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Factors influencing the microbial composition of human milk

Anastasia Mantziari[a,†], **and Samuli Rautava**[b,*,†]

[a]Functional Foods Forum, Faculty of Medicine, University of Turku, Itäinen Pitkäkatu 4A, 20520 Turku, Finland
[b]University of Helsinki and Helsinki University Hospital, New Children's Hospital, Pediatric Research Center, Helsinki, Finland

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ABSTRACT

Aside from nutritional components, human milk is rich in microorganisms. Through breastfeeding these microorganisms are introduced to the infant gut where they may transiently or persistently colonize it. Therefore, the human milk microbiota may be an important factor which shapes the infant gut microbiota further influencing infant health and disease. In the current review we aim to give a brief updated insight into the putative origin of the human milk microbiota, its constituents and the possible factors that shape it. Understanding the factors that determine the human milk microbiota composition and function will aid developing optimal postnatal feeding and intervention strategies to reduce the risk of communicable and noncommunicable diseases.

Introduction

Early life nutrition is associated with the risk of developing noncommunicable diseases (NCDs) including obesity, cardiovascular and chronic respiratory diseases, cancers, and diabetes. While NCDs are now considered the leading causes of death worldwide evidence has shown that breastfeeding can exert beneficial effects for both mother and infant. For instance, breastfeeding may protect mothers from ovarian cancer, breast cancer and diabetes and offer long–term maternal cardiovascular health benefits.[1,2] On the other hand, prolonged breastfeeding for at least eight months has been associated with low average blood glucose levels in infants born to mothers with gestational diabetes mellitus.[3] In addition, multiple studies have highlighted that the use of human milk (HM) has a positive effect on various infant diseases and conditions including respiratory infections and diarrhea as well as necrotizing enterocolitis in preterm neonates.

This protective role of breastfeeding might be mediated through effects on the infant gut microbiome. Increasing evidence links changes in the composition of the intestinal microbiota with adverse health outcomes. Therefore, the development of the gut microbiome during infancy is essential for the maturation and function of the infant's immune system. However, this development is largely modulated by the mode of delivery, perinatal use of antibiotics and infant diet among other factors. Indeed HM is not only viewed as the ideal source of nutrients for infants but also contains a variety of compounds that might affect infant immunity (i.e., human milk oligosaccharides, antibodies, cytokines, human cells and extracellular vesicles).[4,5] Besides essential nutrients and bioactive molecules, HM although at first thought sterile, is found to contain commensal microbes, which have the ability to modulate the colonization of the infant gut. Several

*Corresponding author.
E-mail address: samuli.rautava@helsinki.fi (S. Rautava).
†From University of Turku

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studies have discussed the overlap between human milk and infant gut microbiota. Of special interest is the genus *Bifidobacterium,* which has been identified in both niches and is considered a dominant constituent of the gut microbiota of breastfed infants.[6] Generally, lower abundance of this genus in the infant gut could trigger the overgrowth of *Bacteroides*, which has been associated with the risk of developing of allergies and obesity later in life.[7] The composition of the human milk microbiota (HMM) may play an essential role in shaping the infant gut microbiome and therefore it is important to identify the factors that influence the composition of the human milk microbiome (Figure 1).

Origin of human milk microbiota

Human milk has for long been considered sterile, however over the past decade various reports describe the presence of viable bacteria in milk produced by healthy women free from infection. Currently, human milk is regarded as a continuous source of microbes with more than several hundred different bacterial species identified.[8] Initially, the presence of microbes in milk was thought to be a product of contamination, due to vaginal exposure of the neonate or due to mother's skin and infant's mouth contact.[9]

