REVIEW

Culturomics as a tool to better understand the human milk microbiota and host–microbiota interactions

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Abstract

For almost a century, it has been accepted that human milk contains viable microbial cells. However, for a considerable amount of this period, it was believed that they were the result of exogenous contamination, primarily from the skin or non-sterile handling. Early work using culture-dependent methods, supported by molecular profiling, however, identified the presence of lactic acid bacteria from an endogenous origin. This provided evidence that the human milk microbiota consisted of microorganisms that were not found solely on the skin surface and therefore could not result from contamination. Through the advent of next-generation sequencing, the field of microbiota research has caused a paradigm shift away from a typical focus on the presence of pathogenic microorganisms in human milk. This had led to a broad appreciation that the human milk microbiota consists of several hundred species of non-pathogenic commensal microbes – with many anaerobic microbial taxons being found only in the gastrointestinal tract outside of human milk. Nevertheless, as our appreciation of the complexity and diversity of the human milk microbiota has improved, many questions relating to the functional basis of host–microbiota interactions in the newborn infant's gastrointestinal tract remain outstanding. To address these, mechanistic studies will be required in which the utilisation of isolated microorganisms will be essential. As such, a return to culture-dependent methods in the new paradigm of culturomics will be required. In this review, we bring together the current understanding of the human milk microbiota and how culturomics could play a fundamental role in furthering our understanding.

The human milk microbiota

Human infants are born with relatively immature central nervous, immune, and intestinal systems and as such require a high parental care input (Plantadosi & Kidd 2016). Lactation evolved as a fundamental adaptation that is unique as a reproductive strategy for mammals (Oftedal 2012, Fernandez et al. 2013). Human milk is a highly complex biofluid, which contains thousands of bioactive molecules acting either individually or in synchrony to drive and programme the normal development of each organ system (Andreas et al. 2015, Bode 2015, Lewandowski et al. 2016, Blesa et al. 2019). The World Health Organisation currently recommends that infants receive an exclusive human milk diet for the first 6 months of life, with continued feeding alongside the introduction...
of solids for at least 2 years. The evolutionary pressures that drove species-specific milk composition resulted in enhanced immunity and antimicrobial function, with nutrition a relatively recent secondary function. It is, therefore, unsurprising that the normal duration of breastfeeding by mothers living in cultures untouched by Western lifestyle is at least 2 years, and in societies unaffected by Westernisation continues until children reach immunological maturity (Dettwyler 2001, Goldman 2002).

In addition to essential nutrients, immune factors, bioactive components, and hormones, the transferred microbiota is now being realised as one of the vital components of human milk. Emerging evidence suggests that human milk contains a dynamic microbiota, which helps infants to colonise the gut microbiome development, trains and matures the immune system and prevents colonisation by potential pathogens (Fernandez et al. 2013, Ruiz et al. 2019). For almost a century, it has been accepted that human milk contains viable microbial cells (Dudgeon & Jewesbury 1924). However, human milk was viewed as being sterile in healthy individuals with no infection, such as mastitis, and other microorganisms were introduced because of exogenous contamination. This created a disease-centric view of human milk microorganisms. The presence of any kind of microbe in human milk was traditionally considered as either a potential cause of some diseases, such as mastitis or a potential threat to infants’ health due to the potential transfer of pathogenic microorganisms. Early work using culture-dependent methods, supported by molecular profiling, however, identified the presence of lactic acid bacteria from an endogenous origin (Martin et al. 2003). This provided evidence that the human milk microbiota consisted of microorganisms that were not found solely on the skin surface, and therefore could not result from contamination. This is supported further by work showing that probiotic supplements taken orally during lactation can later be isolated and identified from samples of human milk (Treven et al. 2015, De Andrés et al. 2018). Through the advent of next-generation sequencing, the field of microbiome research has caused a paradigm shift away from a typical focus on the presence of pathogenic microorganisms in human milk. This had led to a broad appreciation that the human milk microbiota consists of several hundred species of non-pathogenic commensal microbes, with many anaerobic microbial taxons being found only in the gastrointestinal tract outside of the human milk microbiota (Fitzstevens et al. 2017).

