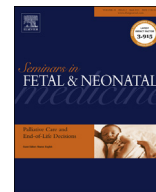




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Review

The human milk microbiome and factors influencing its composition and activity

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S U M M A R Y

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Beyond its nutritional aspects, human milk contains several bioactive compounds, such as microbes, oligosaccharides, and other substances, which are involved in host–microbe interactions and have a key role in infant health. New techniques have increased our understanding of milk microbiota composition, but few data on the activity of bioactive compounds and their biological role in infants are available. Whereas the human milk microbiome may be influenced by specific factors – including genetics, maternal health and nutrition, mode of delivery, breastfeeding, lactation stage, and geographic location – the impact of these factors on the infant microbiome is not yet known. This article gives an overview of milk microbiota composition and activity, including factors influencing microbial composition and their potential biological relevance on infants' future health.

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1. Introduction

Recent reports have revealed the importance of our gut microbiome for optimal health. Accumulating evidence highlights the potential role of microbes in metabolic, immunological, and microbial programming [1]. Microbes are among the most important environmental factors providing specific signals to guide immune system development and maturation [2,3]. Shifts in microbiota composition and activity appear to be related to adverse human health outcomes [4]. The maternal microbial environment impacts the newborn's immune development and, consequently, the infant's health both early and later in life. The maternal microbiota is now recognized as a significant determinant of the maternally transferred factors that impact the child's health [5,6]. Outcomes can be affected by specific perinatal factors that also alter infant microbiome development. For example, excessive use of antibiotics, unbalanced diet, increasing incidence of cesarean section deliveries, unnecessarily stringent hygiene, and continuous stress influence the maternal microbiome. Alterations and disturbances in microbiota composition along with a reduction in microbial

diversity or richness have been described as strong risk factors for the development of lifestyle diseases, such as allergies, diabetes, obesity, and metabolic syndrome, irritable bowel syndrome and other inflammatory-related problems [4,7]. The maternal microbiota and infant diet play a key role in infant growth, adequate microbial colonization, immune system maturation, and metabolic development. Then, the infant stepwise microbial colonization process will have an impact on metabolic and immunological response, and these in turn may have an impact on programming of health later in life [3,8]. This review describes the impact of the maternal microbiota on infant health through breastfeeding, and provides a broad overview of milk microbiota composition and activity, along with the factors influencing microbial composition and their potential biological relevance.

2. Human breast milk: more than infant food

Human milk provides complete nutrition for the infant. Human milk's energy, nutrients, and bioactive components directly influence the development of newborn infants. Among its bioactive factors, HM contains several immune substances, such as immunoglobulins, cytokines and chemokines, growth factors, hormones, and antibodies. It also contains other nonspecific compounds, such as specific peptides, lactoferrin and other whey proteins,

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oligosaccharides, and a large number of bacteria [9]. All these components are transferred to the infant through breastfeeding.

Human milk is the most relevant postnatal element for the metabolic and immunological programming of the infant's health [10,11]. Breastfeeding shapes the infant immune system development and is needed for adequate gut function and immune homeostasis maintenance [12]. Breast-fed infants have been shown to have a reduced risk of necrotizing enterocolitis and diarrhea, allergy and asthma, inflammatory bowel disease, diabetes, and obesity, among other problems [13]. In addition, differences in gut microbiota composition and activity between exclusively breast- and formula-fed infants have been widely reported [14–18]. Breast milk is also a postnatal microbial link as its high diversity of microbes drives the infant's microbial colonization [19–27]. It has been reported that specific maternal gut microbial strains belonging to *Bifidobacterium* and *Staphylococcus* spp. are transmitted to infants [28–31]. These data suggest a unique link between family members as specific strains are present in each mother–infant pair. The findings also suggest that transfer of aberrant microbiota from the mother is possible. Altogether, it is important to define the milk microbiota composition and factors that may be transferrable as commensal bacteria in human milk. For example, transmission of lactic acid bacteria and bifidobacteria from the breastfeeding mother may form a natural protective mechanism that improves development of the infant's gut microbiota for later resilience and reduction in the risk of diarrheal and other dysbiosis-related problems. Improved knowledge in this area may suggest novel means of modulating the maternal and infant microbiota in order to potentially reduce the risk of specific microbiota-associated diseases (Fig. 1).