Three different hypotheses have been proposed to explain how these microbes found themselves in milk: the entero-mammary pathway, the retrograde inoculation pathway and the notion of resident mammary microbiota. The entero-mammary pathway is an endogenous route that is described by translocation of maternal gut bacteria from the gut to the mammary glands. Through this pathway, dendritic cells (and possibly macrophages) can introduce openings in the tight junctions between the epithelial cells of the gut and trap bacteria through dendrites. It is possible that hormonal changes during late pregnancy create a favoring environment for immune cells to open the tight junctions in the intestinal epithelium. Dendritic cells then travel through the lymphatic and blood circulation and finally reach the mammary ducts where they release the bacteria in the milk.[9]

Alternatively, microorganisms present in the milk of healthy women might not only originate from the maternal gut but also from the maternal skin, infant oral cavity or even the environment. For instance, some bacterial genera of the skin, such as *Staphylococcus*, *Corynebacterium* and *Propionibacterium*, were also frequently found in breast milk.[10] Retrograde backflow during breastfeeding may also play a role in the establishment of oral bacteria in human milk. Indeed, human milk and the infant oral cavity were found to share several bacterial genera such as *Streptococcus*, *Veillonella* and *Varioovorax*. [11,12] Nonetheless, typical oral bacterial colonizers were