The taxonomic domains of human milk microbiota

In the past decade, research on the human milk microbiota has focussed on the use of culture-independent sequencing approaches. These include amplicon-based sequencing approaches whereby a marker gene, such as the 16S RNA gene, is targeted and metagenomic sequencing whereby all the double-stranded DNA of a sample is sequenced. Although studies employing these methods have generated much insight into the diversity of the human milk microbiota, they are limited in not allowing physical isolation of microorganisms which prevents further investigation.

Bacteria

Human milk bacteria concentration in studies ranges from $10^4$ to $10^9$ cells/mL when using aseptically collected samples analysed through quantitative polymerase chain reaction (qPCR) (Collado et al. 2009, Boix-Amoros et al. 2016, Sakwinska et al. 2016, Consales et al. 2022), while culture-dependent methods show bacterial loads ranging from around $10^2$ to $10^6$ colony-forming units/mL (Jimenez et al. 2015, Damaceno et al. 2017, Togo et al. 2019c, Zimmermann & Curtis 2020). Previous systematic analysis has shown that a total of 820 bacterial species have been identified from human milk and breast tissue, including 554 only from human milk and 422 only from breast tissue. The 554 species detected in human milk belonged to 13 phyla, 24 classes, 52 orders, 92 families, and 178 genera (Togo et al. 2019c). Firmicutes and Proteobacteria have been reported as the most predominant phyla in the human milk, followed by Actinobacteria and Bacteroidetes (Fig. 1). Some phyla (Cyanobacteria, Deferrribacterota,
Fusobacteria, etc.) have also been identified with much lower relative abundance. At the genus level, commonly identified genera include *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Cutibacterium*, *Lactobacillus*, *Lactococcus*, and *Bifidobacterium*. However, compared to the dominant *Staphylococcus* and *Streptococcus*, the presence of *Lactobacillus* and *Bifidobacterium* appears to be more variable among individuals and sample collection and analysis methods (Table 1).

**Yeast and filamentous fungi**

Research on the human milk mycobiota has been neglected compared to bacteria, despite the likely parallel role that they have with bacteria in infants' gastrointestinal tract colonization during early life (Cui *et al*. 2013, Ward *et al*. 2017, Moossavi *et al*. 2020). This likely results from the traditional costs of sequencing-based approaches and the difficulty of overlap between marker genes for both fungi and the human host. With shotgun sequencing-based metagenomics, many studies have shown that fungal DNA can be detected in human milk samples when it is specifically looked for. The median fungal load which has been reported ranges from 2.5 to 3.5 × 10^4 cells/mL by qPCR. The common/dominant genera have been shown as *Malassezia*, *Davidiella*, *Candida*, *Saccharomyces*, *Basidiomycota*, and *Ascomycota* through a combination of culture-dependent and culture-independent amplicon sequencing of the 28S or 18S or 5.8S rRNA gene (Boix-Amoros *et al*. 2017, 2019, Moossavi *et al*. 2020). Mycobiota compositions have been associated with numerous factors including geographic location and mode of delivery. Studies have shown that fungal populations may be differentially located in the body sites of mother and child, and this can be associated with specific health factors (Ward *et al*. 2017). For example, *Malassezia*-associated DNA was found in all human milk samples from healthy women but not in those women suffering from mastitis, which may indicate a potential role for *Malassezia* in mastitis. Similarly, *Candida* spp. has been shown to play a potential role in nipple and breast pain in lactating women (Amit *et al*. 2013, Jimenez *et al*. 2015). Recent research has shown potential bacterial–fungus interactions in human milk. Bacterial and fungal richness is strongly positively correlated (Moossavi *et al*. 2020), whilst no correlation was found between bacterial and fungal load (Boix-Amoros *et al*. 2017). A co-exclusion relationship between the most abundant fungi (*Candida*) and core bacterial genera has also been shown, but a correlation was found between bacterial load and the relative abundance of *Malassezia* (Boix-Amoros *et al*. 2017, Moossavi *et al*. 2020). In our opinion, future research should characterise the metabolic and functional relationships of bacteria and fungi as well as their interaction effects on mothers’ and infants’ health outcomes.

