0.001$, $\beta = 0.06$)**	
			Ghrelin (ng/mL)		Inversely associated with BMI **($p < 0.001$, $\beta = -0.08$)**	
Zamanillo et al. (40)	86% EBF to 1 month, 85% EBF to 2 months and 78% EBF to 3 months	1, 2 and 3 months	Adiponectin (ng/mL)	1, 2 and 3 months	No associations observed, however, normal weight mothers showed a decrease over time **($p < 0.05$)**	None
			Leptin (ng/mL)		Negative correlation in normal weight mothers **($p < 0.05$)**	
Zielinkska-Pukos et al. (41)	EBF for 3+ months	1, 3 and 6 months	Cortisol (ng/mL)	1, 3 and 6 months	No significant associations with maternal BMI	None

Where confounders were adjusted, data reported is from adjusted analysis. Significant results are depicted with the *p* value in bold. HM, human milk; EBF, exclusive breastfeeding; PBF, predominantly breastfeeding; BMI, Body Mass Index; NS, Not Stated.

such signals, effectively manipulating the infant's brain into over-estimating its fat stores and thus impact its appetite. While studies have indicated that higher leptin in breast milk might indeed reduce infant appetite, this could have both positive and negative implications (32). Positive outcomes include better self-regulation, more appropriate feeding patterns and reduced long term obesity risk, while negatives could be inadequate nutrition and suboptimal growth. Similarly, higher insulin levels in milk were previously found to be associated with lower infant weight and weight-for-length z-scores (14). However, other studies found no link between milk insulin and infant anthropometrics.

This review confirmed that maternal diabetes is associated with significantly higher insulin levels in breast milk. Maternal insulin regulation can be disrupted in type 2 diabetes, leading to increased insulin levels in maternal circulation and potentially influencing its presence in breast milk. A study showed that women with type 2 diabetes had significantly higher insulin levels in their breast milk compared to those with gestational diabetes and normal glucose tolerance, possibly due to insulin therapy and injections (28). Limited research exists on the impact of breast milk constituents on the growth of infants born to mothers with diabetes during pregnancy, especially gestational diabetes. Previous data suggests that breast milk from diabetic mothers may lead to increased relative body weight and obesity at two years of age, while milk from healthy non-diabetic women had a beneficial effect on later body weight and glucose tolerance in childhood (43).

Aging is associated with many changes in the levels of several hormones in maternal plasma which could have an effect on breast

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TABLE 3 Summary of the association between human milk composition and maternal fat mass.

Paper	Feeding type	HM measured time-point	HM component	Maternal fat mass measure time-point	Reported associations and correlations	Confounders adjusted
Brunner et al. (10)	85% EBF and 15% PBF to 6 weeks; 83% EBF and 17% PBF to 4 months	6 weeks and 4 months	Adiponectin (ng mL^{-1})	6 weeks and 4 months	No significant correlations found with fat mass	None
			Leptin (ng/mL)	15th and 32nd week gestation, 6 weeks and 4 months post-partum	Strong correlation with fat mass **($p < 0.001$)**	
Khodabakshi et al. (20)	EBF for 3+ months	6 months	Adiponectin (ng/mL)	6 months	Significant, negative association with fat mass **($p < 0.05$, $r = -0.24$)**	None
			Leptin (ng/mL)		Significant, positive association with fat mass **($p < 0.01$, $r = 0.39$)**	
			Ghrelin (ng/mL)		No significant associations observed with fat mass	
Kunganathan et al. (21)	EBF for 2–5 months	One of 4 timepoints: 2, 5, 9 and 12 months	Adiponectin (ng/mL)	One of 4 timepoints: 2, 5, 9 and 12 months	No significant associations with maternal BMI ($p = 0.81$)	None
			Leptin (ng/mL)		Significant, positive association with fat mass **($p = 0.008$)**	
Larson-Meyer et al. (22)	EBF for 3+ months	1, 6 and 12 months	Leptin (ng/mL)	1 and 6 months	Significant association with fat mass **($p < 0.001$, $r = 0.83$)**	None
Leghi et al. (24)	EBF for 6 to 20 weeks	NS	Adiponectin (ng mL^{-1})	NS	No associations observed with fat mass	None
			Leptin (ng/mL)		Significant association with fat mass **($p < 0.001$)**	
			Insulin (ng/mL)		No associations observed with fat mass	
Pundir et al. (27)	EBF for 2 months	2 and/or 5, 9 and 12 months	Cortisol (ng/mL)	2 and/or 5, 9 and 12 months	No correlation with fat mass ($p = 0.09$)	None
Scheider-Worthington et al. (33)	EBF for 3+ months	NS	Adiponectin (ng mL^{-1})	NS	No significant associations observed with fat mass ($p = 0.65$)	None
			Leptin (ng/mL)		Significant, positive association with fat mass **($p < 0.001$)**	
			Insulin (ng/mL)		Significant, positive association with fat mass **($p = 0.05$)**	

Where confounders were adjusted, data is reported from adjusted analysis. Significant results are depicted with the p value in bold. HM, human milk; EBF, exclusively breastfeeding; PBF, predominantly breastfeeding; BMI, body mass index; NS, not stated.

milk hormone levels. For example, Isidori et al. (44) indicated that serum leptin gradually declines during aging but is independent from BMI and other age-related endocrine changes. However, only one study included in this review found that the concentration of leptin was lower in older women (12) with the remaining studies reporting no association between milk hormones and maternal age.

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TABLE 4 Summary of the association between human milk composition and maternal weight.

Paper	Feeding type	HM measured time-point	HM component	Maternal weight measure time-point	Reported associations and correlations	Confounders adjusted
Cagiran-Yilmaz et al. (11)	EBF for 3+ months	1, 3 and 6 months	Leptin (ng/mL)	1, 3 and 6 months	Significant, positive correlation with weight **($p < 0.01$)**	None
Ellsworth et al. (17)	EBF for 3+ months	Day 16 post-partum	Insulin (ng/mL)	Day 16 post-partum	Significant association with weight **($p = 0.02$)**, higher levels in OW/OB	Infant sex and feeding type (milk only or milk + formula), maternal weight status, pre-pregnancy BMI
Leghi et al. (24)	EBF for 6 to 20 weeks	NS	Adiponectin (ng mL^{-1})	NS	No association observed	None
			Leptin (ng/mL)		Significant, positive association with body weight **($p < 0.001$)**	
			Insulin (ng/mL)		No association observed	
Pundir et al. (28)	EBF for 3+ months	3 months	Cortisol (ng/mL)	3 months	Significant, positive association with normal weight mothers **($p = 0.01$)**	Maternal BMI, education, occupational class, family income, family structure, seasonal variation, maternal distress, pregnancy duration, delivery and gestational diabetes
SadrDadres et al. (31)	EBF for 3+ months	1 and 3 months	Adiponectin (ng mL^{-1})	1 and 3 months	No association observed	None
			Leptin (ng/mL)		Positive association with GWG **($p < 0.001$, $\beta = 0.3$)** and negative association with PPWL **($p < 0.001$, $\beta = -0.18$)**	
			Insulin (ng mL^{-1})		No association observed	
Young et al. (37)	EBF for 3+ months	2 weeks and 1, 2, 3 and 4 months	Insulin (ng/mL)	2 weeks and 1, 2, 3 and 4 months	Significant association with weight **($p = 0.019$)**	Maternal pre-pregnancy BMI group, birth weight, gestational age at delivery, gestational weight gain, maternal age, breastfeeding exclusivity, infant sex and mode of delivery

Where confounders were adjusted, data reported is from adjusted analysis. Significant results are depicted with the *p* value in bold. HM, human milk; EBF, exclusive breastfeeding; PBF, predominantly breastfeeding; BMI, body mass index; GWG, gestational weight gain; PPWL, post-partum weight loss; OW, overweight; OB, obesity; NS, not stated.

The effect of smoking on breast milk hormones might be expected to vary depending on factors such as smoking intensity, duration, and individual differences (45). Research indicates that smoking ten or more cigarettes a day can adversely affect lactation by reducing milk production and altering macronutrient content (45). Smoking during pregnancy and lactation can also impact maternal health, increasing stress and anxiety levels, potentially altering breast milk composition and hormone levels (43). Nonetheless, this review only included two studies on maternal smoking and breast milk hormones, one reporting higher levels of adiponectin in the milk of non-smoking mothers (36), while the other (14) found no association, likely due to low smoking rates in their study population. This highlights the need for further research to comprehensively understand how smoking influences breast milk hormonal composition and its impact on infant outcomes.

Ethnicity could theoretically influence hormone levels in breast milk due to a combination of genetic, cultural, environmental and lifestyle factors that vary among different ethnic groups. However, this review only found one study that mentioned ethnicity in relation to milk hormone levels, concluding that Asian mothers had lower levels of adiponectin and higher levels of insulin in their milk when compared to Caucasian mothers (12). This difference could reflect factors such as body composition as ethnic groups can have distinct

TABLE 5 Summary of the association between human milk composition and maternal age.

Paper	Feeding type	HM measured time-point	HM component	Maternal age measure time-point	Reported associations and correlations	Confounders adjusted
Chan et al. (12)	EBF for 3–4 months	3 months	Adiponectin (ng mL^{-1})	3 months	No significant correlation observed	Maternal BMI, pre-pregnancy weight, gestational weight gain, mode of infant feeding (exclusively or partially breastfed without formula at 3 months postpartum)
			Leptin (ng mL^{-1})		Concentrations were lower in older women ($p = 0.05$)	
			Insulin (ng mL^{-1})		No significant correlation observed	
Pundir et al. (28)	EBF for 3+ months	3 months	Cortisol (ng/mL)	3 months	No significant effect observed	Maternal BMI, education, occupational class, family income, family structure, seasonal variation, maternal distress, pregnancy duration, delivery and gestational diabetes
Zielinkska-Pukos et al. (41)	EBF for 3+ months	1, 3 and 6 months	Cortisol (ng/mL)	1, 3 and 6 months	No significant correlation observed	None

Where confounders were adjusted, data reported is from adjusted analysis. Significant results are depicted with the *p* value in bold. HM, human milk; EBF, exclusive breastfeeding; BMI, body mass index.

body composition characteristics which could impact hormone production and metabolism (46). In addition, cultural dietary practices and certain foods and nutrients vary among different ethnic groups. Diets with anti-inflammatory properties, such as the Mediterranean diet and others emphasizing plant-based foods and healthy fats, have demonstrated the ability to lower leptin levels in circulating blood and enhance leptin sensitivity (47). Conversely, heightened intakes of saturated fatty acids have been linked to inducing leptin resistance by interrupting leptin signaling after chronic overstimulation of the leptin receptor (48). However, research on the specific differences in breast milk composition among various ethnicities is limited.

The review included two studies on post-partum depression and stress in relation to breast milk cortisol levels. One study reported that mothers in a psychiatry-obstetric-pediatric clinic had lower milk cortisol compared to those not in the clinic (30). However, this study had limitations, as it focused on a specific population with a higher risk of psychological distress during pregnancy. On the other hand, another study did not find a significant association between maternal depression and breast milk cortisol (29). Chronic stress can affect the hypothalamic–pituitary–adrenal (HPA) axis, potentially leading to elevated cortisol levels in breast milk. While previous studies have explored the connection between maternal psychological factors and milk cortisol levels, they have yielded inconsistent results. Objective assessments of maternal cortisol levels in plasma appear to provide a more reliable measure of chronic stress compared to subjective methods used in the studies reviewed.

Mother-infant signaling

The results from this systematic review suggest that the mother may communicate important cues through the hormonal composition of breast milk. The studies consistently revealed the presence of diverse hormones such as adiponectin, leptin, insulin, cortisol, and ghrelin in breast milk. The varying concentration of these hormones (specifically leptin and insulin) in relation to maternal factors such as BMI, weight and other health indicators highlight an intricate interplay between maternal physiology and breast milk composition. This supports the notion that maternal factors are somewhat linked to the hormonal makeup of breast milk in particular maternal BMI and weight, which, in turn, could potentially impact infant outcomes such as growth, development and overall health. Notably, associations between maternal nutritional status and offspring development, mediated by milk hormones, have recently been reported in primates (49). Breast milk hormones could potentially influence infant outcomes through epigenetic mechanisms such as changes in the gene expression that could be triggered by various environmental, nutritional and hormonal factors during pregnancy and lactation (50).

However, there are six steps that should be fulfilled for a component to act as a signal (Figure 3), with the results from this review contributing evidence toward one step only in establishing whether a hormone is a signaling component, by assessing which maternal factors affect their concentration in human breast milk. This review did not find any studies assessing the association between maternal factors and hormones such as prolactin, oxytocin and resistin. The lack of current evidence found does not rule out a signaling role of these hormones. Instead, it highlights a notable gap in the existing literature in relation to other hormones within breast milk. In addition, this review solely focused on hormones as potential signaling components, but there are many other possible signals in milk such as bacteria, nutrients, and growth factors. These other components could be investigated using the same approach as this systematic review.

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TABLE 6 Summary of the association between human milk composition and maternal diabetes.

Paper	Feeding type	HM measured time-point	HM component	Maternal diabetes measure time-point	Reported associations and correlations	Confounders adjusted
Choi et al. (13)	EBF for 3+ months	1 and 3 months	Adiponectin (ng mL^{-1})	1 and 3 months	No association observed	Pre-pregnancy BMI, gestational weight gain and breast feeding status (full, partial or none at 3 months)
			Leptin (ng mL^{-1})		No association observed	
			Insulin (ng mL^{-1})		Significant difference found **(p = 0.03 and p = 0.003)**, lower levels of insulin in GDM	
Pundir et al. (28)	EBF for 3+ months	3 months	Cortisol (ng/mL)	3 months	No significant association found	Maternal BMI, education, occupational class, family income, family structure, seasonal variation, maternal distress, pregnancy duration, delivery and gestational diabetes
Rodel et al. (29)	EBF but duration not stated	2 weeks	Insulin (ng/mL)	2 weeks	Significant difference found **(p < 0.001)**, levels 2 times higher in T2DM compared to GDM & NGT	None
Yu et al. (39)	EBF for 3+ months	Colostrum – 72 h after birth, Mature – 1 (42 days) and 3 months (90 days)	Adiponectin (ng/mL)	Colostrum – 72 h after birth, Mature – 1 (42 days) and 3 months (90 days)	Significant, negative correlation with GDM **(pcolostrum < 0.001; pmature = 0.009)**	None
			Leptin (ng/mL)		No significant association found ($p > 0.05$)	
			Insulin (ng/mL)		Significant, positive concentration **(pcolostrum = 0.047; pmature = 0.02)**	
			Ghrelin (ng/mL)		Significant, negative correlation with GDM **(pcolostrum = 0.011; pmature < 0.001)**	

Where confounders were adjusted, data reported is from adjusted analysis. Significant results are depicted with the *p* value in bold. HM, human milk; EBF, exclusive breastfeeding; T2DM, type 2 diabetes mellitus; GDM, gestational diabetes mellitus; BMI, body mass index.

TABLE 7 Summary of the association between human milk composition and maternal smoking.

Paper	Feeding type	HM measured time-point	HM component	Maternal smoking measure time-point	Reported associations and correlations	Confounders adjusted
Chan et al. (12)	EBF for 3–4 months	3 months	Adiponectin (ng mL^{-1})	4 months	No significant association found	Maternal BMI, pre-pregnancy weight, gestational weight gain, mode of infant feeding (exclusively or partially breastfed without formula at 3 months postpartum)
			Leptin (ng mL^{-1})		No significant association found	
			Insulin (ng mL^{-1})		No significant association found	
Weyermann et al. (36)	EBF for 3+ months	33 and 71 days post-partum	Adiponectin (ng mL^{-1})	33 and 71 days post-partum	Levels higher in milk with no smoking (*p* value not stated)	Age, education, nationality, BMI, smoking status of mother
			Leptin (ng mL^{-1})		No association found	

Where confounders were adjusted, data reported is from adjusted analysis. Significant results are depicted with the *p* value in bold. HM, human milk; EBF, exclusive breastfeeding; BMI, body mass index.

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TABLE 8 Summary of the association between human milk composition and other maternal factors.

Paper	Feeding type	HM measured time-point	HM component	Other maternal factors measure time-point	Reported associations and correlations	Confounders adjusted
Cagiran-Yilmaz et al. (11)	EBF for 3+ months	1, 3 and 6 months	Leptin (ng/mL)	1, 3 and 6 months	Significant, positive correlation with hip, waist and mid-upper arm circumference in all months **($p < 0.05$)**	None
Chan et al. (12)	EBF for 3–4 months	3 months	Adiponectin (ng mL^{-1})	4 months	Asian mothers had lower levels compared to Caucasian **($p = 0.01$)**	Maternal BMI, pre-pregnancy weight, gestational weight gain, mode of infant feeding (exclusively or partially breastfed without formula at 3 months postpartum)
			Leptin (ng mL^{-1})		No association observed with ethnicity	
			Insulin (ng mL^{-1})		Asian mothers had higher levels compared to Caucasian **($p < 0.0001$)**	
Gridneva et al. (19)	EBF for 3+ months	2 and/or 5, 9 and 12 months	Adiponectin (ng mL^{-1})	2 and/or 5, 9 and 12 months	No significant association observed with maternal body composition	None
			Leptin (ng/mL)		No significant association observed with maternal body composition	
Leghi et al. (24)	EBF for 6 to 20 weeks	NS	Adiponectin (ng mL^{-1})	NS	Positive association with changes in maternal carbohydrate intake **($p = 0.033$)** and total energy intake **($p = 0.038$)**	None
			Leptin (ng/mL)		No association observed with maternal carbohydrate and energy intake	
			Insulin (ng/mL)		No association observed with maternal carbohydrate and energy intake	
Romijn et al. (30)	EBF for 3+ months	1 month	Cortisol (ng/mL)	1 month	Cortisol was lower in the group who sought consultation at the psychiatry-obstetric-pediatric outpatient clinic **($p = 0.02$)**	None
Pundir et al. (28)	EBF for 3+ months	3 months	Cortisol (ng/mL)	3 months	No association observed with mode of delivery	Maternal BMI, education, occupational class, family income, family structure, seasonal variation, maternal distress, pregnancy duration, delivery and gestational diabetes
Zielinkska-Pukos et al. (41)	EBF for 3+ months	1, 3 and 6 months	Cortisol (ng/mL)	1, 3 and 6 months	No association observed with postpartum depression score and percieved stress scale	None

Where confounders were adjusted, data reported is from adjusted analysis. Significant results are depicted with the *p* value in bold. HM, human milk; EBF, exclusive breastfeeding; BMI, body mass index; NS, not stated.

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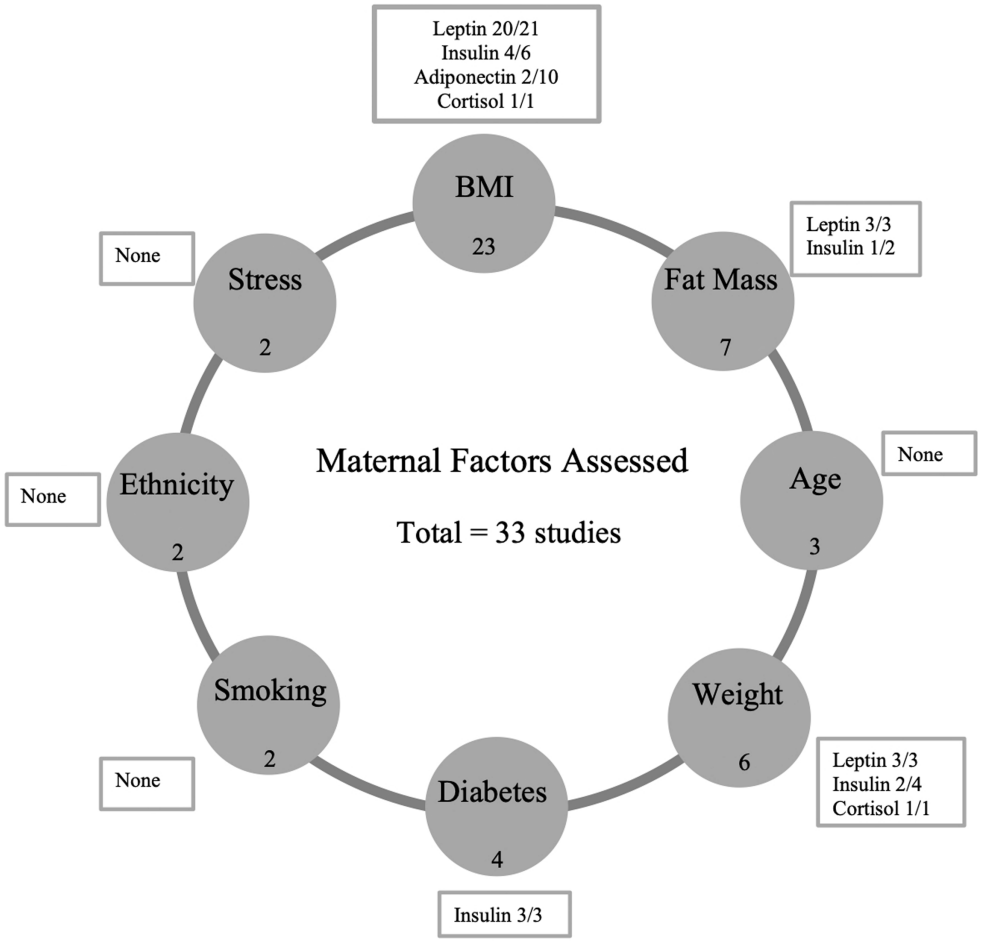

FIGURE 2
Summary of results. This figure shows the positive associations found between specific maternal factors and the concentration of breast milk hormones in this systematic review. The number of studies included which reported each maternal factor (i.e., BMI -23) are represented in filled circles. The number of studies that found a positive association between the maternal factor and hormone are shown in textboxes (i.e., Leptin 20 studies found a positive association out of a total of 21 studies). BMI, body mass index.

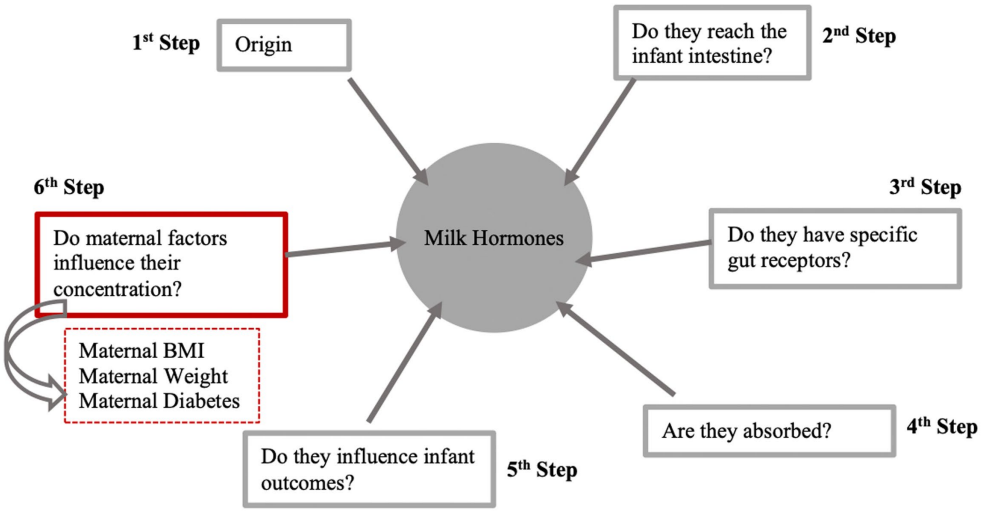

FIGURE 3
Six steps to determine whether a milk hormone acts as a signal between mother and offspring. This figure summarizes the results from this systematic review which aimed to obtain evidence for the sixth stage of the process. BMI, body mass index. Figure adapted from Fewtrell et al., (4), licensed under CC BY 4.0.

Limitations

This systematic review has highlighted limitations stemming from inadequate study design, hindering our understanding of how maternal factors affect the composition of bioactive components in breast milk. The complexity of maternal influences, both genetic and environmental, makes it challenging to draw causal inferences, as randomizing subjects based on many relevant factors is practically impossible. New study designs are needed, such as long-term observational studies that follow mother-infant pairs over time or clustering them by similar characteristics like BMI. Targeted interventions, focusing on factors like maternal diet or stress reduction, can provide insights into how specific changes impact breast milk composition.

Additionally, the variability in sampling methods among different studies, including lactation stage, feeding frequency, and time of day, complicates direct comparisons of findings. Inconsistencies may arise from the lack of standardization in collecting and processing human milk samples. For instance, some hormones show diurnal patterns influenced by the time of day, and variations in sampling techniques, such as analyzing foremilk or hindmilk, can affect results. Notably, the quality of the studies reviewed was generally low or fair, and many studies lacked reporting on maternal factors or breastfeeding patterns, making it difficult to assess the strength of the associations between maternal factors and breast milk hormone concentrations.

Conclusion

This systematic review suggests that higher maternal BMI is linked to increased breast milk leptin and maternal diabetes to higher breast milk insulin. However, it fails to establish clear associations between maternal fat mass, age, smoking, ethnicity, stress, and breast milk hormonal composition due to insufficient data and methodological limitations in prior studies. The use of standardized protocols for sample collection and analysis in future studies would enable more meaningful cross-study comparisons. Further research is required to understand how breast milk hormones affect infant outcomes and their role as signaling components. In conclusion, this study underscores the complex relationship between maternal factors, breast milk composition, and potential infant signaling mechanisms, serving as a starting point for future investigations.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

RQ: Writing – original draft. MF: Writing – review & editing. JW: Writing – review & editing. SD: Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1390232/full#supplementary-material

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Maternal docosahexaenoic acid supplementation during lactation improves exercise performance, enhances intestinal glucose absorption and modulates gut microbiota in weaning offspring mice

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Introduction: Intestinal dysfunction induced by weaning stress is common during breastfeeding period. Docosahexaenoic acid (DHA) is well known for promoting visual and brain development, but its effects on early intestinal development remain unknown. This study investigated the impact of maternal DHA supplementation during lactation on intestinal glucose absorption and gut microbiota in weaning offspring mice.

Materials and methods: Dams were supplemented with vehicle (control), 150 mg/(kg body weight · day) DHA (L-DHA), or 450 mg/(kg body weight · day) DHA (H-DHA) throughout lactation by oral administration. After weaning, pups were randomly divided into three groups for athletic analysis, microbial and proteomic analysis, biochemical analysis, 4-deoxy-4-fluoro-D-glucose (4-FDG) absorption test, and gene expression quantitation of glucose transport-associated proteins and mTOR signaling components.

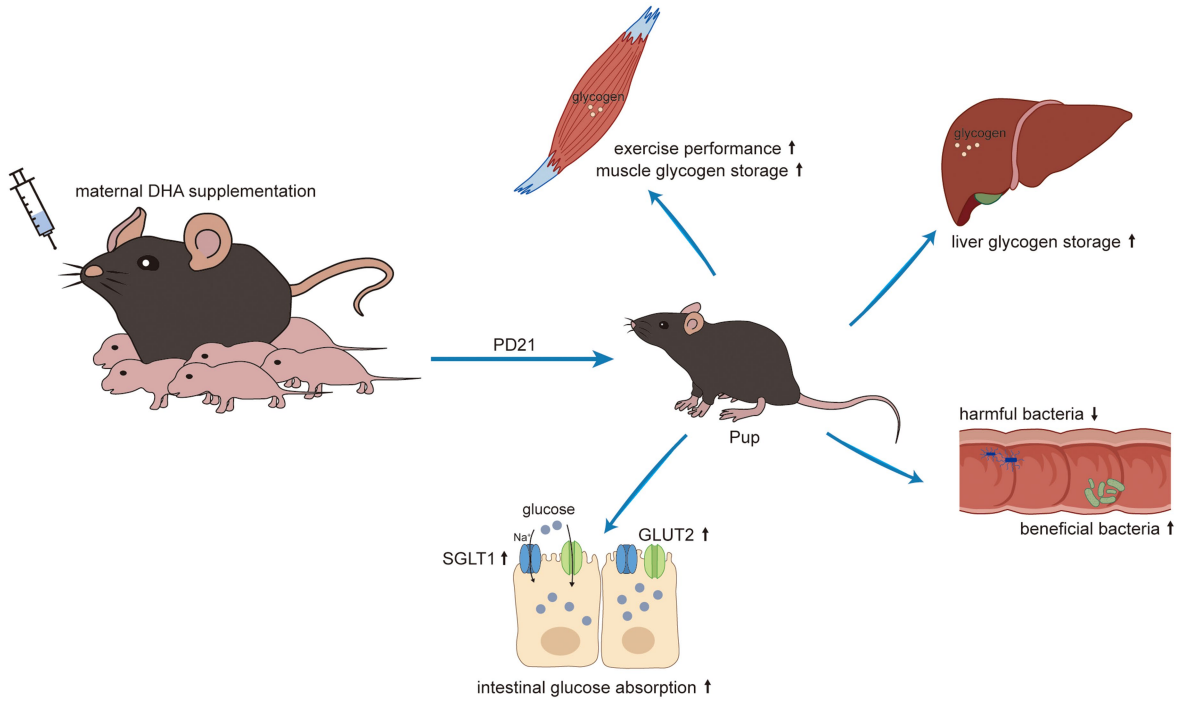
Results: The H-DHA group exhibited enhanced grip strength and prolonged swimming duration compared to the control group. Additionally, there were significant increases in jejunal and ileal villus height, and expanded surface area of jejunal villi in the H-DHA group. Microbial analyses revealed that maternal DHA intake increased the abundance of beneficial gut bacteria and promoted metabolic pathways linked to carbohydrate and energy metabolism. Proteomic studies indicated an increased abundance of nutrient transport proteins and enrichment of pathways involved in absorption and digestion in the H-DHA group. This group also showed higher concentrations of glucose in the jejunum and ileum, as well as elevated glycogen levels in the liver and muscles, in contrast to lower glucose levels in the intestinal contents and feces compared to the control group. The 4-FDG absorption test showed more efficient absorption after oral 4-FDG gavage in the H-DHA group. Moreover, the expressions of glucose transport-associated proteins, GLUT2 and SGLT1, and the activation of mTOR pathway were enhanced in the H-DHA group compared to the control

group. The L-DHA group also showed similar but less pronounced improvements in these aspects relative to the H-DHA group.

Conclusion: Our findings suggested that maternal DHA supplementation during lactation improves the exercise performance, enhances the intestinal glucose absorption by increasing the expressions of glucose transporters, and beneficially alters the structure of gut microbiome in weaning offspring mice.

KEYWORDS

docosahexaenoic acid, lactation, exercise, glucose absorption, microbiota, mTOR pathway



GRAPHICAL ABSTRACT

1 Introduction

Nutrition during the prenatal and infancy stages is considered to be a critical (non-genetic) determinant for growth and development. Breastfeeding recommended by the World Health Organization (WHO) is considered as the best feeding modality for infants (1, 2). It is reported that breast milk composition is closely related to maternal dietary intake (3). Therefore, maternal nutrition during pregnancy and lactation has a momentous impact on the long-term health of offspring. As the ideal nutritional source for infants, especially for the first 6 months of life, breast milk contains a multitude of basic nutrients and bioactive ingredients that are essential for the maturation of the gut in infants, such as immunoglobulin, oligosaccharides, short-chain fatty acids, antimicrobial peptides, and microbes. Moreover, a previous research suggested that the extent of breastmilk's benefits is affected by its fatty acid composition (4). Fatty acids are essential components of breast milk, which provide structure and modulate functions of cell membranes, synthesize signaling molecules as cellular messengers in signal-transduction pathways, and mediate and regulate immune functions (5). Docosahexaenoic acid (DHA), a prominent n-3 long-chain polyunsaturated fatty acids (LCPUFAs) in human breast milk, has been identified to play a central role in the development of the infant visual and brain neural systems and is also known to be involved in membrane fluidity, neurogenesis, synaptogenesis, anti-inflammatory and anti-oxidative activities (6). Although it is possible to convert α-linolenic acid to DHA by elongase and desaturase enzymes in the body, studies have shown that only small amounts of DHA can be synthesized by this process (7). Thus, maternal supplementation with no less than 200 mg of DHA per day is recommended during lactation to meet the requirements both of the mother and the suckling infant (8).

Early life is a vital period for the development of the gut microbiota and the intestinal physical barrier and digestive functions, and the maturation and health of the gut in this period have been found to be associated with programming health and disease later in adulthood (9). In the early stages of rapid development, intestinal maturation in infancy relies heavily on adequate nutritional support from breast milk

for optimal development of digestive system. As the immaturity of the gastrointestinal tract and changes in food composition, post-weaning disturbed digestive function is one of the important problems in infant feeding (10). A growing body of evidence has shown that the weaning period, a critical transition period for mammalian neonates, becomes more susceptible to be accompanied by intestinal bacterial disorders and inflammatory reactions. Prolonged intestinal inflammation caused by pathogens can damage the intestinal epithelium and compromise the nutrient digestion and absorption, which leads to growth retardation and malnutrition in subsequent early childhood (11, 12). The early-life gut microbiota of offspring is primarily shaped by breast milk, which is known as the entero-mammary axis (13). During weaning, the gut microbiota shifts to a new stage of development and evolves towards a composition similar to that of adult individuals due to the introduction of solid food. A stable gut microbiota system can help neonates adapt to such transition and reduce the incidence of dysbiosis. Accordingly, it is necessary to promote the intestinal development and maintain the gut homeostasis of infants to optimize their performance during this critical stage. Previous studies have reported that maternal supplementation of fish oil, a complex consisting mainly of DHA, during pregnancy and lactation significantly facilitates the suckling-to-weaning transition by altering the gut microbial community and improves intestinal nutrient uptake and energy stores in weaning piglets (14, 15). These findings suggested that DHA may positively affect gut development in early life.

