Emerging frontiers in human milk microbiome research and suggested primers for 16S rRNA gene analysis

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Human milk is the ideal food for infants due to its unique nutritional and immune properties, and more recently human milk has also been recognized as an important source of bacteria for infants. However, a substantial amount of fundamental human milk microbiome information remains unclear, such as the origin, composition and function of the community and its members. There is emerging evidence to suggest that the diversity and composition of the milk microbiome might differ between lactation stages, due to maternal factors and diet, agrarian and urban lifestyles, and geographical location. The evolution of the methods used for studying milk microbiota, transitioning from culture-dependent approaches to include culture-independent approaches, has had an impact on findings and, in particular, primer selection within 16S rRNA gene barcoding studies have led to discrepancies in observed microbiota communities. Here, the current state-of-the-art is reviewed and emerging frontiers essential to improving our understanding of the human milk microbiome are considered.

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1. History of milk microbiome

Although many of the nutritional and immune system benefits of human milk have been established [1–4], the influence of the milk microbiome on health is still in the early days of exploration. A recent review [5], while stressing further evidence is needed, has explored the idea that human milk could represent a reservoir of bacteria which influences infant gut microbiome diversity and aspects of health such as allergy prevention. The existence of the human microbiome is well-established [6] and was studied as far back as Antonie van Leeuwenhoek (1632–1723); however, microbiome study in human milk is very recent due to a long-standing belief that human milk was sterile [7]. In the late ‘60 s, the presence of bacteria in human milk was related to the low levels of personal hygiene and environmental sanitation in women from Guatemala [8] (Table 1). Later, diverse methods were employed in order to make milk ‘bacteriologically safe’, such as heating and freezing [9,10]. By the late ‘80 s it had been recognized that human milk contained growth-promoting substances postulated to be involved in the development of microbiota [11]. In the early 2000s, interest and study of the microbiome significantly increased and promptly led to important discoveries, including the existence of commensal bacteria in human milk [12]. Bacteria were recognized as components of the natural microbiota rather than contaminants [13] resulting in notable differences among human body sites that included highly diverse communities in skin, less variable communities within the oral cavity compared to other habitats and highly variable gut communities between individuals but with low variability over time [14].

One of the most important advances in microbiome research began in 2007 with the Human Microbiome Project conducted by the National Institutes of Health, the first large-scale effort to characterize the healthy human microbiome [15]. The study included a population of 242 healthy adults and a description of the microbiome: retrograde flow and the entero-mammary pathway. Retrograde flow is an infant-to-mother transfer in which microbes are transmitted via the skin and saliva from the infant’s oral cavity into the duct during suckling [16]. This could explain how bacteria commonly found in the infant oral cavity, such as anaerobic species, could be acquired by the infant through a vaginal delivery and then transferred to the human milk through retrograde flow. Recent evidence has suggested that the mode of delivery might influence human milk composition [17,19–22]. Some studies show higher bacterial diversity and richness in the milk from vaginal deliveries [17,19,20,22]. Human milk from vaginal deliveries has been reported to have higher Bifidobacterium [21], Streptococcus and Haemophilus, and lower abundance of Finigolda spp., Halomonas spp., Prevotella spp., Pseudomonas spp. and Staphylococcus spp. [22]. However, despite these differences, findings are not consistent as other studies did not report differences [23–26].

2. Sources of the milk microbiome

Research has aimed to explain the sources of the milk microbiome (Fig. 1). Two main pathways have been recognized as sources that might potentially contribute to the human milk microbiome: retrograde flow and the entero-mammary pathway. Retrograde flow is an infant-to-mother transfer in which microbes are transmitted via the skin and saliva from the infant’s oral cavity into the duct during suckling [16]. This could explain how bacteria commonly found in the infant oral cavity, such as those within the genera Veillonella, Leptotrichia and Prevotella [17], or bacteria commonly found in the vagina, such as Lactobacillus [18] are sometimes found in human milk. The vaginal bacteria could be acquired by the infant through a vaginal delivery and then transferred to the human milk through retrograde flow. Recent evidence has suggested that the mode of delivery might influence human milk composition [17,19–22]. Some studies show higher bacterial diversity and richness in the milk from vaginal deliveries [17,19,20,22]. Human milk from vaginal deliveries has been reported to have higher Bifidobacterium [21], Streptococcus and Haemophilus, and lower abundance of Finigolda spp., Halomonas spp., Prevotella spp., Pseudomonas spp. and Staphylococcus spp. [22]. However, despite these differences, findings are not consistent as other studies did not report differences [23–26].

The entero-mammary route has emerged as another potential route for how maternal gut microbiota might enter and enrich the human milk microbiome. The route proposes that maternal gut bacteria are translocated through the intestinal epithelial barrier via dendritic cells which cross the paracellular space of the intestinal epithelium and directly sample bacteria from the intestinal lumen [13,27–29]. Circulation of lymph within the mucosal associated lymphoid tissue could then allow the maternal