3. Human breast-milk microbiome

Historically, human breast milk has been considered an almost sterile fluid, but this dogma has been revised over the years. Studies

in primates have reported that rhesus monkey (*Macaca mulatta*) milk contains culturable strains of at least 19 species of bacteria belonging to at least five different genera [32,33]. Similar results have been described in other mammals [34].

Traditionally, the presence of microbes in human milk has been confirmed by use of culture-dependent techniques. Most of the bacteria isolated from milk belong to *Staphylococcus*, *Streptococcus*, and *Lactobacillus* and *Bifidobacterium* spp., latter two genera having several strains with a long record of use as probiotics [35]. It has been stated that “human milk constitutes one of the main sources of bacteria to the breastfed infant gut since an infant consuming approximately 800 mL/day of milk would ingest between 1×10^5 and 1×10^7 bacteria daily” [26,27]. Moreover, the biological role of these microbes on neonatal health at short and long-term has not yet been identified.

The origin of breast milk bacteria is currently not known, but their presence corresponds to a perinatal period which starts during the third trimester of pregnancy and continues through lactation [25]. Several reports have proposed that the human milk microbiota could derive from colonization from the mother's skin, the infant's oral cavity during suckling, or the mother's gut via the entero-mammary pathway [1,26,27,36]. However, a commensal microbiota has been reported in human breast tissue [37,38], suggesting that specific microbes inhabit the breast tissue and potentially colonize the milk ducts.

With the development and application of culture-independent techniques (most of them based on polymerase chain reaction techniques) and next-generation sequencing platforms, the presence of microbial DNA has been confirmed. These techniques have confirmed the existence of a rich and diverse breast-milk microbial community [20–27,35].

Human milk harbors a unique microbial ecosystem which differs from any other in humans. The milk microbiota is not linked with any mucosal or fecal samples, nor does it seem to be a subset of any other specific human sample [21]. The breast milk microbial