To date, trials that have explored the effect of early DHA supplementation have been mainly focused on brain development and cognitive assessments, but studies on the effects of DHA on intestinal nutrient absorption functions are limited. It is a cost-effective intervention that provides a specific maternal diet to promote the intestinal development of offspring. Consequently, on the basis of previously published research, we further investigated the potential influence of maternal DHA supplementation during lactation on intestinal glucose absorption capacity and gut microbiota colonization of weaning offspring using mice as a model. In addition, we also explored whether such supplementation could enhance tissue glycogen stores and improve exercise performance in offspring.

2 Materials and methods

2.1 Animals and experimental design

Pregnant female C57BL/6 mice at gestational day (GD)16 were purchased from Shanghai Jihui Laboratory Animals (China). All animals were housed in ventilated cages with a 12 h light/dark cycle at a constant temperature (21–23°C) and were provided with food and water *ad libitum*. The compositions of rodent chow diet were provided in Supplementary Table S1. The fatty acid compositions of this chow diet were provided in Supplementary Table S2. C57BL/6 dams were randomly assigned into three groups ($n = 6$): received without DHA supplementation orally (control group), received with 150 mg/(kg body weight · day) DHA supplementation orally (L-DHA group), and received with 450 mg/(kg body weight · day) DHA supplementation orally (H-DHA group). DHA (purity >98%) was purchased from Macklin (China). We dissolved DHA in ddH_2O containing 0.5% CMC-Na (Solarbio, China) to obtain 15 mg/mL and 45 mg/mL DHA solutions. From GD18 to postpartum day (PD) 21, DHA solutions at low and high concentrations were administrated to the dams from L-DHA and H-DHA groups, respectively, by oral gavage daily, while the dams from the control group received an equal volume of vehicle daily. The DHA doses in this study were based on amounts used in previous clinical trials with humans and adjusted for the 12-fold higher metabolic rate in mice (16–19). Accordingly, the doses of 150 and 450 mg/kg body weight per day for mice correspond to approximately 750 and 2,250 mg per day for humans, respectively. After delivery, pups were raised by their own mothers from PD0 to PD21. To guarantee offspring homogeneity, each litter was adjusted to 6 pups with equal numbers of males and females wherever possible. The body weight of pups was recorded every day from PD7 to PD21.

At weaning (PD21), three random selections with one male and one female pup per litter for each selection were carried out. The random sampling method was used in each selection. The pups from the first selection were used for sample collection ($n = 12$). Briefly, fresh stool feces from each pup were first collected after overnight fasting and stored at −80°C. Immediately after feces collection, offspring were sacrificed under anesthesia with sodium pentobarbital (45 mg · kg^{-1}, i.p.) and autopsied. Organs were carefully excised, rinsed in saline solution and blotted dry. The weight of the brain, heart, liver, spleen, kidney and the length of the small intestine were recorded for growth and developmental assessment. The blood samples and the contents in ileum and colon were also collected. Serum was separated from whole blood samples by centrifugation at 5000 rpm for 10 min at 4°C and stored at −80°C. Tissues of liver, gastrocnemius muscle, jejunum, ileum, colon and the contents in ileum and colon canal were collected and stored at −80°C. Part of jejunal and ileal segments was immersed in 4% paraformaldehyde for histochemical staining. The pups from the second selection were used for forelimb grip strength test and weight-loaded swimming test ($n = 12$), while the pups from the third selection were used for glucose/4-deoxy-4-fluoro-D-glucose (4-FDG) absorption test ($n = 8$). A schematic of the experimental design was shown in Figure 1.

2.2 Determination of glycogen content in liver and muscle and glucose content in intestine, intestinal contents and feces

The glycogen level in liver and muscle tissue was determined using the glycogen assay kit (Nanjing Jiancheng, China). The glucose level in jejunum, ileum, colon tissue, and contents in ileum and colon canal, and feces was determined using the glucose assay kit (Nanjing Jiancheng). The final concentration was normalized to the initial wet weight of the specimen.

2.3 Examination of serum biochemical parameters

Total serum cholesterol (TC), triglyceride (TG), protein (TP) and serum glucose were estimated by commercial assay kits (Nanjing Jiancheng). All procedures were conducted according to the manufacturer's instructions.

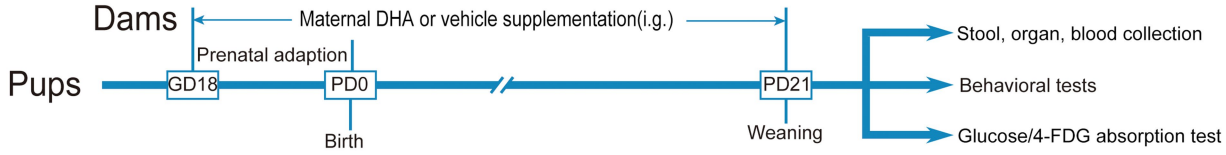


FIGURE 1
A scheme of the experimental design.

2.4 Fatty acid analysis

Fifty milligram of chow diet and jejunum tissue were crushed into fine powder in liquid nitrogen, and total lipids were extracted using chloroform:methanol (2:1). Lipid samples were then saponified with 0.5 M methanolic NaOH for 5 min in boiling water. The hydrolyzed sample was cooled and added boron triflouride solution in methanol (10%). The solution was heated in boiling water bath for 30 min. Extraction was carried out by adding n-hexane and vortex-mixing for 30 s. After centrifugation, the supernatant was collected, and methyl nonadecanoate (C19:0) was added to the extract as an internal standard. Fatty acid methyl esters in the upper hexane layer were analyzed by GCMS-QP2010 Ultra instrument (SHIMADZU, Japan) equipped with a SP-2560 capillary column (100 m × 0.25 mm × 0.2 µm, Sigma-Aldrich, USA). Determination of the fatty acid methyl esters was performed using standard spectrogram from the NIST Mass Spectral Database. Quantitation was determined after normalization to the internal standard. Detailed procedures of fatty acid analysis can be seen in Ochin's study (20).

2.5 Small intestine morphology

After fixation, jejunum and ileum samples were embedded in paraffin and cut into 5-µm-thick sections for hematoxylin and eosin (H&E) staining. The stained sections were observed under light microscopy (Eclipse 80i, Nikon, Japan). The villus height, the villus width and the crypt depth of ten vertically well-orientated villi and crypts were determined using an analysis system (NIS-elements suite; Nikon) by experienced observers who were unaware of treatment group (D.L. and G.H.). The villus height was determined from the crypt opening to the top of the villus and crypt depth from the base of the crypt to its opening. The villus width was defined as the distance from the outside epithelial edge to the outside of the opposite epithelial edge at the half height of the villus. The ratio of villus height to crypt depth (V/C) was calculated to indicate the maturity of villus. The villi surface area was calculated from the villus height and the villus width according to Kolba et al. (21).

2.6 Forelimb grip strength test

The detailed procedure of forelimb grip strength test was performed as described in the previous publication (22). Briefly, a grip strength meter adapted from a digital tension apparatus (SF-3, AIPLI, China) was used to measure the forelimb grip strength of offspring after weaning at PD21. Each pup was allowed to grasp the grid of the device with both forepaws and its tail was gently pulled back until both forepaws released the grid. The maximum pull force was recorded by the tension apparatus. Measurements were discarded when mice only used one paw, used its hind paws, turned backwards during the pull, or released the bar without resistance. Each pup had five times to perform the test with a 5-min rest between each test. The values, excluding the highest and lowest results, were normalized to the body weight of each pup.

2.7 Weight-loaded swimming test

The weight-loaded swimming test was performed 1 h after the forelimb grip strength test, as described previously in the previous publication (23). Each mouse, which was loaded with a lead block weighing 5% of the body weight on its tail, was placed individually in a plastic pool (50 × 40 × 40 cm) filled with water (25 ± 1°C). The pool water was kept flowing by gently stirring with a glass rod, allowing the mice to swim to exhaustion during the swimming test. The weight-loaded swimming time, used as an index of exercise endurance, was recorded when the mice sank into the water and failed to rise to the surface for breath within 10 s. After the test, the mouse was removed from the water and dried immediately.

2.8 Glucose and 4-FDG absorption test

At weaning (PD21), after fasting overnight, 2 g/kg glucose and 10 mg/kg 4-FDG (a glucose analog) were orally administered together followed by taking blood samples from the tail vein at 0, 15, 30, 60 and 120 min and from the mandibular vein at 30 and 60 min. Glucose levels were measured using a glucometer (Bayer Healthcare LLC, Germany). The blood glucose changes over time (0, 15, 30, 60 and 120 min) in response to oral glucose gavage were used to indicate the function of intestinal glucose absorption and overall glucose tolerance. The area under the curve (AUC) of the glucose response curve was calculated to indicate the overall glucose tolerance. The changes of blood 4-FDG concentration in response to oral 4-FDG gavage were used to indicate the function of intestinal glucose absorption. Serum obtained from blood collected at 30 and 60 min was mixed with the same volume of HPLC buffer (55% 100 mM potassium phosphate, pH 7, and 45% methanol), and then analyzed by HPLC for 4-FDG quantification (24).

2.9 Microbial 16S rRNA sequencing

The stool samples in all groups were sent to BGI Genomics (China) for 16S rRNA gene sequencing. Bacterial DNA was extracted

from the fecal contents following the manufacturer's protocol (DNeasy PowerSoil Kit; Qiagen, Germany). The quality and quantity of DNA were verified by agarose gel electrophoresis and NanoDrop. The DNA extracted from all samples was stored at −20°C until sequencing. The extracted DNA was used as a template for the PCR amplification of bacterial 16S rRNA genes with the barcoded primers. The V3-V4 variable regions of the 16S rRNA genes were amplified using the universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5'-GGACTACHVGGGTWTCTAAT-3′). The amplicon quality was visualized using gel electrophoresis, purified using the QIAquick Gel Extraction kit (Qiagen), and quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher, United States) according to the manufacturer's protocols. Equal amounts of purified amplicons were pooled for sequencing using the MGISEQ-2000 platform PE300 (BGI Genomics).

2.10 Protein preparation and LC–MS/MS experiments

Lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris, 65 mM DTT) was added into the jejunum tissue homogenized in liquid nitrogen for protein extraction, and sonicated at 20% amplitude for the total working time of 2 min with 5 s on and 5 s off (JY92-IIDN, Scientz Biotechnology, China). The proteins were denatured at 95°C for 5 min. Lysates were centrifuged at 12,000 rpm for 10 min to remove the insoluble debris and retain the supernatant for proteomic experiment. The protein concentration of the supernatant was determined using the tryptophan-based fluorescence quantification method (21586754). Equal amounts of protein samples were digested by filter-aided sample preparation (FASP) procedure with 10 kDa centrifugal filter tubes (Millipore, United States) and centrifuged at 12,000 g at 22°C. First, the protein was washed with urea buffer (8 M urea in 0.1 M Tris–HCl, pH 8.5) three times, and incubated with 50 mM iodoacetamide for 30 min in the darkness. Then, the iodoacetamide was removed by centrifugation. Continually, the protein was washed with urea buffer three times and then washed thrice with 50 mM ammonium bicarbonate. Next, proteins were digested with Trypsin (Promega, United States) in 50 mM ABC at a concentration of 1:50 (w/w) at 37°C for 18 h. Digested peptides were collected at 12,000 g centrifugation. Concentration of eluted peptides was determined by bicinchoninic acid method. 400 μg peptides were dried by vacuum freeze dryer and resolved in 0.1% formic acid. All the prepared samples were analyzed on a LC–MS platform (Bruker Daltonics, United States) consisting of a nanoElute UHPLC coupled to a timsTOF Pro mass spectrometer, and data acquisition was performed in the data independent acquisition (DIA) mode.

2.11 Quantitative real-time PCR

Total RNA from jejunum tissue was extracted using Trizol reagent (RNAiso Plus, Takara, Japan), following manufacturer's instructions. RT reaction (10 μL) was carried out using the Prime Script RT Reagent Kit (Takara). Two-step reverse transcription-quantitative PCR reactions were performed in a qPCR system (QuantStudio Dx, Applied Biosystems) using SYBR GREEN Master Mix reagent kits (Yeasen, China). The primer sequences for glucose transporter 2 (*GLUT2*), sodium glucose co-transporter 1 (*SGLT1*), peptide transporter 1 (*PEPT1*), alanine-serine-cysteine transporter 2 (*ASCT2*), sodium-dependent neutral amino acid transporter 1 (*B0AT1*), excitatory amino acid transporter 1 (*EAAT1*), excitatory amino acid transporter 3 (*EAAT3*), L-type amino acid transporter 1 (y^+*LAT1*), 4F2 cell-surface antigen heavy chain (*4F2hc*), cationic amino acid transporter 1 (*CAT1*), Niemann Pick C1 like 1 (*NPC1L1*), apolipoprotein A-1 (*APOA1*), apolipoprotein A-4 (*APOA4*), apolipoprotein B (*APOB*), *CD36*, fatty acid binding protein 2 (*FABP2*), fatty acid binding protein 4 (*FATP4*) and *β-actin*, synthesized by Sangon Biotech (China), were provided in Supplementary Table S3. The housekeeping gene β-actin was used for normalization. qPCR results were analyzed using the $2-\Delta\Delta Ct$ method.

2.12 Western blot analysis

Total protein was isolated from jejunum tissues and then lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotech, China) supplemented with a 1% PMSF (Beyotime Biotech) and 1% phosphatase inhibitor mixture (Servicebio, China) on ice. Total protein was quantitated using BCA assay kit (Beyotime Biotech). Aliquots (50 μg total proteins) were loaded into SDS/polyacrylamide gels and transferred on to a PVDF-Plus membrane (Millipore). After transfer, the PVDF membrane was blocked for 1 h in 5% fat-free milk and then was, respectively, incubated overnight at 4°C with dilutions of primary antibodies: GLUT2 (A12307, 1:2000, Abclonal, China), SGLT1 (bs-1128R, 1:2000, Bioss, China), S6 ribosomal protein (abs131865, 1:2000, Absin, China), p-Ser240/244-S6 ribosomal protein (ab215214, 1:2000, Abcam, UK), p70S6 kinase (14485-1-AP, 1,1,000, Proteintech, China), p-Thr389-p70S6 kinase (9,234, 1,1,000, Cell Signaling Technology, United States), mammalian target of rapamycin (mTOR, T55306, 1:2000, Abmart, China), p-S2448-mTOR (5,536, 1,1,000, Cell Signaling Technology), β-actin (T40104, 1:4000, Abmart). The bands were detected with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (Beyotime Biotech), followed by the use of a chemiluminescence system (Pierce, United States) and ChemiDOC XRS+ imaging system (Bio-Rad, United States). Protein expression was normalized to β-actin, whereas phosphorylated protein was normalized to the total target protein. Band intensities were quantified with Image Lab (Bio-Rad).

2.13 Bioinformatic analysis

16S rRNA sequencing data were preprocessed to detect and remove ambiguous bases and low-quality sequences using Trimmomatic software. After trimming, FLASH software was used in the assembly of paired-end reads. Sequences were further denoised using QIIME software, and clustered to generate operational taxonomic units (OTUs) using USEARCH software. The representative read of each OTU was selected using QIIME, and blasted against the RDP database (Release 16). The estimators of α-diversity, including community richness (Chao index) and diversity (Simpson and Shannon index), were calculated using Mothur software. The β-diversity analysis, including principal coordinates analysis (PCoA) and unweighted pair group method

with arithmetic mean (UPGMA), was performed using QIIME. Differences of microbiota from phylum down to genus level among groups were discovered using linear discriminant analysis coupled with effect size measurements (LEfSe), and compared using Kruskal–Wallis test. The functional features of 16S rRNA sequencing data was predicted using PICRUST2 software. The predicted functional profiles were then collapsed into KEGG (Kyoto Encyclopedia of Genes and Genomes) database pathways and the differences between the groups were compared by Kruskal-Wallis test.

Protein identification and quantification were conducted using the library-free search function in DIA-NN with the UniProt mouse reference proteome (release version 2019/10). Proteins identified in more than 50% of the samples in each group were retained for further analysis. Missing values were imputed based on the k-nearest neighbor method. Protein intensity was calculated by summing the intensity of their respective peptides. Student's *t* test was used to compare the differences between two groups by R language. Fold Change (FC) > 1.5 or < 0.67 and $p < 0.05$ were set as the criteria for significant changes between any of the two groups. Bioinformatic analyses including PCoA, Kernel density plot analysis, Pearson correlation coefficient analysis, volcano plot analysis, Gene Ontology (GO) annotation enrichment analysis, and KEGG annotation enrichment analysis were conducted by R language.

2.14 Statistical analysis

All data were presented as the mean ± standard error of the mean (SEM). Two-way repeated-measures analysis of variance (ANOVA) was performed to compare the body weight, blood glucose and 4-FDG levels of glucose/ 4-FDG absorption test at various time points among multiple groups. The differences in other indices among groups were analyzed using Student's t-test when two groups were compared, or one-way ANOVA when multiple groups were compared. Tukey's test was used to assess the statistical significance between groups following ANOVA tests. Correlation analyses between the relative abundance of bacterial taxa and the relative mRNA or protein expression of glucose transporters were performed using the Spearman correlation coefficient test. p value <0.05 was considered to be statistically significant. GraphPad Prism 8.0 was used for statistical analysis.

3 Results

3.1 Maternal DHA supplementation during lactation has no effect on body weight and organ weight of offspring mice

To explore the possible effect of maternal DHA supplementation during lactation on body weight and organ weight of pups during the suckling period, we monitored the body weight every day from PD7 to PD21, and recorded organ weight and calculated relative organ weight at the end of the study. However, as shown in Figure 2A, no statistical difference in body weight by 2-way ANOVA analysis was observed among the three groups. The organ weight and relative organ weight also did not differ among the three groups (Supplementary Table S4).

3.2 Maternal DHA supplementation during lactation improves exercise performance of offspring mice

As the diagram shown in Figures 2B, the two physical performance tests, including forelimb grip strength and weight-loaded swimming, were conducted. In the forelimb grip strength test, we observed a significant increase in the L-DHA group (18% increase) and the H-DHA group (21% increase) compared with the control group (Figure 2C; $p < 0.001$ and $p < 0.001$, respectively). Meanwhile, relative grip strength (%), calculated by normalization to individual body weight, was 7 and 11% greater in the L-DHA group and the H-DHA group than in the control group (Figure 2D; $p < 0.05$ and $p < 0.001$, respectively). In addition, compared to the control group, the L-DHA group (20% increase) and the H-DHA group (32% increase) also performed longer swimming time, and the trend was significant (Figure 2E; both $p < 0.01$). Whereas, no significant difference of grip strength, normalized grip strength and weight-loaded swimming time was found between the L-DHA group and the H-DHA group.

3.3 Effects of maternal DHA supplementation during lactation on serum biochemical indexes of offspring mice

To determine the nutritional effects of maternal DHA supplementation, the serum glucose, TP, TC and TG of pups were examined. The serum biochemical parameters were summarized in Table 1. Markedly, the serum TG concentration was 29 and 36% higher in the L-DHA group and the H-DHA group than that in the control group ($p < 0.05$ and $p < 0.01$, respectively). However, there was no statistical difference between the L-DHA group and the H-DHA group. Moreover, the serum glucose, TP and TC levels were similar among the three groups.

3.4 Maternal DHA supplementation during lactation facilitates intestinal growth and changes intestinal fatty acid composition of offspring mice

It is well known that small intestine is the main site for nutrient absorption. To characterize the effect of maternal DHA supplementation on the morphology of the developing small intestine, we compared jejunum and ileum morphology among groups. Representative H&E-stained jejunal and ileal images of the three groups were shown in Figure 3A. We observed a significant increase in jejunal and ileal villus height in the H-DHA group, a 0.27-fold increase in jejunal villus height ($p < 0.01$) and a 0.24-fold increase in ileal villus height ($p < 0.05$), as compared with the control group. Although jejunal and ileal villus height in the L-DHA group was also increased, it did not reach statistical significance compared to the control group (Figure 3B). The ratio of villus height to crypt depth in the ileum was increased by 20 and 21% in the L-DHA and H-DHA groups compared with the control group ($p < 0.05$ and $p < 0.05$, respectively) (Figure 3D). In addition, the jejunal villus surface in the H-DHA group resulted in a 0.38-fold increase ($p < 0.01$) when compared with the control group

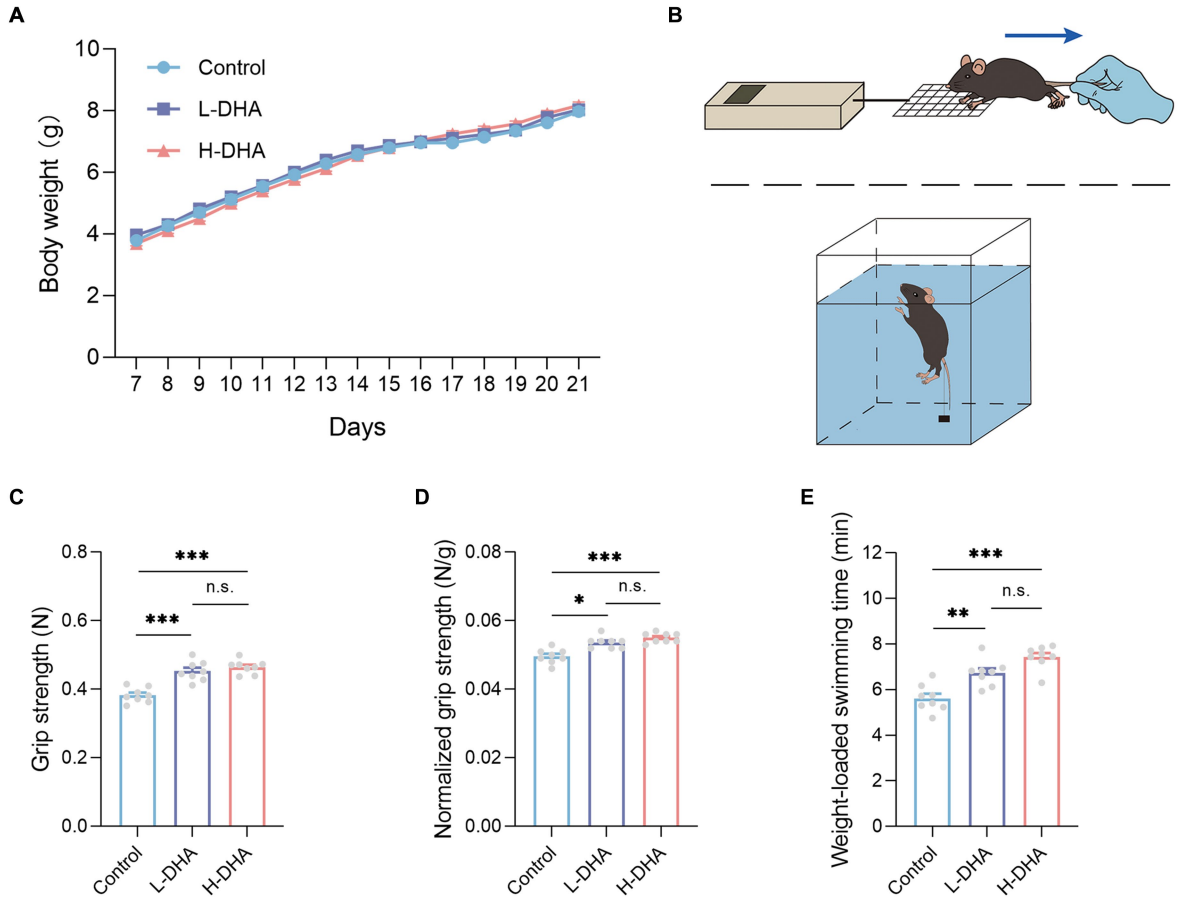


FIGURE 2
Effect of maternal DHA supplementation during lactation on the body weight and the exercise performance in weaning mice. **(A)** Growth curves of mice from different groups during suckling period. $n = 12$ per group. **(B)** Schematics of the grip strength test and the weight-loaded swimming test. **(C)** Forelimb grip strength and **(D)** normalized forelimb grip strength of mice from different groups. $n = 8$ per group. **(E)** Weight-loaded swimming time of mice from different groups. $n = 8$ per group. Data were expressed as the mean ± SEM. Significance was set at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). L-DHA: 150 mg/(kg body weight day) DHA; H-DHA: 450 mg/(kg body weight · day) DHA. DHA, docosahexaenoic acid.

TABLE 1 **Effect of maternal DHA supplementation during lactation on serum biochemical indexes in weaning mice.**

	Control	L-DHA	H-DHA	*p* value
Glucose (mmol/L)	4.882 ± 0.338	4.831 ± 0.360	4.932 ± 0.421	0.9821
TP (g/L)	38.110 ± 2.670	38.681 ± 2.232	37.026 ± 1.851	0.7888
TC (mmol/L)	1.970 ± 0.088	2.025 ± 0.098	2.071 ± 0.083	0.7301
TG (mmol/L)	1.151 ± 0.045[a]	1.488 ± 0.089[b]	1.564 ± 0.066[b]	0.0023

Data were expressed as the mean ± SEM; $n = 8$–10 mice per group. Statistical analysis was performed using one-way ANOVA test. Labeled means without a common letter differ at p value < 0.05. L-DHA: 150 mg/(kg body weight day) DHA; H-DHA: 450 mg/(kg body weight day) DHA. DHA, docosahexaenoic acid; TC, total cholesterol; TG, total triglyceride, TP, total protein.

and resulted in a 0.23-fold increase ($p < 0.05$) when compared with the L-DHA group (Figure 3E). However, the crypt depth of jejunum and ileum, the ratio of villus height to crypt depth of jejunum and villus surface of ileum were similar among the three groups (Figures 3C–E).

We also investigated whether maternal DHA supplementation could change the fatty acid composition of the offspring jejunum. Our findings revealed that the relative levels of eicosapentaenoic acid, DHA, and n-3 PUFAs in the jejunal tissue were significantly elevated (11-fold, 2.8-fold, and 1.6-fold increase), while the relative level of stearic acid was significantly lower (40% decrease) in the H-DHA group compared to the control group (all $p < 0.05$). The detailed fatty acid compositions were provided in Table 2.

3.5 Effects of maternal DHA supplementation during lactation on fecal microbiota of offspring mice

16 s rRNA sequencing was conducted in five fecal samples from each group to explore the effect of maternal DHA supplementation during lactation on the gut bacterial structure of pups. The overlapping and unique OTUs among groups were displayed by Venn diagram (Figure 4A). β-diversity analyses, PCoA and UPGMA based on unweighted Unifrac distance, indicated that the overall microbial compositions in the control group and the DHA-treated groups

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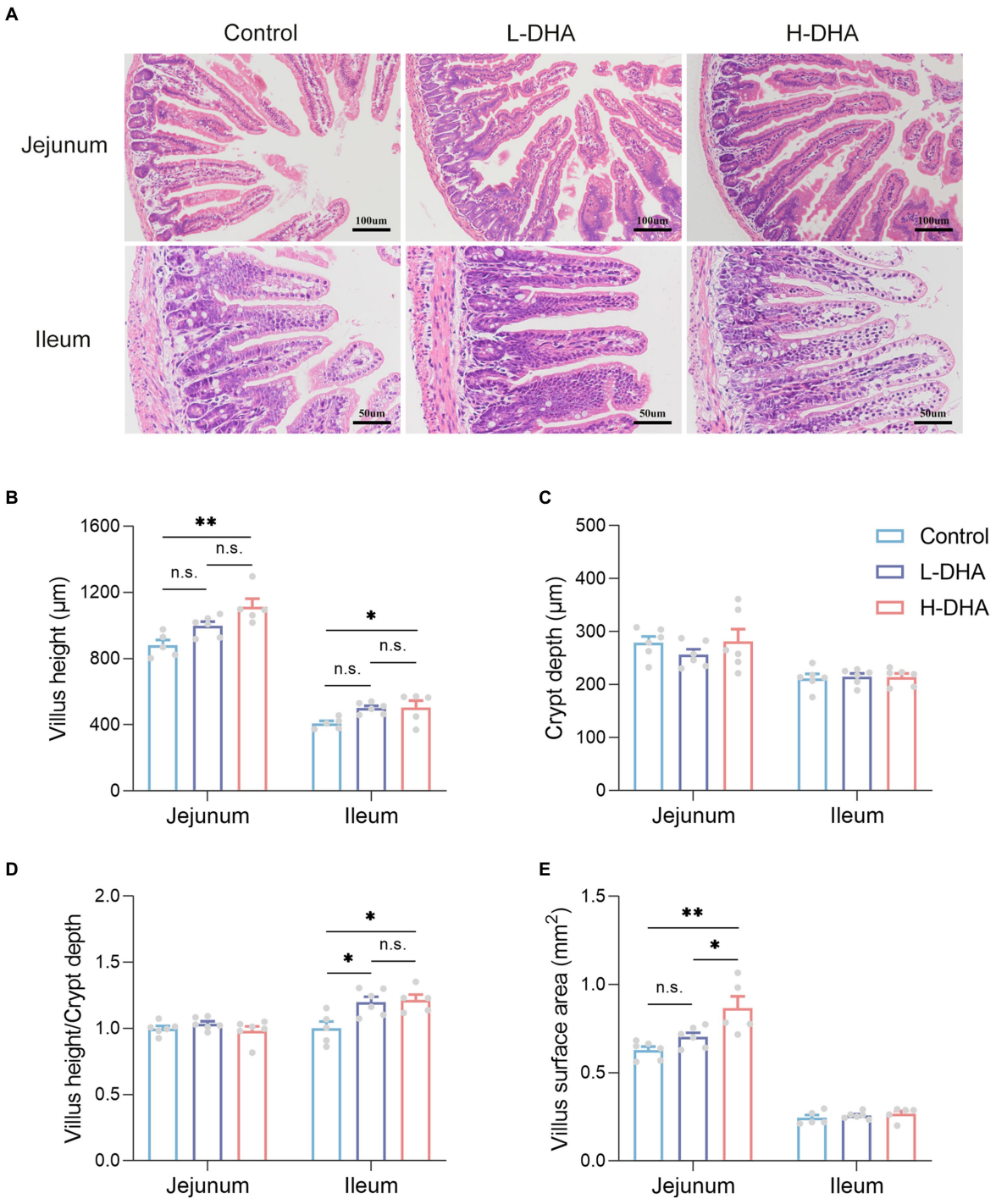

FIGURE 3
Effect of maternal DHA supplementation during lactation on small intestinal morphology in weaning mice. **(A)** Representative HE staining of jejunum (100x) and ileum (200x). **(B)** Quantitation of villus height of jejunum and ileum of mice from different groups. **(C)** Quantitation of crypt depth of jejunum and ileum of mice from different groups. **(D)** The ratio of villus height to crypt depth of jejunum and ileum of mice from different groups. **(E)** Quantitation of villus surface area of jejunum and ileum of mice from different groups. Data were expressed as the mean ± SEM; $n = 5–6$ per group. Significance was set at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). L-DHA: 150 mg/(kg body weight · day) DHA; H-DHA: 450 mg/(kg body weight · day) DHA. DHA, docosahexaenoic acid.

separated from each other (Figures 4B,C). As shown in Supplementary Figure S1A, compared with the control group, the L-DHA group exhibited a significantly lower Chao index ($p < 0.01$) and the H-DHA group also showed a decreased Chao index. However, no differences were found in Simpson index and Shannon index among the three groups. Next, we calculated the relative abundance of bacterial taxa at the phylum and genera level in each pup among the three groups (Supplementary Figures S1B,C). At the phylum level,

TABLE 2 Fatty acid compositions of jejunal tissue in weaning offspring.

