



1 **SUMMARY**

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

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14 *Keywords:* **milk microbiota, lactation, C-section, diet, gestational age**

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## 1 **1. Introduction**

2 Recent reports have revealed the importance of our gut microbiome for optimal health.  
3 Accumulating evidence highlights the potential role of microbes in metabolic, immunological,  
4 and microbial programming [1]. Microbes are among the most important environmental factors  
5 providing specific signals to guide immune system development and maturation [2, 3]. Shifts in  
6 microbiota composition and activity appear to be related to adverse human health outcomes [4].  
7 The maternal microbial environment impacts the newborn's immune development and,  
8 consequently, the infant's health both early and later on in life. The maternal microbiota is now  
9 recognized as a significant determinant of the maternally transferred factors that impact the  
10 child's health [5, 6]. Outcomes can be affected by specific perinatal factors that also alter infant  
11 microbiome development. For example, excessive use of antibiotics, unbalanced diet, increasing  
12 incidence of caesarean section (C-section) deliveries, unnecessarily stringent hygiene, and  
13 continuous stress influence the maternal microbiome. Alterations and disturbances in microbiota  
14 composition along with a reduction in microbial diversity or richness have been described as  
15 strong risk factors for the development of lifestyle diseases, such as allergies, diabetes, obesity,  
16 and metabolic syndrome, irritable bowel syndrome and other inflammatory-related problems [4,  
17 7]. The maternal microbiota and infant diet play a key role in infant growth, adequate microbial  
18 colonization, immune system maturation, and metabolic development. Then, the infant step-  
19 wise microbial colonization process will have an impact on metabolic and immunological  
20 response, and these in turn may have an impact on programming of health later in life [3, 8].  
21 This review aims to describe the impact of the maternal microbiota on infant health through  
22 breastfeeding. The review will also provide a broad overview of milk microbiota composition  
23 and activity, along with the factors influencing microbial composition and their potential  
24 biological relevance.

25

## 26 **2. Human breast milk: More than infant food**

27 Human milk (HM) provides complete nutrition for the infant. HM's energy, nutrients, and  
28 bioactive components directly influence the development of newborn infants. Among its

1 bioactive factors, HM contains several immune substances, such as immunoglobulins, cytokines  
2 and chemokines, growth factors, hormones, and antibodies. It also contains other nonspecific  
3 compounds, such as specific peptides, lactoferrin and other whey proteins, oligosaccharides and  
4 a large number of bacteria [9]. All these components are transferred to the infant through  
5 breastfeeding.

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

25

### 26 **3. Human breast milk microbiome**

27 Historically, human breast milk has been considered an almost sterile fluid, but this dogma has  
28 been revised over the years. Studies in primates have reported that rhesus monkey (*Macaca*

1 *mulatta*) milk contains culturable strains of at least 19 species of bacteria belonging to at least  
2 five different genera [32, 33]. Similar results have been described in other mammals [34].

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

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

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

22 Human milk harbors a unique microbial ecosystem which differs from any other in humans. The  
23 milk microbiota is not linked with any mucosal or fecal samples, nor does it seem to be a subset  
24 of any other specific human sample [21]. The breast milk microbial diversity has been  
25 evaluated by several independent research groups [20-27, 35]. These researches have shown  
26 that *Staphylococcus* spp., *Streptococcus* spp., are the common groups followed by specific lactic  
27 acid bacteria. The first study on the milk microbiome using pyrosequencing demonstrated that

1 milk bacterial communities are generally complex [20]. It was reported a high inter-individual  
2 variation regarding the number and abundance of different species in human milk, however, a  
3 common core of nine bacterial groups were present in the milk samples of each individual  
4 (*Streptococcus*, *Staphylococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Ralstonia*,  
5 *Propionibacterium*, *Sphingomonas*, and *Bradyrhizobiaceae*) [20]. Difference results between  
6 studies may be due to different sampling and process protocols and varied DNA extraction,  
7 selection of specific primers with high bacterial coverage including bacteria with higher content  
8 of G+C as *Bifidobacterium* spp and sequencing platforms; thus, a more standardized approach is  
9 needed in the future [20, 21, 23, 35, 39].