| Time | Microbiome Concept | Historical Human Milk Microbiome Findings |
|------|-------------------|------------------------------------------|
| ‘60 s | Enterobacteriaceae detected | The presence of Enterobacteriaceae in human breast milk is related to the low levels of personal hygiene and environmental sanitation in women from Guatemala. It is concluded that an interchange of breast milk bacteria between the mother’s breast and the infant’s mouth is possible [8]. |
| ‘70 s | Heat sterilization | Bacteria in milk were detected. Breast milk was heated in order to be “bacteriologically safe” [9]. |
| ‘80 s | Microbial growth promotion suggested | Growth-promoting substances in human milk postulated to be involved in the development of microbiota. Components like lactoferrin and a saccharide containing N-acetyl glucosamine could provide an adequate environment for bacterial growth [11]. |
| ‘90 s | Bacteria considered solely contamination | Presence of bacteria considered to be due to contamination in frozen milk. |
| ‘00 s | Commensal bacteria | Contamination levels of human milk compared to pasteurized cow’s milk to develop guidelines for the acceptable microbial quality of human milk [10]. |
| ‘10 s | Vertical transfer | A study looks for commensal bacteria inhibiting Staphylococcus aureus as published reports at that time had only focused on pathogenic bacteria [12]. |
| ‘15 s | Gut and milk link to lymphatic system | Description of the “retrograde infant-to-mother transfer” in which microbes are transmitted via the skin and via retrograde-flow of milk into the duct during suckling [16]. |
| ‘20 s | Anaerobic species | Alternative route for microbial transfer due to the presence of the anaerobic genus Bifidobacterium in breast milk samples [39]. Although this led to important new theories about the origin of milk bacteria, Bifidobacterium is now recognised as containing extensive strain variance in O2 tolerance and sensitivity [121,122]. |
| ‘25 s | Vertical transfer widely accepted | Evidence of an internal microsomal transfer pathway due to the presence of maternal gut and breast milk bacteria in the lymphatic system is strengthened, although more evidence is necessary due to the characteristics of the study. [27]. |
| ‘30 s | Tissue specific microbiome patterning | Identification of distinct patterns of the microbiome among human body sites [14]. |

Table 1

Historical Study of Milk Microbiome.
gastrointestinal tract microbiota to reach distant sites such as the lactating mammary gland.

3. Major factors that influence the human milk microbiome

Milk microbiome composition is influenced by diverse factors such as stage of lactation, maternal BMI, diet and use of antibiotics [17,20,21,23,26,30–35]. However, there are other factors that remain unexplored and might have an important influence on human milk composition including age, parity, geographical location, and interactions with the environment.

3.1. Stage of lactation

There is evidence of substantial shifts in microbiota composition at different stages of lactation. Chen et al. [36] found that colostrum and transitional milk share 48.9% of bacterial genera and 42% of bacterial species, so there are common and unique bacteria between the two stages of milk. Cabrera et al. [17] reported initially that the microbiota of colostrum was dominated by Weissella, Leuconostoc, Staphylococcus spp., Streptococcus spp., and Lactococcus spp. Later, milk samples collected 1–6 months post-partum had higher levels of Veillonella, Prevotella, Leptotrichia, Lactobacillus spp, Streptococcus spp, and increasing levels of Bifidobacterium and Enterococcus spp, which the authors speculated was related to the frequent interaction with the infant's oral microbiota. Similar results were reported by Khodayar et al. [21], in which Bifidobacterium and Enterococcus spp. counts increased throughout the lactation period. Another study reported that colostrum had a higher diversity typical of skin and maternal gut bacteria, and as lactation progressed, mature milk became less diverse but increased in infant oral and skin associated bacteria [37]. While improved technologies will help elucidate the current discrepancies in microbiota across lactation stage, a consistent finding is that the microbiome is dynamic and remodels as lactation progresses [38,39]. Given this, other factors known to change throughout lactation, such as the nutritional and immunological composition of milk, will need to be explored as modifiers of human milk microbiome.

3.2. Maternal BMI and diet

The relationship between BMI and human milk microbiota is not clear. Some studies did not find any significant associations [31,33] but others have reported that maternal BMI and weight gain during pregnancy do impact the diversity of bacterial community in human milk [17,40]. Those studies that found an association reported that obesity and excessive weight gain reduced diversity of the milk microbiome [17,40]. Milk samples of mothers with higher BMI had higher abundance of Lactobacillus in colostrum and higher abundance of Staphylococcus and Akkermansia in mature milk [17] as well as higher Granulicatella [33]. On the other hand, higher BMI has been related to reductions in the genera Bifidobacterium in milk produced at 6 months [17] and Bacteroides [33] and, more broadly, reductions in the phyla Proteobacteria [23] and Firmicutes [35].

Maternal diet, a factor often associated with BMI, could influence microbial composition of human milk directly or as a secondary effect by influencing human milk nutritional content. Some studies have reported that maternal diet influences the nutrient concentration of milk [41–43] and likely shapes its bacterial community [33]. With regards to macronutrients, maternal intake of saturated fatty acids and monosaturated fatty acids were inversely associated with Corynebacterium, and protein consumption was positively correlated with the relative abundance of Gemella [33]. In relation to micronutrients, a negative correlation was observed between pantothenic acid intake and Streptococcus and between Lactobacillus with thiamin, niacin, vitamin B-6 and chromium and a positive correlation was found between riboflavin and calcium with Veillonella [33]. Since the relationship between diet and gut microbiota has been extensively explored [44–47], Fig. 1. Sources of milk microbiota Two potential sources of bacteria which could contribute to the breast milk microbiome: retrograde flow [16] and the entero-mammary pathway [13,27–29]. The retrograde flow pathway suggests that the during suckling, infant oral microbiota and breast skin microbiota can reach the breast milk microbiota. The entero-mammary pathway suggests that dendritic cells in the maternal gut that cross the intestinal epithelium can take up bacteria from the intestinal lumen which are then taken to the mammary gland through the blood and lymphatic systems.
### Table 2
Summary of most abundant bacteria, lifestyle or community considered and methods employed in breast milk microbiome studies.