Fatty acid	Control	L-DHA	H-DHA	*p* value
Percentage of total fatty acid				
C14:0	1.71 ± 0.44	1.01 ± 0.25	1.23 ± 0.33	0.382
C16:0	29.39 ± 1.99	26.39 ± 1.65	25.39 ± 2.76	0.431
C16:1n-9	7.71 ± 1.85	8.62 ± 2.19	9.49 ± 1.79	0.816
C18:0	21.33 ± 3.02[a]	19.27 ± 1.58[a]	12.56 ± 1.47[b]	0.034
C18:1n-9	18.77 ± 1.77	17.73 ± 3.33	13.24 ± 1.24	0.234
C18:2n-6	0.67 ± 0.21	0.61 ± 0.23	0.83 ± 0.22	0.777
C18:3n-3 (ALA)	1.21 ± 0.26[a]	3.11 ± 0.48[b]	2.04 ± 0.61	0.045
C20:0	2.62 ± 0.35	1.95 ± 0.41	2.20 ± 0.51	0.551
C20:4n-6 (AA)	3.41 ± 0.66	3.16 ± 0.71	4.57 ± 1.55	0.613
C20:5n-3 (EPA)	0.20 ± 0.09[a]	0.46 ± 0.16[a]	2.21 ± 0.59[b]	0.004
C22:4n-6	0.38 ± 0.13	0.52 ± 0.24	0.50 ± 0.18	0.862
C22:5n-3 (DPA)	0.57 ± 0.15	0.71 ± 0.29	0.59 ± 0.09	0.883
C22:6n-3 (DHA)	0.67 ± 0.19[a]	0.54 ± 0.16[a]	2.58 ± 0.71[b]	0.011
Total SFA	55.06 ± 5.02	48.61 ± 2.32	41.39 ± 2.68	0.056
Total MUFA	26.48 ± 1.74	26.35 ± 1.91	22.73 ± 2.28	0.353
n-3 PUFA	2.67 ± 0.39[a]	4.81 ± 0.74[a]	7.42 ± 0.92[b]	0.002
n-6 PUFA	4.46 ± 0.77	4.29 ± 0.65	5.91 ± 1.47	0.498

Data were expressed as the mean ± SEM; n = 5 mice per group. Statistical analysis was performed using one-way ANOVA test. Labeled means without a common letter differ at P value < 0.05. L-DHA: 150 mg/(kg body weight day) DHA; H-DHA: 450 mg/(kg body weight day) DHA. AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Bacillota and *Bacteroidetes* were the dominant bacteria in the gut microbiota of weaning mice, and the L-DHA and H-DHA groups compared with the control group insignificantly decreased the relative abundance of *Bacillota*, while insignificantly increased that of *Bacteroidetes*. The ratios of *Bacillota*/*Bacteroidetes* were reduced in the L-DHA and H-DHA groups but did not reach statistical significance when compared with the control group (Supplementary Figure S1D). LEfSe analysis demonstrated significantly different microbiota among the groups at the genus levels (Figure 4D). In addition, maternal DHA supplementation during lactation decreased the relative abundance of some harmful bacteria and increased the relative abundance of several beneficial bacteria at the genus level. The relative abundance of *Acetatifactor* ($p < 0.01$), *Desulfovibrio* ($p < 0.05$), *Harryflintia* ($p < 0.05$), *Alistipes* ($p < 0.01$), *Oscillibacter* ($p < 0.05$), *Intestinimonas* ($p < 0.01$) and *Pseudoflavonifractor* ($p < 0.05$) were distinctly lower in the L-DHA group than those in the control group. Compared to the control group, the relative abundance of *Acetatifactor* ($p < 0.05$), *Desulfovibrio* ($p < 0.01$), *Alistipes* ($p < 0.05$), *Oscillibacter* ($p < 0.05$), *Intestinimonas* ($p < 0.05$) and *Pseudoflavonifractor* ($p < 0.05$) were significantly decreased, while the relative abundance of *Ruminococcus* ($p < 0.05$), *Lactobacillus* ($p < 0.05$) and *Barnesiella* ($p < 0.01$) were markedly increased in the H-DHA group (Figure 4E).

We further conducted functional prediction analysis based on KEGG database to understand the potential difference in fecal microbiota among groups. Functional compositions of the three groups at level 1 and 2 were shown in Figure 5A. Compared with the control group, the DHA-treated groups exhibited up-regulated trend in the abundances of pathways involved in carbohydrate metabolism ($p < 0.05$), metabolism of terpenoids and polyketides ($p < 0.05$), energy metabolism ($p < 0.05$), xenobiotics biodegradation and metabolism ($p < 0.05$) and biosynthesis of other secondary metabolites ($p < 0.05$), and down-regulated trend in the abundances of pathways related to amino acid metabolism ($p < 0.05$), translation ($p < 0.01$) and folding, sorting and degradation ($p < 0.05$). Intriguingly, most of these altered pathways were mainly prominent in the L-DHA group (Figure 5B).

3.6 Maternal DHA supplementation during lactation alters jejunum proteome of offspring mice

In order to understand the underlying biological effects of maternal DHA supplementation during lactation on the intestinal development of pups, we analyzed the proteomics of jejunum tissues from three groups with the DIA-MS method (Figure 6A). There were 9,641, 9,681, and 9,754 identified proteins and 113, 122, and 211 unique proteins in the control, L-DHA, and H-DHA groups, respectively, while 8,534 proteins overlapped among the three groups (Figures 6B,C). PCoA analysis showed that the control, L-DHA, and H-DHA groups were separated from each other (Figure 6D). Kernel density plot and correlation analysis between samples were presented in Supplementary Figures S2A,B.

Subsequently, the jejunal proteome profiles of the L-DHA and H-DHA groups were compared with those of the control group, individually. A total of 1,046 differentially expressed proteins (FC > 1.5, $p < 0.05$) were determined between the control group and the L-DHA group, among which 441 proteins were up-regulated and 605 proteins were down-regulated in the L-DHA group (Figure 7A). There were 873 differentially expressed proteins (FC > 1.5, $p < 0.05$) in total between the control group and the H-DHA group, and 457 proteins up-regulated and 316 proteins down-regulated in the H-DHA group (Figure 7B). Next, hierarchical clustering heat map showed the expression tendency for these differentially expressed proteins. The patterns of differentially expressed proteins were found to be similar in the L-DHA and H-DHA groups, while both were significantly different from the pattern in the control group (Figure 7C).

To further identify the effects of maternal DHA supplementation, GO enrichment analysis, including biological process (BP) analysis, molecular function (MF) analysis and cell component (CC) analysis, and KEGG pathway enrichment analysis were performed using differentially expressed proteins when the control group was compared with the L-DHA group or the H-DHA group, respectively. As shown in Figure 8A, between the control and H-DHA groups, BP analysis revealed that the metabolic and transport-related processes were significantly affected; terms mainly related to membrane, especially brush border membrane, were enriched in CC analysis; MF analysis showed that glucose: sodium symporter activity was significantly enriched. Meanwhile, KEGG pathways analysis showed

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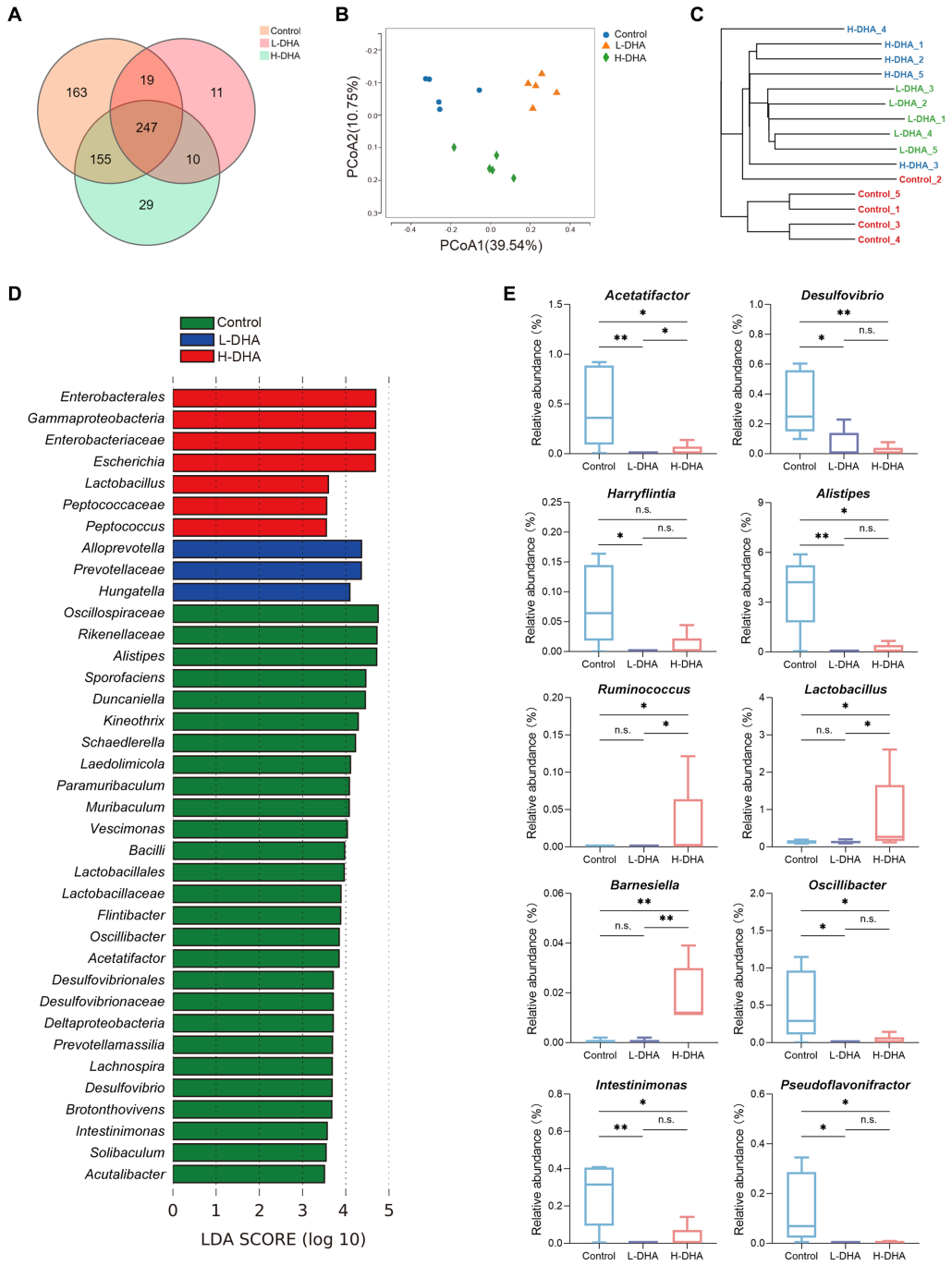

FIGURE 4
Effect of maternal DHA supplementation during lactation on the gut bacterial structure of weaning mice. **(A)** Venn diagram of the OTUs in different groups. **(B)** PCoA plot based on unweighted Unifrac distance in different groups. **(C)** UPGMA cluster tree based on unweighted Unifrac distance in different groups. **(D)** LEfSe analysis (LDA score ≥ 3.5) of the different gut microbiota in the genus level among three groups. **(E)** Significantly different genera in different groups. $n = 5$ per group. Significance was set at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$). L-DHA: 150 mg/(kg body weight day) DHA; H-DHA: 450 mg/(kg body weight · day) DHA. DHA, docosahexaenoic acid; OUT, operational taxonomic units; PCoA, principal coordinates analysis; UPGMA, unweighted pair group method with arithmetic mean; LEFSe, Linear discriminant analysis effect size; LDA, Linear discriminant analysis.

that carbohydrate digestion and absorption, fat digestion and absorption and protein digestion and absorption were significantly enriched (Figure 8B). Between the control and L-DHA groups, enrichment in CC analysis was similar to the results between the control and H-DHA groups, and protein digestion and absorption was also enriched in KEGG pathways analysis, but carbohydrate digestion and absorption as well as fat digestion and absorption were not affected (Supplementary Figures S2C,D). Heat map analysis on the representative proteins associated with nutrient absorption and metabolism in transport-related classifications among the three groups suggested that the abundance of these proteins was increased in DHA-treated groups (Figure 8C). These results indicated that

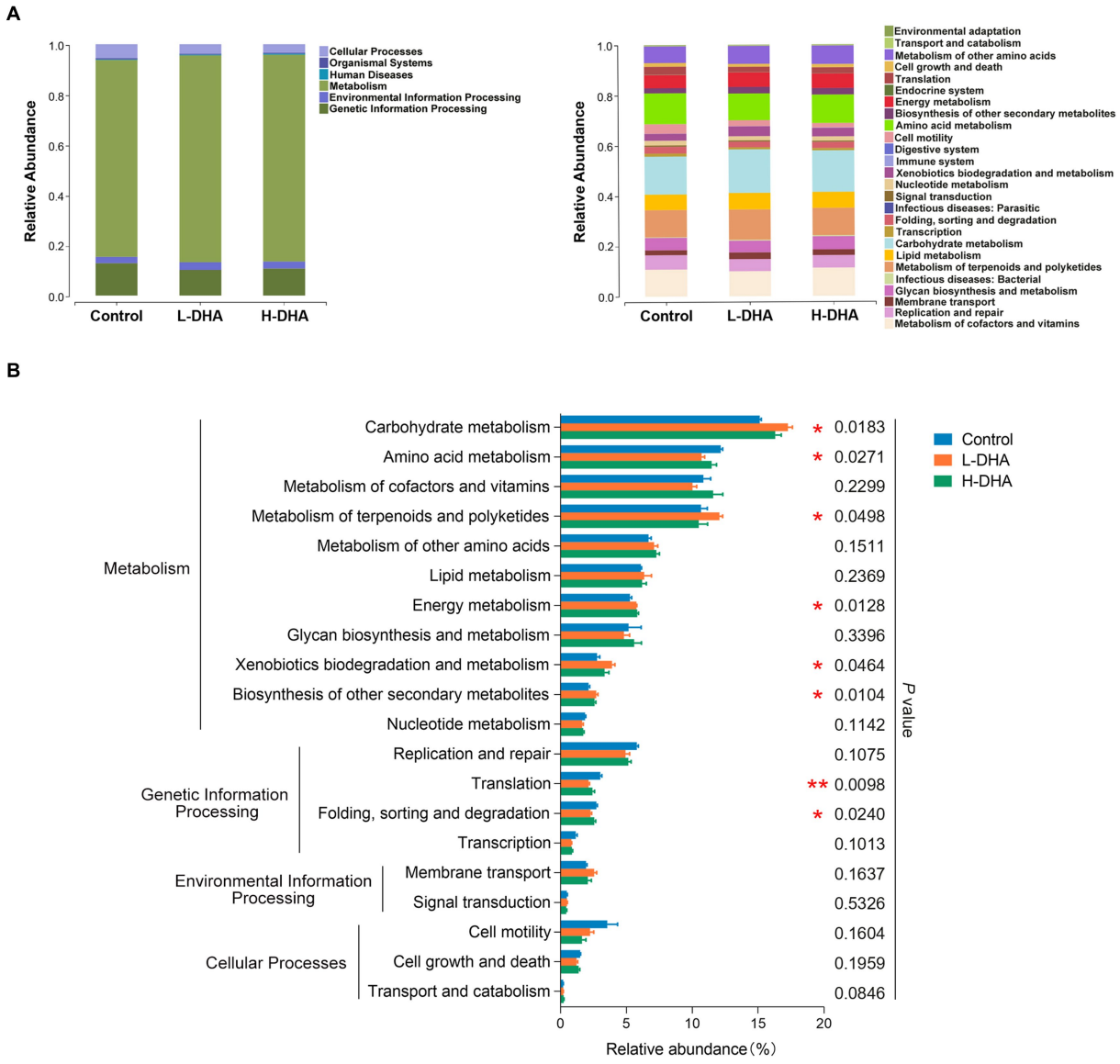

FIGURE 5
PICRUST functional prediction based on KEGG database in weaning mice. **(A)** Relative functional abundance at levels 1 and 2 among the control group, L-DHA group and H-DHA group. **(B)** Comparisons of KEGG pathways among the control group, L-DHA group and H-DHA group. $n = 5$ per group. Significance was set at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$). L-DHA: 150 mg/(kg body weight · day) DHA; H-DHA: 450 mg/(kg body weight day) DHA. DHA, docosahexaenoic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes.

maternal DHA supplementation during lactation may improve the capacity of jejunum absorption and transport in weaning mice.

3.7 Maternal DHA supplementation during lactation enhances the expressions of jejunal glucose transport-associated proteins of offspring mice

We further detected the expressions of genes related to glucose absorption and transport in the jejunum. Western blot analysis indicated that the expression of GLUT2 protein in the H-DHA group was significantly increased by 227% compared with the control group (Figures 9A,D; $p < 0.05$). Additionally, the *GLUT2* mRNA level in the L-DHA group and H-DHA group was 34 and 43% greater than that in the control group (Figure 9C; $p < 0.05$ and $p < 0.01$, respectively). The protein and mRNA expressions of SGLT1 in the jejunum were notably 275 and 57% higher in the H-DHA group than that in the control group (Figures 9B,E,F; both $p < 0.01$). Although not reaching statistical significance, the L-DHA group tended to elevate the mRNA expression of *SGLT1* (36% increase) and the protein expressions of GLUT2 (159% increase) and SGLT1 (94% increase), as compared with the control group (Figures 9D–F; $p = 0.0661$, $p = 0.1685$ and $p = 0.0711$, respectively). Meanwhile, we also determined the mRNA expressions of genes involved in lipid and protein transport in the jejunum, and the results showed that maternal DHA supplementation significantly modulated some of them. In the L-DHA and H-DHA groups, compared with the control group, the mRNA expression of *NPC1L1*

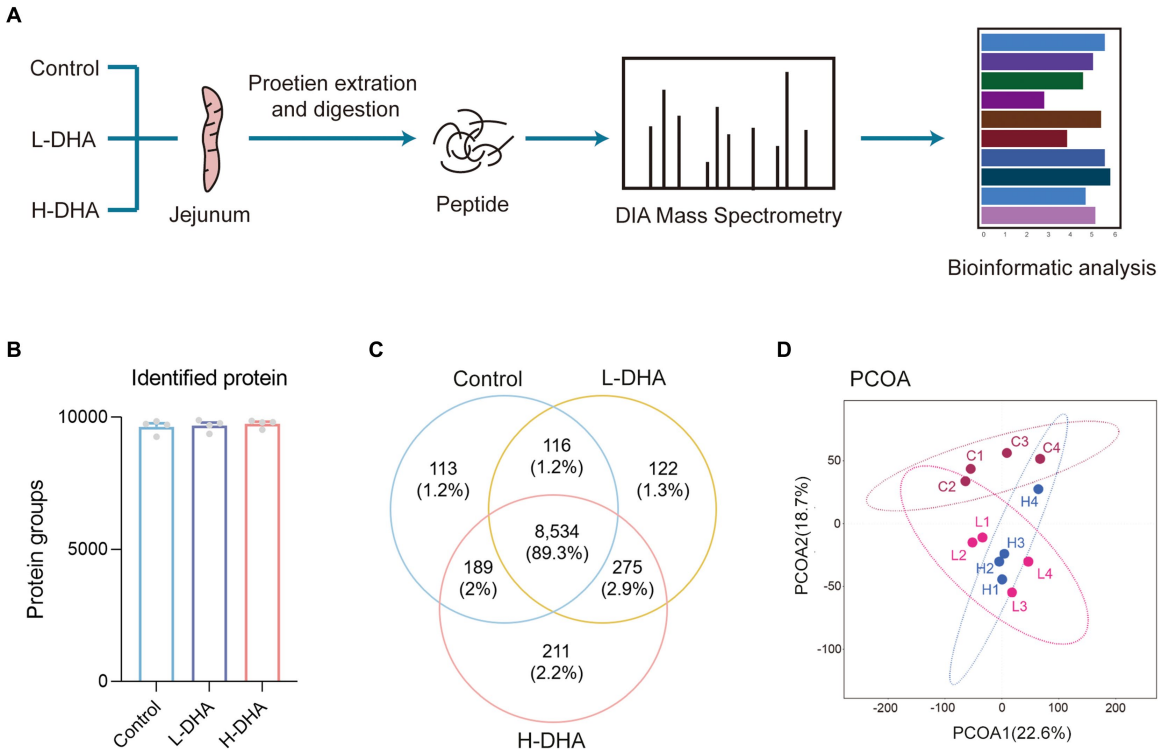

FIGURE 6
The workflow and profiling of weaning mice jejunum proteomes using LC–MS/MS. **(A)** Schematic diagram of LC–MS/MS experimental procedure in this study. **(B)** The total identified protein number in each group. **(C)** Venn diagram showing the total numbers of identified proteins among the three groups. **(D)** PCoA of all groups. Data were expressed as the mean ± SEM; $n = 4$ per group. L-DHA: 150 mg/(kg body weight day) DHA; H-DHA: 450 mg/(kg body weight · day) DHA. DHA, docosahexaenoic acid; PCoA, principal coordinates analysis.

was increased by 97 and 113% (Supplementary Figure S3A; $p < 0.05$ and $p < 0.01$, respectively), the mRNA expression of *CD36* was 87 and 101% greater (Supplementary Figure S3A; $p < 0.01$ and $p < 0.001$, respectively), and the mRNA level of *ASCT2* was up-regulated by 82 and 72% (Supplementary Figure S3B; both $p < 0.05$). Besides, compared with both the control group and L-DHA group, the H-DHA group exhibited a remarkable 234% and a 64% increase in the mRNA expression of *APOB* (Supplementary Figure S3A; $p < 0.001$ and $p < 0.05$, respectively). Intriguingly, the mRNA level of *APOA1* was 52 and 48% lower in the L-DHA group and the H-DHA group than in the control group (Supplementary Figure S3A; both $p < 0.01$).

3.8 Maternal DHA supplementation during lactation increases intestinal glucose absorption and tissue glycogen storage of offspring mice

We observed a significant increase in jejunum glucose concentrations in the L-DHA and H-DHA groups, a 0.36-fold increase and a 0.38-fold increase individually, as compared with the control group (Figure 10A; $p < 0.05$ and $p < 0.01$, respectively). The ileum glucose concentration in the H-DHA group was 22% greater than in the control group, and 25% higher than in the L-DHA group (Figure 10B; both $p < 0.05$). Notwithstanding, no significant difference in colon glucose concentrations was found among groups (Figure 10C). The glucose concentrations of ileum contents (29% decrease), colon contents (33% decrease) and feces (42% decrease) in the H-DHA group were dramatically lower than in the control group ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively); the colon contents glucose was also strongly reduced by 38% in the L-DHA group when compared with the control group ($p < 0.01$), whereas there were no significant differences in glucose concentrations of ileal contents and feces (Figures 10D–F). Furthermore, the glycogen levels of the liver and muscle were 0.17-fold and 0.88-fold higher in the H-DHA group than those in the control group (Figures 10G,H; both $p < 0.01$). In the L-DHA group, the liver glycogen concentration was increased by 13% compared to the control group (Figure 10G; $p < 0.05$), and the muscle glycogen level also exhibited a 57% increase, although not reaching statistical difference (Figure 10H).

To further demonstrate the reliability of our outcomes, the glucose and 4-FDG absorption test was conducted *in vivo*, using a co-load of 2 g/kg glucose and 10 mg/kg 4-FDG. According to the oral glucose tolerance test (OGTT), the blood glucose levels were higher at 15 min in the L-DHA group and the H-DHA group than in the control group (Figure 10I; both $p < 0.05$), but there was no significant difference in the AUC of OGTT among groups (Figure 10J). Compared with the control group, the L-DHA group increased the concentrations of 4-FDG at 30 and 60 min (Figure 10K; $p < 0.01$ and $p < 0.05$, respectively). Similarly, the H-DHA group exhibited higher serum 4-FDG contents than those in the control group (Figure 10K; both $p < 0.05$).

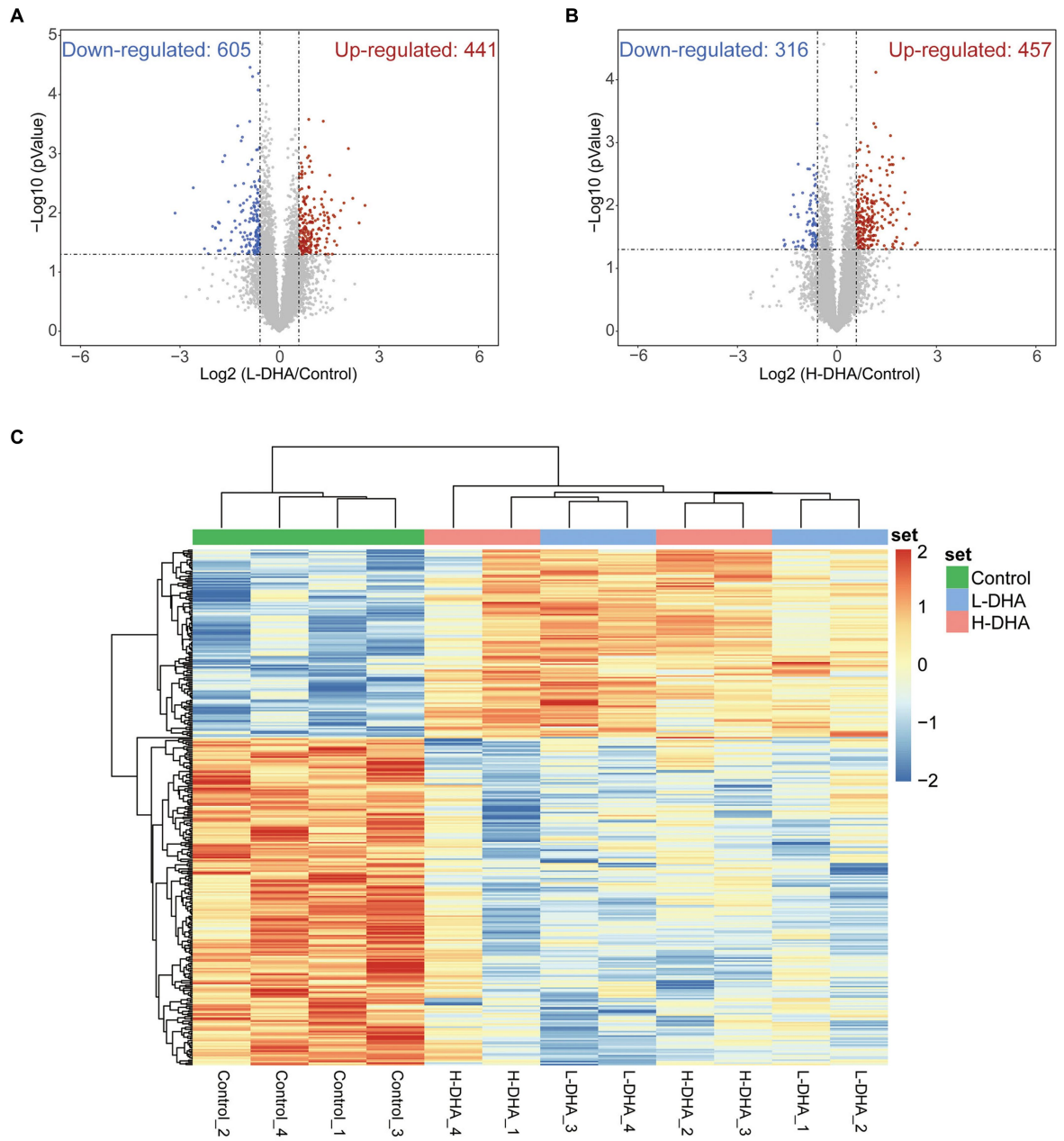

FIGURE 7
Identification of differentially expressed proteins in weaning mice. **(A)** Volcano plot of up-regulation or down-regulation of differentiated proteins for the L-DHA group compared with the control group. **(B)** Volcano plot of up-regulation or down-regulation of differentiated proteins for the H-DHA group compared with the control group. Red points represent up-regulated proteins in the L-DHA or H-DHA group, blue points represent down-regulated proteins in the L-DHA or H-DHA group (fold change >1.5, *p* value <0.05) and gray points represent unchanged proteins. **(C)** The heat map of hierarchical clustering analysis on the differentially expressed proteins in the three groups. $n = 4$ per group. L-DHA: 150 mg/(kg body weight day) DHA; H-DHA: 450 mg/(kg body weight day) DHA. DHA, docosahexaenoic acid.

3.9 Maternal DHA supplementation during lactation up-regulates the mTOR signaling pathway of offspring mice

Phosphorylation levels of mTOR and downstream p70S6 kinase and S6 ribosomal protein were determined to further explore the possible molecular mechanism underlying the effect of maternal DHA supplementation during lactation to promote intestinal glucose uptake in pups. The results revealed that the phosphorylation of mTOR (S2448), p70S6 kinase (T389) and S6 ribosomal protein (S240/244) were notably up-regulated in the H-DHA group, 103% increase, 100% increase and 257% increase respectively, compared with the control group (Figures 11A–D; $p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively). A marked 56% higher level of phosphorylation of p70S6 kinase (T389) was observed in the L-DHA group than in the control group (Figure 11C; $p < 0.05$), whereas no differences were found in the phosphorylation levels of mTOR (S2448) and S6 ribosomal protein (S240/244) between the control and L-DHA groups.

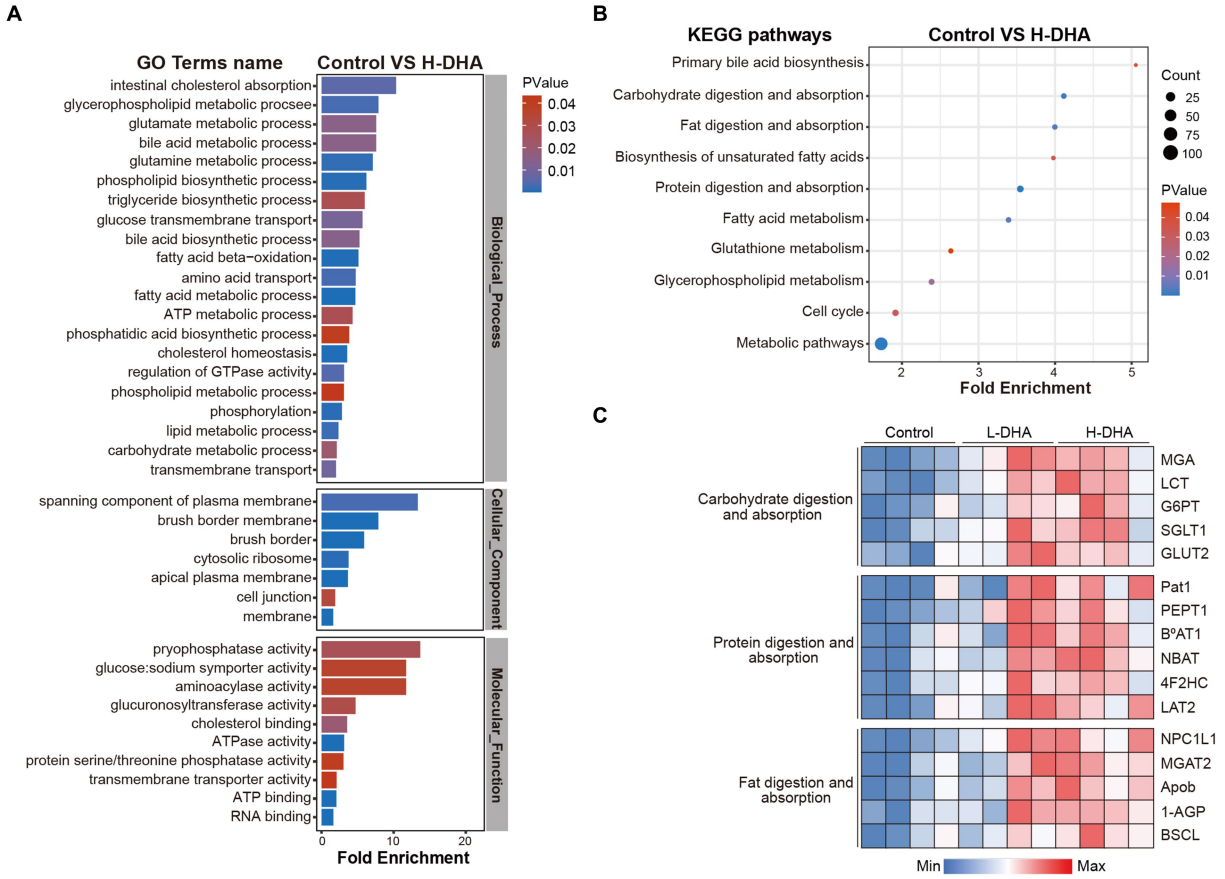

FIGURE 8
Effects of maternal DHA supplementation during lactation on the jejunum proteomic level in weaning mice. **(A)** GO analysis and **(B)** KEGG pathways analysis in control and H-DHA groups. **(C)** Heat map analysis based on the intensity of representative proteins in classifications related to nutritional absorption and metabolism for the control, L-DHA, and H-DHA groups. $n = 4$ per group. L-DHA: 150 mg/(kg body weight day) DHA; H-DHA: 450 mg/(kg body weight day) DHA. DHA, docosahexaenoic acid; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

4 Discussion

Early life nutrition plays a crucial role in shaping the development of bodily systems and programming the gut microbiota, ultimately influencing lifelong health (25, 26). The transition from suckling to weaning is a pivotal period prone to intestinal dysfunction and dysbiosis, often resulting in growth retardation. Therefore, mitigating the adverse effects of weaning stress on offspring has become a significant public health concern. Nowadays, perinatal maternal nutrition is gaining increased attention due to its close association with the early nutrition of offspring. DHA, produced only in marginal amounts by the body, is recognized as a vital nutrient crucial for visual and brain development. Daily supplementation with DHA is a common clinical practice for perinatal women (7, 8). Despite numerous studies focusing on maternal DHA supplementation during pregnancy and lactation, very few researches have investigated its impact on gut development, including the underlying biochemical and molecular mechanisms. In this study, we found that maternal DHA feeding improved exercise performance, modulated gut microbiota, increased intestinal glucose absorption capacity and enhanced tissue glycogen storage in pups.

Considering the mice used in the present study were normal and non-defective, the effect of maternal DHA supplementation on the body and organ weights of the pups was likely to be negligible. Numerous studies have reported several beneficial effects of fish oil and DHA, such as increased muscle mass and function in healthy older adults following the oral administration of fish oil (27), and improved physical exercise performance in adult mice fed ethanol extract from the red seaweed *Gloiopeltis furcata*, in which DHA is the prominent active ingredient (28). It is essential to underscore that our investigation specifically addresses the influence of maternal DHA supplementation during lactation, distinguishing it from prior research that focused on DHA intake during pregnancy. The experimental design used in this study is based on Richard's study on DHA supplementation during lactation, considering 1–2 days before parturition as the adaptation period for lactational exposure (29, 30). The parturition period for mice is GD19-GD21, and GD19 is considered full term for fetal mice. By GD18, the major organs of fetal mice are almost completely developed, and the treatment beginning at GD18 is expected to have limited impact on fetal mice at late pregnancy stage.