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

21 Shotgun metagenomics analysis of human milk by total DNA sequencing using Illumina next-  
22 generation sequencing technology has been done using 10 pooled milk samples [41]. This study  
23 reported that human milk contains over 360 prokaryotic genera, with Proteobacteria (65%) and  
24 Firmicutes (34%) as the predominant phyla, and with *Pseudomonas* (61.1%), *Staphylococcus*  
25 (33.4%), and *Streptococcus* (0.5%) as the predominant genera [41]. This study also showed that  
26 the most abundant open reading frames (ORFs) within human milk encoded proteins for basic  
27 cellular functions (respiration, cell signaling, RNA, DNA, and amino acid metabolism).

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

7 Molecular techniques have some limitations. For example, the viability of milk microbes cannot  
8 be analyzed, and total bacteria counts may be over- or underestimated because of cell-wall  
9 composition, DNA extraction methods, and the number of microbial 16S gene copies which  
10 possible lead the over- or underestimation of bacteria counts. The DNA contamination in  
11 extraction kits and reagents has also been reported [35]. Other potential factors influencing milk  
12 microbiota composition include sampling methods (e.g., aseptic methods, time of day, breast  
13 cleaning methods, sampling before or after suckling, manual extraction or breast pump  
14 extraction), DNA extraction methods (e.g., from whole or defatted milk, use of commercial kits,  
15 enzymatic lysis, bead-beating step), sequencing platforms (Illumina, Solid, Ion Torrent, 454  
16 Roche), 16S bacterial gene region studies (V1–V3; V3–V4, V4, etc.), and the 16S database and  
17 bioinformatics pipeline used. Thus, there is a need to standardize protocols and validate  
18 methods.

19

#### 20 **4. Potential factors influencing the milk microbiome**

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

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

7 The mode of delivery affects human milk microbiota composition. High microbial diversity and  
8 high prevalence of *Bifidobacterium* and *Lactobacillus* spp. are found in colostrum and milk  
9 following vaginal delivery, while the contrary is observed following caesarean delivery [21, 22,  
10 44-46] although other studies did not report differences in microbial profiles based on gestation,  
11 mode of delivery or infant gender [47]. Breast milk microbiota composition is also influenced  
12 by gestational age, with significant differences between term- and preterm-delivered mothers.  
13 Lower counts of *Enterococcus* in colostrum and higher counts of *Bifidobacterium* in milk have  
14 been detected in samples from mothers with term deliveries [44]. In addition, changes in the  
15 milk microbiota composition are associated with maternal physiological status, including  
16 obesity, celiac disease, and HIV [21, 48-50]. Obesity is reflected in the levels of  
17 *Bifidobacterium* spp and cytokines in human milk [50], as well as increased *Staphylococcus*  
18 spp., leptin, and pro-inflammatory fatty acid levels [50-53] and reduced microbial diversity  
19 [21]. Mothers with celiac disease have reduced levels of cytokines, *Bacteroides* spp. and  
20 *Bifidobacterium* spp. in their milk [48]. Finally, the milk from HIV-positive women from  
21 Africa has been found to show higher bacterial diversity and higher prevalence of *Lactobacillus*  
22 spp. than the milk of non-HIV women [49].

23 Analysis of the human milk microbiota shows that, in general, *Staphylococcus* and  
24 *Streptococcus* genus and also, lactic acid bacteria strains are present in milk [19- 24], but their  
25 relative amounts and the presence of other bacteria, could be dependent on geographical  
26 location [37, 49]. However, large-scale studies with breast milk samples from different  
27 geographic locations are needed. It is also evident that perinatal use of antibiotics has an impact  
28 on the maternal microbiota, including HM microbiota, affecting the prevalence of