| Most Abundant Taxa | Cohort location | Method | Region | Primer\(a\)(common label) | Primer sequences (including degeneracy variants) | Template free PCR controls\(b\) | Reference |
|--------------------|----------------|--------|--------|-----------------------------|--------------------------------------------------|---------------------------------|-----------|
| Staphylococcus, Streptococcus, Serratia, Pseudomonas, Corynebacterium, Rabiotia, Cutibacterium/Propionibacterium, Sphingomonas and Bradyrhizobiaceae | USA | 16S rRNA gene sequencing | V1-V2 | 27F\(2\) and 338R | 27F: '5'–AGAGTTGTATCATCTGGTĂG-3' 338R: '5'-TTCCTGTCGTCCCCGTAGAGT-3' | Yes | [52] |
| Weisella, Leuconostoc, Staphylococcus, Streptococcus, Lactobacillus, Veillonella, Lepotrichia, and Prevotella | Finland | 16S rRNA gene sequencing | V1-V3 | 27F and 533R | N/A | Yes | [17] |
| Cutibacterium acnes, Staphylococcus epidermidis, Streptococcus (S. salivarius, S. thermophilus, S. vestibularis, S. mitis, S. pneumoniae, Staphylococcus (S. lugdunensis, S. aureus, S. haemolyticus, S. hominis, S. pasteuri, S. warneri), Veillonella (V. atypica, V. parvula), Rothia mucilaginosa, Propionibacterium granulosum, Bifidobacterium breve, Klebsiella pneumoniae, Escherichia/Shigella, Lactobacillus (L. gasseri, L. brevis), Enterococcus (E. faecalis, E. gallinarum) | Switzerland | 16S rRNA gene sequencing | V5 – V6 | 784F and 1061R | 784F: '5'–AGGATTAGATACCCCTGGA-3'1061R: '5'-CRRCCAGGACTCAGAC-3' | No | [65] |
| Staphylococcus spp., Streptococcus spp., Veillonella spp., Corynebacterium spp., Rothia spp., Enterococcus spp., Lactobacillus spp., Escherichia/Shigella spp., Klebsiella spp. | Switzerland | 16S rRNA gene sequencing | V5 – V6 | 784F and 1061R | 784F: '5'–AGGATTAGATACCCCTGGA-3'1061R: '5'-CRRCCAGGACTCAGAC-3' | No | [123] |
| Eubacterium, Lactobacillus, Acinetobacter, Xanthomonadaceae, Stenotrophomonas | Canada | 16S rRNA gene sequencing | V6 | N/A | Fw: '5'-CWAAGCCGARGAACCCTTACC-3'Rv: '5'-ACRAACAGGACGCAC-3' | Yes | [36] |
| Staphylococcus, Streptococcus, Bacteroides, Faecalibacterium, Ruminoococcus, Lactobacillus, Cutibacterium/Propionibacterium, Staphylococcus aureus and Staphylococcus epidermidis | Spain | Shotgun metagenomic sequencing | – | – | – | – | [53] |
| Streptococcus, Staphylococcus, and Neisseria | USA | 16S rRNA gene sequencing | V4 | 515F and 806R | 515F: GTGCGACGMCGCGCGGTAAB086R: GCCATACHVGGGTWTCAAT 27F: '5'-AGAGTTGTATCATCTGGGCTCAG-3' 533R: '5'-GCCCTGACCGCCCGCTCAGGC-3' | No | [30] |
| Streptococcus, Pseudomonas, Streptococcus and Acinetobacter | Spain | 16S rRNA gene sequencing | V1-V3 | 27F and 533R | 27F: '5'-AGAGTTGTATCATCTGGGTACAG-3' 533R: '5'-GCCCTGACCGCCCGCTCAGGC-3' | No | [50] |
| Staphylococcus, Pseudomonas, Enterobacteriaceae, Streptococcus and Lactobacillus | Canada | 16S rRNA gene sequencing | V6 | N/A | Fw: '5'-CWAAGCCGARGAACCCTTACC-3'Rv: '5'-ACRAACAGGACGCAC-3' | Yes | [105] |
| Staphylococcus, Streptococcus, Pseudomonas and Acinetobacter | Spain, Finland, South Africa and China | 16S rRNA gene sequencing | V4 | 515F and 806R | 515F: '5'-GTGCGACGMCGCGCGGTAAB086R: GCCATACHVGGGTWTCAAT 533R: '5'-GCCCTGACCGCCCGCTCAGGC-3' | No | [35] |
| Streptococcus Staphylococcus and Acinetobacter | China | 16S rRNA gene sequencing | V4 | 515F and 806R | 515F: '5'-GTGCGACGMCGCGCGGTAAB086R: GCCATACHVGGGTWTCAAT 8F/27F/mol: '5'-AGRGTGTATGATMTTGGGTACAG-3' 533R: '5'-GCCCTGACCGCCCGCTCAGGC-3' | No | [31] |
| Streptococcus, Pseudomonas, Staphylococcus, Lactobacillus, Propionibacterium, Herbaspirillum, Rothia, Stenotrophomonas, Acinetobacter, Bacteroides, Halomonas, Veillonella, Sphingomonas, Delftia, and Corynebacterium | Taiwan and China | 16S rRNA gene sequencing | V1 – V2 | 27F and 338R | 27F: '5'-AGAGTTGTATCATCTGGGCTCAG-3' 338R: '5'-GCCCTGACCGCCCGCTCAGGC-3' | No | [59] |
| Pseudomonas, Staphylococcus and Streptococcus | Ireland | 16S rRNA gene sequencing | V3 – V4 | Bakl3114F-Bakl805R | Bakl3114F: '5'-CTACGGGNGGCWGGCAG-3' Bakl805R: '5'-GACTAHHVVGGGTATCTAATCC-3' | No | [54] |
| Streptococcusaceae, Staphylococcusaceae, Gamellaceae, Alcylobacillaceae, Enterobacteriaceae, Pseudomonadaceae, Moraxellaceae, Xanthomonadaceae, Bradyrhizobiaceae, Caulobacteriaceae, Neisseriaceae and Weeksellaceae | USA | 16S rRNA gene sequencing | V4 | 515F and 806R | 515F: '5'-GTGCGACGMCGCGCGGTAAB086R: GCCATACHVGGGTWTCAAT | Yes | [24] |
the diet induced alterations of maternal gut microbiota might also influence human milk microbes, and therefore vertical transfer to the infant [48]. Nonetheless, there is limited evidence about the direct relationship of maternal diet on the human milk microbiome, or the interactions between maternal diet, maternal microbiota and infant microbiota. Despite the limited evidence, it is valuable that research suggests that maternal diet can influence human milk microbial composition, as maternal diet is one of the most modifiable factors by which interventions could be explored for modulating the human milk microbiome.