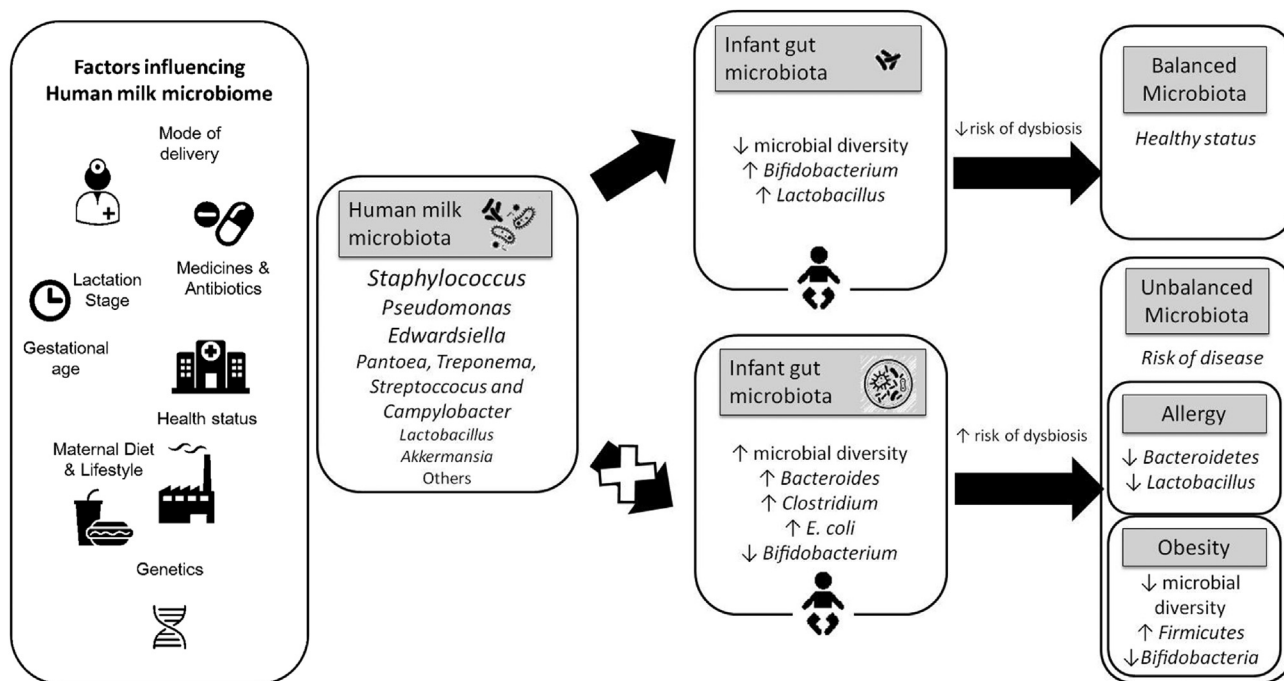


Fig. 1. Human milk microbiota composition and comparison between breastfeeding and formula-feeding microbiota. Metagenome analysis of human milk shows that human milk microbiota is mainly dominated by *Staphylococcus*, *Pseudomonas* and *Edwardsiella*, but other groups are also represented in minor amounts [41,42]. Breast-fed and formula-fed infants have different bacterial populations which seems to modulate the susceptibility of non-communicable diseases (NCDs) such as allergy and/or obesity during infancy and/or in adult life.

diversity has been evaluated by several independent research groups [20–27,35]. These researches have shown that *Staphylococcus* spp., *Streptococcus* spp., are the common groups followed by specific lactic acid bacteria. The first study on the milk microbiome using pyrosequencing demonstrated that milk bacterial communities are generally complex [20]. A high inter-individual variation was reported regarding the number and abundance of different species in human milk; however, a common core of nine bacterial groups were present in the milk samples of each individual (*Streptococcus*, *Staphylococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Ralstonia*, *Propionibacterium*, and *Sphingomonas* spp., plus *Bradyrhizobiaceae*) [20]. Different results between studies may be due to different sampling and process protocols and varied DNA extraction, selection of specific primers with high bacterial coverage including bacteria with higher content of G+C as *Bifidobacterium* spp. and sequencing platforms; thus, a more standardized approach is needed in the future [20,21,23,35,39].

The isolation and detection of live bacteria and the presence of strictly anaerobic species, such as *Bifidobacterium*, *Clostridium*, and *Bacteroides* spp. [23,25] usually associated with gut environments and unable to survive in aerobic locations, have resulted in a scientific debate on the origin of milk-associated bacteria. Recently, high-throughput sequencing has indicated the presence of gut-associated, strictly anaerobic microbes belonging to the Clostridiaceae (*Blautia*, *Clostridium*, *Collinsella*, and *Veillonella* spp.) in breast milk. The presence of specific microbes shared between the maternal microbiota, breast milk, and infant intestinal microbiota has been reported [24,28–31], and in other maternal–neonatal niches such as meconium, placenta, and amniotic fluid [40]. Furthermore, the presence of butyrate-producing bacteria, including *Coprococcus*, *Faecalibacterium*, and *Roseburia* spp., has been confirmed and shown to be shared in both maternal feces and human milk.

Shotgun metagenomics analysis of human milk by total DNA sequencing using Illumina next-generation sequencing technology has been done using 10 pooled milk samples [41]. This study reported that human milk contains >360 prokaryotic genera, with Proteobacteria (65%) and Firmicutes (34%) as the predominant phyla, and with *Pseudomonas* spp. (61.1%), *Staphylococcus* spp. (33.4%), and *Streptococcus* spp. (0.5%) as the predominant genera [41]. This study also showed that the most abundant open reading frames within human milk encoded proteins for basic cellular functions (respiration, cell signaling, RNA, DNA, and amino acid metabolism).

A recent study [42] has reported on the metagenome and microbiota of healthy human milk ($n = 10$ mothers) and compared it with the milk metagenome of women suffering from mastitis. A healthy core microbiome included *Staphylococcus*, *Streptococcus*, *Bacteroides*, *Faecalibacterium*, *Ruminococcus*, *Lactobacillus*, and *Propionibacterium* spp., as well as fungal, protozoa-related, and viral-related sequences, whereas the milk microbiota of women with mastitis was dominated by *Staphylococcus aureus* [42].