1 *Lactobacillus*, *Bifidobacterium*, and *Staphylococcus* spp. [45,54] and decreasing the abundance  
2 of *Bifidobacterium*, *Staphylococcus*, and *Eubacterium* spp. in milk samples [37]. Chemotherapy  
3 has also been associated with alteration in the milk microbiome and with a reduction in bacterial  
4 diversity [55]. Studies on the impact of maternal diet on milk microbiota are few in number, but  
5 it is likely that nutritional habits, which are able to modulate intestinal microbiota and human  
6 milk nutritional composition, may exert changes in the milk microbiota. For example, shared  
7 microbial features have been reported between bacteria present in local foods and other-  
8 fermented foods and mother-infant microbial gut and breast milk [56].  
9 Moreover, diet clearly influences the lipid profile of human milk, modulating the concentration  
10 of long-chain polyunsaturated fatty acids and the ratio of  $\omega$ -3 to  $\omega$ -6 [57, 58]. These fatty acids  
11 have immunomodulatory properties in nursing children [59], and it has recently been  
12 demonstrated in animal models that they are able to modulate gut microbiota composition in  
13 early life [60]. The consumption of probiotics and prebiotics during pregnancy could influence  
14 the human milk microbiota, but more studies are necessary to document the potential transfer of  
15 gut bacteria to the mammary glands and the impact of specific strains of bacteria [61]. On the  
16 other hand, it has been reported that nonnutritive sweeteners, such as saccharin, sucralose, and  
17 acesulfame potassium, were present in 65% of breast milk samples analyzed [62]. Those data  
18 suggest that maternal diet could modulate the bioactive compounds and microbes present in  
19 breast milk. Studies are urgently needed to investigate interactions among nutrients in the  
20 maternal diet and the breast milk microbiota and their health effects on infants.

21

## 22 **5. Potential strategies to modulate the maternal breast milk microbiota**

23 As mentioned above, diet is likely a powerful tool to alter gut microbiota. Dietary strategies,  
24 then, could be devised to modulate the microbial composition in order to affect human  
25 physiology and reduce the risk of diseases related to imbalances of microbiota composition. A  
26 recent study [63], with Japanese macaques (*Macaca fuscata*) as animal model, has reported the  
27 impact of maternal diet on neonatal microbiome composition. This study showed the effect of  
28 maternal high fat diet affects infant microbiome composition and activity and also, affects the

1 metabolic health. Thus, deciphering the contribution of specific gut bacteria and promoting  
2 nutrition and lifestyle counseling may open new tools to reduce the risk of diseases related to  
3 microbial composition shifts. Accumulating evidence shows that early dietary interventions and  
4 nutrition counselling would support health programming effects (immunological, metabolic and  
5 microbial effect) on adult health.

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

21

## 22 **6. Conclusions**

23 Several perinatal factors influence microbial transfer from mother to infant via breast milk. The  
24 lactation period may provide a new target for devising novel dietary and nutritional tools to  
25 modulate the milk microbiota and thereby reduce the risk of noncommunicable diseases  
26 (NCDs), while at the same time promoting breastfeeding. To continue to increase our  
27 knowledge of the milk microbiota, further studies from different geographical regions and  
28 among varying population groups with different genetic backgrounds and environmental and

1 nutrition conditions are required to fully understand the impact of the microbiome and its  
2 potential in infant health promotion.

3

#### 4 **Practice Points**

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

13

14

#### 15 **Research**

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

24

1 **Conflict of interest statement**

2 None declared.

3 **Funding sources**

4 This review has been written within the European Research Council starting grant, MAMI  
5 project under grant agreement No. 639226. M.C. Collado is involved in the “ISCH COST  
6 Action IS1405” entitled Building Intrapartum Research Through Health e an interdisciplinary  
7 whole system approach to understanding and contextualising physiological labour and birth  
8 (BIRTH). C. Gomez- Gallego is a recipient of the Seneca Postdoctoral Grant from the Seneca  
9 Foundation, the Regional Agency of Science and Technology of the Region of Murcia (funded  
10 by the Education and Universities Council e Autonomous Community of the Region of  
11 Murcia).

12

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