3.3. Use of antibiotics and probiotics

Results regarding the use of antibiotics are contradictory. For instance, in a study of women living in Germany and Austria abundance of lactobacilli or bifidobacteria was lower in women who had received antibiotics during pregnancy or lactation [26]. However, another study reported that the effect of antibiotic exposure on human milk microbiota at 1 month postpartum appeared to be an increase in bacterial richness and diversity [20]. Similarly, it has also been reported that mean bacterial counts in milk produced by women receiving antibiotics were higher than in milk from women taking probiotics [32]. Although probiotics are largely considered to support microbiome diversity, a gut microbiome study found a similar pattern and reported that after the use of antibiotics, the introduction of probiotics actually delayed and impaired mucosal microbiome reconstitution [49]. However, the impact of the consumption of probiotics during pregnancy and lactation on the milk microbiome and infant health following antibiotic use has not been widely explored.

4. Inconsistencies in dominant milk microbiome bacteria persist

Although lactation stage, maternal BMI, diet and use of antibiotics are thought to drastically influence the microbiota present in human milk, a number of taxa are consistently observed across studies. The genera often found include *Staphylococcus*, *Corynebacterium*, *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Escherichia/Shigella*, *Bacillus*, *Stenotrophomonas*, *Achromobacter*, *Escherichia/Shigella*, *Achromobacter*, *Sphingomonas*, *Serratia*, *Ralstonia*, *Bradyrhizobaceae* [31,50–56]. According to the latest systematic review 590 different genera have been detected via sequencing studies. The genera often found include *Lactobacillus*, *Prevotella*, *Micrococcus* and *Bifidobacterium*, *B. infantis* with no degeneracy (genera found in 97% of studies; range of relative abundance 5–83%) [34].

**Table 2 (continued)**

| Most Abundant Taxa | Cohort location | Method | Region | Primer label (common primer) | Primer sequences (including degeneracy variants) | Template free PCR controls | Reference |
|--------------------|----------------|--------|--------|-------------------------------|-----------------------------------------------|--------------------------|----------|
| *Pseudomonas*, unclassified Enterobacteriaceae, Enterobacter, unclassified Pseudomonadaceae, Klebsiella, Ralstonia, Acinetobacter and *Serratia*, *Bacillus*, *Staphylococcus*, *Enterococcus*, *Bacillus*, unclassified Lachnospiraceae, Streptococcus | India | 16S rRNA gene sequencing | V2 – V3 | 101F and 518R | 101F: 5'-ACTGGCCGGACGGTATGA3'-518R: 5'-CGTATTACCGCGGCTGCTGG-3' | No | [99] |
| *Streptococcus*, *Staphylococcus*, Veillonella, *Corynebacterium*, Rhodococcus, *Dyella*, *Lactobacillus*, *Prevotella*, *Micrococcus* and *Hafnia* | Central African Republic | 16S rRNA gene sequencing | V1 – V3 | 27F-YM + 3 and 534R | 27F-YM + 3: 5'-AGMGTTYGATYMTGGCTACG-3'/534R: 5'-ATTACCGCGGCTGCGG-3' | Yes | [92] |
| *Streptococcus*, *Staphylococcus*, *Ralstonia*, *Acidovorax*, *Acinetobacter*, *Aquabacterium*, *Massilia*, *Agrobacterium*, *Rheinheimera*, *Veillonella*, *Vogesella*, *Nocardioae* and *Pseudomonas* | Canada | 16S rRNA gene sequencing | V4 | 515F and 806R | 515F: 5'-GTCGACGCMGCCGCGTAA-3'/806R: 5'-GGAACATACVGGTCTTAAT-3' | Yes | [23] |
| *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Cutibacterium*/*Propionibacterium*, *Rhizobium*, *Lactobacillus*, *Dyella*, * Rothia*, *Kocuria*, *Veillonella*, *Bifidobacterium*, *Acinetobacter*, *Klebsiella*, *Gemella*, *Achromobacter*, *Escherichia/Shigella*, *Bacillus*, *Stenotrophomonas*, *Enterococcus*, *Janthinibacterium*, *Anaerococcus*, *Acidocella*, *Enterobacter*, *Bacteroides*, *Pseudomonas*, *Chryseobacterium*, *Tatumella*, *Psychrobacter*, *Clostridium sensu stricto* | Ethiopia, Kenya, Ghana, Gambia, Peru, Spain, Sweden and USA | 16S rRNA gene sequencing | V1 – V3 | 27F-YM + 3 and 534R | 27F-YM + 3: 5'-AGMGTTYGAYMTGGCTACG-3'/534R: 5'-ATTACCGCGGCTGCGG-3' | Yes | [89] |
| *Staphylococcus*, *Kaistobacter*, *Paracoccus*, *Pseudomonas*, *Bradyrhizobium*, *Methylbacterium*, *Acinetobacter*, *Cutibacterium/Propionibacterium*, *Corynebacterium* and *Microbacterium*; three families *Phyllobacteriaceae*, *Sphingomonadaceae*, *Gemellaceae* | Mexico | 16S rRNA gene sequencing | V3 | 341F and 518R | 341F: CCTACGGAGGGCACCAG518R: ATTACCGCGGCTGCGG | Yes | [98] |