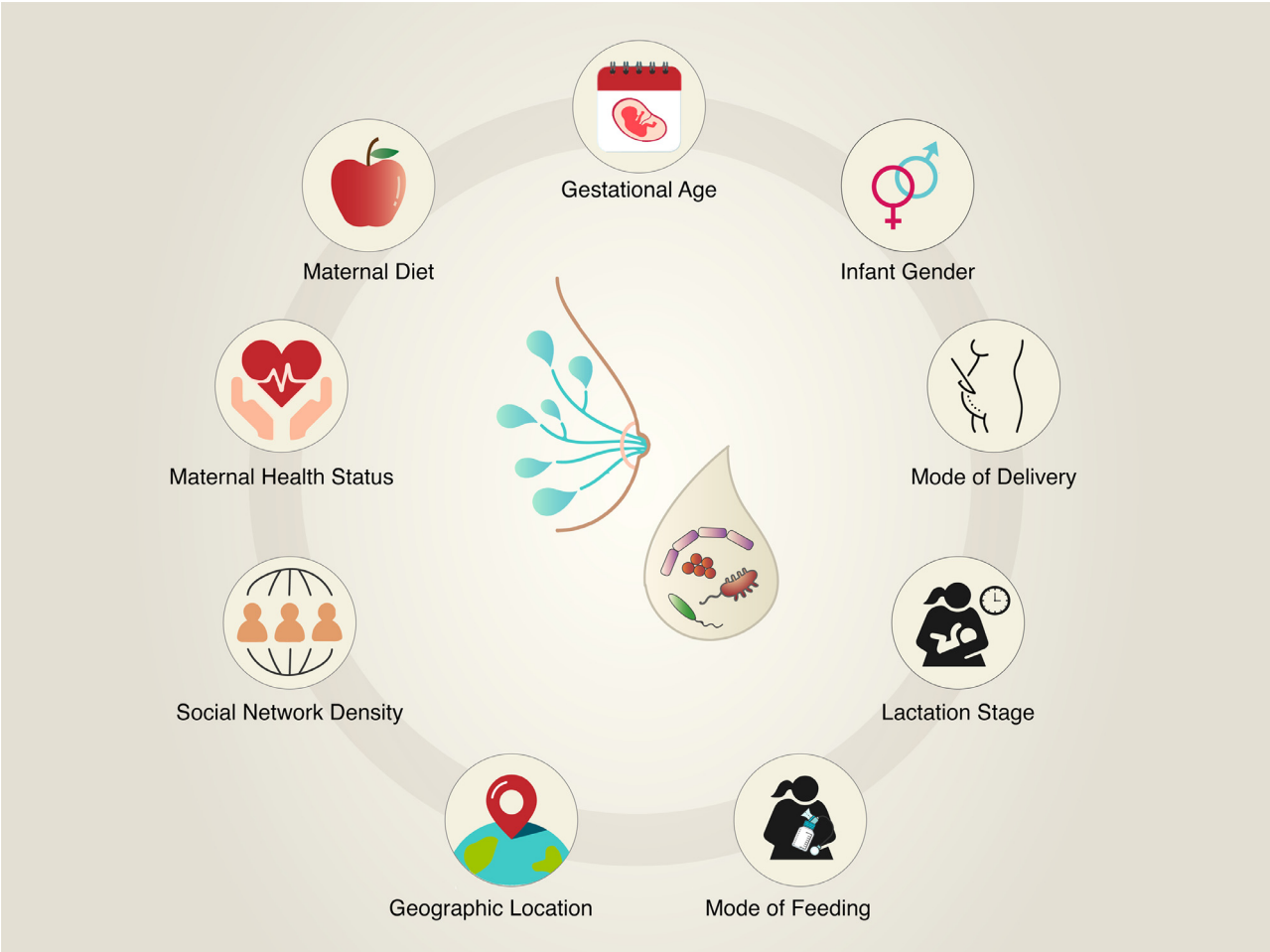

Fig. 1 – Factors influencing the microbial composition of human milk.

found in precolostrum before delivery when the breast had not yet been exposed to the infant's mouth.[13] In addition, the retrograde inoculation pathway can not fully explain the fact that some bacteria in human milk are strictly anaerobic (eg. *Bifidobacterium* and *Faecalibacterium* strains). These observations suggest the possibility that both pathways may be potential sources of the human milk microbiota although the mechanisms are not yet clear.[14] Finally, it has long been known that mammary tissue of non-lactating women has its own microbiota although previously described as similar to the one on the skin. Specifically, in their recent systematic review, Togo and colleagues identified seven species including *C. acnes*, *S. epidermidis* and *S. agalactiae* that are also present in human milk supporting the thought of a commensal mammary microbiota that could affect the HMM composition.[11]

Human milk microbiota

The presence of bacteria in human milk has been explored in a number of studies using culture-dependent and culture-independent techniques. While culture-dependent methods enable the identification of viable and culturable bacteria in human milk, they also lead to an underestimation of the total bacterial count since dormant or unculturable microbes remain undetected. To overcome this limitation culture-independent techniques, such as quantitative polymerase chain reaction (qPCR) and next generation sequencing (NGS) have been employed. These molecular approaches have revealed that the diversity and the inter-individual variability of HMM is higher than previously thought. Nevertheless, a recent systematic review highlighted the importance of using a combination of the afore mentioned methodologies to accurately assess the HMM composition.[11] In short, molecular methods allowed the detection of twice the number of bacterial species compared to culture methods with only 26% species detected by both approaches. In addition, 62% species were found only by molecular methods, while 12% were found only by traditional culturing.