Notably, our findings reveal an enhancement in exercise performance among offspring with maternal DHA supplementation

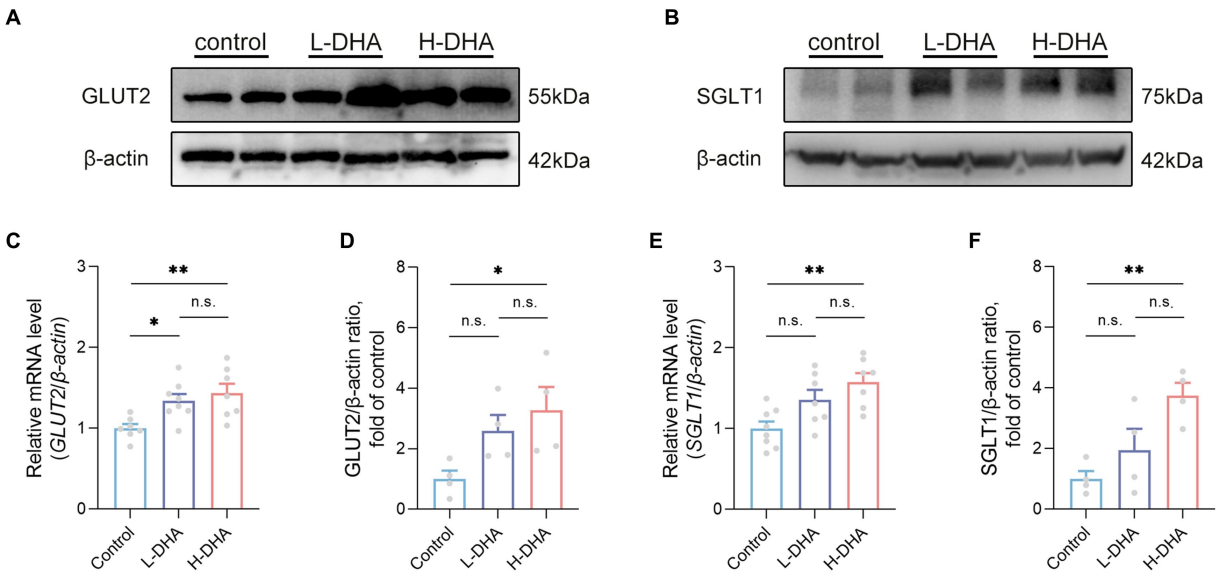

FIGURE 9
Effect of maternal DHA supplementation during lactation on the jejunal glucose transport-associated gene expression in weaning mice. Representative Western blots of **(A)** GLUT2 and **(B)** SGLT1, and **(D,F)** their relative band intensities in jejunal tissue of mice from different groups. n = 4 per group. Relative mRNA levels of **(C)** *GLUT2* and **(E)** *SGLT1* in jejunal tissue of mice from different groups. $n = 7–8$ per group. Data were expressed as the mean ± SEM. Significance was set at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$). L-DHA: 150 mg/(kg body weight day) DHA; H-DHA: 450 mg/(kg body weight · day) DHA. DHA, docosahexaenoic acid; GLUT2, glucose transporter 2; SGLT1, sodium glucose co-transporter 1.

during lactation, aligning with distinct results from another study that demonstrated maternal DHA intake during pregnancy mitigating grip strength impairment induced by MeHg in mouse pups (31). This emphasizes the critical consideration of the specific timing of DHA supplementation. Physical fatigue primarily stems from energy expenditure and a deficiency of energy during exercise. Carbohydrates serve as the principal energy source when skeletal muscles engage in activity, primarily stored in the liver and muscles as glycogen, with a minor amount circulating in the bloodstream as glucose (32). During exercise, muscle glucose utilization is expedited and liver glycogen may break down to glucose, transported through the bloodstream to working muscles to support physical activity (33). Thereby, as the predominant source of glycolysis for energy production, maintaining optimal tissue glycogen concentrations in both muscle and liver is recognized as crucial for increasing endurance capacity and delaying the onset of fatigue (34). Our study indicated that maternal DHA supplementation during lactation could increase energy storage in mouse pups, providing a potential explanation for their improved exercise performance.

Although carbohydrate metabolism takes precedence, catabolized fat also could supply partial energy in the phase of exercise (34). TG would be hydrolyzed to free fatty acids and then provided for working muscles as fuel during exercise (35). In agreement with the research reported by Drouin et al., we found that the offspring from the DHA-treated groups also exhibited a higher serum TG, which may result from the content of DHA, mostly as the form of TG, was increased in milk after maternal DHA intake (30, 36). It is worth mentioning that, distinguished from adults, infants are in a period of negative energy balance and require greater lipid accumulation to support the high metabolic demands of physical development, especially for the development of the brain (37). Therefore, we deemed that the acceptable elevation of serum TG in our result did not adversely impact the pups.

During the early stages of life, gut maturation is essential to provide adequate nutritional support for growth development, and abnormal intestinal development commonly leads to feeding intolerance, impaired nutrient absorption and even necrotizing enterocolitis (38). Villus height, villus surface area and V/C ratios are widely used to reflect the healthy state of intestinal function and absorption in the small intestine and are correlated with the intestinal capacity to absorb and transport dietary nutrients (39). In the DHA-treated groups, we observed increased villus height, villus surface area as well as V/C ratios. Previous studies have reported that a diet supplemented with fish oil could mitigate LPS-induced intestinal morphology injury, evidenced by increased jejunal and ileal villus height and V/C ratios (40). Additionally, maternal fish oil supplementation has been shown to improve intestinal morphology in weaning pigs (41). DHA has also been demonstrated to ameliorate gut morphology injury following heatstroke (42). Unlike previous studies, our results demonstrated that maternal DHA supplementation during lactation may facilitate the intestinal absorption function of offspring mice under normal conditions, which provides a novel and viable intervention to promote infant gut development and health.

The gut microbiota is strongly associated with gut health and is involved in diverse functions of the host, including digestion, nutrition absorption, metabolism, pathogen protection, and immunity. There is growing evidence that supplementation with DHA is beneficial to gut flora (43, 44). The decreased richness index of gut microbiota in the L-DHA group is inconsistent with the study reported by Han et al., which demonstrated maternal DHA intake insignificantly affected the richness index of pups (14). Although not significant, our data showed a decreasing trend in the ratio of *Bacillota*/*Bacteroidetes* reported as a

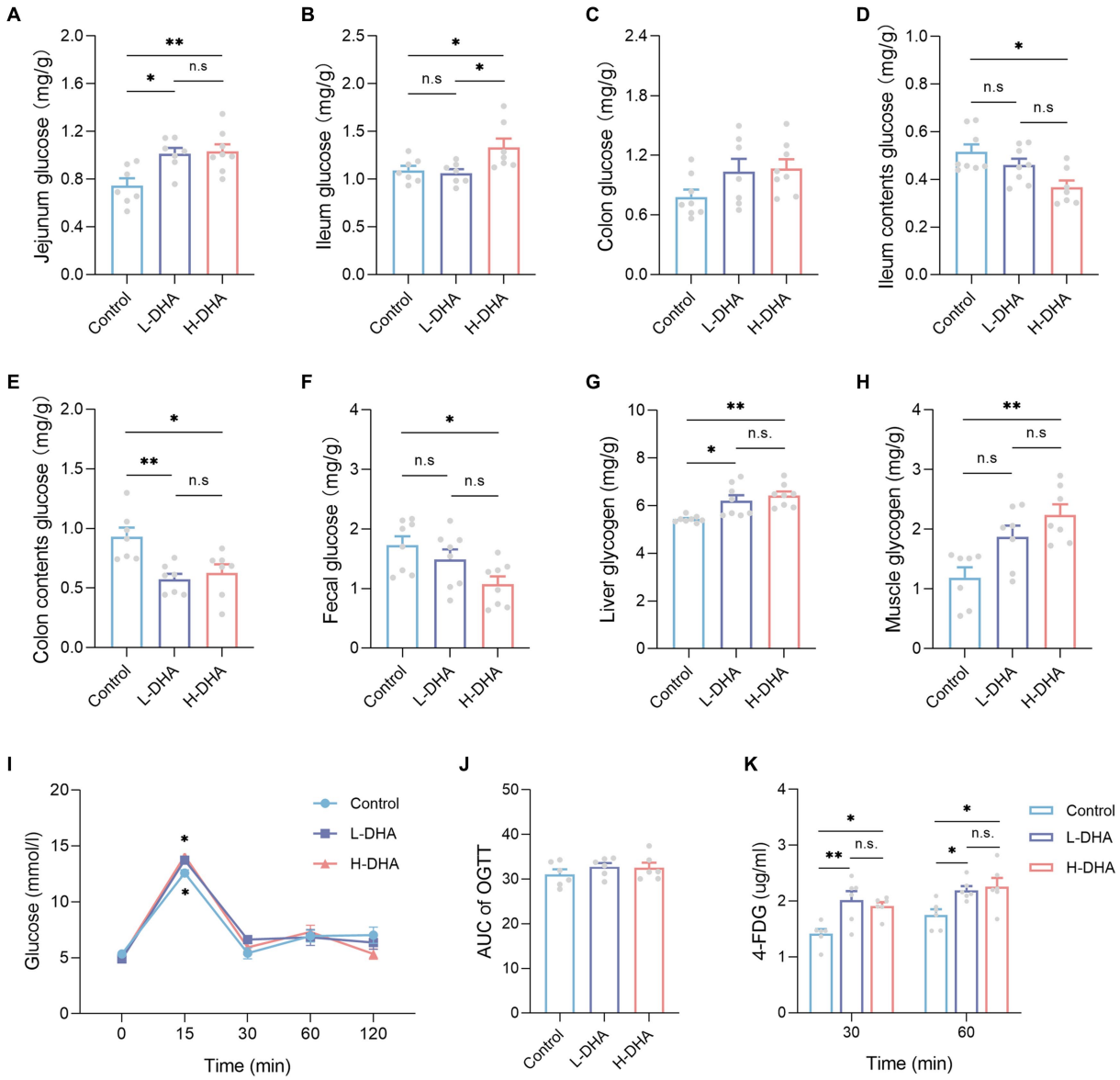

FIGURE 10
Effect of maternal DHA supplementation during lactation on intestinal glucose absorption and tissue glycogen storage in weaning mice. **(A-H)** Mice untreated with glucose and 4-FDG before sacrifice. $n = 7–8$ per group. The levels of **(A)** jejunum glucose, **(B)** ileum glucose, **(C)** colon glucose, **(D)** ileum contents glucose, **(E)** colon contents glucose, **(F)** feces glucose, **(G)** liver glycogen, and **(H)** muscle glycogen in weaning mice. **(I-K)** 2 g/kg glucose and 10 mg/kg 4-FDG were orally co-administered followed by blood glucose and 4-FDG quantitation at various time points. $n = 6$ per group. **(I)** OGTT and **(J)** AUC of weaning mice subjected to glucose/4-FDG oral gavage. **(K)** Serum 4-FDG levels of weaning mice subjected to glucose/4-FDG oral gavage. Data were expressed as the mean ± SEM. Significance was set at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$). L-DHA: 150 mg/(kg body weight day) DHA; H-DHA: 450 mg/(kg body weight day) DHA. DHA, docosahexaenoic acid; OGTT, oral glucose tolerance test; AUC, area under the curve; 4-FDG, 4-deoxy-4-fluoro-D-glucose.

hallmark of obesity in the DHA-treated groups (45). Additionally, the relative abundance of specific bacteria changed significantly among the groups at the genus level. In the H-DHA group, the relative abundances of several genera, such as *Ruminococcus* associated with short-chain-fatty-acid-producing, *Lactobacillus* that improves gut integrity and counteracts endotoxemia and inflammation, and *Barnesiella* related to taurine-conjugated bile acids, were increased, which are consistent with previous studies (44, 46, 47). In contrast, we found the relative abundances of *Acetatifactor*, *Desulfovibrio*, *Harryflintia*, *Alistipes*, *Oscillibacter*, *Intestinimonas* and *Pseudoflavonifractor* identified as harmful inflammation-associated or obesity-associated bacteria were lower in the DHA-treated group (48–51). The decrease in the number of harmful bacteria was more than the increase in the number of beneficial bacteria, which may account for the reduction of the gut flora richness. The variations of *Desulfovibrio* and *Alistipes* agree with the studies reported by Ran et al. and Zhang et al. (52, 53), but the alterations of *Oscillibacter* and *Intestinimonas* are inconsistent with the results reported by Qian et al. and Zhuang et al. (54, 55). Furthermore, the KEGG functional prediction of fecal microbiota revealed that the metabolic pathways

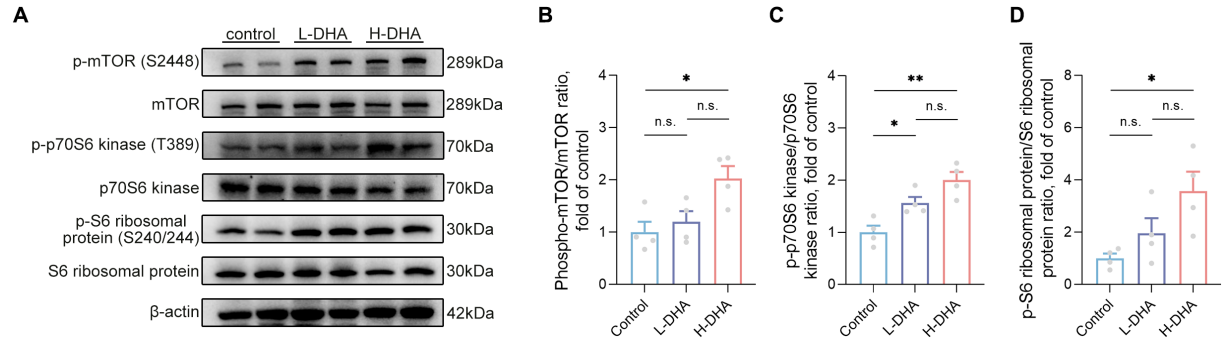

FIGURE 11
Effect of maternal DHA supplementation during lactation on the mTOR signaling pathway in weaning mice. **(A)** Representative Western blots of p-mTOR (S2448), total mTOR, p-p70S6 kinase (T389), total p70S6 kinase, p-S6 ribosomal protein (S240/244) and total S6 ribosomal protein, and **(B, C, D)** the relative band intensities of phosphorylation levels normalized to their respective total proteins in jejunal tissue of mice from different groups. Data were expressed as the mean ± SEM; $n = 4$ per group. Significance was set at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$). L-DHA: 150 mg/(kg body weight day) DHA; H-DHA: 450 mg/(kg body weight day) DHA. DHA, docosahexaenoic acid; mTOR, mammalian target of rapamycin.

involved in carbohydrate metabolism and energy metabolism were up-regulated in the DHA-treated groups, suggesting an enhanced metabolism *in vivo*, which was conducive to growth and development in early life. In the present study, DHA maternal intervention showed an advantageous effect on programming the gut microbiota of pups.

The transition from breastfeeding to weaning is challenged by dietary changes that predispose to intestinal digestive and absorption disorders (12). There is mounting evidence that DHA could mainly promote neurogenesis and synaptogenesis in the brain (56, 57). However, our results revealed that maternal DHA supplementation during lactation also seemed to facilitate intestinal development in pups, which is different from the primary role of DHA found in previous studies. Therefore, we used emerging non-targeted proteomics to systematically investigate the potential biological effects of maternal DHA supplementation on the intestine of weaning mice. Previous study has reported that increased nutrient digestibility is associated with increased expression of enteric nutrient transporter protein genes (58). In the jejunal proteome results, KEGG and GO functional enrichment analyses that showed up-regulation of intestinal nutrients, including carbohydrate, lipid and protein, digestion and absorption indicated that maternal DHA supplementation during lactation affected jejunal membrane transport function, more pronounced in the H-DHA group, implying an improved nutrient transport and absorption capacity in the offspring, which was consistent with changes in intestinal morphology. It was further verified at the mRNA level that the expression of genes associated with glucose transport were all increased, whereas genes related to lipid and protein transport were only partially up- or even down-regulated. Meanwhile, the alterations in the functional annotation of the intestinal flora were primarily focused on carbohydrate metabolism, hence we speculated that DHA supplementation during lactation in dams more predominantly affected intestinal glucose absorption in the offspring. SGLT1 is responsible for the transport of glucose from the lumen to the epithelial cells, and subsequently promotes the glucose transporter protein, GLUT2, to mediate the transport of glucose through the basolateral membrane to the mesenchyme and thus into the blood circulation (59). Glucose uptake in the intestine is accomplished by the interplay of SGLT1 and GLUT2 in collaboration with each other. Moreover, we found the abundance of glucose transport proteins, both GLUT2 and SGLT1, in the jejunum of weaning offspring mice were enhanced in the DHA-supplemented groups, and there appeared to be a dose-dependent effect of this gain. Similar promotion of intestinal glucose absorption has been documented in previous studies where sows exposed to an n-3 LCPUFA enriched diet showed enhanced intestinal absorption in their weaning offspring (15, 60). However, our study more directly elucidates the role of maternal DHA intake during lactation and provides insights into the potentially effective dosage of supplementation.

Intestinal tissue is a pivotal organ in the regulation of glucose metabolism. We found that glucose levels were elevated in small intestinal tissues but decreased in intestinal contents and feces in the DHA-treated groups. Although commonly employed to assess insulin regulation of blood glucose, the OGTT is an important tool for assessing intestinal glucose absorption. Nevertheless, the OGTT is not a robust representation of intestinal glucose absorption capacity because it is related to the process of insulin regulation of blood glucose. The glucose analogue 4-FDG, a substrate for both GLUTs and SGLTs, is not metabolized *in vivo,* and could more exactly reflect intestinal glucose absorption (61). The data of our glucose uptake test *in vivo* further confirmed that maternal DHA supplementation could enhance intestinal glucose absorption in pups without disrupting glucose homeostasis. Glycogen, a branched polymer of glucose, acts as an energy reserve when nutritional sufficiency for consumption in times of need (62). Since gluconeogenesis and glucose utilization are expedited during exercise, high concentrations of muscle and liver glycogen prior to exercise are believed to be essential for exercise performance (33). Consistently, the increased muscle and liver glycogen contents in the DHA-supplemented groups indicated tissue energy storage in pups was enhanced, which in turn supported the outcomes of our behavioral tests.

The mTOR signaling pathway is well known for its crucial function in regulating translation, protein synthesis and catabolism, nutrient sensing, energy utilization and growth factor signaling. It consists of two distinct multi-subunit complexes known as mTOR complex 1/2 (mTORC1/2) (63). Previous studies have shown that mTORC1-driven translational regulation is mainly dependent on catalyzing the phosphorylation of eukaryotic translation initiation factor 4E

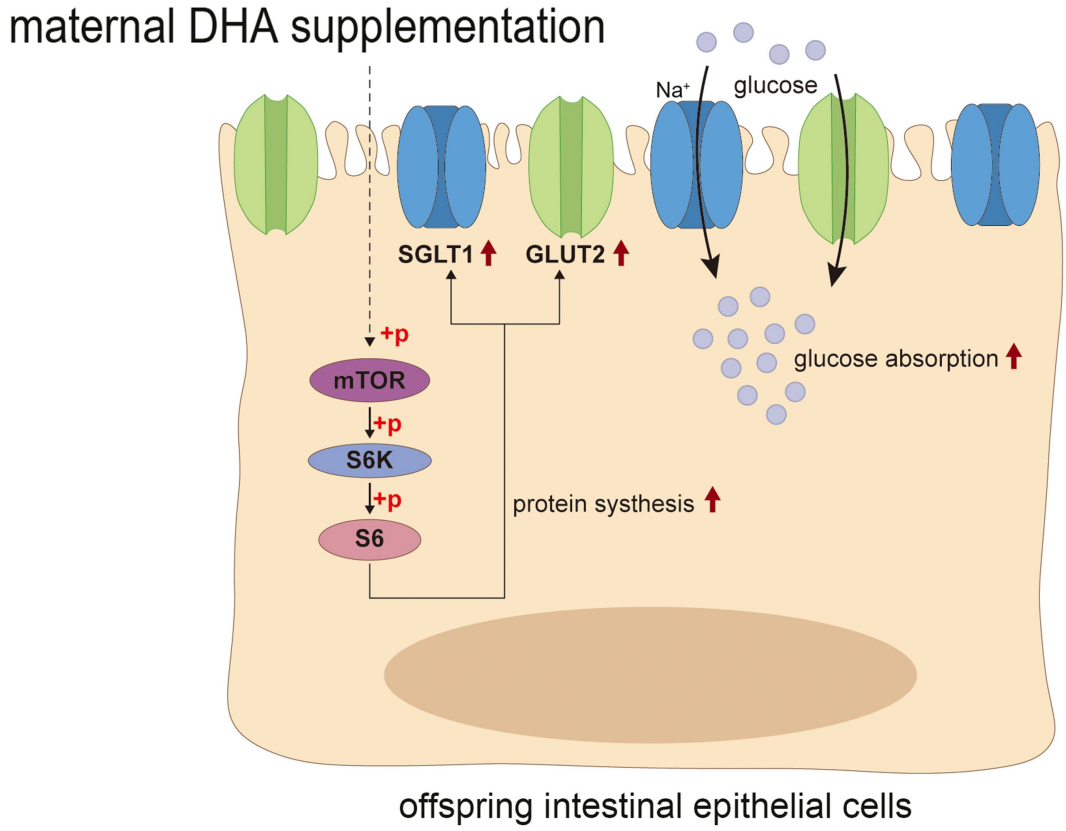

FIGURE 12
Possible mechanism of maternal DHA supplementation during lactation promotes intestinal glucose absorption in weaning mice. DHA, docosahexaenoic acid.

(eIF4E)-binding protein (4E-BP), p70S6 kinase, and its downstream players to enhance translation and protein synthesis, which is essential for mediating cellular proliferation and growth (64). Moreover, mTOR is believed to be closely linked to intestinal epithelium growth and proliferation. Systemic administration of rapamycin, a canonical mTOR inhibitor, has been shown to reduce intestinal surface area and disrupt intestinal regeneration (65). Activation of mTOR plays a critical role in promoting intestinal adaptation and regulating intestinal epithelial homeostasis and regeneration (65, 66). Notably, some studies suggested that the expression of GLUT2 could be regulated by the Akt/mTOR signaling pathway (67, 68). In the present study, we found that the improved intestinal tissue morphology, the enhanced intestinal glucose absorption, and the increased expression of glucose transport-associated proteins in offspring with maternal DHA supplementation may be attributed to the activation of the mTOR signaling pathway. This finding suggested that maternal DHA supplementation during lactation might modulate the mTOR signaling pathway in the intestines of breastfed offspring, thereby promoting intestinal glucose absorption (Figure 12). However, the specific mechanisms require further investigation.

5 Conclusion

In summary, the results presented in this work demonstrated that maternal DHA supplementation during lactation enhances the intestinal glucose absorption. This effect is achieved by up-regulating the mTOR pathway and increasing the expressions of glucose transporters in the jejunum of weaning offspring. Furthermore, maternal DHA supplementation increases glycogen storage within the liver and muscle, leading to improved exercise performance, and beneficially alters the structure of gut microbiome in weaning offspring. These findings lay the foundation for advocating maternal DHA supplementation during lactation as a viable strategy to foster the intestinal development of weaning offspring. However, it is important to emphasize that our study was conducted on mice, and while the results are promising, any further application to lactating women should be rigorously tested through clinical trials in human populations.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject; PRJNA1102858, https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1102858 and ProteomeXchange; PXD052063, https://www.ebi.ac.uk/pride/archive/projects/PXD052063.

Ethics statement

The animal study was approved by the Institutional Review Board and the Animal Care and Use Committee of Shanghai Xinhua

Hospital. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DL: Writing – original draft, Investigation, Methodology. DY: Writing – original draft, Methodology. GH: Investigation, Writing – original draft. JZ: Writing – original draft, Data curation. XS: Writing – review & editing, Funding acquisition. LQ: Writing – review & editing, Funding acquisition.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1423576/full#supplementary-material

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Glossary

DHA	docosahexaenoic acid
LCPUFAs	long-chain polyunsaturated fatty acids
PUFAs	polyunsaturated fatty acids
GD	gestational day
PD	postpartum day
4-FDG	glucose/4-deoxy-4-fluoro-D-glucose
TC	total cholesterol
TG	total triglyceride
TP	total protein
H&E	hematoxylin and eosin
V/C	villus height to crypt depth
AUC	area under the curve
FASP	filter-aided sample preparation
DIA	data independent acquisition
GLUT2	glucose transporter 2
SGLT1	sodium glucose co-transporter 1
PEPT1	peptide transporter 1
ASCT2	alanine-serine-cysteine transporter 2
B0AT1	sodium-dependent neutral amino acid transporter 1
EAAT1	excitatory amino acid transporter 1
EAAT3	excitatory amino acid transporter 3
y + LAT1	L-type amino acid transporter 1
4F2hc	4F2 cell-surface antigen heavy chain
CAT1	cationic amino acid transporter 1
NPC1L1	Niemann Pick C1 like 1
APOA1	apolipoprotein A-1
APOA4	apolipoprotein A-4
APOB	apolipoprotein B
FABP2	fatty acid binding protein 2
FATP4	fatty acid binding protein 4
RIPA	radioimmunoprecipitation assay
OTUs	operational taxonomic units
PCoA	principal coordinates analysis
UPGMA	unweighted pair group method with arithmetic mean
LEfSe	linear discriminant analysis coupled with effect size measurements
KEGG	Kyoto Encyclopedia of Genes and Genomes
FC	fold change
GO	gene ontology
SEM	standard error of the mean
ANOVA	analysis of variance
BP	biological process
MF	molecular function
CC	cell component
OGTT	oral glucose tolerance test
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
eIF4E	eukaryotic translation initiation factor 4E
4E-BP	eIF4E-binding protein

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The association of different types of human milk with bronchopulmonary dysplasia in preterm infants

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Objective: To evaluate the association between different types of human milk feeds and bronchopulmonary dysplasia (BPD) in preterm infants.

Methods: Data on dispensed mother's own milk (MOM) and donor human milk (DHM) from Leipzig Milk Bank for hospitalized infants with a gestational age (GA) ≤32 weeks observed from birth to 36 weeks' postmenstrual age or prior discharge were used. BPD was assessed based on documented International Statistical Classification of Diseases and Related Health Problems (ICD) diagnosis and on electronic hospital records (EHR) of data on ventilation and oxygen supplementation. Associations of dispensed milk feed variations with BPD were investigated using logistic regressions in crude and adjusted models.

Results: 866 infants were included with a BPD prevalence of 15.4% (EHR) and 23.2% (ICD). The mean GA was 29.1 weeks. The majority (84.4%, $n = 746$) of infants were nurtured with a mix of MOM, DHM supplemented by formula or parenteral (other) nutrition during hospitalization. For which, MOM comprised the highest median [Q1–Q3] percentage proportion (53[31–81] %) of this mix. Exclusive fresh milk and exclusive MOM feeds were dispensed on a mean of 40 and 34% patient-days, respectively. Statistically significant associations with lower BPD incidence were only observed for 70–80% MOM vs. DHM, and 60% fresh vs. frozen milk, in crude and adjusted models.

Conclusion: Our findings suggest a protective association of MOM and fresh milk with lower odds of BPD, which may be dependent on the proportion of MOM or fresh milk administered. These results highlight the importance of MOM as an ideal source of nutrition during early infancy.

KEYWORDS

bronchopulmonary dysplasia (BPD), donor human milk (DHM), human milk, Leipzig Donor Human Milk Bank (LMB), pasteurization, mother's own milk (MOM)

1 Introduction

Human milk is the gold standard of enteral nutrition for preterm infants, promoting their health. When mother's milk is not enough or available, donor human milk (DHM) is preferred over preterm formula (PF) (1, 2). The beneficial effects of human milk among very low birth weight (VLBW) infants, and particularly in reducing risks of necrotizing enterocolitis (NEC),

late-onset-sepsis (LOS), and bronchopulmonary dysplasia (BPD) are well documented (3–8). These benefits are attributed to its high content of bioactive components, antioxidant agents and immune modulating factor that support the infant's immune system (9, 10).

BPD, a chronic lung disease and common complication after preterm birth, especially affects infants born at <28 weeks' gestational age (GA), with higher risk of mortality, morbidity, and long-term pulmonary consequences (11, 12). In addition to the required parenteral nutrition for preterm infants, optimal and early enteral nutrition is considered to be essential for normal lung development and might have a protective effect against BPD (13–15). However, it is uncertain whether a specific type of human milk (e.g., MOM or DHM) has an influence on the development of BPD.

Studies have shown that the macronutrient and micronutrient composition of MOM and DHM differs (16, 17), which might have adverse infant outcomes such as BPD. However, comparisons between MOM and DHM feeds do not show MOM's superiority against BPD (18, 19). Studies comparing raw (unpasteurized) human milk with pasteurized human milk are scarce and findings are inconclusive (20, 21). Pasteurization may compromise human milk's immunological components, increasing the risk of infection-related complications, including BPD (22, 23). Additionally, freezing and thawing human milk may have similar effects (24). Overall, evidence is limited, with few studies comparing different human milk types and their associations with infant outcomes, as noted in our previous scoping review (25).

A limitation of previous studies is that the assignment into comparison groups by percentage or patterns of human milk fed is heterogeneous and making it challenging to interpret and compare results (26). Furthermore, the relative proportions of MOM and DHM to categorize infants by their nutrition are usually not reported in these studies (17). The use of arbitrary cut-offs (e.g., 75% DHM) may not be applicable in otherwise different populations and settings (27).

The definition of BPD has been revised several times over the past years, based on several additional parameters (28). Of note, there is currently no worldwide accepted or practiced BPD definition (29). Thus, we used two distinct operationalizations of BPD in this study: (i) a main or secondary diagnosis of BPD using the International Statistical Classification of Diseases and Related Health Problems (ICD-10-GM, code P27.1) in administrative hospital data and (ii) classification of BPD cases based electronic hospital record (EHR) data on ventilation and oxygen needs following the National Institute of Child Health & Human Development (NICHD) consensus definition from 2000 (30), similar to previous studies (3, 19, 31). Therefore, this study's objective was to investigate the associations of fresh, frozen, raw and pasteurized MOM and/or DHM milk feeds dispensed from the Leipzig Donor Human Milk Bank (LMB) with BPD in infants born at ≤32 weeks GA, using different cutoffs and the two afore-mentioned operationalizations of BPD.

2 Methods

2.1 Data sources and study variables

The LMB of Leipzig University Medical Center provides a supply of human milk for infants whose mothers cannot express human milk. Retrospective data on daily dispensed milk feeds from 2012 up to 2019 comprising MOM, DHM, fresh, frozen, raw and pasteurized milk were used for the current analysis (32). These data were merged with electronic hospital records by the Data Integration Center (Datenintegrationszentrum; DIZ), which contained additional information on general characteristics like child sex, age at admission, birth weight, morbidity, and parameters required to derive the BPD outcome. All data were restricted to infants that were born at ≤32 weeks GA. The observational period for infants <32 weeks GA was from birth to 36 weeks' postmenstrual age or discharge, whichever occurred first, i.e., the time at which BPD diagnosis is established. For children with exactly 32 weeks GA, per NICHD consensus definition, the observational period was >28 but <56 days' postnatal life or discharge. The Ethics board of the Medical Faculty at Leipzig University approved this study (#026/23).

In principle, frozen milk is stored for a maximum of 6 months at −20°C and chilled milk is kept for a maximum of 3 days at 4°C. DHM is pasteurized if the donor is cytomegalovirus (CMV) positive or the skin microbial count is over 10^4/mL, whereas MOM is pasteurized for preterm infants with a GA of <28 weeks and CMV positive mothers until 32 weeks GA. For MOM, a maximum skin microbial count of 10^5 is acceptable. The milk is processed by Holder Pasteurization (62.5 degrees for 30 min) (32). In addition, the milk is fortified with a bovine based multi-component fortifier for preterm infants, mostly with a birth weight < 1,500 g, when an enteral intake of 70–100 mL/kg/day milk is reached. However, there was no information on actual milk volume, actual milk fed to the infant and/or biological composition of MOM or DHM. Thus, data on dispensed human milk feeds were weighted by the inverse of the count of different milk types dispensed on a given day as a proxy for the changing feeding type. The numerical contributions of these milk types were then subsequently determined. Although these data may be prone to some error, for instance, if clinically required nutrition requirements changed during the day, we previously showed plausible complex intra-individual patterns during the first 100 days of hospitalization (32). These findings highlighted the importance of selecting cut-offs based on the underlying research population and the desired contrast. Thereby potentially improving accuracy in grouping infants into distinct human milk feeds accounting for co-exposure to each milk feed.