Molecular techniques have some limitations. For example, the viability of milk microbes cannot be analyzed, and total bacteria counts may be over- or underestimated because of cell-wall composition, DNA extraction methods, and the number of microbial 16S gene copies which may lead to the over- or underestimation of bacteria counts. The DNA contamination in extraction kits and reagents has also been reported [35]. Other potential factors influencing milk microbiota composition include sampling methods (e.g. aseptic methods, time of day, breast cleaning methods, sampling before or after suckling, manual extraction or breast pump extraction), DNA extraction methods (e.g. from whole or defatted milk, use of commercial kits, enzymatic lysis, bead-beating step), sequencing platforms (Illumina,

Solid, Ion Torrent, 454 Roche), 16S bacterial gene region studies (V1–V3; V3–V4, V4, etc.), and the 16S database and bioinformatics pipeline used. Thus, there is a need to standardize protocols and validate methods.

4. Potential factors influencing the milk microbiome

It is known that genetic factors, mode of delivery, maternal nutrition, time of day, lactation stage, and geographical region influence human milk composition, and considerable inter-individual variation has also been reported [43]. Similarly, all the factors that could modulate the microbiota of the mother's skin, oral cavity, vagina and intestine, and the microbiota of the infant are potentially able to modulate the human milk microbiota. Therefore, the lactation period, maternal dietary habits and nutritional status, mode of delivery, gestational age, geographical location, and the use of antibiotics or other medicines can all have an influence on the milk microbiota.

Higher microbial diversity has been reported in colostrum samples than in mature milk. Lactation stage has been described as a factor influencing milk microbes [20,21]. Initially, the microbiota is dominated by *Weissella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus*, and *Lactococcus* spp [21]. Later, the microbiota contains high levels of *Veillonella*, *Prevotella*, *Leptotrichia*, *Lactobacillus*, *Streptococcus* spp., and increasing levels of *Bifidobacterium* and *Enterococcus* spp [21,44].

The mode of delivery affects human milk microbiota composition. High microbial diversity and high prevalence of *Bifidobacterium* and *Lactobacillus* spp. are found in colostrum and milk following vaginal delivery, whereas the contrary is observed following cesarean delivery [21,22,44–46] although other studies did not report differences in microbial profiles based on gestation, mode of delivery or infant gender [47]. Breast-milk microbiota composition is also influenced by gestational age, with significant differences between term- and preterm-delivered mothers. Lower counts of *Enterococcus* spp. in colostrum and higher counts of *Bifidobacterium* spp. in milk have been detected in samples from mothers with term deliveries [44]. In addition, changes in the milk microbiota composition are associated with maternal physiological status, including obesity, celiac disease, and human immunodeficiency virus (HIV)-positive status [21,48–50]. Obesity is reflected in the levels of *Bifidobacterium* spp. and cytokines in human milk [50], as well as increased *Staphylococcus* spp., leptin, and pro-inflammatory fatty acid levels [50–53] and reduced microbial diversity [21]. Mothers with celiac disease have reduced levels of cytokines, *Bacteroides* spp. and *Bifidobacterium* spp. in their milk [48]. Finally, the milk from HIV-positive women from Africa has been found to show higher bacterial diversity and higher prevalence of *Lactobacillus* spp. than the milk of non-HIV-positive women [49].

Analysis of the human milk microbiota shows that, in general, *Staphylococcus* and *Streptococcus* spp. as well as lactic acid bacteria strains are present in milk [19–24], but their relative amounts and the presence of other bacteria could be dependent on geographical location [37–49]. However, large-scale studies with breast-milk samples from different geographic locations are needed. It is also evident that perinatal use of antibiotics has an impact on the maternal microbiota, including human milk microbiota, affecting the prevalence of *Lactobacillus*, *Bifidobacterium*, and *Staphylococcus* spp. [45,54] and decreasing the abundance of *Bifidobacterium*, *Staphylococcus*, and *Eubacterium* spp. in milk samples [37]. Chemotherapy has also been associated with alteration in the milk microbiome and with a reduction in bacterial diversity [55]. Studies on the impact of maternal diet on milk microbiota are few in number, but it is likely that nutritional habits, which are able to

modulate intestinal microbiota and human milk nutritional composition, may exert changes in the milk microbiota. For example, shared microbial features have been reported between bacteria present in local foods and other fermented foods, as well as mother–infant microbial gut and breast-milk [56].