**Viruses and bacteriophages**

In recent years, a growing number of studies have demonstrated that viruses are capable of transmission to infants through human milk and that these play a particularly significant role in shaping the infants' gastrointestinal microbiome and immune maturation (Pannaraj *et al*. 2018, Dinleyici *et al*. 2021). It has been reported that the virome of human milk is dominated by bacteriophages, followed by viruses affecting eukaryotic cells. The common bacteriophages in human milk are Podoviridae, Myoviridae, and Siphoviridae. These bacteriophages can affect bacterial ecology through their lytic and lysogenic cycles and are considered to have potentially beneficial functions (Richardson *et al*. 2016, Pannaraj *et al*. 2018, Mohandas & Pannaraj 2020, Dinleyici *et al*. 2021, Maqsood *et al*. 2021). To date, research on the human milk virome is still limited, particularly when compared to bacterial and fungal microorganisms. Research methods for virome analysis are largely limited to sequencing-based approaches due to the difficulty of viral culture, and these are typically biased towards DNA viruses.

**Archaea**

Originally considered bacteria, but now considered a distinct domain of life, archaea in human microbiota studies have received minimal attention (Woese *et al*. 1978). Archaea were initially discovered in extreme environments, such as hot springs and salt lakes. More recent research has found archaea in various non-extreme environments including marine and freshwater environments, soils, and the gastrointestinal tracts of humans and animals. They participate in recycling and utilizing metabolic products including carbon, hydrogen, nitrogen, phosphorus, and sulphur. Archaea were traditionally grouped into methanogens, extreme halophiles (haloarchaea), and thermoacidophiles (Cavicchioli 2010, Dridi *et al*. 2011). To date, there have been very few studies that have shown only occasional
### Table 1