2.2 BPD definition

The first BPD operationalization was the extraction of a documented physician's diagnosis based on the ICD-10-GM, code P27.1 from the electronic discharge summary used for administrative purposes. For the second operationalization, the ventilation parameters and oxygen supply over the course of hospitalization were extracted from the patients' electronic hospital records (EHR). These included high-resolution data from medical ventilation devices and patient monitors. The data were subjected to an automated algorithm to identify BPD cases according to the consensus definition formed by the NICHD Workshop in 2000 (30). Specifically, treatment with oxygen >21% for at least 28 days meant that the infant received oxygen >21% for more than 12 h on each of the 28 days, which had to be achieved cumulatively but not necessarily consecutively in the observational period (see above) from birth. BPD severity based on EHR data was investigated, although sample size was very low. However, BPD severity was not reflected in the other operationalization by ICD code. Data on dispensed milk feeds were available from a total of 2,562 infants, but only 866 infants born at ≤32 gestational weeks

had complete data set of dispensed milk, BPD outcomes, and other relevant characteristics for analysis (Figure 1).

2.3 Statistical analysis

Data on daily dispensed milk feeds were analyzed during the observational period according to the milk type, classified by MOM/DHM, fresh/frozen and pasteurized/raw (non-pasteurized). On some patient-days, mixed milk types (e.g., a portion MOM and a portion DHM) were dispensed which was accounted for by applying weights (e.g., 0.5 in case of two components). On some patient-days, no human milk was dispensed because infants received parenteral or other forms of enteral nutrition; these patient-days were coded as days with other nutrition. Numerical contributions of each of the dispensed human milk types to the overall nutrition during the observational period were calculated as percentages of the patient-days on which the respective human milk type was dispensed. These percentages were used to determine relevant cut-offs for subsequent analyses (e.g., 50% MOM vs. 50% DHM). Kernel density plots were used to visualize the distribution of the percentages of the different milk types, analogous to a histogram. Infants were categorized into one of five groups, the first four were based on cut-offs for the distributions of the different milk types that ranged from 20 to 80%; and the fifth group was based on fresh MOM vs. any DHM in the first 14 days. Associations between dispensed milk feeds and BPD were assessed using logistic regression in crude and adjusted models. Gestational age, birth weight, child sex, surfactant therapy, and pre- and postnatal steroid therapy were identified as factors that have an influence on the development of BPD (12, 28, 30, 31) and were entered into the models as confounding factors. Gestational age is highly associated with the risk of BPD (12). Therefore, infants were stratified into two groups (GA <28 weeks and GA between 28 and 32 weeks) for subsequent analysis. All statistical analyses were done using R (version 3.5.1; R Foundation for Statistical Computing) and SAS version 9.4 (The SAS Institute, Cary, NC, United States).

3 Results

Baseline characteristics of the infants ($n = 866$) are presented in Table 1. Of these, 201 (23.2%) had BPD according to the ICD-based operationalization and 133 (15.4%) had BPD according to the EHR-based operationalization; $n = 123$ had both. The disparity between the methods is due to the differences in the local practice to judge a need for positive airway pressure ventilation with ambient air equivalent as a need for oxygen >21%.

The mean GA was 29.1 weeks; with 26.1% ($n = 226$) born before 28 weeks. The majority of infants (84.4%, $n = 746$) had a mix of MOM, DHM, and other nutrition dispensed for them, with MOM comprising the highest median percentage proportion (53%, Q1–Q3, 31–81%); i.e., 50% of the infants had MOM covering at least 53% of their hospitalized days, while for 25% of infants, MOM covered at least 81% of their hospitalized days. Dispensing exclusive MOM and DHM feeds

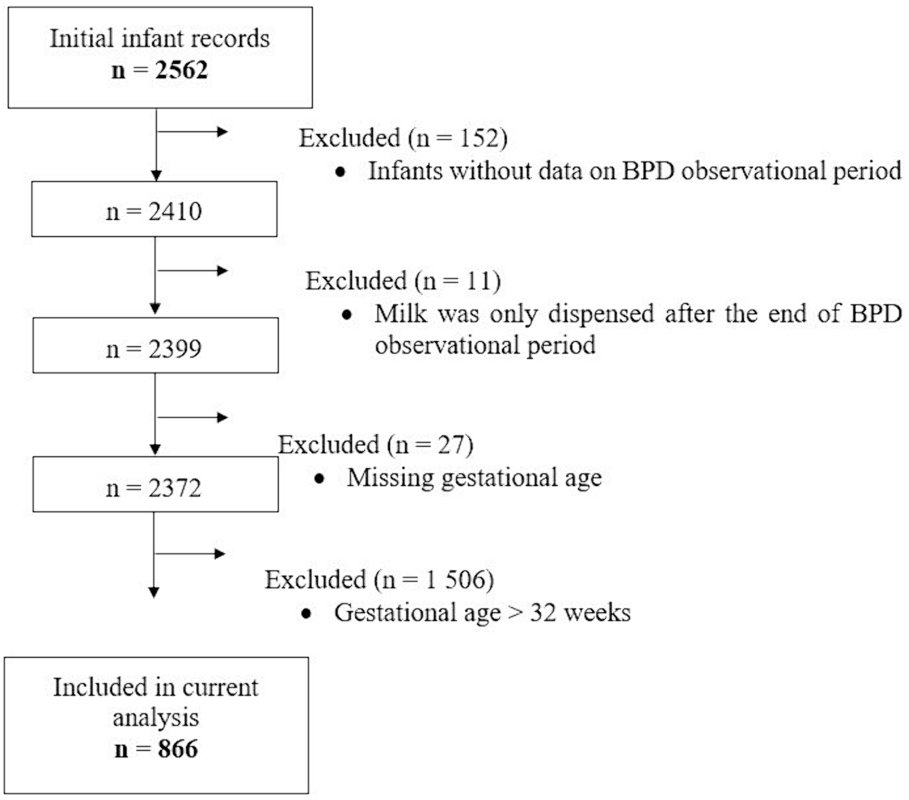

FIGURE 1
Flowchart of hospitalized infants whose records on dispensed milk types were used for the current analysis. BPD, Bronchopulmonary Dysplasia.

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TABLE 1 Characteristics of infants included in the current analysis.

	Category	Frequency (*n*)	% or mean
Infant sex	Boys	466	53.8%
	Girls	400	46.2%
Birth weight	ELBW(<1,000 g)	270	31.2%
	VLBW (≥1,000 and < 1,500 g)	286	33.0%
	LBW (≥1,500 and < 2,500 g)	308	35.6%
	Other (≥2,500 g)	2	0.2%
Gestational age (weeks)		866	29.1
Gestational age	Less than 28 weeks	226	26.1%
	28–32 weeks	640	73.9%
Milk type[1]	MOM and DHM only	15	1.7%
	DHM + other nutrition	65	7.5%
	MOM + other nutrition	55	6.4%
	MOM + DHM + other nutrition	746	84.4%
Proportions of MOM and DHM[2]	MOM and DHM only	83% [62–90%]	17% [10–38%]
	DHM + other nutrition	.	53% [21–89%]
	MOM + other nutrition	47% [32–74%]	.
	MOM + DHM + other nutrition	53% [31–81%]	16% [8–36%]
BPD: ICD operationalization	Yes	201	23.2%
	No	665	76.8%
BPD: EHR operationalization	Yes	133	15.4%
	No	733	84.6%
BPD: both ICD and EHR	Yes	123	14.2%
Antenatal corticosteroids	None	87	10.0%
	Betamethason/Dexamethason	672	77.6%
	Other	75	8.7%
	Unknown	32	3.7%
Multiples	1	586	67.7%
	2	232	26.8%
	3 or greater	48	5.5%
Surfactant application	None	192	22.2%
	LISA	114	13.2%
	Tube	545	62.9%
	LISA + Tube	15	1.7%
Sepsis	Yes	114	13.2%
	No	752	86.8%
Systemic and inhaled steroid therapy (postnatal)	Yes	204	23.6%
	No	662	76.4%

Surfactant variable includes initial care after birth and application during the further hospital stay. [1]Refers to the children who received the respective milk type during the period of admission until end of BPD period. [2]The median and [Q1–Q3] percentage of days, which were covered by either MOM or DHM, respectively, in each of the four categories shown. Other nutrition refers to other forms of parental and/or enteral nutrition that could have been ordered for the infants. BPD, Bronchopulmonary dysplasia; ELBW, Extremely low birth weight; LBW, Low birth weight; VLBW, Very low birth weight; GA, Gestational age; ICD, International Statistical Classification of Diseases and Related Health Problems; EHR, Electronic hospital records; LISA, Less Invasive Surfactant Administration, MOM, Mothers' own milk; and DHM, Donor human milk.

was rare, observed in only 15 (1.7%) infants during hospitalization until the end of the BPD period.

Table 2 shows the average [mean (SD)] and median [min, max] number of days on which exclusive human milk feeds were dispensed. Infants had a median hospital stay of 47 days, with a median of 56 days until the end of the BPD observation period. For infants born at <28 weeks GA, both the average duration of hospitalization and the time to the end of the BPD period were longer compared to those born

TABLE 2 Days on which exclusive feeds were dispensed during hospitalization in the observational period.

	Infants (*n*)	Mean (SD)	Median [min, max]
Number of days of the whole hospitalization	866	54 (33)	47 [2, 430]
Number of days of hospitalization in the observational period until classification of BPD	866	51 (14)	56 [29, 91]
% of days of hospitalization until classification of BPD with…			
Exclusive human milk feeds	866	74% (27%)	89% [1, 100%]
Other nutrition	850	26% (27%)	12% [1, 99%]
Fresh milk*	788	40% (26%)	37% [1, 95%]
Fresh raw milk	632	22% (19%)	16% [1, 84%]
Fresh raw MOM	580	17% (19%)	17% [1, 84%]
Fresh raw DHM	118	5% (5%)	3% [1, 25%]
MOM	758	34% (30%)	47% [1, 96%]
DHM	793	20% (22%)	11% [1, 99%]
MOM and DHM	563	20% (21%)	10% [1, 88%]

BPD, Bronchopulmonary dysplasia; DHM, Donor human milk; GA, Gestational age; MOM, Mothers' own milk. *Fresh milk, Milk is stored cold for a maximum of 3 days, both MOM and DHM included. Fresh raw milk refers to fresh unpasteurized MOM or DHM.

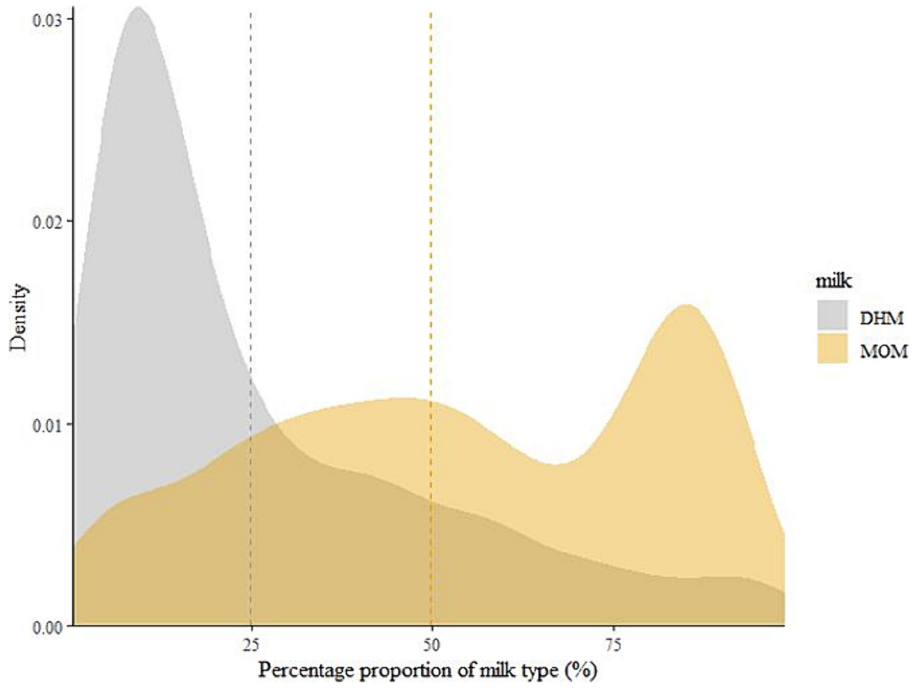

FIGURE 2
The distribution of mother's own milk (MOM) and donor human milk (DHM) contributions to the overall dispensed milk feeds for an infant during hospitalization until the end of the observational period to classify BPD. Dotted lines represent the means of the contribution of the respective human milk feed as a percentage of the days on which any human milk was dispensed. DHM, Donor human milk; MOM, Mothers' own milk.

between 28 and 32 weeks (Supplementary Table S1). All infants had at least 1 day on which exclusive human milk feeds were dispensed. For instance, exclusive MOM was dispensed on at least 1 day for $n = 758$ infants, on an average of 34% of the days of hospitalization until BPD classification (Table 2). Exclusive fresh milk feeds were dispensed for at least 1 day to $n = 788$ infants covering a median of 37% of hospitalization days during the observation period. On average, exclusive fresh raw human milk was dispensed on 22% of the days.

Density plots were created to visualize the distribution of the dispensed human milk feeds (Figure 2; Supplementary Figures S1–S3). DHM contributed less to overall dispensed milk feeds compared to MOM. More than 75% DHM was dispensed for only a few infants, while most infants had less than 50% DHM dispensed. Conversely, the average proportion of MOM was balanced and slightly shifted toward higher percentages (>50%) with a peak well above 75% MOM.

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TABLE 3 Proportions of dispensed milk feeds within the different human milk groups used in the current analysis.

Group	Proportion of milk type	Average (%)	% Median [Q1–Q3]
Fresh milk	MOM	78	82 [68–88]
	DHM	15	11 [7–19]
	Pasteurized Milk	42	41 [20–65]
	Raw Milk	50	52 [29–72]
Frozen milk	MOM	32	32 [18–44]
	DHM	65	63 [53–78]
	Pasteurized Milk	54	54 [42–68]
	Raw Milk	36	35 [21–49]
Pasteurized milk	MOM	58	58 [38–81]
	DHM	40	37 [15–58]
	Fresh milk	52	53 [34–74]
	Frozen milk	41	38 [20–58]
Raw milk	MOM	73	82 [65–88]
	DHM	21	11 [8–21]
	Fresh milk	62	69 [50–78]
	Frozen milk	30	23 [16–38]
MOM	Pasteurized Milk	40	39 [18–62]
	Raw Milk	50	51 [31–70]
	Fresh milk	67	70 [56–79]
	Frozen milk	23	21 [13–30]
DHM	Pasteurized Milk	55	59 [43–70]
	Raw Milk	34	31 [19–47]
	Fresh milk	22	22 [9–35]
	Frozen milk	69	67 [57–79]
Raw MOM	Fresh milk	59	61 [43–77]
	Frozen milk	21	18 [11–27]
Raw DHM	Fresh milk	29	27 [12–46]
	Frozen milk	59	58 [42–75]

Infants were categorized into one of five human milk categories: fresh vs. frozen milk, pasteurized vs. raw milk and MOM vs. DHM, and raw MOM vs. raw DHM. Percentages shown refer to the contribution of each milk type to the respective categories in bold. MOM, Mother's own milk; DHM, Donor human milk.

Infants were categorized into one of five human milk categories: fresh vs. frozen milk, pasteurized vs. raw milk, MOM vs. DHM, and raw MOM vs. raw DHM. Table 3 shows the percentage contributions of each milk type within in each milk group to which the infants were assigned. Infants belonging to the fresh milk group had a higher fraction of MOM compared to the frozen group, while the frozen group had a larger DHM proportion. Percentage contributions of pasteurized and raw milk also differed between these subgroups. Within the pasteurized milk group, the average MOM proportion and the proportion of fresh milk were lower compared to the raw milk group. In the MOM group, the proportion of fresh milk and raw milk was greater than in the DHM group.

In addition to the above-mentioned four human milk groups, infants were further grouped into cut offs ranging between 20 and 80% of the respective milk feeds. The fifth group was a comparison between fresh MOM vs. any DHM in the first 14 days (Figure 3). In crude models, statistically significant ($p < 0.05$) protective associations of 50–80% fresh vs. frozen milk, 60–80% MOM vs. DHM, and 20–50% raw MOM vs. raw DHM were observed using both BPD operationalizations (Figure 3). Interestingly, 50–60% pasteurized milk showed lower odds of developing BPD ($p < 0.05$). However, following adjustment for gestational age, birth weight, child sex, surfactant therapy, and pre- and postnatal steroid therapy, most associations were not statistically significant ($p > 0.05$) except for 70–80% MOM vs. DHM, 60% fresh vs. frozen milk using the ICD- and EHR-based BPD operationalizations, respectively (Figure 4). In infants with a GA <28 weeks, 60% fresh milk vs. frozen milk was associated with lower odds of BPD using the EHR-based definition, in both crude and adjusted models (Supplementary Figures S4, S5).

4 Discussion

The current study investigated associations between dispensed human milk feeds and the odds of BPD in preterm infants,

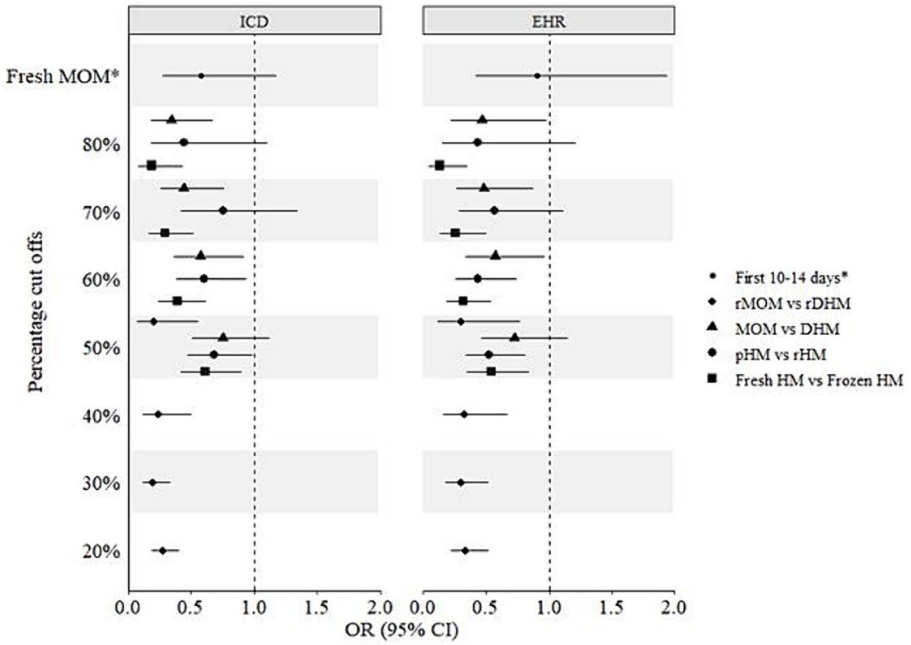

FIGURE 3
Crude associations of different milk types and bronchopulmonary dysplasia (BPD) in preterm infants. ICD, International Statistical Classification of Diseases and Related Health Problems; EHR, Electronic hospital records; CI, Confidence interval; DHM, Donor human milk; HM, Human milk; MOM, Mothers' own milk; OR, Odds ratio; pHM, Pasteurized human milk; rDHM, Raw donor human milk; rHM, Raw human milk; rMOM, Raw mothers' own milk; *Fresh MOM in the first 10–14 days vs. any DHM in the first 10–14 days.

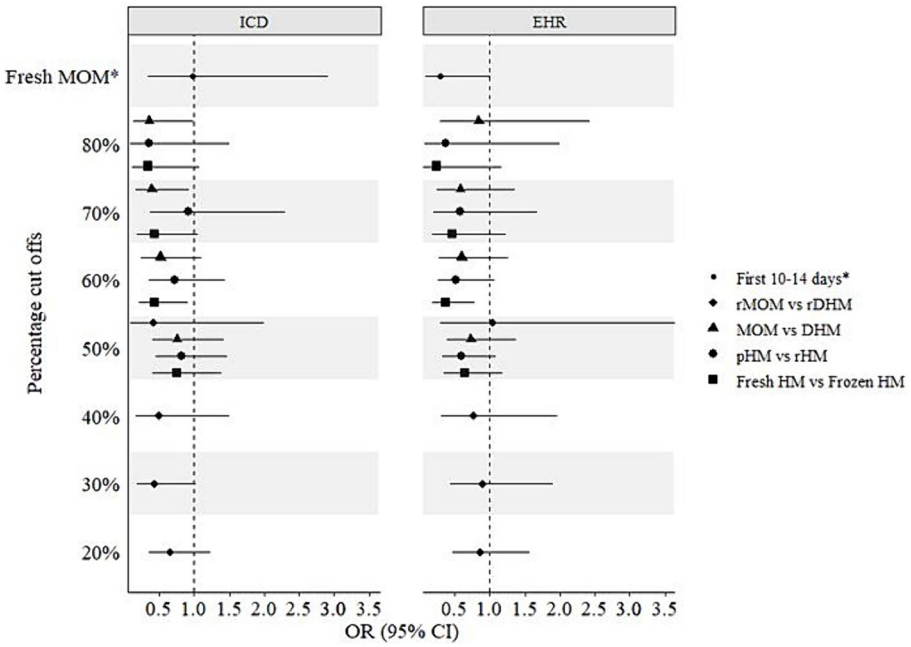

FIGURE 4
Adjusted associations of different milk types and bronchopulmonary dysplasia (BPD) in preterm infants. ICD, International Statistical Classification of Diseases and Related Health Problems; EHR, Electronic hospital records; CI, Confidence interval; DHM, Donor human milk; HM, Human milk; MOM, Mothers' own milk; OR, Odds ratio; pHM, Pasteurized human milk; rDHM, Raw donor human milk; rHM, Raw human milk; rMOM, Raw mothers' own milk; *Fresh MOM in the first 10–14 days vs. any DHM in the first 10–14 days.

considering different milk types and percentage cut-off values, along with two BPD operationalizations. Results showed a significant protective association of fresh human milk compared to frozen, as well as MOM compared to DHM. Interestingly, pasteurized milk showed a protective association against BPD compared to raw milk, although not statistically significant.

We found lower odds of BPD with fresh compared to frozen milk across different percentage cutoffs, particularly notable with higher proportions of fresh milk. Notably, the fresh milk group contained more MOM and raw milk, while the frozen milk group had more DHM and pasteurized milk. Thereby potentially influencing these associations. With a similar limitation, a randomized controlled trial (RCT) comparing frozen MOM to both fresh and frozen MOM and found no difference in BPD rates, though BPD was not the primary focus (24). Similarly, Sun et al. (33) observed faster weight gain, lower incidence of retinopathy of prematurity (ROP), and reduced BPD in the fresh milk group, suggesting potential benefits of fresh human milk for improving infant outcomes.

Conversely, our study shows a protective association of pasteurized human milk, although not statistically significant, following adjustment for confounding factors. A systematic review (34), found no BPD difference between unpasteurized and pasteurized human milk, while a recent prospective study (35) showed that preterm infants fed fresh MOM compared to pasteurized, non-frozen MOM, had a higher survival rate without severe complications, including BPD. In contrast to our study, that study (35) implemented a MOM-only nutrition regime (supplemented with preterm formula if necessary). Similarly, two other studies (20, 21) found a reduced risk of BPD in the raw MOM group compared to the pasteurized MOM group. In our study, the proportion of MOM and fresh milk dispensed among pasteurized milk were more than 50%, although not higher than those proportions among dispensed raw milk. It is therefore plausible that our results were confounded by the proportion of dispensed MOM and fresh milk.

Moreover, in contrast to previous studies that showed inconclusive results and no beneficial effects of MOM (19, 31, 36–38), we report a protective association of MOM vs. DHM. However, it must be noted that BPD was only considered as a secondary outcome in most of these studies (36–38), which comes with different observational periods compared to our study design. On the other hand, one study (19) stood out for its detailed information on actual human milk intake during the first 28 days of life, as well as proportion of MOM on total breast milk intake. But these results, similarly to ours, should be interpreted with caution as the sample size was very small and only few infants received DHM.

Xu et al. (31) demonstrated a protective effect of >50 mL/kg/day of human milk, both MOM and DHM, against BPD compared to preterm formula. Similarly, another study (39), found a 9.5% reduction in the odds of BPD for every 10% increase in MOM dose. Fonseca et al. (40) reported a protective effect of 7 mL/kg/day of human milk in the first 42 days of life regarding BPD. However, all human milk in that study (40) was frozen and pasteurized. Although we lacked actual volume data and infant intake, we reported a protective association of fresh MOM dispensed in the first 10–14 days of life against BPD. While not statistically significant, this highlights the importance of early exposure to MOM in the first few days of life.

This study has several limitations that should be acknowledged. Firstly, the retrospective nature and observational design of the study inherently limit the ability to establish causality. Secondly, the data on dispensed human milk feeds, does not accurately reflect actual infant feeding practices and there is no information on the volume of actual human milk intake. Thus, a potential dose dependent association could not be investigated. Still, we accounted for the potential changes in feeding type by applying weighting to the data on dispensed human milk feeds. This resulted in plausible intra-individual feeding patterns during the first 100 days of hospitalization (32). Additionally, due to small numbers of infants with BPD in the gestational age categories, our stratified analysis lacked sufficient power, limiting the possibility to draw robust conclusions. Moreover, most BPD cases were mild ($n = 174$), with even fewer moderate ($n = 17$) and severe BPD ($n = 19$), making it challenging to assess BPD severity accurately. While we did evaluate severity, (Supplementary Figures S6, S7), caution when interpreting these results is warranted due to limited sample sizes. Further, there is no data on calorie and/or protein intake and growth parameters in the study population. Of which, protein intake and growth could have a positive impact on BPD. Despite this, we provide valuable insights and highlight the need for further prospective studies that can incorporate comprehensive data on nutritional intake and growth parameters to better understand their impact on BPD. Our study's strength includes the determination of the relative contribution of each milk type to the respective group, providing clearer insights of the feed mix compared to previous research. This tackles a key issue of merging MOM and DHM in single matrices, reducing inclusion bias and enhancing generalizability in observational analyses. We also employed various cutoff values tailored to our population and clinical setting (32). Additionally, we used two distinct operationalizations of BPD aligning with previous studies (one stricter based on NICHD consensus and one more relaxed based on administrative data), which yielded similar results. However, residual confounding from unaccounted factors (as they were not available) may influence results, although we believe their impact to be minimal.

In conclusion, our findings suggest potential protective associations of higher contribution of fresh human milk and MOM to overall feeds during hospitalization against developing BPD. Our results highlight the importance of MOM as an ideal source of nutrition during early infancy. The dose dependent effect of these associations still remains to be studied. For future research, data on the actual amount of milk feeds should be analyzed to verify these findings. Future research could examine whether the initiation time and duration of MOM feedings has an impact on short-term outcomes including BPD.

Data availability statement

The data analyzed in this study are subject to the following licenses/restrictions: due to data protection laws, we may not be able to share the raw data. However, the authors are open to sharing aggregate data (for instance, relative concentrations of the different milk types). Requests to access these datasets should be directed to jon.genuneit@medizin.uni-leipzig.de.

Ethics statement

The studies involving humans were approved by the Ethics Board of the Medical Faculty at Leipzig University. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

EP: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. RA: Data curation, Investigation, Methodology, Writing – review & editing. TW: Data curation, Investigation, Methodology, Writing – review & editing. UT: Data curation, Investigation, Methodology, Writing – review & editing. CG: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. JG: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. LS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing.

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Conflict of interest

JG was the project manager and LS was a scientist on unrestricted research grants from Danone Nutricia Research to Ulm University and to Leipzig University for research into human milk composition within the Ulm SPATZ Health Study and the Ulm Birth Cohort Study.

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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1408033/full#supplementary-material

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Association between breastfeeding duration and BMI, 2009–2018: a population-based study

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Background: In the 21st century, childhood overweight and obesity have become major public health issues worldwide. Previous studies have shown that breastfeeding helps prevent overweight or obesity in children. Despite the significant advantages of breastfeeding, the global exclusive breastfeeding rate for infants under 6 months old is only 40%, while in the United States, the rate is only 25%. The aim of this study is to explore the relationship between breastfeeding duration and BMI in children aged 2 to 6 in the United States, and to raise awareness of breastfeeding.

Methods: A cross-sectional study included 2,769 participants between the ages of 2 and 6 from a sample that represented the entire NHANES 2009–2018. Data was analyzed using EmpowerStats, (www.empowerstats.com) linear regression as well as Chi-square test, t-tests, multivariate regression analysis and smooth cure fitting were done.

Results: Breastfeeding duration long-term group exhibited a statistically significant negative association with BMI, with a regression coefficient of −0.21 ($P < 0.05$). The continuous analysis of breastfeeding duration by tertile also demonstrate a statistically significant negative association with BMI. Subgroup analysis revealed that the potential benefits of breastfeeding on BMI were more obvious in low-income environments and maternal age 18 to 35 years, with a regression coefficient of −0.57 and −0.24, respectively (all $P < 0.05$).

Conclusion: The findings emphasize the importance of breastfeeding in reducing childhood overweight/obesity and preventing associated diseases, both in clinical and public health settings.

KEYWORDS

breastfeeding, BMI, obesity, children, NHANES

1 Introduction

In the 21st century, childhood overweight and obesity have emerged as significant public health challenges in many developed countries (1–3), and posing serious health threats globally (4). In 2022, the World Health Organization (WHO) reported that around 37 million children under the age of 5 are obese, with over 390 million children and

teenagers aged 5 to 19 also being overweight. In addition, estimated 160 million children suffer from obesity (5). The global incidence of childhood obesity is increasing, and it is expected that by 2030, the numbers of overweight and obese individuals will reach 40 million and 254 million, respectively (6). In the past forty years, the mean Body Mass Index(BMI) for American adults between 18 and 25 years old increased from 23.0 to 27.5 (95% CI: 22.8–23.2), while the worldwide rate of adult obesity doubled, highlighting the enduring effects of childhood obesity (7). Children who are overweight or obese are likely to continue being overweight or obese as adults (3, 8), making early overweight and obese a strong predictor of adult overweight and obesity (6). Overweight and obesity might lead to a series of serious health problems, which may appear in the short or long-term, including type 2 diabetes, cardiovascular problems (3, 8), increased mortality, premature death, disability (6, 8), and mental health problems (9). Childhood obesity has both health and economic impacts, leading to expenses like medical costs and reduced productivity due to increased absenteeism (10).

Breastfeeding provides many advantages for infants and young children, and is widely regarded as the optimal nutrition source (11). Breast milk has the effects of promoting somatic growth, regulating postnatal intestinal functions, boosting immunity, and supporting brain development (12). Breast milk is acknowledged for its role as the primary source of nourishment for infants, offering ideal nutrition, boosting the immune system, and fostering special connections between mother and child. The WHO, the American Academy of Pediatrics, and the American College of Obstetricians and Gynecologists have all endorsed the suggestion of exclusive breastfeeding for the initial six months of a baby's life (11, 13, 14). The WHO also recommends that breastfeeding should continue even after adding complementary foods, until the child is two years old or older, in order to achieve optimal growth, development, and health (14).

In Singapore, a cohort study discovered that children breastfed for less than four months had a 0.18 (−0.01, 0.38) (β (95% CI) increase in BMI at age 6 compared to those breastfed for over four months, after adjusting for confounding factors, accompanied by a total of 1.83 mm (0.05, 3.61) (β(95% CI) of larger skin folds (15). A cohort study of low-income Mexican/Mexican American mothers revealed significant differences between breastfed and formula-fed children under 3 years of age ($F = 4.644$, $P < 0.05$), and breastfed children were 18% less likely to be obese compared to their formula-fed counterparts (16). In the United States, a research project examined children aged 4 to 8 and discovered a dose-response relationship between how long they were breastfed and the likelihood of developing obesity in early childhood. Exclusive breastfeeding at 6 months reduced the risk of obesity by 60% compared to non-breastfeeding (95% CI: 0.18–0.91) (17).

Early breastfeeding in children has a potential correlation with overweight and obesity. Despite the clear advantages of breastfeeding, the worldwide rate of exclusive breastfeeding for infants under 6 months is just 40% (18). In a 2020 study conducted by the Centers for Disease Control and Prevention in the U.S., it was found that just 25% of infants are advised to exclusively breastfeed until they reach six months of age (19). The low breastfeeding rate makes research on breastfeeding more urgent. In addition, recent research has mainly focused on the impact of overweight and obesity on older children and adolescents, with less research on children aged 2 to 6 years old. The age range of 2 to 6 is a period of rapid physical, cognitive, and social development for a child. The developments that have occurred in these four years will affect children's lives in various ways in the future. The objective of this research is to investigate the impact of the length of breastfeeding on the BMI of children between 2 and 6 years old in the U.S. The significance of this study is multifaceted. Firstly, it helps more and more literature explore the long-term effects of early nutrition on health, with a particular focus on how breastfeeding affects BMI trajectory. Secondly, by utilizing extensive and detailed NHANES datasets, this study provides strong epidemiological analysis that helps clarify inconsistencies and gaps in existing studies. Finally, the results of this research could offer evidence-supported suggestions for breastfeeding techniques and influence efforts to prevent obesity beginning in early childhood.

2 Materials and methods

2.1 Study population and design

NHANES is a nationally representative comprehensive cross-sectional study designed to assess the health and nutritional status of the adult and child populations in the United States (20). The survey consists of questions about demographics, dietary habits, socioeconomic status, and health factors (21). The research procedures were all authorized by the National Center for Health Statistics Ethics Review Board, and informed consent was obtained from all participants prior to data collection (22). For more detailed information, please visit the official website.[1]

A total of 2,769 individuals aged 2 to 6 years were involved in this survey, which was part of a comprehensive NHANES 2009–2018 study. We excluded 40,897 participants who did not meet the age criteria from the study sample. We further excluded 3,793 participants with missing BMI data or 1,436 participants with incomplete breastfeeding duration samples. In addition, 799 participants missing co-variates data were excluded, leaving a total of 2,769 participants in the present analysis. The detailed screening process is illustrated in Figure 1.