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* Primer labels are often inconsistent in the literature and so exact sequences are provided where reported.
* The use of no template PCR controls does not preclude the possibility that any observed sequences were contamination.
* “27F” with no degeneracy is sometimes called 8F.
Some studies have attempted to define a “core” microbiome of human milk [51,52], but the presence of some bacteria in human milk remains controversial as different studies have considered specific genera as either contaminant or as part of the “core” microbiome. Salter et al. [57] found that laboratory reagents were commonly contaminated with species from genera that are repeatedly reported as present in human milk, such as Acinetobacter, Cutibacterium, Novosphingibium, Pseudomonas, Ralstonia, Sphingomonas and Streptococcus, and which can become spurious signals in samples with low bacterial load during 16S rRNA gene amplification. Jiménez et al. [53] speculated that while species within genera such as Cutibacterium and Streptococcus have been isolated using culture-dependent techniques, species from genera such as Sphingomonas and Pseudomonas (containing many readily culturable species) had not, suggesting these genera could commonly be contamination from laboratory reagents. More recently, similar warnings that reagent contamination can substantially influence low bacterial load human milk microbiome samples were raised within Douglas et al. [58], which provided evidence that species within Pseudomonas are common contaminants and also likely genuinely present in human milk (highlighting the current challenges within the field). Similarly, Acinetobacter, a bacteria commonly found in soil, has been reported previously in human milk [35,50,56,59], Sakwinska et al. [59] and Urbaniai et al. [56] both detected Acinetobacter in their milk samples where milk collection was done without aseptic cleansing of the breast and also rejection of foremilk. However, Sakwinska et al. [56] found it unlikely that the predominance of Acinetobacter was due to the collection protocol and argued that Acinetobacter might be a specific feature of breastfeeding associated microbiota. Other studies that have reported bacteria commonly associated with soil in their samples correlated it to the maternal diet based in legumes [60] or to the proximity to a soil environment [61].

Care should be taken to clearly report the use of PCR controls in microbiome studies as the presence of species from certain bacterial genera in human milk remains controversial. Because of this, interpretation of observed genera should also include their potential as contamination while not precluding novel discovery within this understudied field. Few studies analysing human milk microbiome have been conducted at species level (Table 2), despite the advantages improved resolution would have by providing more information about functionality and in facilitating biological interpretation. However, research at species level is increasing as 16S rRNA gene amplification approaches [66], although research suggests 50% of bacteria may be a more reasonable rough estimate in the human gut [67]. This difficulty to culture can be due to the lack of specific required nutrients in the culture medium, toxicity of the culture medium, inhibition by other bacteria or a dependence on association with other species (such as present in bacterial consortia or eukaryote host interactions) [68]. Therefore, although culture-based techniques are vital for the study of specific bacteria of clinical importance or functional interest [69,70], they can be heavily biased and drastically underestimate the diversity when used to assess a microbiome community.

To overcome these challenges, culture-independent metagenomic approaches have been developed in which high-throughput sequencing is harnessed to identify bacteria within microbiome samples using shotgun metagenomics or the 16S ribosomal RNA gene [70]. The major advantage of these approaches is the possibility to detect difficult or yet-to-be-cultured bacteria in addition to improved sample throughput without a requirement of viable cells (allowing the use of frozen samples) [25]. Shotgun metagenomics attempts to sequence DNA directly from DNA fragments derived from all the genomic material present within a microbiome sample and attempts de novo assembly of as many entire genomes or large contiguous sequences as possible in order to infer taxonomic and functional information [70,71]. As this approach does not target a specific gene for PCR, it does not include the same amplification biases associated to 16S rRNA gene barcoding and is widely considered the gold standard of microbiome research. However, in addition to the high expense currently associated with high depth shotgun metagenomics (which is rapidly dropping [72]), the approach generates large amounts of reads from complex samples which are challenging to assemble as de novo [73,74]. While de novo shotgun metagenomics has yet to become common for the study of the human milk microbiota, recent research has used shotgun metagenomics sequence reads as markers for mapping to reference libraries [53,75–77]. While read mapping approaches are still improving in terms of accuracy for quantitative analysis, made difficult by database limitations, very low mapping rates and extensive sequence ambiguity, these rapid improvements in sequencing and bioinformatics methodologies suggest an exciting future for high-resolution identification of metagenomes which includes distinct inventories of genes between human milk microbiome communities. Alternatively, the 16S ribosomal RNA (rRNA) gene has been the most popular approach used for microbial community assessment over recent decades [78–82]. The value of the 16S rRNA gene as a ‘barcode’ for the identification and phylogenetic classification of bacterial species lies in the very highly conserved function of 16S rRNA leading to regions of hyper-conservation within the gene [83,84]. These regions of conservation can then be targeted by primers in order to amplify proximal hyper-variable sequence regions (an amplicon) used as a potentially unique barcode of life [85].

In human milk research, 16S rRNA is still the most popular tools for profiling microbiome samples for quantitative comparison of groups or treatments (Table 2). While some limitations of the 16S rRNA gene barcoding approach are generally well recognised in the field, including the impact of low bacterial load in human milk [57] and poor utility for inference of biological functions in the community [70], primer specificity has been less well addressed. Certain primers have, in the past, been considered “universal” to prokaryotes and thought to amplify hypervariable regions from all bacteria. Research including that conducted by Klindworth et al. [85] has now demonstrated that no known primer pair is universal in amplifying 16S rRNA gene regions across all currently known and well-characterised bacterial species, although many have coverage of over 90% of known bacteria. Given that the observed absence or presence of certain bacteria can be

5. Methods shape observed bacterial taxa in human milk

The methods used to study the human milk microbiome continue to advance. The initial studies used bacterial culture techniques followed by phenotyping of isolated strains using morphological and biochemical characteristics. These studies isolated only a limited number of genera, predominantly facultative anaerobes such as members of the Staphylococcus spp., Streptococcus spp., Propionibacterium spp. (now Cutibacterium sp. in the case of C. acnes), Rothia spp., Enterococcus spp. and Lactobacillus spp. [62–64]. Studies that have used culturing techniques have generally reported that human milk harbours relatively low mean viable bacterial counts often < log 3 cfu/ml [12,27,65]. While still a powerful tool for assessing viability of specific bacterial strains, culture-dependent analyses are limited in revealing only taxa capable of surviving sampling procedures and growth under laboratory conditions [25]. This selection can potentially reduce the observed microbiota community in complex habitats to below 1% of the diversity currently estimated by culture-independent approaches [66], although research suggests 50% of bacteria may be a more reasonable rough estimate in the human gut [67]. This difficulty to culture can be due to the lack of specific required nutrients in the culture medium, toxicity of the culture medium, inhibition by other bacteria or a dependence on association with other species (such as present in bacterial consortia or eukaryote host interactions) [68]. Therefore, although culture-based techniques are vital for the study of specific bacteria of clinical importance or functional interest [69,70], they can be heavily biased and drastically underestimate the diversity when used to assess a microbiome community.