Despite these variations, both methods have shown that human milk is characterized by a core microbiota comprised of 7-9 taxa depending on the selected methodology and study population.[15,16] The majority of performed studies have identified *Staphylococcus* and *Streptococcus* as the most consistently and frequently observed genera in human milk. Interestingly, breast-fed infants also have higher levels of *Staphylococcus* in their feces compared to formula-fed ones.[17] While growing in the gut, *Staphylococcus* and *Streptococcus* utilize oxygen creating a favorable environment for the proliferation of beneficial anaerobic bacteria. A few anaerobes which are also detected in human milk include *Bifidobacterium*, *Faecalibacterium* and *Akkermansia* and may reduce the risk of dysbiosis-related diseases through the production of short chain fatty acids. For instance, *Bifidobacterium* and *Akkermansia muciniphila* produce acetate, which may help prevent pathogenic infection while *Faecalibacterium prausnitzii* produces butyrate, which can modulate inflammation.[18,19] Other identified genera include lactic acid bacteria (i.e., *Lactobacillus*), skin (i.e., *Propionibacterium, Cutibacterium* and *Corynebacterium*) and gut commensals (i.e. *Acinetobacter*, *Bacteroides*, *Blautia*, *Clostridium*, *Dorea*, *Enterococcus*, *Escherichia*, *Mucispirillum*, *Pseudomonas*, *Rothia*, *Salmonella*, *Serratia*, *Shigella*, and *Veillonella*).[7,11] Further characterization of the HMM has revealed the presence of fungi (*Malassezia*, *Candida*, *Saccharomyces*, *Rhodotorula*, *Davidiella*, *Sistotrema*, and *Penicillium*), archaea (*Haloarcula*, *Halorhabdus*, and *Halomicrobium*), protozoa (*Giardia* and *Toxoplasma*), and viruses in the milk of healthy mothers.[15,20,21] Specifically regarding viruses, HMM of healthy mothers consists mainly of lytic bacteriophages (*Myoviridae*, *Podoviridae* and *Siphoviridae*) and a minority of eukaryotic viruses (*Phycodnaviridae*, *Iridoviridae* and *Imiviridae*).[22] Lytic bifidophages in particular have been shown to suppress the growth of dominant *Bifidobacterium* in the gut.[23] Thus, the presence of bacteriophages could not only affect the bacterial diversity and relative abundance in human milk but also in the gut.

Factors shaping the human milk microbiota

Maternal health status

While obesity has mainly been considered a problem of developed countries, its prevalence has now reached global epidemic proportions. Besides being one of the major risk factors for premature death, maternal obesity is a major risk factor for childhood obesity. Although the mechanisms behind this are poorly understood, differences between HMM diversity and composition between obese and normal weight mothers may play a role. Indeed, overweight and obese mothers reportedly produce milk with a lower microbial diversity and lower counts of Bifidobacterium and a higher level of *Staphylococcus*, *Akkermansia* and *Granulicatella* than normal-weight women.[8,24,25] Obese mothers have also been observed to display a higher incidence of *Staphylococcus*, *Corynebacterium* and *Brevundimonas* in their milk when compared to overweight or normal weight mothers[26] Consequently, these changes may affect the human milk metabolome and further influence the infant microbiome development. For instance, a recent study showed that human milk metabolite levels differ significantly between overweight or obese and normal weight mothers.[27] Specifically, milk hormones such as insulin and leptin are found in elevated levels in milk of obese mothers and may impact the development of the infant intestinal microbiome.[28] Gestational prehypertensive status is another factor that has been reported to contribute to lower bacterial diversity and lower relative abundance of *Lactobacillus* in human milk.[29] If this translates to colonization of the infant gut with lower numbers of *Lactobacillus*, it could limit the protective functions associated with this genus.

HMM composition is influenced by chronic illnesses such as celiac disease and allergic disorders. Recently, Spanish researchers found that the milk of healthy mothers was richer in *Bifidobacterium* and *Bacteroides fragilis* and had higher concentration of the tolerogenic cytokine TGF-β1 than mothers with celiac disease.[30] Higher TGF-β1 levels in milk during the first month of lactation, have been associated with reduced risk of eczema in infants.[31] Milk of healthy mothers was also found to be richer in *Bifidobacterium* compared to the milk of allergic mothers.[32] It has been suggested that the immune responses associated with these chronic diseases could be responsible for the lower levels

of *Bifidobacterium* in the milk of mothers with celiac disease or allergic disorders.[30,33]