2.2 Assessment of BMI (outcome)

The article includes details about the weight status categories and percentiles for BMI-for-age, which are based on Centers for Disease Control and Prevention (23). This information highlights the categorical representation of the BMI variable, providing a standardized method for assessing weight status in children and teenagers. The weight status categories are divided into four distinct groups: underweight, healthy weight, overweight, and obesity. These categories are determined based on the percentile range of the BMI-for-age measurements. The specific percentiles for each category are as follows in Table 1.

1 https://www.cdc.gov/nchs/nhanes/

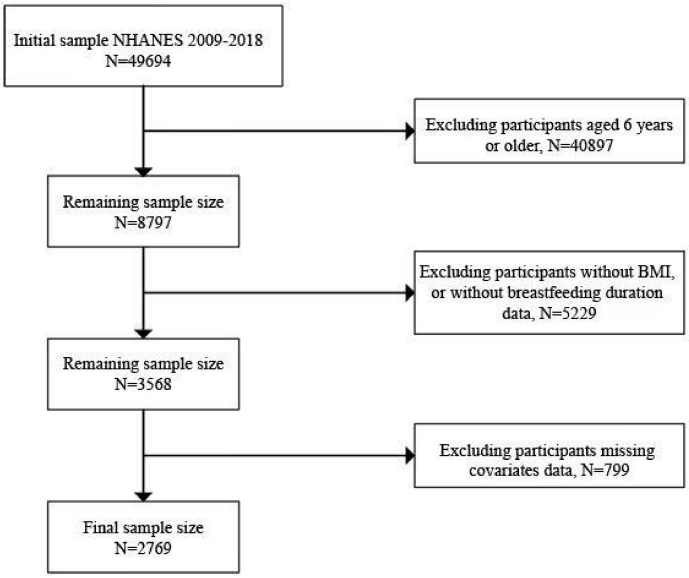

FIGURE 1
Flowchart of sample selection from the NHANES 2009–2018. We included a total of 49,694 samples, and after excluding variables with incomplete data, such as missing BMI, breastfeeding duration, and other covariates, we screened a total of 2,769 samples.

TABLE 1 The weight status categories. Reproduced from (60).

Weight status category	Percentile range
Underweight	Less than the 5th percentile
Healthy Weight	5th percentile to less than the 85th percentile
Overweight	85th to less than the 95th percentile
Obesity	Equal to or greater than the 95th percentile

2.3 Assessment of breastfeeding duration (exposure)

We define breastfeeding as any form of breastfeeding that is reserved for direct breast feeding or consumption of extruded breast milk, and was not limited to exclusive breastfeeding. The timing of when solid food was introduced was assessed by asking questions raised at 3 and 9 months (2). Introducing solid foods might influence on the changes in breastfeeding patterns for infants and young children, so the breastfeeding duration in this study is set at 3 and 9 months as critical points. Participants were asked to provide information on the duration of breastfeeding (in days) and were divided into three groups depending on the duration: short-term ($0 \leq$ days < 90), medium-term ($90 \leq$ days < 270), and long-term ($\geq$ 270 days).

2.4 Covariates assessment

Information on age, maternal age at conception, sex, birthweight, maternal smoking status, and ethnicities was self-reported. The classification of ethnicities included Mexican American, other Hispanic, non-Hispanic White, non-Hispanic Black, and other ethnicities, such as multi-ethnical individuals (24, 25). Physical activity was divided into 2 groups: inactive (the number of days with at least 60 minutes of physical activity per day was $\leq$ 3 days in the past 7 days), active ($\geq$ 4 days). According to the annual family income questionnaire, income level was divided into 3 groups: Under $20,000, $20,000 to 75,000 and Over $75,000. Divide into two groups based on whether smoking during pregnancy: never smoker and smoker (26).

2.5 Statistical analysis

The study population's baseline characteristics were evaluated using a weighted linear regression model and weighted Chi-square test. The study utilized Multiple Regression analysis to evaluate the relationship between the length of breastfeeding and BMI as a continuous variable. Smooth curve fitting was utilized to examine the correlation between the length of breastfeeding and BMI. All analytical processes were performed by using EmpowerStats[2] version 5.0, with a significance threshold set at a $P < 0.05$. Additionally, to mitigate large fluctuations in the dataset, we employed a weighting strategy.

3 Results

3.1 Baseline characteristics

This article included 2,769 participants (1410 male and 1359 female) from NHANES 2009–2018, the mean(SD) age was 3.76 (1.4) years; the gender ratio is balanced, with 50.92% of people

2 www.empowerstats.com

TABLE 2 Baseline characteristics of the study population.

Characteristic	Tertile of breastfeeding duration(N = 2,769)			*P*-value
	Short-term	Medium-term	Long-term	
Age, years	3.77 ± 1.34	3.88 ± 1.38	3.90 ± 1.33	0.1110
Maternal age at conception, years	26.77 ± 6.28	28.01 ± 5.83	30.01 ± 5.51	< 0.0001
Birthweight, pounds	6.84 ± 1.31	6.76 ± 1.23	7.08 ± 1.21	< 0.0001
BMI, (kg/m^2)	16.50 ± 2.06	16.49 ± 1.99	16.22 ± 1.75	0.0012
BMI				0.0019
Underweight	23 (3.10)	47 (4.35)	26 (2.70)	
Healthy weight	394 (54.62)	568 (52.82)	552 (56.78)	
Overweight	179 (24.78)	236 (21.98)	252 (25.93)	
Obesity	126 (17.50)	224 (20.85)	142 (14.59)	
Sex				0.4356
Male	376 (52.09)	555 (51.59)	479 (49.30)	
Female	346 (47.91)	520 (48.41)	493 (50.70)	
Ethnicities				< 0.0001
Mexican American	123 (17.20)	190 (17.66)	140 (14.42)	
Other Hispanic	62 (8.50)	103 (9.59)	67 (6.93)	
Non-Hispanic White	381 (52.76)	550 (51.21)	590 (60.71)	
Non-Hispanic Black	93 (12.89)	124 (11.52)	70 (7.17)	
Other Ethnicities - Including Multi-Ethnical	63 (8.64)	108 (10.02)	105 (10.78)	
Annual family income				< 0.0001
Under $20,000	159 (21.99)	206 (19.13)	121 (12.42)	
$20,000 to 75,000	383 (53.01)	499 (46.42)	426 (43.81)	
Over $75,000	180 (25.00)	370 (34.45)	425 (43.77)	
Physical activity				0.0610
Inactive	42 (5.82)	93 (8.68)	83 (8.57)	
Active	680 (94.18)	982 (91.32)	889 (91.43)	
Maternal smoking status				< 0.0001
Smoker	82 (11.42)	86 (7.99)	33 (3.41)	
Never smoker	640 (88.58)	989 (92.01)	939 (96.59)	

For categorical variables, *P*-value was calculated by the weighted chi-square test. For continuous variables, weighted linear regression model was used to calculate *P*-value.

were males. Compared with the lowest tertile of breastfeeding duration, higher tertile groups were more likely to be elder, mothers give birth to children at a much older age, non-Hispanic White, income over $75,000, and mother never smoked when pregnant (all $P < 0.05$). Highest tertile group has the highest number of people with a healthy weight ($P < 0.05$). The detailed characteristics of participants were shown in Table 2.

3.2 Association between breastfeeding duration and BMI

Compared to the lowest tertile of breastfeeding duration, the middle tertile had a non-significant association with BMI, while the highest tertile exhibited a statistically significant negative association with BMI, with a regression coefficient of −0.21 ($P < 0.05$). The continuous analysis of breastfeeding duration by tertile also demonstrate a statistically significant negative association with BMI. These findings suggest that a longer duration of breastfeeding may be associated with a lower BMI in the population studied, as shown in Table 3.

In addition, the results of the smooth-fitting curves further confirmed the non-linear correlation between BMI and the duration of the breastfeeding period, as shown in Figure 2. The breastfeeding duration days (in red) with 95% CIs (in blue) determined using the generalized additive model. Within the range of 0 days to approximately 1000 days, we can observe a slight U-shaped trend. The BMI value slightly decreases during the early stages of breastfeeding, then reaches its lowest point around 500 days, and then slowly increases. After a longer feeding period, the relationship becomes less clear.

TABLE 3 Regression Coefficients (β) in the association between breastfeeding duration and BMI on a continuous scale.

Exposure	Non-adjusted model			Adjust I model			Adjust II model		
	β	OR (95%CI)	*P*	β	OR (95%CI)	*P*	β	OR (95%CI)	*P*
Breastfeeding duration	−0.00	(−0.00, −0.00)	0.0012	−0.00	(−0.00, −0.00)	0.0044	−0.00	(−0.00, −0.00)	0.0104
Tertile of breastfeeding duration									
Short-term		1 (ref)			1 (ref)			1 (ref)	
Medium-term	−0.02	(−0.20, 0.17)	0.8723	−0.02	(−0.20, 0.17)	0.8554	0.03	(−0.15, 0.22)	0.7272
Long-term	−0.29	(−0.47, −0.10)	0.0024	−0.25	(−0.43, −0.06)	0.0091	−0.21	(−0.39, −0.02)	0.0305
P for trend	−0.15	(−0.24, −0.06)	0.0011	−0.13	(−0.22, −0.04)	0.0051	−0.11	(−0.21, −0.02)	0.0172

Non-adjusted model: none. Adjust I model: ethnicities and gender-adjusted. Adjust II model: ethnicities, gender, age, income, physical activity, mother age, mother smoking status, and birthweight-adjusted.

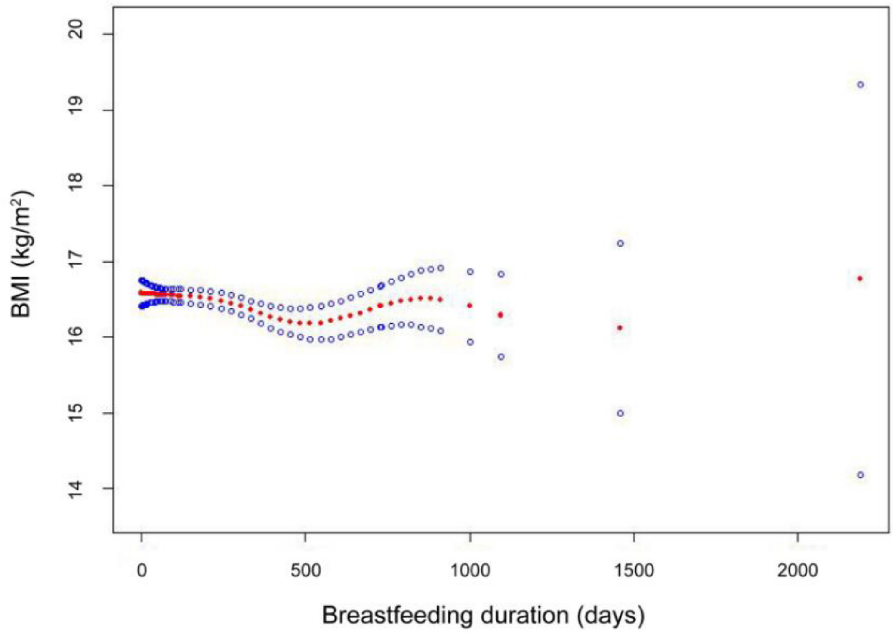

FIGURE 2
The smooth-fitting curves between breastfeeding duration and BMI. Smooth curve fitting results indicate that the breastfeeding duration is non-linear associated with BMI among participants. Adjust model: ethnicities, gender, age, income, physical activity, mother age, mother smoking status, and birthweight-adjusted.

3.3 Subgroup analyses

We conducted a subgroup analysis of annual family income level. The detailed content is shown in Table 4. For households with annual incomes below $20,000, we analyzed the relationship between breastfeeding duration and BMI. Compared to the reference group (breastfeeding duration < 90 days), the middle-duration group showed no significant correlation with BMI. However, the longest-duration group exhibited a statistically significant negative correlation with BMI, with a regression coefficient of −0.57 ($P < 0.05$). The continuous analysis of breastfeeding duration by tertile also demonstrate a statistically significant negative association with BMI. The above conclusion is consistent with the conclusion when subgroup analysis was not conducted. However, analysis of subgroup with an annual family income between $20,000 and $75,000, as well as subgroup above $75,000, showed no statistical correlation between breastfeeding duration and BMI.

35 years old is a threshold for female fertility. After 35 years old, women's fertility potential gradually decreases, and the number and quality of primordial follicles in oocytes decrease (27). In our subgroup analysis, we divided them into three groups: under 18 years old, between 18 and 35 years old, and 35 years old and above. We conducted subgroup analysis according to the age of the mother at the time of delivery, as shown in Table 5. The analysis of mothers with delivery age between 18 and 35 years showed that breastfeeding was a protective factor for BMI, with a regression coefficient of −0.24 ($P < 0.05$). A continuous analysis of the duration of breastfeeding in three equal groups also showed that there was a statistically significant negative correlation between the duration of breastfeeding and BMI. The above conclusions were consistent with those without subgroup analysis. However, this

TABLE 4 Regression Coefficients (β) in the association between breastfeeding duration and BMI in annual family income under $20,000 on a continuous scale.

Exposure	Non-adjusted model			Adjust I model			Adjust II model		
	β	OR (95%CI)	*P*	β	OR (95%CI)	*P*	β	OR (95%CI)	*P*
Breastfeeding duration	−0.00	(−0.00, −0.00)	0.1068	−0.00	(−0.00, −0.00)	0.0544	−0.00	(−0.00, −0.00)	0.0529
Tertile of breastfeeding duration									
Short-term		1 (ref)			1 (ref)			1 (ref)	
Medium-term	−0.15	(−0.54, 0.25)	0.4667	−0.16	(−0.55, 0.24)	0.4369	−0.18	(−0.57, 0.22)	0.3827
Long-term	−0.46	(−0.89, −0.02)	0.0392	−0.50	(−0.94, −0.07)	0.0235	−0.57	(−1.01, −0.12)	0.0130
P for trend	−0.23	(−0.44, −0.01)	0.0410	−0.25	(−0.46, −0.03)	0.0250	−0.28	(−0.50, −0.06)	0.0143

Non-adjusted model: none. Adjust I model: ethnicities and gender-adjusted. Adjust II model: ethnicities, gender, age, physical activity, mother age, mother smoking status, and birthweight-adjusted.

conclusion was not found in the group with delivery age less than 18 years or age equal to or older than 35 years.

4 Discussion

In this cross-sectional study of 2,769 children aged 2 to 6 in the U.S., we found that children who breastfed for a longer period of time were less likely to be overweight or obese at the age of 2 to 6. The observed associations were independent of major lifestyle risk factors, self-reported lifestyle habits, and socio-demographic factors. One possible explanation is that formula fed infants always overeat (28), compared to breastfeeding, formula feeding can lead to faster growth in infancy (29, 30). Formula-fed infants are typically bottle-fed (31), which may lead to poor self-regulation of intake and reduced prolonged satiety (32). Mothers who bottle-feed their infants often have greater control over their baby's intake, potentially leading to practices that pressure infants to consume more, such as encouraging them to finish the bottle (33). This may hinder the development of infants' ability to self-regulate their intake, leading to overfeeding, poor satiety response, and excessive weight gain (34). Compared to children who are directly breastfed, children who are breastfed through bottles are 67% less likely to experience high satiety, and early direct breastfeeding is associated with better appetite regulation in later childhood (32). Breastfeeding facilitates infant self-regulation of milk intake, as mothers have limited control over the process, while formula feeding, often managed by nursing staff, may be more passive, potentially hindering infants' ability to self-regulate intake (34). Another possible explanation is that there are differences in the composition between breast milk and formula milk. While infant formula is primarily derived from bovine milk, its composition differs significantly from human milk in terms of fats, proteins, vitamins, and minerals (12). Formula milk contains higher protein content than breast milk, with bovine milk protein concentrations ranging from 1.80 to 2.0 g/L (35). Research suggests that increased protein intake during infancy correlates with accelerated weight gain and elevated obesity risk in later life (35, 36). Bovine milk fat globule membrane (MFGM) has demonstrated beneficial effects on infant development (37, 38), leading to its inclusion in most infant formulas (39). As cholesterol is a component of MFGM, infant formula contains higher concentrations of milk cholesterol compared to human milk (40). Hypercholesterolemia, a common lipid abnormality in obesity, often results from lipid metabolism disorders associated with overweight and obesity. These disorders can lead to elevated levels of low-density lipoprotein (LDL) and total cholesterol in the body. These findings emphasize the potential clinical and public health significance of adhering to breastfeeding as a way to reduce childhood overweight or obesity and prevent related diseases. The association between longer breastfeeding and reduced early childhood obesity observed in our study is consistent with previous clinical trial results in children and adolescents. In a large-scale cross-sectional study conducted in Qingdao, China. Research has found that children who breastfeed for more than a year have a lower risk of overweight and obesity, especially boys aged 9 to 11 (41). A study by Croatian on second and third grade student groups showed that breastfeeding for more than 6 months is a protective factor for overweight and obesity (42). In addition, a cross-sectional survey study in Shanghai showed that exclusive breastfeeding is associated with a reduced risk of central obesity (RR:0.76; 95% CI: 0.60, 0.96, $P < 0.05$) (43).

Curve fitting analysis revealed a complex, non-linear relationship between breastfeeding duration and children's BMI, suggesting an optimal range of breastfeeding duration associated with lower BMI. This complex, non-linear relationship aligned with previous research findings. Harder found a non-linear dose-response relationship between breastfeeding and later obesity risk (44), while Rito emphasized the complex, multifactorial nature of this relationship (45). We observed a slight decrease in BMI during the first 500 days of breastfeeding, potentially reflecting its protective effect against rapid weight gain in infants and young children. The review by Woo Baidal suggested that early life nutritional interventions, particularly breastfeeding, may play a key role in preventing childhood obesity (46). In addition, Ong and Loo's research suggested that breastfeeding may reduce the risk of obesity in the future by regulating early growth rate (47). The BMI value reaches its nadir at approximately 500 days (16.5 months) of breastfeeding before gradually increasing. This trend may be attributed to changes in dietary behavior after 18 months, as children transition from complementary foods to adult-like dietary patterns, potentially impacting their BMI. Emmett and Jones found that between 18 and 24 months, children begin consuming foods similar to family members, potentially increasing exposure to energy-dense foods and affecting BMI (48).

TABLE 5 Regression Coefficients (β) in the association between breastfeeding duration and BMI in maternal age during delivery 18–35 years old on a continuous scale.

Exposure	Non-adjusted model			Adjust I model			Adjust II model		
	β	OR (95%CI)	*P*	β	OR (95%CI)	*P*	β	OR (95%CI)	*P*
Breastfeeding duration	−0.00	(−0.00, −0.00)	0.0116	−0.00	(−0.00, −0.00)	0.0285	−0.00	(−0.00, −0.00)	0.0187
Tertile of breastfeeding duration									
Short-term		1 (ref)			1 (ref)			1 (ref)	
Medium-term	−0.04	(−0.25, 0.16)	0.6807	−0.05	(−0.26, 0.16)	0.6459	−0.02	(−0.22, 0.18)	0.8461
Long-term	−0.27	(−0.47, −0.06)	0.0130	−0.23	(−0.44, −0.03)	0.0277	−0.24	(−0.45, −0.03)	0.0242
P for trend	−0.14	(−0.24, −0.03)	0.0092	−0.12	(−0.23, −0.02)	0.0218	−0.13	(−0.23, −0.02)	0.0175

Non-adjusted model: none. Adjust I model: ethnicities and gender-adjusted. Adjust II model: ethnicities, gender, age, physical activity, mother age, mother smoking status, and birthweight-adjusted.

Nicklaus emphasized that 18–24 months is a critical period for dietary behavior formation, with children adopting family diets, potentially leading to increased energy intake and subsequent BMI changes (49).

In our study, we examined the relationship between the duration of breastfeeding and BMI at different annual family income levels. The results of subgroup analysis revealed that the potential benefits of breastfeeding on BMI are more pronounced in low-income environments. However, this association was not observed in the two subgroups with higher annual income (from $20000 to $75000 and above). In social epidemiology and public health research, income has been recognized as a factor that increases the risk of developing subsequent obesity (50). Research showed that in developed countries, lower social classes are associated with a higher risk of obesity, which even affects infants and young children (51). This may be because babies born in impoverished backgrounds are more likely to be exposed to poor breastfeeding practices (51). Low socio-economic status is associated with high parental pressure and restrictive feeding practices (39), which may lead to overfeeding. In addition, high-income families are more likely to acquire knowledge about nutrition and have more opportunities to engage in other behaviors that help maintain a healthy BMI, thereby reducing the relative impact of breastfeeding duration.

Long-term breastfeeding is not only a protective factor for obesity in children and adolescents, but also a protective factor for obesity in adults (52–54). Previous studies by others have shown that breastfeeding protects from diarrhea and respiratory infections, seasonal allergic rhinitis, and diabetes (55–57). Overall, these findings contribute to the growing body of evidence supporting the multifaceted influence of breastfeeding duration, socio-demographic factors, and lifestyle variables on BMI outcomes in early childhood. The implications of these results extend to public health interventions aimed at promoting breastfeeding and addressing disparities in BMI outcomes across diverse demographic groups. Future research should continue to explore the longitudinal effects of breastfeeding duration on BMI and elucidate the underlying mechanisms driving these associations to inform targeted interventions and policies aimed at improving childhood obesity prevention and management.

This article has some limitations, including the overall small sample size might result in bias, particularly in certain ethnic categories, in which the smallest number of participants are included. Then, the study did not delve into the impact of mixed feeding on BMI. It is important to take into account these restrictions when analyzing the findings of a study. Furthermore, due to incomplete information in the NHANES database, several important covariates could not be included in our analysis. These include maternal body mass index, maternal weight fluctuations before and during pregnancy, gestational diabetes, and mode of delivery. The absence of these factors may introduce potential bias to our findings. A limitation of our study is the modest practical significance of our findings, despite achieving statistical significance. The regression coefficients exhibit relatively small effect sizes, despite being statistically significant. This may be attributed to our sample size which, although substantial, might not have been sufficient to detect more pronounced practical effects. We also observed wide confidence intervals in several instances, some of which included zero. This pattern indicates uncertainty in our estimates and potentially constrains the practical implications of our results. These limitations necessitate cautious interpretation of our findings and emphasize the need for future studies with larger sample sizes to more accurately estimate the relationship between breastfeeding duration and BMI. Lastly, this article was only based on the database of the U.S. and has not surveyed the entire population worldwide, so it might not be applicable to developing countries.

An interesting result of our study is that we found no correlation between breastfeeding duration and BMI in the maternal age group more than 35 years old and above ($P > 0.05$). This may be related to the presence of other confounding factors. Research conducted in Taiwan revealed that mothers' BMI ($\beta = 0.240$; $P < 0.05$), and education level ($\beta = -0.141$; $P < 0.05$) are important factors affecting children's BMI, and together with monthly family income, it explains an additional 8% variance in children's BMI, which is statistically significant (58). Obesity is also influenced by many other factors, such as child gender (59), parental inheritance, etc., (50). In addition, there was no correlation between breastfeeding and BMI in the group under 18 years old ($P > 0.05$). We believe this may be due to the strong support of American families and the low number of young mothers. Only 3.47% of the respondents were mothers under the age of 18.

5 Conclusion

The research suggests that the correlation between the duration of breastfeeding and early childhood BMI outcomes. These findings emphasize the potential clinical and public health implications of adhering to breastfeeding as a way to reduce childhood overweight or obesity and prevent related diseases. This conclusion is particularly evident in households with an annual income of less than $20,000 and maternal aged between 18 and 35. It is necessary to conduct larger scale prospective cohort studies to further replicate this finding and elucidate the potential role of extending breastfeeding time in reducing overweight and obesity in young children.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cdc.gov/nchs/nhanes/.

Author contributions

JS: Methodology, Software, Writing – original draft, Writing – review and editing, Formal analysis. SL: Methodology, Software, Writing – original draft, Writing – review and editing, Supervision. JH: Supervision, Writing – review and editing. XJ: Software, Writing – review and editing. YY: Methodology, Software, Supervision, Writing – review and editing.

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Conflict of interest

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

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Interactions between the human milk oligosaccharide 2′-fucosyllactose and *Bifidobacterium longum* subspecies *infantis* in influencing systemic immune development and function in piglets

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Introduction: The oligosaccharide 2′-fucosyllactose (2′-FL) is a predominant component of human milk, serving as a prebiotic for gut microbiota and influencing immune development in infants. *Bifidobacterium longum* subspecies *infantis (B. infantis)* is a commensal bacterium found in breastfed infants. Both 2′-FL and a specific strain of *B. infantis*, Bi-26™, are commercially available. This study investigates the potential synbiotic relationship between 2′-FL and Bi-26™ on immune development.

Methods: Two-day-old piglets ($n = 53$) were randomized in a 2×2 design, receiving either a commercial milk replacer *ad libitum* without (CON) or with 1.0 g/L 2′-FL (FL). Piglets in each diet were further randomized to receive either glycerol stock alone or Bi-26™ (10^9 CFU) (BI and FLBI) orally once daily. On postnatal day (PND) 34/35, animals were euthanized, and blood was collected for serum cytokine analysis. Additionally, peripheral blood mononuclear cells (PBMCs) were isolated for *ex vivo* stimulation and flow cytometry analysis. Serum and *ex vivo* cytokines were analyzed using a multivariate model. All other outcomes were analyzed using a two-way ANOVA, considering prebiotic and probiotic fixed effects. The significance level was set at a p value <0.05, with trends reported for $0.05 < p < 0.1$.

Results: Immune cell populations in PBMCs were unaffected by the experimental treatment. However, serum interleukin (IL)-1RA, IL-1β, IL-12, and IL-18 were all higher ($p < 0.05$) in the FL group than in the CON group. In isolated PBMCs, lipopolysaccharide (LPS) stimulation resulted in higher IL-1RA and a trend for higher IFN-γ secretion in the FL group vs. the CON group.

Conclusion: 2′-FL stimulates a balanced cytokine profile in healthy piglets without changing immune cell populations. When immune cells are stimulated *ex vivo* with LPS, 2′-FL primes T-cells for a proinflammatory response, which is moderated by co-administration of Bi-26™.

KEYWORDS

B. infantis Bi-26, immune, cytokine, human milk oligosaccharides, 2-fucosyl-lactose

1 Introduction

The newborn infants' immune system is functionally naïve, leaving them vulnerable to pathogens. Morbidity and mortality are higher in non-breastfed infants (1), which is attributed to the bioactive components in human milk that stimulate immune maturation and development of the gut microbiome (2). Among these bioactive components are the multifunctional human milk oligosaccharides (HMOs), which serve as prebiotics and inhibitors of pathogen attachment to the epithelial lining (3). Infant formulas are virtually devoid of these glycans since they are in low concentration in bovine milk, which is the starting material for most infant formulas (3).

In human milk, 2′-fucosyllactose (2′-FL) is one of the most abundant HMOs in mothers who express the 2-fucosyltransferase gene (4). A small amount of 2′-FL is absorbed into the systemic circulation in human infants (5) and rats (6). There is evidence that 2′-FL modulates cytokine production and receptor signaling, but data are not entirely consistent. Co-culturing cord blood mononuclear cells with neutral HMOs had little effect on intracellular production of IFN-γ, IL-4, and IL-13, but acidic HMOs increased the production of these cytokines (7). Preclinical and clinical studies have demonstrated that 2′-FL alone directly impacts immune development and inflammation. Infants fed formula supplemented with 0.2 or 2.0 g/L 2′-FL had serum IL-1RA, IL-1β, IL-1α, tumor necrosis factor-α, and IL-6 concentrations that were not statistically different from those of breastfed infants (8). In human infant and mouse intestinal explants, 2′-FL decreased the severity of necrotizing enterocolitis by binding to the TLR4-myeloid differentiation factor 2-[MD2] complex, which prevented lipopolysaccharide (LPS) binding (9). Furthermore, 2′-FL ameliorated inflammatory response to rotavirus (RV) infection in the small intestine of rat pups and reduced IL-β, IL-6, and IFN-γ production at the peak of infection (10). In addition, in healthy suckling rat pups, an oral gavage of 2′-FL increased plasma IgG and IgA levels as well as T-helper cell populations in the mesenteric lymph nodes (MLNs) (11). Thus, 2′-FL has shown a range of immunomodulatory characteristics that may benefit infants.

Bifidobacterium longum subspecies *infantis (B. infantis)* is a common resident of the gut microbiota of breastfed, but not formula-fed infants. A body of literature also supports the ability of *B. infantis* to influence cytokine production and health outcomes. In human intestinal NEC models, *B. infantis* reduced markers of inflammation, such as IL-6 and IL-8 production, and decreased the upregulation of NFκB-related genes *in vitro* (12). In a clinical trial evaluating the enteric immunomodulatory effects of *B. infantis* EVC001, IL-8, IL-22, IL-1β, and IFN-γ were lower in infants receiving probiotic supplementation than in control infants only being breastfed (13). In addition, in a placebo-controlled, double-blind, randomized study in human infants, supplementation with *B. infantis* increased the IL-10/IL-12 ratio toward an anti-inflammatory effect (14). In a piglet model of *Staphylococcus aureus* infection, post-infection, *B. infantis* supplementation increased serum IL-10 level concentrations and decreased memory T-cell populations (15). Thus, *B. infantis* holds promise as an immunomodulatory probiotic supplement for human infants.

Recent evidence also supports a synbiotic potential for combined administration of 2′-FL and *B infantis*. In addition to direct effects on the immune system, 2′-FL stimulates the growth of *Bifidobacterium*, and *B. infantis* can use 2′-FL for growth and metabolite production (16, 17). Bi-26™ is a commercially available strain of *B. infantis* in the Danisco Global Culture Collection. Its genome reveals that Bi-26™ contains the enzymes necessary to digest 2′-FL. Utilization of 2′-FL by Bi-26™ produces a more diverse group of metabolites than those generated from lactose utilization (18). However, the potential symbiotic effects of Bi-26 and 2′-FL on immune function and development *in vivo* are not well studied.

Therefore, we aimed to better understand the individual and synbiotic effects of dietary 2′-FL and Bi-26™ on piglet systemic immune development. Because of their individual effects, we hypothesized that both 2′-FL and Bi-26™ would promote healthy development of systemic immunity. We further hypothesized that piglets exposed to the combination of 2′-FL and Bi-26™ would have lower levels of serum pro-inflammatory cytokines than CON or 2′-FL, but immune cells isolated from these piglets would demonstrate a more robust response to *ex vivo* LPS stimulation than cells from piglets fed either 2′-FL or Bi-26™ alone.

2 Materials and methods

2.1 Experimental design

The Institutional Animal Care and Use Committee at the University of Illinois approved all animal care and experimental procedures. The experimental design, dietary treatments, and probiotic administration were previously described by Daniels et al. (19). Briefly, naturally farrowed piglets ($n = 63$) from a commercial swine herd received colostrum from the sow for up to 2-day (PND2) after birth before being transported to a specialized neonatal pig rearing system at the Piglet Nutrition and Cognition Laboratory (PNCL) on the University of Illinois campus. Piglets were randomized into the four treatment groups in a 2 × 2 design. Two experimental milk replacer diets (CON and 2′-FL) were manufactured in powder form by TestDiet (Purina Mills, St. Louis, MO). The composition was identical to ProNurse (Land O'Lakes, North Arden Hills, MN) except that the CON diet had added lactose equal to the 2′-FL content in the test diet (1 g/L in the reconstituted formula). The 2′-FL (CARE4U®) was provided by International Flavors & Fragrances (New York, NY, United States). CARE4U® is a high purity (≥94%) infant-grade 2′-FL containing ≤300 EU endotoxins/g product. Liquid milk replacer was reconstituted daily at 20% solids and automatically delivered to piglets beginning at 1,000 h each day and ending at 0600 h the following day (i.e., *ad libitum* access to treatment diets over a 20-h feeding period each day). Milk disappearance was calculated per individual pig based on the initial and final reservoir weights collected before and after the daily feeding cycle. Piglets in the CON and 2′-FL diet groups were further randomized to probiotic or no probiotic, resulting in four experimental treatment groups

(CON, FL, BI, and FLBI). Aliquots of the probiotic treatment (Bi-26™; Danisco Global Culture collection DGCC11473 Niebüll, Germany) at the dose of 10^9 CFU/day were solubilized before the start of the study in bacteria glycerol stock (12.1% glycerol) and frozen until administration. The treatment was thawed and orally dosed daily. Piglets not receiving probiotic treatment received an equal dose of glycerol stock.

2.2 Sample collection

On PND 34 or 35, piglets were sedated with an intramuscular injection of Telazol® (Tiletamine HCl and Zolazepam HCl, 3.5 mg/kg BW each, Pfizer Animal Health, Fort Dodge, IA). Blood samples were collected by intracardiac puncture into heparin-laced vials (BD Biosciences, Franklin Lakes, NJ, United States) to isolate peripheral blood mononuclear cells (PBMCs). In addition, blood samples were collected and prepared as serum and or plasma by centrifugation. Piglets were then euthanized by an intracardiac injection of sodium pentobarbital (72 mg/kg BW; Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI).