To overcome these challenges, culture-independent metagenomic approaches have been developed in which high-throughput sequencing is harnessed to identify bacteria within microbiome samples using shotgun metagenomics or the 16S ribosomal RNA gene [70]. The major advantage of these approaches is the possibility to detect difficult or yet-to-be-cultured bacteria in addition to improved sample throughput without a requirement of viable cells (allowing the use of frozen samples) [25]. Shotgun metagenomics attempts to sequence DNA directly from DNA fragments derived from all the genomic material present within a microbiome sample and attempts de novo assembly of as many entire genomes or large contiguous sequences as possible in order to infer taxonomic and functional information [70,71]. As this approach does not target a specific gene for PCR, it does not include the same amplification biases associated to 16S rRNA gene barcoding and is widely considered the gold standard of microbiome research. However, in addition to the high expense currently associated with high depth shotgun metagenomics (which is rapidly dropping [72]), the approach generates large amounts of reads from complex samples which are challenging to assemble as de novo [73,74]. While de novo shotgun metagenomics has yet to become common for the study of the human milk microbiota, recent research has used shotgun metagenomics sequence reads as markers for mapping to reference libraries [53,75–77]. While read mapping approaches are still improving in terms of accuracy for quantitative analysis, made difficult by database limitations, very low mapping rates and extensive sequence ambiguity, these rapid improvements in sequencing and bioinformatics methodologies suggest an exciting future for high-resolution identification of metagenomes which includes distinct inventories of genes between human milk microbiome communities. Alternatively, the 16S ribosomal RNA (rRNA) gene has been the most popular approach used for microbial community assessment over recent decades [78–82]. The value of the 16S rRNA gene as a ‘barcode’ for the identification and phylogenetic classification of bacterial species lies in the very highly conserved function of 16S rRNA leading to regions of hyper-conservation within the gene [83,84]. These regions of conservation can then be targeted by primers in order to amplify proximal hyper-variable sequence regions (an amplicon) used as a potentially unique barcode of life [85].

In human milk research, 16S rRNA is still the most popular tools for profiling microbiome samples for quantitative comparison of groups or treatments (Table 2). While some limitations of the 16S rRNA gene barcoding approach are generally well recognised in the field, including the impact of low bacterial load in human milk [57] and poor utility for inference of biological functions in the community [70], primer specificity has been less well addressed. Certain primers have, in the past, been considered “universal” to prokaryotes and thought to amplify hypervariable regions from all bacteria. Research including that conducted by Klindworth et al. [85] has now demonstrated that no known primer pair is universal in amplifying 16S rRNA gene regions across all currently known and well-characterised bacterial species, although many have coverage of over 90% of known bacteria. Given that the observed absence or presence of certain bacteria can be
determined by primer pair selection and the extensive range of primers used in human milk microbiome research (Table 2), it is perhaps not unsurprising that the “core” human milk microbiome genera or species have been reported as inconsistent in systematic literature reviews [86,87].

Using the TestPrime tools in the Silva rRNA database [88] and the research by Klindworth et al. [85], it is possible to assess the specific utility of primers commonly used in human milk microbiome research to amplify the putative “core” milk genera in silico. Interestingly, the primers 27F/533R (V1 region targeting), which are often used in human milk microbiome studies [17,33,52,89,90], have high coverage for amplification of the genus Cutibacterium but not species within the genus Bifidobacterium (with the most common 27F variant,Fig. 2), both of which are considered putative “core” genera [52,53,65] (Table 2). This short-fall in the coverage of Bifidobacterium of the commonly used 27F primer was reported by Frank et al. [91], who designed a high degeneracy variant of seven primers (27F YM + 3) with improved coverage in the genera which has been used in human milk microbiome research [92,89]. Conversely, the other most commonly used primers 515F/806R primer pair (V4 region targeting) will likely amplify species within the genus Bifidobacterium but have very low coverage and do not amplify species from the genera Cutibacterium. To overcome these discrepancies, the 27F YM + 3 high degeneracy variant (within 27F/533R, V1-V3 targeting) or the 784F/1061R primer pair (V5-V6 targeting) should be considered more suitable for human milk research due to high coverage within all of the genera currently considered “core” in human milk (Fig. 2). It is important to recognize that these primers might still fail to amplify yet-to-be-identified species but will allow for the quantitative assessment of relative changes in most species within these important genera when experimentally comparing groups of mothers. While there is a desperate need to increase research into the human milk microbiome using tools such as 16S rRNA gene

Fig. 2. 16S rRNA gene primers pair coverage of major breast milk microbiome genera Common 16S rRNA gene primer pairs 515F-806R, 27F-338R, 27F-533R and 784F-1061R [34] were tested in silico against all sequences within major breast milk microbiome genera in the SILVA database using the TestPrime tool (set to allow 1 mismatch outside the 3’ first 5 nucleotides; https://www.arb-silva.de/search/testprime/) [34]. Percentage of sequences (coverage) within each genus is reported. High and low coverage primer pairs are illustrated in green and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
barcoding, care needs to be taken with all culture-independent techniques to not assume a perfect snapshot of the community, similar to the lessons previously learned with culture-based community assessment [64,65].