Moreover, diet clearly influences the lipid profile of human milk, modulating the concentration of long-chain polyunsaturated fatty acids and the ratio of ω -3 to ω -6 [57,58]. These fatty acids have immunomodulatory properties in nursing children [59], and it has recently been demonstrated in animal models that they are able to modulate gut microbiota composition in early life [60]. The consumption of probiotics and prebiotics during pregnancy could influence the human milk microbiota, but more studies are necessary to document the potential transfer of gut bacteria to the mammary glands and the impact of specific strains of bacteria [61]. On the other hand, it has been reported that non-nutritive sweeteners, such as saccharin, sucralose, and acesulfame potassium, were present in 65% of breast-milk samples analyzed [62]. Those data suggest that maternal diet could modulate the bioactive compounds and microbes present in breast milk. Studies are urgently needed to investigate interactions among nutrients in the maternal diet and the breast-milk microbiota and their health effects on infants.

5. Potential strategies to modulate the maternal breast-milk microbiota

As mentioned above, diet is likely a powerful tool to alter gut microbiota. Dietary strategies, then, could be devised to modulate the microbial composition in order to affect human physiology and reduce the risk of diseases related to imbalances of microbiota composition. A recent study [63], with Japanese macaques (*Macaca fuscata*) as animal model, has reported the impact of maternal diet on neonatal microbiome composition. This study showed the effect of maternal high fat diet affects infant microbiome composition and activity and also, affects the metabolic health. Thus, deciphering the contribution of specific gut bacteria and promoting nutrition and lifestyle counseling may open new tools to reduce the risk of diseases related to microbial composition shifts. Accumulating evidence shows that early dietary interventions and nutrition counselling would support health programming effects (immunological, metabolic and microbial effect) on adult health.

It has been demonstrated that specific probiotic strains are effective in the prevention and treatment of infectious diseases in early life [64] and that they may reduce the risk of eczema in infants in at-risk populations [65]. In addition, a specific probiotic bacterium, *Lactobacillus reuteri*, has been detected in breast milk and infant feces from mothers consuming this probiotic [66]. In a placebo-controlled study, probiotics ingestion during pregnancy and breastfeeding period has been describe to modulate in infant *Bifidobacteria* colonization and also to modulate breast milk microbiome [67,68]. Recently, it has been suggested that perinatal probiotic supplementation affects breast-milk composition in terms of microbes, including *Bifidobacterium* and *Lactobacillus* spp., also affects other bioactive compounds likewise human milk oligosaccharides and lactoferrin [69]. Moreover, the same study found only beneficial effects of probiotic supplementation in vaginal birth, while no significant differences were found in milk samples from C-section deliveries, suggesting probiotic-specific dependent modulation depending on mode of delivery. A recent workshop report on the use of specific probiotics during the first 1500 days of life supports healthy perinatal life with lowered risk of infections and autoimmune problems later in life [70].

6. Conclusions

Several perinatal factors influence microbial transfer from mother to infant via breast milk. The lactation period may provide a new target for devising novel dietary and nutritional tools to modulate the milk microbiota and thereby reduce the risk of non-communicable diseases (NCDs), while at the same time promoting breastfeeding. To continue to increase our knowledge of the milk microbiota, further studies from different geographical regions and among varying population groups with different genetic backgrounds and environmental and nutrition conditions are required to fully understand the impact of the microbiome and its potential in infant health promotion.

Practice points

- Alterations in microbiota composition have been described as strong risk factors for the development of lifestyle diseases, such as allergies, diabetes, obesity, and metabolic syndrome, irritable bowel syndrome, and other inflammatory-related problems.
- The maternal microbiota is recognized as a significant determinant of the maternally transferred factors that impact the child's health, providing specific signals to guide immune system development and maturation.
- The principal bacteria present in human milk are *Streptococcus*, *Staphylococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Ralstonia*, and *Propionibacterium* spp.

Research directions

- The origin of the bacteria in breast milk (colonization from the mother's skin, the infant's oral cavity during suckling, or the mother's gut).
- Clarification of the potential impact of each different species of milk bacteria on infant health.
- Standardized protocols and methodology to analyze milk microbiota.
- Influence of the geographical location, genetic background, and diet in milk microbiota.
- Use of prebiotics, probiotics, and symbiotics to modulate breast-milk microbiota and improve infant health.

Conflict of interest statement

None declared.

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