2.3 Immune cell isolation

Peripheral blood mononuclear cells were isolated on PND 34 or 35 following necropsy as previously described (20). Blood was initially diluted with DPBS (Dulbecco's PBS, no Ca^{++}, no Mg^{++}, Life Technologies, Thermo Fisher, Carlsbad, CA) at a 1:1 ratio, then layered onto Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ), and spun at $400 \times g$ for 40 min at 20°C. PBMCs were collected from the gradient interface and washed three times in wash buffer (Hanks Buffered Salt Solution, no Ca^{++}, no Mg^{++}, Life Technologies) supplemented with 2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), 0.01 M EDTA (Sigma-Aldrich), 50 µg/mL Gentamycin (Life Technologies), 1,000 U/mL penicillin (Sigma-Aldrich), and 100 µg/mL streptomycin (10 mg/mL stock, Sigma-Aldrich). The remaining red blood cells in the pellet were lysed with lysis buffer (0.15 M of NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA). PBMCs were suspended in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 µg/mL gentamycin, 1 mM sodium pyruvate (Life Technologies), 20 mM HEPES (Life Technologies), and 20 mM 1,000 U/mL penicillin/100 µg/mL streptomycin prior to quantification. The number of viable cells was assessed with Countess® Automated Cell Counter (Life Technologies). Cells were then used for phenotypic cell identification by flow cytometry and *ex vivo* stimulation as described below.

2.4 Phenotypic identification of immune cells in PBMC

Phenotypes of PBMC subpopulations were analyzed by flow cytometry (BD™ LSRII, Biosciences) in the Roy J Carver Biotechnology Center Flow Cytometry facility at the University of Illinois. A panel of fluorescein (FITC) or Phycoerythrin (PE)-labeled mAbs was following a previously established staining procedure (20, 21). Briefly, T-lymphocytes were identified by mouse anti-pig CD4 (FITC, Clone 74-12-4) and mouse anti-pig CD8 (PE, Clone 76-2-11) antibodies (Southern Biotech, Birmingham, AL). B-lymphocytes were identified by mouse anti-pig SLA class II (FITC, Clone 2E9/13) and mouse anti-pig CD21 (PE, Clone BB6-11C6.9). A total of 10 µL of each antibody was added to 1×10^6 cells from each sample. Staining procedures were carried out on ice, and the samples were protected from light when possible. In brief, all cells were initially incubated with 5 µg/mL of unlabeled mouse anti-pig CD16 (20 µL; AbD Serotec, Raleigh, NC) to prevent non-specific binding. Each well was blocked with 5% mouse serum (Southern Biotech) for 5 min each. After centrifugation, CD3 was added to the wells, incubated for 20 min (50 µL: CD3:PE-Cy5; Southern Biotech), and centrifuged again. CD4:FITC and CD8:PE were added or SLAII:FITC and CD21:PE (10 µL each) and incubated for an additional 15 min until centrifuged. Cells were washed with PBS/1% BSA/0.1% sodium azide and then fixed with 2% paraformaldehyde. Cells were assessed using an LSRII flow cytometer (BD™, Biosciences). The percentage of T-cell subpopulations and B-cell populations was determined using FCS 4 Express software (DeNovo Software; Glendale, CA). $CD3^+$ events were considered T-cells. $CD3^+CD4^+CD8^-$ events were considered T-helper cells, and CD3 + CD4-CD8+ and CD3 + CD4 + CD8+ were considered cytotoxic T and memory T-cells, respectively. B-cells were identified at $CD21^+$ and SLA class II^+ dual positive cells using SLAII:FITC- and CD21PE-labeled antibodies.

2.5 Serum cytokine concentrations

The concentrations of 13 cytokines, namely, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFNγ), tumor necrosis factor-alpha (TNFα), interleukin (IL)-1α, IL-1RA, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and IL-18 were measured using a Millipore (Milliplex) porcine cytokine array at the University of Illinois Flow Cytometry Center. When the value for a sample fell below the limit of detection (Supplementary Table 1), values were set to 0.

2.6 *Ex vivo* cell stimulation and cytokine production

Isolated PBMC were cultured to assess *ex vivo* cytokine secretion in response to LPS and phytohemagglutinin (PHA) stimulation as previously described (20, 21) Briefly, cells (2×10^5 cells/well) were plated in 96-well plates in a final volume of 200 µL in complete RPMI and were maintained at 37°C under 5% CO_2. Triplicate wells per cell type and experimental treatment were immediately stimulated with 2 µg/mL LPS and 2.5 µg/mL PHA, a lymphocyte activation and proliferation modulator. Cell culture supernatants were collected 72 h after culture initiation and frozen at −80°C until analyzed. Supernatants from the *ex vivo* assay were analyzed for 13 cytokines using a Millipore (Milliplex) pig cytokine array described for serum. In addition, IL-17 was analyzed using an ELISA kit (Raybiotech, Norcross, GA) according to the manufacturer's instructions, with the exception that PBS was used as the basis for the assay buffers.

2.7 Statistical analyses

All data, except for the cytokine data, were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC) using the PROC MIXED procedure. Immunoglobulin and immune cell data were analyzed using a two-way ANOVA. The statistical model was nested for random effects with replicate nested within sow. Both models included fixed effects of diet (prebiotic), probiotics, and the interaction between diet and probiotics with Dunnett's *post hoc* test to compare the statistical differences between the control and experimental treatment groups. If samples were ± 3 standard deviations from the mean, they were excluded as outliers. Significance was defined at a p value <0.05 and trends are reported as a p value <0.10. Data are presented as means ± standard error of the mean (SEM) unless otherwise stated.

Serum and *ex vivo* cytokine concentrations were analyzed using a PROC LCA model using SAS version 9.4 adapted to the respective design. The design for serum samples was a basic design with a split-plot structure. Whole plots were characterized by random effects of sow ID nested within the cohort. Split plots were characterized by piglet ID with the fixed effect of diet. The diet was composed as a 2-by-2 factorial from factors Bi-26™ and 2′-FL. The response is multivariate with manifestations of each cytokine (IFN-γ, IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and IL-18). The design for the *ex vivo* analyses was a one-split of the basic design, by a treatment (unstimulated, LPS, PHA) for PBMC. Whole plots were characterized by random effects of sow ID nested within the cohort. Split plots were characterized by piglet ID with a fixed effect of diet. The diet was composed as a 2-by-2 factorial from factors Bi-26™ and 2′-FL. Split–split plots were characterized by random residual with nested fixed effect of treatment interaction between treatment and diet.

3 Results

3.1 Serum immunoglobulin and cytokine concentrations

No differences were found in serum IgG or IgM. Serum IL-1RA, IL-1β, IL-2, and IL-18 were all significantly higher in FL but not BI alone or FLBI than in CON (Table 1). Serum IL-12 was greater in FL than CON and FLBI but not BI alone. There were trends ($p < 0.1$) for IL-1α, IL-4, IL-6, and IL-10 to also be affected by diet or the interaction of diet and probiotics. Serum IFN-γ and IL-8 were unaffected by experimental treatment (Table 2). Serum IL-17A was below the level of detection.

3.2 PBMC immune cell populations

No treatment effect was observed in T-cell subpopulations or B-cell populations in PBMC (Table 3).

3.3 PBMC *ex vivo* cytokine secretion

To investigate the impact of *in vivo* exposure to 2′-FL or Bi-26™ on cytokine secretion in response to LPS or PHA stimulation, the secretion of cytokines into the media was compared between unstimulated to stimulated PBMCs (Table 4). PBMC isolated from piglets fed FLBI stimulated with LPS *ex vivo* secreted higher concentrations of IL-1RA than CON ($p \leq 0.05$) (Table 4). There was a trend ($p < 0.1$) for the effect of 2′-FL on IFN-γ secretion. There was also a trend ($p < 0.1$) for the effect of 2′-FL on IL-6 secretion. No other cytokines were affected by experimental treatment with stimulation from LPS or PHA compared to CON.

4 Discussion

Oligosaccharides in milk play an important role in modulating the development of the neonatal immune system directly and indirectly through altering the gut microbiota (22). A recent study by Rosa et al. demonstrated the direct effects of HMO in the absence of microbiota (23). Germ-free 1-day-old mice were gavaged daily with pooled HMOs (15 mg) for 7 or 14 days. Samples were collected on days 28, 35, or 50. HMO-administration induced differences in immune cell populations, antibody-secreting cells, and intestinal tissue gene expression in germ-free animals. Cytokine secretion was not measured in that study. Given that pooled HMOs were administered, it is not possible to attribute outcomes to specific individuals or categories of HMOs.

TABLE 1 Serum immunoglobulin concentrations (mg/mL).

Treatment	IgG	IgM
CON	4.88 ± 0.66	0.84 ± 0.11
FL	4.61 ± 0.77	0.73 ± 0.09
BI	4.64 ± 0.52	0.72 ± 0.07
FLBI	6.26 ± 0.45	0.86 ± 0.10
p values		
Diet	0.7529	0.0.5956
Probiotic	0.7280	0.0.4991
Interaction	0.8828	0.0.7462

Data are expressed as means ± SEM. $n = 9$–12.
BI, control diet + 10^9 CFU Bi-26™/day; CON, Control diet; FL, Control formula + 1.0 g/L 2′-FL; FLBI, Control formula + 1.0 g/L 2′-FL + 10^9 CFU Bi-26™/day; Ig, Immunoglobulin; SEM, Standard error of the mean.

TABLE 2 Serum cytokine concentrations (pg/mL).

					p value		
	CON	FL	BI	FLBI	Diet	Probiotic	Interaction
IFN-γ	8,032 ± 2,729	7,265 ± 2,493	6,396 ± 2,506	8,863 ± 2,860	0.825	0.896	0.219
IL-1α	28.1 ± 17.2	73.6 ± 22.7	51.3 ± 21.6	46.6 ± 21.7	0.077	0.976	0.112
IL-1β	52.2 ± 48.5^{a}	236.1 ± 84.3^{b}	168.0 ± 79.1^{ab}	129.7 ± 73.7^{ab}	0.076	0.952	0.043
IL-1RA	94.5 ± 47.3^{a}	274.1 ± 64.4^{b}	192.4 ± 62.3^{ab}	133.6 ± 56.4^{ab}	0.053	0.803	0.034
IL-2	32.7 ± 40.7^{a}	301.3 ± 98.2^{b}	152.2 ± 82.2^{ab}	129.3 ± 79.2^{ab}	0.091	0.944	0.039
IL-4	96.7 ± 121.2	795.2 ± 282.1	530.5 ± 261.0	354.8 ± 229.0	0.099	0.968	0.091
IL-6	53.2 ± 34.4	175.8 ± 50.7	94.0 ± 43.3	88.4 ± 43.4	0.103	0.960	0.067
IL-8	228.2 ± 48.4	252.8 ± 46.7	263.9 ± 50.5	271.9 ± 52.7	0.575	0.976	0.984
IL-10	107.4 ± 89.9	490.9 ± 155.2	353.6 ± 149.6	269.5 ± 139.8	0.087	0.976	0.069
IL-12	439.7 ± 53.2^{a}	552.0 ± 49.7^{b}	550.1 ± 52.6^{abc}	461.0 ± 53.8^{ac}	0.139	0.652	0.005
IL-18	447.5 ± 196.6^{a}	1,078 ± 244.3^{b}	923.0 ± 252.3^{ab}	731.5 ± 243.7^{ab}	0.076	0.992	0.044

Data are expressed as means ± SEM. $n = 9–12$. Different letter superscripts indicate differences between groups at $p \leq 0.05$.
BI, Control diet + 10^9 CFU Bi-26™/day; CON, Control diet; FL, Control formula + 1.0 g/L 2′-FL; FLBI, Control formula + 1.0 g/L 2′-FL + 10^9 CFU Bi-26™/day; IFN, Interferon; IL, Interleukin; PND, Postnatal day; SEM, Standard error of the mean; TNF, Tumor necrosis factor.

TABLE 3 Immune cell populations in peripheral blood mononuclear cells.

					p value		
	CON	BI	FL	FLBI	Diet	Probiotic	Interaction
T-cells (% $CD3^+$)	52.9 ± 3.69	54.8 ± 3.25	58.1 ± 0.92	55.1 ± 1.92	0.2044	0.6596	0.7493
Helper $CD4^+$	11.4 ± 1.29	10.7 ± 1.11	9.3 ± 1.04	12.0 ± 1.04	0.9775	0.9194	0.4175
Cytotoxic $CD8^+$	15.9 ± 2.39	14.6 ± 1.99	14.1 ± 1.41	13.8 ± 1.41	0.4935	0.8872	0.3323
Memory $CD4^+$ $CD8^+$	4.29 ± 0.82	4.01 ± 0.49	3.83 ± 0.83	3.90 ± 0.72	0.9296	0.9003	0.7979
B-cells (% $MHCII^+CD21^+$)	52.9 ± 3.69	54.8 ± 3.25	58.1 ± 0.92	55.1 ± 1.92	0.2044	0.6596	0.7493

Data are expressed as means ± SEM. $n = 9–12$.
BI, Control diet + 10^9 CFU Bi-26™/day; CD, Cluster differentiation; CON, Control diet; FL, Control formula + 1.0 g/L 2′-FL; FLBI, Control formula + 1.0 g/L 2′-FL + 10^9 CFU Bi-26™/day; PBMC, Peripheral blood mononuclear cells; SEM, Standard error of the mean.

Herein, the individual and combined effects of 2′-FL and *B. infantis* were studied for the following reasons. Supplementing infant formula with all HMOs at the current time is not feasible. 2′-FL is a predominant HMO in the milk of secretor mothers (4), it has been supplemented to infant formulae for several years with no adverse effects (24) and has documented effects on infant immunity (8). *B. infantis* was studied as formula-fed infants are devoid of *B. infantis*, a predominant bifidobacterial strain in breastfed infants, it can use 2′-FL and has immunomodulatory effects and has documented effects on infant immunity (16, 18). Given the demonstrated benefits of supplemental 2′-FL (8, 25) and *B. infantis* (13, 26), documented in previous clinical trials, and their potential for symbiotic activity, we investigated the individual and combined effects of dietary 2′-FL at 1 g/L and Bi-26™ at 10^9 CFU on immune development and function in piglets. Our findings show the effects of 2′-FL systemic immunity and potential interactive effects when supplemented with Bi-26™.

We found no effect of dietary 2′-FL and/or Bi-26™ on circulating immune cell populations; all levels were within the range previously reported in our laboratory for healthy piglets (27, 28), suggesting that there was no impact on T-cell or B-cell percentages. In a clinical trial of 2′-FL supplementation, circulating $CD4^+$ and $CD8^+$ T-lymphocyte populations were similar between breastfed infants and infants fed formula supplemented with 2′-FL (8). Since we saw no differences in T-cell subsets or B-cells in PBMC, 2′-FL could be affecting innate immunity. Although dendritic cells make up a small percentage of circulating immune cells, they present antigens to T-cells, leading to the upregulation of cytokines to recruit immune cells to clear the pathogen (29). IL-10 and IL-6 are upregulated in dendritic cells treated with HMOs isolated from human milk (30). Increased dendritic cell activity could recruit T-helper cells, which could explain why the serum cytokines were increased in our study; however, more research is required to establish the precise mechanism at hand.

A key finding of the present study was that 2′-FL increased the concentrations of IL-1RA, IL-1β, IL-2, IL-12, and IL-18 in serum compared to control piglets. The group receiving both 2′-FL and Bi-26™ did not differ from the control group, suggesting that the co-administration of Bi-26™ may moderate the effect of 2′-FL impact on serum cytokines. Studies in human infants have shown that breastfeeding is associated with lower circulating concentrations of proinflammatory cytokines (8, 31). In a clinical trial of 2′-FL supplementation, infants fed formula alone had higher IL-1RA, IL-1β, IL-1α, tumor necrosis factor-α, and IL-6 than infants fed formula

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TABLE 4 Cytokine concentrations (pg/mL) in conditioned media from *ex vivo* stimulated peripheral blood mononuclear cells.

					p value		
	CON	FL	BI	FLBI	Diet	Probiotic	Interaction
IFN-γ							
Unstimulated	22.5 ± 0.77	20.2 ± 0.60	11.3 ± 0.27	5.92 ± 0.11	0.841	0.208	0.944
Stimulated	23.6 ± 0.82	166.6 ± 12.8	43.0 ± 1.95	48.5 ± 2.4	0.072	0.317	0.082
Fold-stimulation	1.05	8.32	3.80	8.20	0.121	0.834	0.165
IL-1RA							
Unstimulated	366.8 ± 7.26	453.0 ± 9.34	450.9 ± 9.71	813.7 ± 23.9	0.407	0.897	0.841
Stimulated	777.7 ± 22.1 [a]	1,145 ± 36.7 [a]	1,042 ± 33.4 [a]	1,677 ± 68.5 [b]	0.050	0.204	0.484
Fold-stimulation	2.12	2.53	2.31	2.06	0.136	0.070	0.509
IL-6							
Unstimulated	114.9 ± 3.66	166.0 ± 5.71	74.4 ± 1.88	25.2 ± 0.39	0.944	0.453	0.749
Stimulated	311.3 ± 15.7	643.3 ± 39.7	486.3 ± 29.1	504.7 ± 31.9	0.097	0.447	0.674
Fold-stimulation	2.71	3.87	6.54	20.1	0.089	0.112	0.430

Data are expressed as means ± SEM. $n = 9–12$. Different letter superscripts indicate differences between groups at $p \leq 0.05$.
BI, Control diet + 10^9 CFU Bi-26™/day; CON, Control diet; FL, Control formula + 1.0 g/L 2′-FL; FLBI, Control formula + 1.0 g/L 2′-FL + 10^9 CFU Bi-26™/day; IFN, Interferon; IL, Interleukin; PND, Postnatal day; SEM, Standard error of the mean.

supplemented with 0.2 g/L and 2.0 g/L 2′-FL. Infants supplemented with 2′-FL had inflammatory cytokine concentrations similar to that of breastfed infants (8). Similar observations have been made in rat studies, with 2′-FL lowering IL-1β, IL-4, IL-6, IL-12, IFN-γ, and TNF-α in the small intestine (11) and serum IL-18 in male rats compared to control (32). The study of Goehring et al. (8) demonstrated similar effects of 0.2 g/L and 2.0 g/L on serum cytokines, supporting a lack of dose dependency on cytokine concentrations (8); so, it is unlikely we would have seen lower cytokine concentrations with a higher or lower dose. Intact HMOs can be absorbed into the blood, and it is likely that immune cells are directly influenced in circulation.

In addition to measuring circulating cytokines, we assessed whether *in vivo* exposure to 2′-FL and/or Bi-26™ would impact cytokine secretion by immune cells isolated from blood (PBMC) when exposed to LPS or PHA *ex vivo*. Cytokines are predominantly produced by T-helper cells and macrophages (29). Although, in the present study, T-cell and B-cell populations in PBMC were unaffected by 2′-FL and Bi-26™, these supplements impacted immune response to LPS *ex vivo*. We found that PBMC, isolated from piglets fed 2′-FL alone, demonstrated increased secretion of IL-1RA, after LPS stimulation. In addition, *in vivo* exposure to 2′-FL tended to increase IFN-γ and IL-6 secretion. IFN-γ induces a wide array of immune responses. It can promote macrophage activation, facilitate antiviral and antibacterial activities, increase antigen presentation, and influence activation of the innate immune system (33) while IL-1RA is an inhibitor of pro-inflammatory IL-1 (34). LPS is a TLR-4 agonist, which leads to the production of IL-1, so the increase in IL-1RA and IL-6 could be a response to ameliorate inflammation caused by LPS challenge since 2′-FL and *B infants* have been shown to have anti-inflammatory effects. These results may be due to receptors that recognize HMOs, such as galectins and TLRs. Since HMOs have been found in the blood (6, 35), binding of HMOs to galectins in the blood could result in the regulation of TLR expression. In addition, 2′-FL has been shown to bind directly to TLR-4 *in silico* and modulate response to LPS by reducing TLR-4 expression in mice (9). An *in vitro* model of necrotizing enterocolitis in immature human enterocytes, *B. infantis* (ATCC 15697) conditioned medium reduced expression of inflammatory markers, TLR-2 and TLR-4, as well as concentrations of IL-6 and IL-8 (36). In a piglet model of *Staphylococcus aureus* infection, piglets supplemented with *B. infantis* (ATCC 15697) at 3×10^9 CFU/day had increased serum IL-10, potentially as an attempt by the immune system to reduce the inflammatory response (15). Although no increase in IL-10 was observed following LPS stimulation, an increase in IL-1RA secretion by PBMC from piglets in the combined group was observed, suggesting that 2′-FL and Bi-26™ may still exhibit an anti-inflammatory effect. In addition, PBMC stimulated with PHA showed no effect of experimental treatment compared to the control formula. PHA is a lectin that requires monocytes, which are antigen-presenting cells, to induce T-cell proliferation (37). Our data suggests that neither 2′-FL nor Bi-26™ enhances T-cell proliferation in response to PHA compared to the control formula. Our study showed that dietary exposure to 2′-FL elicited a more robust cytokine response to the LPS challenge than from cells isolated from piglets fed the control formula. This could translate to improved immune response in infants receiving these HMOs.

Piglets were not immunologically challenged *in vivo*, therefore this study may not be translational to infants enduring infection. Although *ex vivo* stimulation shows a robust response to LPS stimulation, *ex vivo* conditions do not exactly mirror *in vivo* inflammation (38); therefore, an *in vivo* immune challenge could better evaluate the potential protective effects of 2′-FL and Bi-26™. A limitation of the study is that all immune analyses were from samples taken at a single time point at the end of the study, which limits our ability to evaluate the influence 2′-FL and Bi-26™ have on early immune development.

To summarize, human milk is the ideal feeding mode for infants; however, the World Health Organization (WHO) reports that globally, 66% of infants are not exclusively breastfeeding by 6 months of age, in accordance with the recommendations from 2015–2020 (39). As a result, many families must rely on infant formula during this critical period of development. While 2′-FL and *B. infantis* are commercially available, this is the first study to our knowledge to assess their combined effect on immune development in the piglet model. We have shown that the addition of 2′-FL and/or Bi-26™ could provide formula-fed infants with certain protective benefits associated with bioactive components that are typically lacking in infant formulae. Future investigations are needed to evaluate the effects of 2′-FL and Bi-26™ in response to immune stimuli during development and to explore the underlying mechanisms of these immune responses. This study builds upon previous observations demonstrating that supplementation of formula with 2′-FL and/or Bi-26™ is well tolerated and supports normal growth of piglets (19). Additionally, supplementation with 2′-FL and/or Bi-26 has been associated with changes in exploratory behaviors in novel object recognition tests, as well as changes to brain macro/micro-structures (40). Taken together, these findings support the multifunctional activities of 2′-FL and/or Bi-26™ in neonates.

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 animal study was approved by University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committtee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

VD: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. MM: Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Methodology, Supervision. JH: Writing – review & editing, Conceptualization, Resources. AO: Conceptualization, Writing – review & editing, Formal analysis. HJ: Conceptualization, Writing – review & editing. RM: Conceptualization, Writing – review & editing. NC: Writing – review & editing, Formal analysis. ML: Formal analysis, Writing – review & editing. RD: Writing – review & editing, Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft. SD: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing, Formal analysis, Investigation.

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Conflict of interest

SMD and RND received grant funding from IFF, the producer of *B. infantis* Bi-26™ and 2′-FL. JH, HMJ, NC, MJL, and ACO are employees of IFF. RM was employed by IFF at the time the study was conducted.

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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1444594/full#supplementary-material

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Associations between human milk EV-miRNAs and oligosaccharide concentrations in human milk

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Introduction: Human milk contains human milk oligosaccharides (HMOs) and microRNAs (miRNAs), which are key bioactive components. HMOs are indigestible carbohydrates that impact infant growth and development. miRNAs are small, non-coding RNAs that regulate post-transcriptional gene expression. miRNAs are abundant in human milk and can be contained in extracellular vesicles (EVs). There is evidence that miRNAs are synthesized in the mammary epithelium and may influence mammary gland development and milk synthesis. However, the relationships between miRNAs and HMOs have yet to be fully characterized.

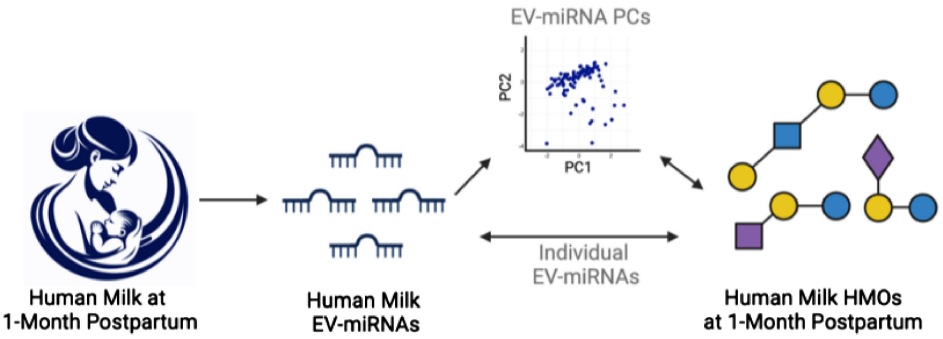
Methods: This study examined the associations between 210 human milk EV-miRNAs and 19 HMOs in a cohort of 98 Latina mothers. HMO measures included summary measures and concentrations of 19 HMOs. Relationships between EV-miRNAs and HMOs were examined using principal components analysis and associations between individual EV-miRNAs and HMOs were assessed.

Results: Overall patterns of EV-miRNA levels, summarized using principal components, were associated with HMO summary measures and concentrations. Levels of individual EV-miRNAs were associated with HMO summary measures and individual concentrations of 2'FL, 3FL, 3'SL, 6'SL, FLNH, LNFP I, and LNH.

Discussion: Results from this study suggest that human milk EV-miRNAs are associated with the concentration of HMOs, which may have important effects on infant growth and development.

KEYWORDS

microRNA, human milk (HM), human milk oligosaccharides, extracellular vesicles, EV-microRNAs



GRAPHICAL ABSTRACT
Images created with Dalle AI and BioRender.com.

Introduction

Human milk is the optimal source of nutrition for term infants and is associated with a wide variety of positive health outcomes (1–8). Human milk oligosaccharides (HMOs) are indigestible carbohydrates (9, 10) which are found in colostrum, transitional, and mature milk. HMOs are the third-most abundant solid component of human milk and are composed of five fundamental monosaccharides: glucose, galactose, N-acetylglucosamine, fucose, and sialic acid. The structure of nearly all HMOs features lactose at their reducing end (11). HMOs resist digestion to have prebiotic effects in the infant gut (12–14), serve as antiadhesive antimicrobials (15, 16), mediate epithelial cell responses (14, 17), are immune modulators (18), and support brain development (19). Additionally, HMO diversity, a summary measure of overall richness and evenness of HMO levels, has previously been associated with child growth measures including with child height and weight (20). Nevertheless, numerous aspects of HMO biosynthesis have yet to be fully characterized.

HMO diversity and concentrations of specific HMOs are primarily determined by genetics, and genetic variation can explain up to 55% of variability in HMO concentrations (21). For example, individuals with an active *Se* locus are defined as Secretors. These individuals produce milk abundant in 2'-fucosyllactose (2'FL), Lacto-N-Fucopentaose I (LNFP I), and other alpha1-2-fucosylated HMOs. In contrast, individuals without an active *Se* locus lack the FUT2 enzyme and their milk contains few to no alpha1-2-fucosylated HMOs (9). Similarly, the Lewis gene regulates expression of Le^a and Le^b antigens. Human milk from individuals without enzymes from the Lewis gene produce milk with low LNFP II (22). In addition to genetics, HMO concentrations change throughout lactation (23–25). Though the highest concentrations of most HMOs are present at the colostrum stage, the concentration of some HMOs increase with infant age (23, 26). Further, HMO concentrations have been associated with season and geographic location (which may be closely linked with genetics) (10, 27), maternal body mass index and obesity (10, 24, 28, 29), maternal age (10, 24), parity (27), ethnicity (27), and exclusive breastfeeding (27). Despite the identification of these factors that influence HMO concentrations, their biosynthesis and determinants of their concentration have not yet been fully elucidated.

In addition to HMOs, human milk contains several other bioactive components, which include hormones, cytokines, leukocytes, immunoglobulins, and microRNAs (miRNAs) (30). miRNAs are small, non-coding RNAs that regulate gene expression post-transcriptionally. Human milk is one of the most abundant sources of miRNAs and there is evidence that these miRNAs are synthesized in the mammary epithelium (31) and may influence mammary gland development (32). miRNAs can be contained inside of extracellular vesicles (i.e., EV-miRNAs), which confer resistance to harsh environments. Importantly, EV-miRNAs can survive digestion *in vitro* (33–36) and may be taken up by epithelial cells in the infant gut (35, 36). Therefore, EV-miRNAs in human milk may impact postnatal gut maturation, nutrient uptake, and infant health via local regulation of post-transcriptional gene expression. Similar to HMOs, human milk miRNAs have been shown to vary by gestational age and pre-pregnancy BMI (37, 38) and may also be influenced by breastfeeding (39). Importantly, many of the miRNAs that are abundant in human milk are involved in milk synthesis (e.g., triacylglycerol in milk fat) (39). However, whether human milk miRNAs may also influence HMO composition in human milk is currently unknown.

Although HMOs are essential for infant nutrition and health, the factors that determine HMO concentrations remain incompletely understood. We hypothesize that human milk EV-miRNAs influence HMO concentrations. This hypothesis is supported by the observation that miRNAs play a critical role in regulating gene expression at the post-transcriptional level and many shared factors that affect miRNA abundance are also linked with HMO concentrations, such as maternal BMI and breastfeeding (10, 24, 27–29, 37–39). Given this, the primary aim of this study was to determine whether human milk EV-miRNAs at 1-month postpartum are associated with summary measures of HMOs (i.e., diversity, sum of all HMOs, and sum of HMO-bound sialic acid and HMO-bound fucose) and concentrations of 19 HMOs that our analytical platform can quantify with confidence.

Methods

Study participants

The Mother's Milk Study is a longitudinal cohort of Latino mother-infant dyads from Southern California, which is investigating the associations between HMOs, the infant gut microbiota, and early life growth and development. Detailed methods have been previously described (40, 41). Briefly, participants were recruited from Los Angeles County maternity clinics affiliated with the University of Southern California. Individuals were eligible to participate if they were 18 years of age or older at time of delivery; had a healthy, singleton birth; were enrolled by approximately one month postpartum; and were able to read at the 5th grade level in either Spanish or English. Individuals were excluded if they had any medical diagnoses or were taking medications known to affect physical or mental health, nutritional status, or metabolism; were current tobacco users (>1 cigarette in the past week); reported recreational drug use; had pre-term or low birth weight infants; or had infants with any clinically diagnosed fetal abnormalities. The Institutional Review Boards of the University of Southern California, Children's Hospital of Los Angeles, and the University of Colorado Boulder approved study procedures. Written informed consent was obtained from participants prior to study enrollment.

Study design

Participants had visits at 1-, 6-, 12-, 18-, and 24-months postpartum. The Mother's Milk Study had 219 mother-infant dyads, 209 of whom contributed a human milk sample at the 1-month visit. Of these, funding was available to assess EV-miRNA levels in 110 human milk samples. Among these 110, 11 were removed since they were classified as HMO "non-secretors" (produced under 500 nmol/mL of the HMO 2'FL). We elected to exclude non-secretors because of their low abundance in our sample. An additional participant was excluded as they produced an intermediate amount of 2'FL (559 nmol/mL, considered a "non-secretor" by some definitions), and had HMO diversity 3 standard deviations (SD) from the mean. This resulted in a final sample size of 98. Participants who were excluded from EV-miRNA analysis were similar to those who were included (40).

Clinical assessments

The clinical measures used for this study were derived from the 1-month postpartum visit. Maternal weight (kg) was measured using an electronic scale and standing height was measured using a stadiometer (m) to calculate maternal body mass index (BMI, kg/m^2). Additionally, maternal age at delivery, infant sex, delivery mode, days postpartum, and number of breast feedings per day were collected at the 1-month postpartum visit. Questionnaires were used to determine breast feedings per day, where participants selected 0-1, 1, 2, 3, 4, 5, 6, 7, or ≥ 8 feedings/day and this was treated as a continuous variable. Non-consecutive 24-hour dietary recalls were performed to represent average maternal dietary intake, and from this, the healthy eating index (HEI) was calculated (42). HEI is a composite dietary measure which assesses how well dietary intake aligns with the Dietary Guidelines for Americans (43). Socioeconomic status was measured using a modified version of the Hollingshead Index as previously reported (44).