A primary concern for women in the postpartum period is maternal postnatal distress, which is characterized by symptoms of depression or anxiety. Mild postpartum depressive symptoms have been associated with increased cortisol levels, which could alter the fecal microbiota diversity through the recently proposed gut-brain axis.[34,35] In accordance with the enterommamary pathway hypothesis, this could also influence the HMM composition. Indeed, in a previous study, Browne and coworkers found that maternal postnatal distress can also influence the HMM composition.[36] Mothers with high psychosocial distress had a less diverse HMM at 3 months postpartum compared to those with low psychosocial distress. Moreover, both groups saw a significant decrease in the relative abundance of *Staphylococcus,* while milk from mothers in the low group had a significant increase in the relative abundance of *Lactobacillus, Acinetobacter,* and *Flavobacterium*. Finally, no significant changes in the relative abundance of Actinobacteria (eg. *Bifidobacterium*) were noted. Mothers who adhered to postpartum confinement practices such as "doing-the-month" programs in Taiwan had lower postpartum physical and depressive symptoms and a higher prevalence of *Lactobacillus*, *Bifidobacterium* and Archaea in their milk compared to mothers who did not.[37,38] However, these are complex 20–30 day programs, which include a unique combination of dietary and herbal therapies and it is still not yet clear which factor contributes to this modulation of the HMM.

Other important factors that seem to shape the HMM are bacterial and viral infections. For example, lactational mastitis and breast abscess, which may develop as a complication of mastitis, most often caused by *Staphylococcus aureus*, have been associated with dominance of *S. aureus*, complete absence of *Bifidobacterium breve* and overall lower bacterial diversity in the milk of infected mothers.[11,15] In addition, *Salmonella enterica* and *Burkholderia ambifaria* are only detected in human milk of mothers with breast abscess, while several Bifidobacteria and Lactobacilli are associated with absence of both mastitis and breast abscess. Lower diversity, increased bacterial loads, and altered microbiota composition were also confirmed in human milk of mothers with sub-acute mastitis by Amorós and colleagues.[39]

Changes in HMM composition triggered by viral infections have also been reported. A study on neonatal rotavirus (RV) infection in India, showed that the milk of mothers of symptomatic infants had a higher relative abundance of *Enterobacter/Klebsiella*. In contrast, the milk of mothers of asymptomatic and RV negative infants was dominated by *Staphylococcus* and *Streptococcus*.[40] Increased abundance of *Staphylococcus* was also noted in the milk of healthy African mothers when compared to the milk of HIV infected mothers that was more diverse and had higher counts of *Lactobacillus*.[41] In conclusion, accumulating evidence suggests that eukaryotic viruses often directly and indirectly interact with bacteria and thus, their presence in human milk may not only modulate its virome but also work synergistically with the milk bacteriome.[42,43]

Antibiotic therapy is common during pregnancy and particularly during delivery either as prophylaxis to protect the mother and her infant or treatment of infection. Numerous reports indicate that the use of antibiotics has significant effects on several HMM parameters. Recently, Hermansson and coworkers described an increase in the levels of different bacterial species and their diversity in the milk of mothers who received antibiotics during delivery.[44] In addition, previous studies have reported lower relative abundance of *Lactobacillus* and *Bifidobacterium* in the milk of mothers who received antibiotics during pregnancy or lactation.[45,46] Besides antibiotherapy, chemotherapy for the treatment of maligagncies can also modulate HMM composition. Before and after treatment analysis of HMM of a woman with Hodgkin's lymphoma revealed reduced levels of certain genera, especially *Bifidobacterium, Eubacterium, Staphylococcus* and *Cloacibacterium*. In contrast, when compared to the milk of healthy mothers, the milk collected during chemotherapy was characterized by a significant increase of *Acinetobacter* and *Xanthomonadaceae*.[47]