6. Emerging frontiers in human milk microbiome research

6.1. Overlooked maternal health issues: age and parity

Two overlooked factors that could be impacting the human milk microbiome include maternal age and parity. To date, only one study has analysed the relationship with maternal age, finding no association with human milk microbiota at family level resolution [23]. However, as there are established differences in the development of breast immunity [93], nutritional requirements and human milk nutrient composition [94] throughout a woman’s lifetime, these factors could also affect the human milk microbiota and should be assessed at higher taxonomic resolution. Parity has also been reported only in one study finding a sex-dependent association with milk microbiome diversity [23]. This study reported an association of human milk microbiota composition with multiparity in female infants but did not characterize the microbiome. Parity could be a factor influencing milk microbiome if we consider the interactions of milk microbiome such as the retrograde flow from the infant [16]. Assuming retrograde flow, it could be expected that a multiparous mother will have a mammary gland with more diverse microbiota since it has been previously inoculated by the bacteria transferred by her previous infant(s) during earlier pregnancies [95].

6.2. Geographical location

Another factor to consider when identifying dominant bacteria in human milk is geographical location, lifestyle, and community. Multi-country studies confirm that human milk microbiome composition differs between and within countries as well as the presence of unique bacteria exclusively related to some study sites [35,89,96]. Some human milk microbiome studies have characterized their study population based on their lifestyle and community, such as rural [92], urban [35,59,97,98], rural and urban [89,99] or low socioeconomic [30,100]. These population characteristics have been proposed as factors that might potentially affect the human milk microbiota composition [35,59,89,92,97]. A recent study by Lackey et al. [89] aimed to elucidate if a “core” microbiome in human milk exists in mothers of different countries. The sample included 413 mothers and their infants from 8 countries: Ethiopia, Gambia, United States, Ghana, Kenya, Peru, US and Sweden [89]. Studies have most frequently been conducted in Spain (15 studies) [19,21,35,38,50,53,63,64,89,97,124,125,127,129,143].

Fig. 3. Geographical distribution of breast milk microbiome studies Fifty-seven studies into the breast milk microbiome and factors that influence its composition grouped by geographical region. Thirty-two studies were conducted in Europe [12,17,19–22,26,35,38,50,53,54,60,63–65,76,89,97,103,124–135], 11 studies in Asia [31,35,36,59,62,97,99,104,108,136,137], 11 studies in North America [23,24,30,33,52,56,77,89,105,138,139], 6 studies in Africa [35,55,89,92,97,140] and 5 studies in Latin-America [89,98,100,141,142]. Five multi-country studies included: Germany and Austria [26], China, Finland, South Africa and Spain [35,97], China and Taiwan, [31], and Ethiopia, Gambia, Ghana, Kenya, Peru, US and Sweden [89]. Studies have most frequently been conducted in Spain (15 studies) [19,21,35,38,50,53,63,64,89,97,124,125,127,129,143]
within countries. On the other hand, some countries showed unique bacteria. For example, only milk collected in rural Ethiopia contained Acidothermus, Demequina, Flaviflexus and Pediococcus, milk from urban Gambia uniquely contained Chroococcidiopsis and Isoptericolar and milk from urban Ghana uniquely contained Akkermansia and Butyricoccus. Importantly, none of the developed countries included in the study reported unique bacteria. The authors interpreted these results as the confirmation of the existence of a small “core” microbiome among all countries consisting of Staphylococcus and Streptococcus since they were found in 98.7 and 97.7% of all samples respectively, which aligns to previous conclusions [86]. However, while reporting that a “core” microbiome might exist across different populations, Lackey et al. [89] concluded that geographical location was not the only factor influencing the structure and diversity of microbiota in human milk. They emphasized that the existence of additional factors such as antibiotic use, infant age, parity, infant sex and exclusive breastfeeding status may also play an understudied role in milk microbiome.

The variance observed between countries could be related to disparities between rural and urban populations or socioeconomic status, characteristics previously proposed as factors that might potentially affect the microbiota composition [45,99]. However, evidence is limited because the majority of published studies characterizing microbial communities have been conducted in developed countries and urban settings (Fig. 3). The few studies available in rural communities have reported that human milk composition differs in these populations and suggested this could be due to their agrarian lifestyles’ activities. Meehan et al. [92] compared foragers with horticulturalist women in the Central African Republic, and observed that even though both groups spent considerable time in proximity to each other, milk microbial communities varied significantly within populations and between ethnic groups. Vaidya et al. [99] concluded that human milk microbiota of rural Indian women was more diverse than the milk microbiota of urban women; at phyla level women from rural communities had more species from Firmicutes while human milk from urban women had more Proteobacteria. Lackey et al. [89] noted differences in the milk microbiota of mothers from developed countries and two developing countries (Ethiopia and Gambia), which were also sub-divided into rural and urban populations. Milk from rural-Ethiopia differed from the other populations and was characterized by a relatively high abundance of Rhizobium and Achromobacter; intermediate abundances of Streptococcus and Staphylococcus; and very little Cutibacterium/Propionibacterium, Dyella, and Rothia. However, the methodology used for collection and processing of the milk in Ethiopia differed from other populations, which calls into question that the uniqueness of the Ethiopian results relates to the rural aspect of the community.

There is only one study that has described its population as a low socioeconomic community. Dave et al. [30] reported an uncommonly high abundance of Streptococcus and therefore a reduced diversity when compared to other populations, suggesting that differences might be related either to ethnicity, socioeconomic status or other factors [17] related to culture including dietary patterns, rituals and customs particular to certain region or geographical location that could affect the interaction of the mother and infant with their environment.