The varied identified bacteria from the human milk with different sample collection and analysis methods.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study size</th>
<th>Sample collection method</th>
<th>Transport temperature</th>
<th>Analysis method</th>
<th>Method</th>
<th>Identified genus or species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jiménez et al. 2008</td>
<td>36</td>
<td>Aseptic method</td>
<td>4°C</td>
<td>Culture and PCR</td>
<td>16S RRNA sequencing and qPCR</td>
<td>Staphylococcus epidermidis, Enterococcus, Lactobacillus aciduregens, Lactobacillus paracasei, Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium breve, Staphylococcus lugdunensis, Streptococcus salivarius, Staphylococcus epidermidis, Propionibacterium acnes, Lactobacillus paracasei, L. gasseri, L. casei, L. plantarum, L. reuteri, L. salivarius, L. mesenteroides, Bifidobacterium breve, B. breve, B. adolescentis, B. bifidum</td>
</tr>
<tr>
<td>Solís et al. 2009</td>
<td>23</td>
<td>Aseptic method</td>
<td>4°C</td>
<td>Culture and PCR</td>
<td>16S RRNA sequencing and 16S rRNA sequencing</td>
<td>Bifidobacterium breve, Bifidobacterium adolescentis, Bifidobacterium bifidum</td>
</tr>
<tr>
<td>Martin et al. 2012</td>
<td>20</td>
<td>Aseptic method</td>
<td>NM</td>
<td>Culture and PCR</td>
<td>qRTi-PCR</td>
<td>Staphylococcus epidermidis, Staphylococcus hominis, Lactobacillus (L. casei, L. gasseri, L. aciduregens, L. plantarum, L. salivarius, L. reuteri, L. gasseri), Bifidobacterium breve, B. breve, B. longum, C. krampferi, C. krampferi, Actinomyces neuii, Bifidobacterium breve in a few samples</td>
</tr>
<tr>
<td>Solís et al. 2010</td>
<td>20</td>
<td>Aseptic method</td>
<td>NM</td>
<td>Culture and PCR</td>
<td>16S rRNA gene sequencing and specific qPCR</td>
<td>Dominant: Streptococcus and Staphylococcus in both collection methods. Acinetobacter sp. is also common in non-aseptic method. Bifidobacterial and Lactobacilli abundance in a few samples</td>
</tr>
<tr>
<td>Non-aseptic, n</td>
<td>60</td>
<td>Aseptic collection</td>
<td>On ice</td>
<td>Culture and MALDI-TOF MS</td>
<td></td>
<td>Staphylococcus (S. epidermidis, S. hominis, S. caprae, S. lugdunensis, S. aureus, S. hominis, S. saccharolyticus, S. capitis), Lactobacillus gasseri, Streptococcus (S. salivarius, S. mitis, S. paraeorrhinosus, S. agalactiae), Corynebacterium (C. tuberculostearicum, C. krampferi), Actinomyces neuii, Staphylococcus, Streptococcus, Entroccoccus, Lactobacillus, Propionibacterium (0.2%), Actinomyces (1.52%)</td>
</tr>
<tr>
<td>Schwab et al. 2019</td>
<td>21</td>
<td>Aseptic method</td>
<td>Anaerobic jar</td>
<td>Culture and 16S RNA sequencing and DNA sequencing</td>
<td>16S rRNA gene sequencing</td>
<td>Streptococcus (S. salivarius, S. mitis, S. paraeorrhinosus, S. agalactiae), Enterococcus (E. faecalis, E. faecium), Lactobacillus (L. casei, L. gasseri, L. aciduregens, L. plantarum, L. reuteri, L. salivarius, L. mesenteroides), Bifidobacterium breve, B. breve (15/19 samples), Lactobacillus (L. casei, L. gasseri, L. aciduregens, L. plantarum, L. reuteri, L. salivarius, L. mesenteroides), Enterococcus (E. faecalis, E. faecium)</td>
</tr>
<tr>
<td>Ding et al. 2019</td>
<td>6481</td>
<td>Aseptic collection</td>
<td>Icebox</td>
<td>Culture and 16S RNA sequencing</td>
<td>16S rRNA gene sequencing</td>
<td>Staphylococcus, Streptococcus, Enterococcus, Lactobacillus, Bifidobacterium breve, B. breve, B. longum, C. krampferi, Actinomyces neuii, Staphylococcus, Streptococcus, Entroccoccus, Lactobacillus, Propionibacterium (0.2%), Actinomyces (1.52%)</td>
</tr>
<tr>
<td>Liu et al. 2020</td>
<td>40</td>
<td>Aseptic method</td>
<td>4°C</td>
<td>Culture and 16S RNA sequencing</td>
<td>16S rRNA gene sequencing</td>
<td>Staphylococcus, Streptococcus, Enterococcus, Lactobacillus, Bifidobacterium breve, B. breve, B. longum, C. krampferi, Actinomyces neuii, Staphylococcus, Streptococcus, Entroccoccus, Lactobacillus, Propionibacterium (0.2%), Actinomyces (1.52%)</td>
</tr>
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</table>

NM, not mentioned.
detection of archaea in human milk, even though archaea are ubiquitous in human body sites, including the human gastrointestinal tract, oral cavity, and skin (Horz & Conrads 2011, Lurie-Weinberger & Gophna 2015, Moissl-Eichinger et al. 2017, Chaudhary et al. 2018). Methanogenic archaea species (Methanobrevibacter smithii and Methanobrevibacter oralis) had been isolated from human colostrum and milk by using co-culture with Bacteroides thetaiotaomicron and supplemented with antioxidants. The findings indicated that around 30% of the colostrum and/or milk samples from mothers tested positive for M. smithii when examined using culture and qPCR (Togo et al. 2019b). Using pyrosequencing-based amplicon sequencing, Haloarcula marismortui was identified from 80% of human milk samples from healthy women and none from women with symptoms of lactational mastitis. This may indicate a potential role for some archaeal species in diseases associated with lactation (Jimenez et al. 2015). Understanding of the abundance and diversity of human milk-associated archaea, as well as their metabolism and functions, is limited. Many culture-independent methods using 16S rRNA gene sequencing are unable to detect archaeal sequences due to insufficient primer coverage. As methodologies improve, the abundance and relative importance of archaea in the human milk microbiota will be better realised.