HMO analysis

Mothers fasted for at least 1 hour before human milk samples were collected, and sample collection occurred at least 1.5 hours after the most recent feeding. Participants were instructed to provide a single full expression from the right breast using an electric breast pump, ensuring the collection of fore-, mid-, and hind-milk as previously described (45). Milk was frozen and stored at -80°C until analysis. The Bode Lab at the University of California San Diego conducted HMO analysis. For each participant, one 500μL aliquot of human milk was shipped on dry ice. HMOs are extremely stable, and do not degrade during repeated freeze/thaw cycles or pasteurization (46). The HMO analysis has been previously described in detail (23). Briefly, HPLC after fluorescent derivatization (Vanquish Quaternary HPLC with fluorescent detection, Thermo Fisher Scientific) was used for HMO analysis. Lipids, proteins, salts, etc., were removed using solid phase extraction over C18 and Carbograph. Next, the reducing end of oligosaccharides were labeled with 2-aminobenzamide and removed excess label by solid phase extraction over silica. To account for analyte loss during the extraction procedure, raffinose was added at the beginning of sample processing. Overall, 19 HMOs were identified and quantified: 2′-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 3′-sialyllactose (3'SL), 6′-sialyllactose (6'SL), difucosyllactose (DFLac), difucosyllacto-*N*-hexaose (DFLNH), difucosyllacto-*N*-tetrose (DFLNT), disialyllacto-*N*-hexaose (DSLNH), disialyllacto-*N*-tetraose (DSLNT), fucodisialyllacto-*N*-hexaose (FDSLNH), fucosyllacto-*N*-hexaose (FLNH), lacto-*N*-fucopentaose (LNFP) I, LNFP II, LNFP III, lacto-*N*-hexaose (LNH), lacto-*N*-neotetraose (LNnT), lacto-*N*-tetrose (LNT), sialyl-lacto-*N*-tetraose b (LSTb), and sialyl-lacto-*N*-tetraose c (LSTc). These are 19 of the most abundant HMOs, and they represent more than 95% of the total HMO concentration and all structural features of HMOs, including chain elongation, branching, and all known types of fucosylation and sialylation. HMO concentrations are reported in nmol/mL. HMO diversity was estimated using Simpson's diversity. The summary measure, sum of all HMOs in a sample, is the sum of all HMOs quantified in each sample. HMO-bound fucose and HMO-bound sialic acid are the sum of all sialic acid and all fucose molecules bound to HMOs in a sample, respectively (e.g., each molecule of 2'FL contains 1 molecule of fucose, and each molecule of DFLNT contains 2 molecules of fucose).

EV-miRNA sequencing, processing, and expression

EVs were isolated from stored human milk samples as previously described (47). Milk samples were thawed on ice prior to analyses.

Next, samples were centrifuged to remove the lipid layer, then again to remove cellular debris and apoptotic bodies. EVs were extracted using the ExoEasy Maxi KIT (Qiagen, Germantown, MD) and total RNA was isolated with the miRNeasy Serum/Plasma Maxi KIT (Qiagen, Germantown, MD). Samples were cleaned using the RNA Clean & Concentrator-5 Kit (Zymo Research, Irvine, CA) and sample purity and quantity were measured on an Implen NanoPhotometer spectrophotometer (München, Germany). As previously described (40), concentration, sizes, and distribution were assessed in four randomly selected samples using nanoparticle tracking analysis on the ViewSizer 3000 (Horiba Scientific, Piscataway, NJ). The Exo-Check Exosome Antibody Assay (System Biosciences, Palo Alto, CA) was used to confirm the presence and purity of isolated EVs on four sets of three pooled EV samples and three sets of three matched EV-depleted samples. All relevant EV characterization data have been submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV220416) (48).

Sequencing and library preparation was performed at the University of California San Diego. The NEBNext Small RNA Library Prep Set for Illumina (NEB, Ipswich, MA) was used to construct sequencing libraries with optimization to account for low input and cell-free RNA. Reactions were conducted at one-fifth the recommended volume, adapters were diluted 1:6, and library amplification PCR used 17 cycles. Libraries were cleaned with the DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA) and the concentrations were quantified using the Quant-iT PicoGreen dsDNA Assay (Invitrogen, Waltham, MA). Samples were pooled with equal volumes. The pool's size distribution was determined with a DNA HS Chip on a BioAnalyzer (Agilent Technologies, Santa Clara, CA) before size selection (115-150 base pairs [bp]) on a Pippin Prep instrument (Sage, Beverly, MA) to remove adapter dimers and fragments larger than the target miRNA population. Libraries were sequenced to ~1 million total reads per pool using a MiSeq instrument with a Nano flow cell (Illumina Inc, San Diego, CA). This sequencing data was used to balance the samples into new pools for deeper sequencing on a HiSeq4000 instrument using single-end 75 bp runs.

Sequencing data were mapped using the ExceRpt small RNA sequencing data analysis pipeline on the Genboree Workbench (49). Samples were mapped using default parameters, except for filtering to a minimum read length of 15 nucleotides with zero mismatches. Quality control was performed according to External RNA Controls Consortium guidelines (49). One sample had <100,000 transcriptome reads and was removed from subsequent analysis. Raw EV-miRNA read counts were normalized using the trimmed mean of M (TMM) method from the EdgeR package (50).

Statistical analysis

Descriptive statistics were calculated using the mean and standard deviation (SD) for continuous variables and frequencies for categorical variables. We examined the associations between human milk EV-miRNAs and HMOs using two approaches. First, due to the high-dimensional EV-miRNA data, we summarized EV-miRNA levels using principal components (PC) analysis. PCs were then used to assess whether EV-miRNA profiles were associated with HMOs (i.e., diversity, HMO-bound sialic acid, HMO-bound fucose, and individual HMO concentrations). To accomplish this, multivariable linear regression was used to estimate the independent associations between our primary predictors (PC1 and PC2) with HMO measures. Prior to principal component transformation, values were centered and scaled to have unit variance. As a second approach, multivariable linear regression models were used to estimate the associations between individual EV-miRNAs with human milk HMOs. Based on our previous work, all models adjusted for technical covariates (i.e., proportion of rRNA, volume of skim milk) as well as days postpartum, time of day of milk collection, and number of breast feedings per day (40). As a sensitivity analysis, we additionally adjusted for maternal variables age, BMI, and HEI. Unadjusted p-values are reported; however, all analyses were also adjusted for multiple testing using the Benjamini-Hochberg procedure with a threshold of $P_{BH} < 0.10$ (51).

Pathway analysis was used to characterize putative mRNA targets of EV-miRNAs that were associated with HMO expression using DIANA MirPath version 4 (52). For pathway analysis, we converted all precursor EV-miRNAs into their mature counterparts, which applied only to hsa-mir-378c. Tarbase v8.0 (53), a catalogue of experimentally validated miRNA-gene interactions, and microT-CDS (54), which predicts *in silico* miRNA-gene interactions, were used to identify Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with significant enrichment.

Results

Population characteristics

Table 1 shows the mean physical and social characteristics of the 98 mothers included in the analysis. The Hollingshead Index was used to measure the participant's socioeconomic status, where 54% had low or very low SES (Hollingshead score < 26.5). On average, mothers were 27.9 years of age at the time of birth (range: 18-42 years) and overweight at 1-month postpartum (BMI: 29.9 ± 4.8 kg/m^2). Additionally, at 1-month postpartum, 16.3% of mothers had a healthy weight, 38.8% had overweight, and 44.9% had obesity. Mothers self-reported that they were breastfeeding an average of 6.3 times per day (range: 0-8) and were in the mature milk stage where, on average, human milk samples were collected 32.5 days after delivery. The top five most abundant EV-miRNAs were miR-148a-3p, miR-146b-5p, miR-200a-3p, let-7g-5p, and let-7b-5p, which is largely consistent with previous studies (55). These EV-miRNAs were detected in every sample and comprised 15.5% of the total reads. As expected, the concentrations of several human milk HMOs were both positively and negatively correlated with one another at 1-month postpartum (Figure 1). Table 2 shows the characteristics of HMO summary measures and individual concentrations at 1-month postpartum.

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TABLE 1 Characteristics of 98 mothers from the Southern California Mother's Milk study at 1-month postpartum.

Maternal Characteristics	Mean ± SD or N, %
Maternal age at birth (years)	27.9 ± 5.7
Socioeconomic status[a]	26.0 ± 12
Infant sex (female, male, % female)	55, 43, 56%
Delivery (vaginal, caesarean, % vaginal)	75, 23, 76.5%
Days post-partum	32.5 ± 3.1
Number of breastfeedings per day	6.3 ± 2.4
Maternal BMI (kg/m^2)	29.9 ± 4.8
Health Eating Index	67.9 ± 11.4

Baseline (1-month) characteristics of 98 Latina mothers from the Southern California Mother's Milk Study. Data are reported as mean and standard deviation (SD) unless otherwise noted.
[a]SES was estimated using a modified version of the Hollingshead index. Range (3,63).

EV-miRNA principal components are associated with HMO measures

PC analysis was used to assess whether EV-miRNA profiles were associated with HMO summary measures and concentrations (Supplementary Figure 1). Here, we focused on PC1 and PC2, which explained a total of 34.7% of the variation in EV-miRNA measures (Supplementary Figure 2). We did not include subsequent PCs because they explained little additional variance (e.g., PC3 explained 5.9%). Using multivariable linear models, we constructed models that included both PC1 and PC2 while adjusting for our *a priori* covariates. PC2 was inversely associated with HMO diversity (P = 0.046), positively associated with the sum of all HMOs (P = 0.0001), and positively associated with HMO-bound fucose (P = 0.02). The EV-miRNAs that were most positively associated with PC2 included miR-183-5p, miR-151a-3p, miR-511-5p, miR-99b-5p, and miR-3615. Conversely, the EV-miRNAs that were most inversely associated with PC2 included miR-502-3p, miR-629-5p, miR-146a-5p, miR-500a-3p, and miR-21-5p (Supplementary Table 1). Additionally, PC2 was positively associated with the concentration of three HMOS, 2'FL, 6'SL, and FLNH (Table 3, all P_{BH} < 0.10). We did not observe any statistically significant associations between PC1 with HMO measures. PC1 was most positively associated with levels of miR-183-5p, miR-151a-3p, miR-511-5p, miR-99b-5p, and miR-3615 and was most negatively associated with levels of miR-21-5p, miR-500a-3p, miR-146a-5p, miR-629-5p, and miR-502-3p.

In sensitivity analyses, we additionally adjusted for maternal BMI, maternal healthy eating index, and maternal age (Supplementary

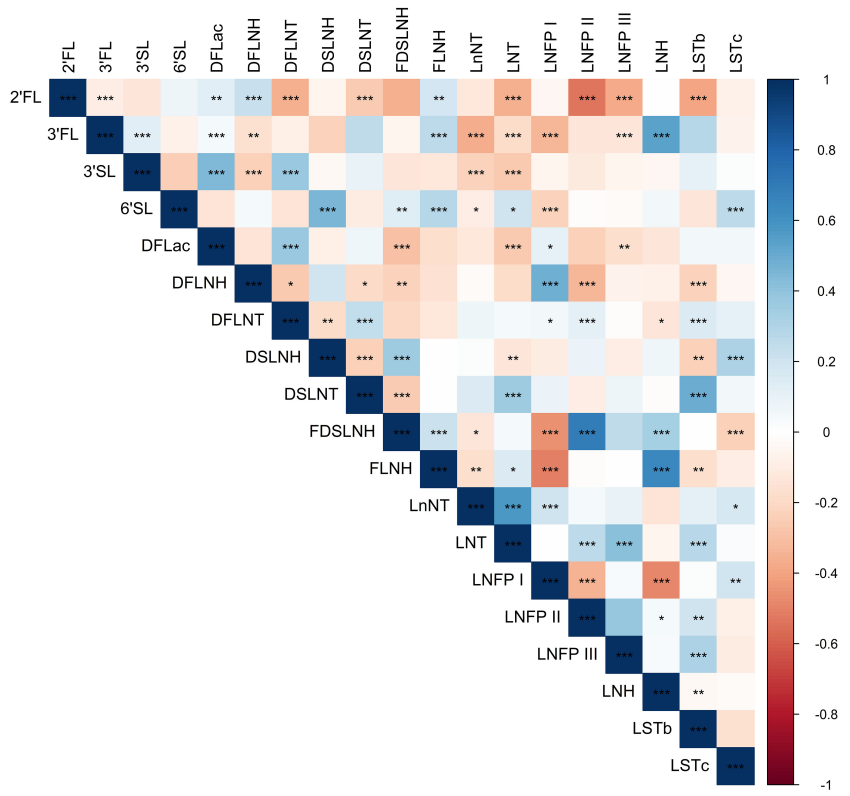

FIGURE 1
Heatmap showing Spearman correlations among HMO concentrations. Blue shading indicates positive Spearman correlation and red shading indicates negative Spearman correlation; color saturation indicates the strength of the correlation. Stars indicate statistical significance, with *** showing correlations with P < 0.001, ** showing correlations with P < 0.01, and * showing correlations with P < 0.05.

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TABLE 2 Characteristics of HMOs at 1-month postpartum.

HMO Summary Measures (nmol/mL)	Mean ± SD
Diversity*	5.1 ± 1.9
Sum of HMOs	16883.9 ± 1813.0
HMO-bound sialic acid	3431.5 ± 888.4
HMO-bound fucose	14583.0 ± 2036.6
HMO Concentrations (nmol/mL)	**Mean ± SD**
2'FL	6771.4 ± 3175.6
3FL	748.2 ± 1151.5
3'SL	592.5 ± 658.9
6'SL	956.5 ± 315.9
DFLac	475.6 ± 292.5
DFLNH	159.0 ± 122.2
DFLNT	1491.4 ± 815.2
DSLNH	159.0 ± 122.2
DSLNT	389.1 ± 179.7
FDSLNH	114.2 ± 98.1
FLNH	137.6 ± 112.0
LnNT	625.0 ± 322.7
LNT	1130.1 ± 667.2
LNFP I	1656.7 ± 1056.4
LNFP II	842.4 ± 387.7
LNFP III	60.4 ± 29.1
LNH	92.4 ± 61.4
LSTb	88.1 ± 54.0
LSTc	318.4 ± 152.3

Baseline (1-month) summary of HMOs among 98 Latina mothers from the Southern California Mother's Milk Study. Data are reported as mean and standard deviation (SD). *Diversity was estimated using Simpson's Diversity measure.

Table 2). Results from these analyses were largely similar to results from the main analyses, where the sum of all HMOs was positively associated with PC2 (P = 0.0004) and positively associated with HMO-bound fucose (P = 0.04). While not statistically significant (P = 0.09), the estimated association between PC2 and HMO diversity was negative. We also found that PC2 was positively associated with 2'FL and 6'SL.

Individual EV-miRNAs are associated with HMO measures

As shown in Table 4, multivariable linear regression analysis, which adjusted for technical covariates, days postpartum, time of day of human milk collection, and number of breast feedings per day, revealed that 26 EV-miRNAs were associated with HMO diversity (all P < 0.05), 55 with the sum of all HMOs, 9 with HMO-bound sialic acid, and 33 with HMO-bound fucose. Complete results are shown in Supplementary Table 3. When examining individual HMO concentrations of 2'FL, 3FL, 3'SL, 6'SL, FLNH, LNFP I, and LNH, each were statistically significantly associated with levels of 7, 17, 1, 1, 4, 1, and 5 individual EV-miRNAs (respectively) after correction for multiple testing ($P_{BH} < 0.1$).

In sensitivity analyses where we additionally adjusted for maternal BMI, maternal healthy eating index (HEI), and maternal age, findings were largely consistent (Supplementary Tables 4, 5). Among the 26 EV-miRNAs that were significantly associated with HMO diversity, 19 remained significantly associated after these additional adjustments. Among the 55 EV-miRNAs significantly associated with the sum of all HMOs in the main analysis, 49 remained significant in the sensitivity analysis. Among the 9 EV-miRNAs associated with HMO-bound sialic acid in the main analysis, 4 remained significant in the sensitivity analysis. Among the 33 EV-miRNAs which were associated with HMO-bound fucose in the main analysis, 27 of those remained significantly associated in the sensitivity analysis. Among the 7 EV-miRNAs associated with 2'FL concentration, 6 remained significant in the sensitivity analysis. Among the 17 EV-miRNAs which were associated with 3'FL concentration in the main analysis, 16 remained significant in the sensitivity analysis. Results for the 3'SL, 6'SL, and LNFP I were unchanged in sensitivity analyses. However, among the 4 EV-miRNAs associated with FLNH in the main analysis, only one EV-miRNA remained significant in the sensitivity analysis. Among the 5 EV-miRNAs associated with LNH in the main analysis, 4 remained significantly associated with LNH in the sensitivity analysis.

Putative pathways of EV-miRNAs associated with HMO measures

Finally, we explored the functional pathways of predicted target genes for the EV-miRNAs that were associated with HMO expression. For example, we found that EV-miRNAs that were associated with HMO diversity, 2'FL, FLNH, LNFP I and LNH were also target genes involved in the Hippo signaling pathway (Table 5). EV-miRNAs associated with sum of HMOs, HMO-bound fucose, 3FL, 6'SL, FLNH, and LNH also target genes involved in the cell cycle pathway. Using the micro-T database, similar pathways were identified as being potential targets of EV-miRNAs that were associated with HMO measures (Supplementary Table 6).

Discussion

To our knowledge, this is the first study to explore the associations between EV-miRNAs and HMOs. We hypothesized that EV-miRNA levels in human milk may be associated with HMO concentrations and assessed this hypothesis using three distinct statistical approaches. We first utilized principal components as a data reduction technique to summarize EV-miRNA levels in human milk samples and found that PC2 was associated with HMO measures including HMO diversity, sum of HMOs, HMO-

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TABLE 3 PC2 was associated with several HMO measures.

HMO Summary Measures (nmol/mL)	PC1		PC2	
	β (95% CI)	P	β (95% CI)	P
Diversity[+]	-0.02 (-0.09, 0.05)	0.55	-0.08 (-0.16, -0.001)	**$4.6x10^{-2}$**
Sum of HMOs	11.79 (-52.17, 75.75)	0.72	151.40 (77.57, 225.23)	**$1.0x10^{-4}$**
HMO-bound fucose	10.76 (-63.81, 85.32)	0.78	103.99 (17.91, 190.07)	**0.02**
HMO-bound sialic acid	20.21 (-12.23, 52.65)	0.21	-6.25 (-43.70, 31.19)	0.74

HMO concentrations (nmol/mL)	PC1			PC2		
	β (95% CI)	P	P_{BH}	β (95% CI)	P	P_{BH}
2'FL	36.11 (-69.52, 141.74)	0.50	0.56	233.90 (111.96, 355.84)	$3.0x10^{-4}$	**$2.0x10^{-3}$**
3FL	24.32 (-11.11, 59.76)	0.18	0.33	-29.12 (-70.02, 11.79)	0.16	0.23
3'SL	17.81 (-6.41, 42.04)	0.15	0.31	-30.39 (-58.36, -2.43)	0.03	0.11
6'SL	4.20 (-6.67, 15.07)	0.44	0.56	25.41 (12.86, 37.96)	$1.0x10^{-4}$	**0.002**
DFLac	5.44 (-5.64, 16.52)	0.33	0.45	-9.80 (-22.59, 2.99)	0.13	0.22
DFLNH	1.61 (-2.80, 6.03)	0.47	0.56	2.16 (-2.93, 7.26)	0.40	0.49
DFLNT	-22.90 (-52.82, 7.02)	0.13	0.31	-27.35 (-61.89, 7.19)	0.12	0.22
DSLNH	2.11 (-1.55, 5.77)	0.26	0.37	3.41 (-0.82, 7.64)	0.11	0.22
DSLNT	-3.38 (-9.17, 2.40)	0.25	0.37	-6.71 (-13.39, -0.03)	$4.9x10^{-2}$	0.13
FDSLNH	2.67 (-0.63, 5.97)	0.11	0.31	2.93 (-0.88, 6.74)	0.13	0.22
FLNH	3.83 (-0.06, 7.71)	0.05	0.28	5.97 (1.48, 10.45)	0.01	**0.06**
LnNT	-11.52 (-23.39, 0.36)	0.06	0.28	1.15 (-12.56, 14.86)	0.87	0.87
LNT	-21.78 (-45.96, 2.41)	0.08	0.29	19.10 (-8.82, 47.02)	0.18	0.24
LNFP I	-11.48 (-50.44, 27.47)	0.56	0.59	-51.51 (-96.48, -6.53)	0.03	0.11
LNFP II	-13.68 (-27.91, 0.56)	0.06	0.28	12.31 (-4.13, 28.75)	0.14	0.22
LNFP III	0.68 (-0.42, 1.78)	0.22	0.37	-0.52 (-1.79, 0.75)	0.42	0.49
LNH	2.33 (0.07, 4.59)	0.04	0.28	0.99 (-1.62, 3.59)	0.45	0.51
LSTb	-0.30 (-2.04, 1.45)	0.74	0.74	-2.17 (-4.18, -0.15)	0.04	0.11
LSTc	-4.30 (-9.87, 1.27)	0.13	0.31	1.63 (-4.80, 8.06)	0.62	0.65

Multivariable linear regression analysis was used to examine the associations between PC1 and PC2 with HMO summary measures and HMO concentrations. Models adjusted for technical covariates (i.e., proportion of rRNA, volume of skim milk) as well as days postpartum, time of day of human milk collection, and number of breast feedings per day. Regressions where the outcome was HMO concentration were adjusted for multiple testing using the Benjamini-Hochberg procedure (BH). Bold indicates $P_{BH} < 0.1$.
[+]Diversity was estimated using Simpson's Diversity measure.

bound fucose, and concentrations of 2'FL, 6'SL, and FLNH. Next, we used multivariable linear regression to characterize the associations between individual EV-miRNA levels with HMO summary measures and individual HMO concentrations. We found that several EV-miRNA levels were associated with summary HMO measures including diversity, HMO-bound sialic acid, and HMO-bound fucose. We then used pathway analysis to explore the putative pathways of EV-miRNAs that were associated with HMOs. We identified several functional pathways through which EV-miRNAs may modulate HMO expression, including the cell cycle pathway and the hippo signaling pathway.

In this study, we found that PC2, which summarized levels of human milk EV-miRNAs, was associated with HMO summary measures and individual HMO concentrations. The highest loading scores for PC2 came from miR-183-5p and miR-151a-3p; miR-183-5p is highly expressed during lactation and is upregulated in mature human milk, compared to colostrum (56, 57). Levels of miR-183-5p have also been indicated in loss of breast epithelial cell polarity, which is associated with plasticity in early breast carcinoma (58). Additionally, miR-151a-3p may affect the growth hormone receptor (GHR), which controls human growth hormone (hGH), a key regulator of human lactation (39). Our study also identified several EV-miRNAs whose levels were associated with HMO summary measures and concentrations. For example, miR-30d-5p was negatively associated with HMO diversity and was also positively associated with sum of HMOs, HMO-bound fucose,

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TABLE 4 Summary of the number of EV-miRNA associated with HMOs measures and concentrations via multivariable linear regression analysis.

HMO Summary Measures (nmol/mL)	P < 0.05 Count	P_{BH} < 0.10 Count
Diversity[+]	26	–
Sum of HMOs	55	–
HMO-bound sialic acid	9	–
HMO-bound fucose	33	–
HMO Concentrations (nmol/mL)	**P < 0.05 Count**	**P_{BH} < 0.10 Count**
2'FL	52	7
3'FL	63	17
3'SL	34	1
6'SL	52	1
DFLac	34	0
DFLNH	7	0
DFLNT	39	0
DSLNH	20	0
DSLNT	34	0
FDSLNH	26	0
FLNH	52	4
LnNT	21	0
LNT	29	0
LNFP I	29	1
LNFP II	22	0
LNFP III	10	0
LNH	58	5
LSTb	19	0
LSTc	13	0

Multivariable linear regression analysis was used to examine the associations between individual EV-miRNAs with HMO summary measures and HMO concentrations. Models adjusted for adjusted for technical covariates (i.e., proportion of rRNA, volume of skim milk) as well as days postpartum, human milk collection time, and breast feedings per day. "P < 0.05 Count" indicates the number of EV-miRNAs that were associated with each HMO measure based on P < 0.05. "P_{BH} Count" indicates the number of EV-miRNAs that were associated with each HMO measures based on P_{BH} < 0.10.
[+]Diversity was estimated using Simpson's Diversity measure.

and the concentration of 2'FL. miR-30d-5p is one of the most abundant human milk miRNAs (59) and has been shown to inhibit cell proliferation via cell cycle modulation in gallbladder (60) and pancreatic cancer (61). In our study, pathway analysis also indicated that miR-30d-5p is involved in pathways including cell cycle and hippo signaling. The hippo signaling pathway plays a key role in modulating cell proliferation (62, 63) and has previously been linked with maternal stress. For example, biological pathways related to hippo signaling were enriched among EV-miRNAs in mothers with high lifetime stress (including miR-30d-5p and miR-148a-3p) (63), suggesting that maternal stress may impact the composition of HMOs in milk via alterations in EV-miRNAs.

Our previous work in the Mother's Milk Study has found that ambient air pollution exposure, including $PM_{2.5}$ (i.e., particulate matter < 2.5 microns) and PM_{10}, was associated with lower HMO diversity, and a higher sum of HMOs (64). We also found that greater exposure to PM was associated with higher 2'FL, 3FL, LNH, FLNH and lower HMO HMO-bound fucose, LNFP I, LNFP II, and DFLNT concentrations at 1-month postpartum. Our recent work in the same cohort also found that higher exposure to particulate matter during pregnancy (i.e., $PM_{2.5}$ and PM_{10}) was associated with several EV-miRNAs at 1-month postpartum; specifically, PM_{10} was positively associated with miR-200b-3p, miR-200c-3p and miR-125b. In the current study, we found that higher miR-200b-3p and miR-200c-3p levels were each associated with lower HMO diversity. Though not statistically significant after adjustment for multiple testing, we also saw that higher levels of miR-200b-3p and miR-200c-3p were associated with higher 2'FL, and lower DFLNT, LNnT, and LNFP I. We also found that miR-200c-3p was positively associated with sum of HMOs and HMO-bound fucose. Lastly, higher levels of miR-125b-5p were associated with lower HMO diversity and higher levels of the sum of HMOs, HMO-bound fucose, and 2'FL. Though not statistically significant after adjustment for multiple testing, we saw that levels of miR-125b-5p were associated with lower DFLNT and LNFP I and higher FLNH. Collectively, this body of work suggests a potential link between environmental exposures and HMO concentrations that may be mediated by specific EV-miRNAs; further studies should therefore continue to consider environmental impacts on gene expression and HMO regulation.

The present study is novel in its investigation of the relationships between levels of EV-miRNAs and HMO concentrations in human milk. However, this study also has important limitations worth noting. Firstly, this cross-sectional study was conducted on samples collected at 1-month postpartum and therefore causality and temporality of the associations cannot be assessed. However, this study can provide hypothesis generation for future, longitudinal studies. Additionally, the original aim of this study was to assess human milk for HMOs. Thus, milk was frozen for storage, which could have resulted in contamination from lysed cells or significant loss of milk EVs (65). However, a representative subset of samples utilized in this analysis underwent additional testing and were determined to be positive for eight EV markers and showed minimal contamination by GM130, a Golgi matrix protein which can be a marker for cellular contamination (40). Lastly, our sample was fully Latino and was largely comprised of mothers with overweight and obesity and who intended to breastfeed for at least 6 months. We additionally excluded participants who were classified as non-secretors. Each of these factors may limit the generalizability of our findings.

Conclusions

This study provides preliminary evidence that EV-miRNA levels may influence HMO summary measures and concentrations in human milk at 1-month postpartum. While further analyses are needed, this work contributes to the growing literature characterizing maternal factors that impact HMO biosynthesis. Understanding

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TABLE 5 Top 5 most statistically significant putative pathways for miRNAs significantly associated with HMO characteristics.

HMO Summary Measures	Pathway	Merged P_{FDR}
Diversity	Ubiquitin mediated proteolysis	$1.2x10^{-47}$
	Pathways in cancer	$8.7x10^{-47}$
	Hippo signaling pathway	$1.5x10^{-43}$
	Colorectal cancer	$8.7x10^{-33}$
	Proteoglycans in cancer	$4.0x10^{-31}$
Sum of HMOs (nmol/mL)	Cell cycle	$2.5x10^{-104}$
	Pathways in cancer	$3.7x10^{-102}$
	p53 signaling pathway	$2.1x10^{-96}$
	Proteoglycans in cancer	$4.3x10^{-92}$
	FoxO signaling pathway	$1.2x10^{-84}$
HMO-bound sialic acid (nmol/mL)	Focal adhesion	$1.4x10^{-8}$
	p53 signaling pathway	$1.4x10^{-8}$
	Colorectal cancer	$5.3x10^{-8}$
	Autophagy-animal	$1.5x10^{-7}$
	Small cell lung cancer	$4.5x10^{-7}$
HMO-bound fucose (nmol/mL)	Ubiquitin mediated proteolysis	$1.7x10^{-63}$
	Pathways in cancer	$3.2x10^{-63}$
	p53 signaling pathway	$3.0x10^{-58}$
	Proteoglycans in cancer	$3.9x10^{-49}$
	Cell cycle	$2.7x10^{-47}$
HMO Concentrations		
2'FL (nmol/mL)	Ubiquitin mediated proteolysis	$7.5x10^{-11}$
	Hippo signaling pathway	$5.9x10^{-8}$
	Colorectal cancer	$4.2x10^{-7}$
	Pathways in cancer	$6.7x10^{-7}$
	Oocyte meiosis	$1.4x10^{-6}$
3'FL (nmol/mL)	Proteoglycans in cancer	$4.4x10^{-27}$
	Cell cycle	$2.7x10^{-23}$
	Pathways in cancer	$5.1x10^{-23}$
	Hepatocellular carcinoma	$2.5x10^{-20}$
	p53 signaling pathway	$7.0x10^{-19}$
3'SL (nmol/mL)	Autophagy-animal	0.04
6'SL (nmol/mL)	Cell cycle	$9.0x10^{-10}$
	Chronic myeloid leukemia	$2.1x10^{-5}$
	FoxO signaling pathway	$2.1x10^{-5}$
	Prostate cancer	$6.5x10^{-5}$
	Colorectal cancer	$8.1x10^{-5}$
FLNH (nmol/mL)	Hippo signaling pathway	$2.9x10^{-6}$
	Salmonella infection	$4.3x10^{-5}$

(Continued)

TABLE 5 Continued

HMO Concentrations		
	Cell cycle	$1.4x10^{-4}$
	Regulation of actin cytoskeleton	$1.4x10^{-4}$
	Focal adhesion	$4.9x10^{-4}$
LNFP I (nmol/mL)	Regulation of actin cytoskeleton	$1.0x10^{-3}$
	Hippo signaling pathway	$1.4x10^{-3}$
	RNA transport	$2.1x10^{-2}$
	Proteoglycans in cancer	$4.4x10^{-2}$
	MAPK signaling pathway	$4.4x10^{-2}$
LNH (nmol/mL)	Pathways in cancer	$4.9x10^{-10}$
	Proteoglycans in cancer	$7.7x10^{-8}$
	Hippo signaling pathway	$7.9x10^{-8}$
	FoxO signaling pathway	$7.9x10^{-8}$
	Cell cycle	$2.7x10^{-7}$

Putative pathways estimated using miRPath v4.0, KEGG pathway annotation, and TarBase v8.0 targets.

these influences is crucial, as HMOs play a significant role in shaping the infant gut microbiome, supporting immune development, and protecting against infections, thereby impacting infant health and development.

Data availability statement

The datasets presented in this article are not readily available because they include potentially identifying information on human subjects. Requests to access the datasets should be directed to taldere1@jhu.edu.

Ethics statement

The studies involving humans were approved by Institutional Review Boards of the University of Southern California, Children's Hospital Los Angeles, and the University of Colorado Boulder. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

EH: Formal analysis, Visualization, Writing – original draft, Writing – review & editing. WP: Validation, Writing – original draft, Writing – review & editing. BW: Writing – original draft, Writing – review & editing. SK: Validation, Writing – original draft, Writing – review & editing. AK: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. CH: Writing – original draft, Writing – review & editing. LB: Data curation, Writing – original draft, Writing – review & editing. MG: Conceptualization, Writing – original draft, Writing – review & editing. TA: Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

MG receives book royalties and is a scientific advisor for Yumi. LB is a co-inventor on patent applications related to the use of HMOs in preventing necrotizing enterocolitis and other inflammatory diseases.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1463463/full#supplementary-material

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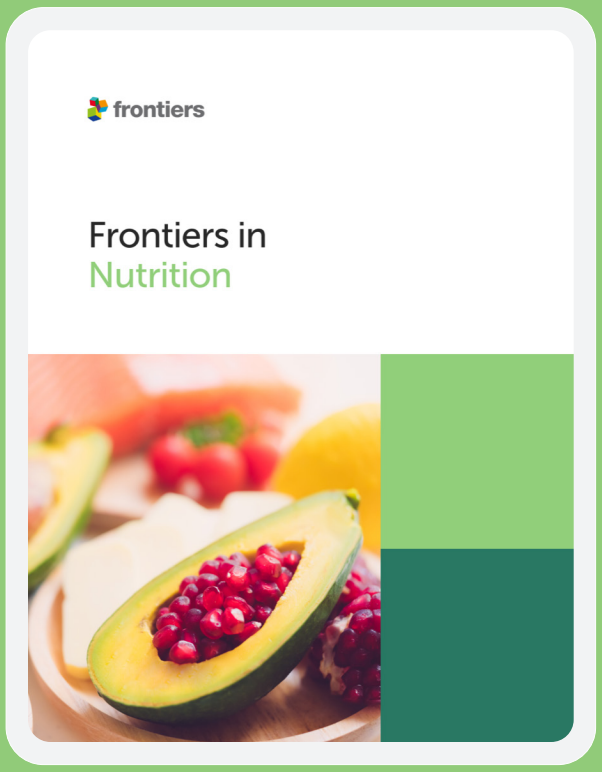

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