Maternal diet

It is well known that maternal diet can influence the bacterial taxonomic composition of human milk since many of its nutrients may be utilized by bacteria. For example, a study on healthy lactating mothers from Brazil showed that the presence of *Staphylococcus* in their milk was positively correlated with Vitamin C intake during pregnancy. During the lactation period, increased sugar intake is associated with a decrease of *Pseudomonas* while an increased Vitamin B9 intake is associated with an increase its numbers in milk. Another positive correlation was found between *Bifidobacterium* and the intake PUFAs and linoleic acid.[48] Conversely, Kumar and coworkers found a negative correlation between *Bifidobacterium* and *Lactobacillus* and MUFA and n-3 PUFA in milk phospholipids. In addition, in the same study, saturated fatty acids were negatively associated with *Corynebacterium* and *Streptococcus*.[25,49] Moreover, greater protein consumption was related to an increased abundance of *Gemella, Bacillus, Peptoniphilus*, and *Anaerococcus*.[25,50] It is also possible that other milk components are able to modulate its microbial composition. For example, concentrations of the polyamine putrescine are correlated with the levels of Gammaproteobacteria and a strongly negative correlation with other Proteobacteria, Clostridia and Actinobacteria.[51] A recent *in vitro* study indicated that Bifidobacteria which utilize specific human milk oligosaccharides (HMOs) can enhance the growth of non-HMO degrading Bifidobacteria.[52] This could potentially lead to their dominance in human milk and thus affect its microbial composition. In line with this notion, correlations between human milk HMO composition and bacteria including Bifidobacteria have been reported.[53] It is also important to bear in mind that maternal secretor status profoundly affects the specific HMO content of milk. The milk of secretor mothers was found to contain a specific HMO composition distinct from secretor mothers. This observation could also partially explain why infants of secretor mothers have higher bifidobacterial abundance in their gut microbiota.[54]

Finally, a number of studies indicates that although pre and postnatal maternal administration of specific probiotics can result in their presence in human milk, the intervention does

not affect HMM diversity and composition.[44,55(p12)] In contrast, other probiotic strains, were shown to be effective in reducing the numbers of *S .aureus* in the milk of mothers with mastitis or increasing the relative abundance of lactobacilli and bifidobacteria.[56,57] This difference could be due to the ability of human milk isolated probiotics to directly metabolize HMOs or other factors such as mode of delivery.

Gestational age, infant gender and mode of delivery

Preterm infants have immune systems characterized by developmental immaturities. This makes them susceptible to detrimental infections and necrotizing enterocolitis (NEC). Interestingly, preterm infants fed with human milk instead of infant formula are less likely to develop NEC. A potential mechanism behind this finding is that some beneficial human milk bacteria may colonize the infant gut, assisting in the development of the immune system and thus provide protection against infections.[58] However, significant differences in the HMM according to the gestational age have been reported, with higher levels of *Enterococcus* and lower levels of *Bifidobacterium* observed in the milk of preterm-delivering mothers compared to those who gave birth on the expected birth date.[59] Nonetheless, despite differences in the relative abundances, *Bifidobacterium, Lactobacillus, Staphylococcus, Streptococcus* and *Enterococcus*, were all present in milk from mothers who had delivered preterm or at full term. When examining the different degrees of prematurity, the same group detected an overall lower bacterial load in the milk of mothers of extremely preterm as compared with those of late preterm infants. On the other hand, Urbaniak and colleagues did not detect any changes in the HMM composition correlating with gestational age.[60] Similar contrasting results have been reported for infant gender, which in some studies seemed to affect HMM composition, but in others no correlation was observed. For instance, a higher relative abundance of *Rothia* was detected in the milk from mothers of female infants compared with mothers of male infants.[55(p12)] In contrast, Urbaniak and coworkers, found no differences in microbial profiles based on gender of the infant.[60] Although the exact cause of these inconsistences is unknown, different collection practices, sample sizes and analytical methods could be considered possible factors.