6.3. Environmental factors

The potential for the environment to modify the human milk microbiome has not been widely researched. Few studies have discussed the source of the bacteria in human milk and have commonly focused on identifying taxa potentially originating from oral, skin or gut habitats [17,37]. Togo et al. [101] analysed where each species identified in human milk studies (820 species from 242 articles, 38 countries, 11,124 women and 15,489 samples) were originally isolated. The study found that only 40% of breast milk bacteria were first isolated from human tissue (including gut, respiratory tract, oral cavity, urinary tract, skin, vagina, milk) while the other 60% was first observed in association with the environment, plants, animals and food. From this 60%, environment associated bacteria were the most prevalent with 34% [101]. This same study recognized that only one species was initially associated with milk, suggesting extensive interaction between the microbiome of humans and their environment. These observations highlight the potential influence of the environment in shaping the breast milk microbiome, which to-date has most often been perceived as contaminants rather than being normally present in the breast milk [57,102].

The presence of soil and water associated bacteria has consistently been observed in breast milk studies. These include Acinetobacter [35,59,103–105], Bradyrhizobiaceae [52], Novosphingobium [106], Pseudomonas [23,24,52,59,104], Ralstonia [23,33,52,99], Sphingobium [106,107], Sphingomonas [20,30,31,52,53,56,108], Stenotrophomonas [56], and Xanthomonadaceae [30,56]. Bacteria commonly found in the environment and also found in breast milk samples have the potential to simply be contamination, particularly as contaminants from PCR reagents as suggested by Ruiz et al. [109]: Douglas et al. [58] has provided evidence that, for at least some of these species, their presence in human milk may be genuine.

Soil bacteria observed in breast milk microbiota have been related to the maternal diet, seasonality, the environment and occupation, such as horticulturalists [92] or hunter-gatherers [61,110,111], rather than as a product of cross-contamination during the analysis [56,57,59]. On the other hand, soil microbes can differ enormously from region to region. There are only a few species that can be found in all soils, while there are numerous rare species that only occur in particular soils or geographical areas [112]. Therefore, generalisations among soil bacteria are difficult to do and further research in milk microbiome that aims to study the source of bacteria should include soil analysis.

Soil is considered to harbour one of the most diverse microbial populations, with several thousand of species often observed in samples. This large microbial diversity in soil results in diverse functional ecology, which includes primary productivity and nutrient cycling such as increased nutrient use efficiency and uptake, which may improve plant resilience and resistance against stressors [61]. For instance, bacteria identified in soil can produce more than 50 antibiotics to protect plants from pathogenic bacteria and rhizobia can associate with plant hosts like alfalfa, soybeans and clover to help provide the plants with nutrients [113]. As soil is part of the habitat of humans providing space for living, recreation and food production [114], it is probable that the soil contributes to the human microbiome due to its close contact as opposed to similar provision of functional diversity. It has been observed that hunter-gatherers have a gut microbiome with a higher species richness than that of humans consuming westernized food or from an urbanized society [61,99,110,111,115]. While these observations could be related to genetics, diet and their unique environments [116], the findings highlight the potential for largely unexplored links between agricultural practices, soil transmitted parasites and protozoa and human health [117].

6.4. Benefits of environmental bacteria

A milk microbiome enriched in diversity and associated functionality, in contrast to assuming certain observed species are de facto contamination, could be explored as potentially conferring some benefit to mother or infant. For instance, Chan et al. [118] have speculated that Sphingobium yanoikuyae found in nipple aspirate fluid might activate a pathway that could inhibit cancer pro-

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gression. A human microbiome enriched by soil bacteria could be beneficial through functions well established in soil communities, such as suppression of soil-borne pathogens, exposure to immunoregulation-inducing soil microorganisms, immune tolerance and increase of microbiota diversity [116]. The influence of bacteria associated with soils and water-types have been somewhat explored in the gut microbiome but research in the breast milk microbiome is still scarce. Blum et al. [61] linked the soil microbiome and the human intestinal microbiome as “superorganisms” which, by close contact (soil, faeces and food), can replenish each other as inoculants and provide beneficial microorganisms which could positively impact human health. It is also speculated that urbanization and industrialization of agriculture may have decreased the richness of an overlapping of soil and human microbiota [61]. Additionally, there is evidence that microbes from diverse habitats like soil can colonize the germ-free gut [119] and contribute commensal microbes which enrich gut microbiota diversity, which can reduce inflammatory disease risk, reduce asthma and improve child health [116].

Although the previous studies linked the benefits of environmental bacteria specifically to the gut microbiome, it is plausible that similar relationships exist with the breast milk microbiome. Further research is essential to explore the relationship of breastfeeding practices, such as the introduction of water, beverages and food, as a factor for introducing soil and water bacteria into breast milk microbiome as microbial diversity might influence neonatal gut colonisation, impact the maturation of the immune system, suppress pathogenic bacteria such as Staphylococcus aureus and therefore prevent maternal and neonatal infections as well as increase breast milk production.

7. Conclusion

Human milk is the first source of nutrients and immunity that the infant receives, supplying microbes to the newborn infant during a critical period of growth and development. Despite this important role for human development and health, there is scarce evidence of how some factors, such as maternal age and diet, geographical area and environment, might influence milk microbiota composition. Contemporary high-resolution 16S rRNA gene amplification and shotgun metagenomics are powerful approaches capable of more accurate genus and species-level microbiome assessment. These tools should be used alongside controls for contamination to study the source of environmental bacteria in breast milk and whether they originate from breastfeeding practices, maternal diet or environmental proximity. Additionally, expansion of human milk research to developing countries and rural areas represents low-hanging fruit for important discovery, as the vast majority of available evidence is in developed countries and urban areas. While the study of the breast milk microbiome has faced diverse challenges, there are extensive new strategies and opportunities to advance our understanding and promote future interventions in maternal and infant health.

CRediT authorship contribution statement

Lilian Lopez Leyva: Conceptualization, Writing - original draft. Nicholas J.B. Breereton: Conceptualization, Writing - original draft. Kristine G. Koski: Conceptualization, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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