**Methodologies for exploring the human milk microbiota**

The experimental methodology chosen plays a pivotal role in uncovering the human milk microbiota. Similarly, the formulation of the research question also has an impact since the composition of the human milk microbiota can be affected by various endogenous and exogenous factors. As a relatively small and emerging research field, there are no specific methodology standardisations that are routinely followed. For example, many researchers use an aseptic technique to collect samples, which usually involves sterilisation of the nipple and areola area. Others follow a breastfeeding routine protocol to maximally mimic the real-life human milk which would feed a nursling. Typically, this method is referred to as a ‘breastfeeding-associated microbiota’ (Sakwinska et al. 2016). Furthermore, the techniques for culturing, isolating, and detecting microbes accurately in human milk are also a challenge because the biofluid typically contains low biomass of human milk microbiota. To date, common analytical techniques, Fig. 2, for human milk microbiota analysis include culture-dependent (which physically isolates microorganisms on a solid growth media) and culture-independent methods (which typically rely on DNA sequencing-based approaches to
taxonomically identify microorganisms, but which do not physically isolate them).

**Culture-dependent**

The first method to characterise microbes involved conventional culture-dependent techniques, but due to its narrow incubation conditions, the method could only culture and isolate microorganisms that were dominant, non-fastidious, and rapidly growing (Ruiz et al. 2019, Fernandez et al. 2020). This is one of the reasons why culture-based methods can successfully culture and identify only a much smaller diversity of microbial taxa than molecular techniques. For instance, Soto et al. [39] using culture-dependent methods isolated *Lactobacilli* and *Bifidobacteria* from 27 (40.91%) and 7 (10.61%) of 66 human milk samples, respectively, while these bacteria were identified by culture-independent techniques in 108/160 samples (67.50%) and 41/160 samples (25.62%), respectively. In a recent systematic analysis, of a total of 554 identified microbial species from human milk, only 210 species (38%) have been successfully isolated by culture-dependent methods, while molecular methods detected 487 (88%) (Togo et al. 2019c). In addition, culture-dependent methods have a limitation on viable but non-culturable (VBNC) microorganisms (Stinson et al. 2021). When microbes are exposed to extreme stressors, they may be forced to enter into a VBNC state. VBNC bacteria are not dead but cannot grow on routine culture media, which would normally be suitable for their growth. Since the discovery of the VBNC of *Escherichia coli* in 1982, many bacterial species have been found to exist in a VBNC state. If VBNC microbes exist in human milk, this may lead to an underestimation of its total microbes and a misunderstanding of microbial metabolism and functionality. Therefore, the uncovering of viability and cultivability of microbes in human milk is extremely critical to analyse the presence of VBNC microbes (Li et al. 2014).

**Culture-independent**

With the development of multiple ‘-omics’ molecular approaches at the DNA, RNA, protein, and metabolite levels, numerous studies have characterised the human milk microbiota in recent years. This has boosted our understanding of microbial complexity and functionality in human milk. However, these culture-independent methods cannot differentiate viable or non-viable microbes and can even amplify DNA/RNA from dead cells which can give a misleading view of prevalence and activity. Nevertheless, recent research has used membrane dyes or fluorescent probes to bind to DNA before viability assessment by fluorescence microscopy or qPCR. These are membrane-impermeable dyes, so they can only enter cells with compromised membranes, after which they bind to DNA with high affinity and cause staining. The mechanisms of some widely used membrane dyes are remarkably similar. Additionally, different DNA extraction methods or pre-treatments before DNA extraction have been shown to introduce biases in the accuracy and coverage of DNA-based molecular techniques. For example, Gram-positive bacteria of human milk (such as *Staphylococcus*, *Lactobacillus*, and *Bifidobacterium*) have a thick and strong peptidoglycan layer in their cell wall which are difficult to lyse during DNA extraction and in an extraction would likely be underestimated when compared to Gram-negative species, which are easier to lyse. In our view, however, the major drawbacks of culture-independent methods are the lack of physical isolation of microorganisms.