Mode of delivery is reportedly associated with profound compositional changes of the HMM. Although human milk from C-section mothers is correlated with higher total bacterial counts (especially *Streptococcus,* Proteobacteria and *Carnobacteriaceae*), it is less diverse with lower levels of *Bifidobacterium, Lactobacillus* and *Leuconostocaceae* compared to the milk of mothers who underwent a vaginal delivery.[8,44] Conversely, another report showed no association between the composition of the HMM and delivery mode.[60]

Lactation stage and mode of feeding

It has long been observed that the composition of human milk adapts to the immediate needs of the infant and is divided in three distinct stages: colostrum, transitional milk, and mature milk. The microbiota during the first stage, colostrum, is characterized by increased bacterial diversity with predominant bacteria belonging to the *Weisella, Leuconostoc, Staphylococcus, Streptococcus*, and *Lactococcus* genus.[8] As lactation progresses, total bacterial concentration increases while bacterial diversity decreases. In transitional and mature milk, *Bifidobacterium*, *Enterococcus*, *Veillonella, Leptotrichia, Prevotella, Lactobacillus* and *Staphylococcus* exhibit an upward trend in their relative abundances when compared to colostrum.[59] This variation across the lactation period may be partly explained by the retrograde inoculation pathway since increased abundance of typical oral bacteria has been reported in milk from the later stages. Indeed, levels of oral bacteria in milk (mainly *Streptococcus* and *Rothia*), are on the rise after each breastfeeding session accompanied by an increased bacterial diversity.[61]

Differences in microbial composition have been observed between the two breasts of the same mother.[62] Regarding the milk collection protocol, mothers who expressed milk through a pump had lower abundance of cultivable staphylococci and higher content of bacterial DNA than mothers who expressed their milk manually.[63] Moreover, mothers who fed their infants pumped milk had lower abundance of *Bifidobacterium*, and higher abundance of *Enterobacteriaceae* and *Pseudomonadaceae* in their milk.[64] Conversely, mothers who fed their infants through direct breastfeeding had higher numbers of *Gemellaceae*, *Vogesella*, and Nocardioides.

Geographic location and social network density

Various studies have explored the impact of geographic location on the HMM composition. In a recent pilot study, the HMM of the Old Order Mennonites (OOMs) population was found to be more diverse compared to the one of mothers from urban areas. This could be due to the different dietary and environmental exposure patterns of this community, mainly characterized by cultivation of own food, consumption of raw milk and preference for home births.[65] Similarly, population differences were also found in Iran, with total counts of *Lactobacillus* being higher in the milk of mothers residing in rural areas compared to those in urban areas.[66] Meehan and colleagues examined the HMM of hunter–gatherer and horticulturalist women in the Central African Republic, reporting lower levels of *Lactobacillus* in the milk of hunter-gatherers than horticulturalists. The most abundant genera were similar to the ones found in the milk of Western women.[67] However, despite the overlap of several genera, lower relative abundances of *Bifidobacterium, Propionibacterium, Veillonella* and *Serratia* were reported in the milk of Central African Republic mothers compared to US or Swiss mothers.[16,68] In addition, in the same study, the social and caregiving environment showed a significant association with HMM diversity with high HMM diversity exhibiting a positive correlation with a simultaneous increase in allomaternal and decrease in maternal care. Another study compared the HMM of ethnically distinct mothers.[49] When the HMM composition was studied in mothers who had delivered vaginally, Spanish mothers had the highest relative abundance of Bacteroidetes in milk when compared to mothers from Finland, China or South Africa. In the same study, Chinese women who delivered by C-section harbored the highest levels of Actinobacteria in their milk compared to their

European or African counterparts. Finally, and in accordance with the previous reports, Lackey and colleagues showed that the composition of HMM varies significantly within and across cohorts from international sites (Ethiopia, Gambia, US, Kenya, Peru, Spain, and Sweden).[69]

Conclusion

Several factors including maternal health status and diet, gestational age, infant gender, mode of delivery, lactation stage, mode of feeding, geographic location and social network density influence the composition of the human milk microbiota and therefore which microorganisms are transferred to the infant through breastfeeding. However, many questions still remain unanswered not only related to the HMM composition but also its function. Lastly, breastfeeding is not always possible and, in many cases, banked milk is the next optimal option. Therefore, further research is needed to determine how factors associated with human milk banking such as storage and pasteurization affect the composition of these microbial communities.

Disclosures

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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