**Culturomics: a return to fundamentals**

Microbial culture methods have been fundamental in understanding the abundance and function of microorganisms in a range of environments. Nevertheless, many of these methods have been strongly led by requirements in diagnostic and food/product safety microbiology. As such, it is believed that the majority of microorganisms from nature cannot be cultivated by currently used routine culture methods in the laboratory. To counter this, in 2002 Lewis and Epstein (Kaeberlein et al. 2022) proposed to move microbial cultivation from the laboratory back to their natural habitats under the assumption that this would be the best environment for them to grow. Therefore, they designed a diffusion chamber for in situ cultivation of microorganisms in their natural marine sediment environment. After 4 weeks of incubation, 70% of 438 species were isolated exclusively by using the diffusion chamber, compared with 23% using conventional methods, and just 7% from both approaches. This indicated that the use of microorganisms’ natural environment, through the diffusion chamber, provides a more substantive growth environment than traditional laboratory-based methods (Bollmann et al. 2007). Subsequently, they optimised this low-throughput single diffusion chamber into a high-throughput platform which has been named an ‘isolation chip’ (ichip) (Nichols et al.
2010, Berdy et al. 2017). Although the ichip method was successfully employed for environmental samples, such as marine sediment and soil, its main limitation is in its application to human or animal systems. This results from the requirement to return the ichip to the original habitat for incubation and access to essential growth nutrients (Liu et al. 2021, Polrot et al. 2022). Nevertheless, the ichip has inspired a renewed focus on improving microbial growth conditions for fastidious microorganisms by better reflecting the environment from which they were sampled. For microbiologists with a focus on human microbiota, the resulting development of microbial culturomics opens many new research opportunities.

Culturomics is a microbial culturing technique that uses high-throughput culture conditions for culture and isolation and rapid and effective matrix-assisted laser desorption mass spectrometry (MALDI-ToF) for identification, followed by DNA sequencing-based approaches if further taxonomic identification, such as strain level, is required. The field of culturomics has largely been enabled by the low-cost and quick turnaround time for results allowed by MALDI-ToF mass spectrometry. Culturomics workflows aim to develop comprehensive conditions that maximally simulate, reproduce, or mimic the entirety of the natural conditions that microorganisms collected from a sample would have been exposed to (Lagier et al. 2015, Togo et al. 2019c). In the last 10 years, culturomics in the human gut microbiota has successfully demonstrated that a variety of previously unculturable bacteria can be isolated if appropriate culture media and conditions are provided. Therefore, culturomics offers a valuable and distinct perspective for further research on the human gut ecosystem, compared to metagenomics, as these isolated microorganisms can be utilised in in vitro work to establish mechanisms of host–microbiota interactions. In 2012, the Lagier research group applied the first culturomics research workflow in the human gastrointestinal tract by designing 212 different culture conditions and using MALDI-ToF mass spectrometry and 16S rRNA gene sequencing for identifying isolates. They identified 174 previously undescribed species in the human gastrointestinal tract (Lagier et al. 2012). The same group further identified 11 new bacterial species from faecal samples by using culturomics with 88 culture conditions (Pfeiderer et al. 2013). Within the gastrointestinal tract samples, culturomics workflows have also been used to isolate novel fungal species (Hamad et al. 2017). The main weakness of culturomics is arguably the workload of culture conditions, requiring substantial resources to be invested in optimisation, standardisation, and miniaturisation.

Additionally, there is the unavoidable risk that culturomics methods may miss VBNC microorganisms (Lagier et al. 2015, 2016). Recent work has shown that the use of selective pre-incubation media allows faster and cheaper workflows (Naud et al. 2020). Combining pre-incubation with enrichments and using multiple time points of sampling has also been shown to increase the diversity of isolated species (Chang et al. 2019).

To date, culturomics has mostly been applied to samples from the human gastrointestinal tract, which contains 10^{10}–10^{12} bacteria per gram of faeces. Human milk, however, has a much lower microbial load of 10^2–10^6 bacteria per ml (Lagier et al. 2015, 2018). This likely necessitates a requirement for selective and/or enrichment culture steps to maximise the diversity of isolated microorganisms. To the best of our knowledge, there are very few instances of culturomics workflows being applied to human milk samples within the published literature. The Lagier group used their developed culturomics workflow to successfully isolate novel species (Lactimicrobium massiliense, Anaerolactibacter massiliensis, Galactobacillus timonensis, and Lactomassilus timonensis) from the human milk of healthy mothers from Mali, which was as part of their project about deciphering the bacterial diversity of colostrum and breast milk (Togo et al. 2018, 2019a). Nevertheless, this single example provides good support for the role that culturomics workflows can have in improving our understanding of the human milk microbiota.

**What questions in human milk research could be answered through culturomics?**

Microbiological analysis of human milk has been around since the 1970s, but for a long time, it was only performed in cases of infections, so the presence of non-pathogenic bacteria was not well understood. However, in recent years, there has been a growing interest in studying the human milk microbiome using more sophisticated culture-dependent and -independent techniques, as well as various ‘omic approaches’. It is important to move beyond the studying of individual components of human milk and instead recognise its importance as a whole within which these components exist. A comprehensive approach to studying human milk needs to be considered, involving the chronobiology and systems biology of human milk microbiome origination and feeding, as well as environmental factors that impact the microbiome and function. It is imperative to incorporate standardised...
and optimal sampling and quantification methods when studying human milk composition and its association with infant growth. As human milk composition changes extensively according to the stage of lactation (Lyons et al. 2022), making it essential to use standardised collection methods and longitudinal study designs to obtain a comparable result. A comprehensive approach from individual recruiting and sample collection to analyse methods should be focussed on to understand the human milk microbial complete taxonomical, metabolic, and functional profiles. However, this comprehensive approach will be pointless if the microbes cannot be cultured, isolated, and identified in vitro. Previous evidence in the human gastrointestinal microbiome has shown that culturomics workflow is much more efficient than the traditional culture method in cultivating and isolating microbes.

As our understanding progresses, it is clear that human milk is more than just food for infants. It provides immune protection and helps in the development of an infant’s gastrointestinal tract microbiome. Fortunately, there is evidence to suggest that interventions targeting the gut microbiota in early life can promote long-term health. For example, breastfeeding (Van den Elsen et al. 2019) and early exposure to diverse types of foods (Armet et al. 2022) have been shown to have a positive impact on gut microbiota development and reduce the risk of obesity and other chronic diseases. It has also been linked to metabolism and even behaviour (Grey et al. 2013). Culturomics techniques can enhance our ability to analyse microbiota–metabolite interactions in vitro by providing a broader and more detailed understanding of the microbial communities in human milk. By maximising the isolation of diverse microbes from this complex environment, researchers can gain insight into the metabolic interplay between microorganisms and metabolites, contributing to our knowledge of human milk composition and its impact on the health.

The presence of microbial communities in human milk has been confirmed, which are proposed to originate from various sources, including the mother’s gut, neonates’ oral cavity, and mother’s skin (Fernandez et al. 2013, Pannaraj et al. 2017). However, the exact species that link human milk to the gut microbiota and other sources are still unclear. Some studies have found that certain bacteria, such as Bifidobacterium and Lactobacillus (Granger et al. 2021), are more abundant in breastfed infants than formula-fed infants, suggesting these may be important species for the development of the infant gut microbiota. Other studies have identified a wide range of bacteria in breast milk, including Staphylococcus, Streptococcus (Li et al. 2017), and various species of Proteobacteria and Bacteroidetes (Toscano et al. 2017), but their role in colonising the infant’s gut is not well understood. Sequencing technologies can help us pinpoint the presence and absence of these bacteria but to better understand the functionality, culturomics methodology allows us to isolate bacteria, and research their physical, metabolic, and biological characteristics in vitro.

One of the most studied components of human milk is human milk oligosaccharides (HMOs). It is a prebiotic suggested to promote the growth and activity of beneficial bacteria in the infant’s gut microbiome. It is considered a critical component of human milk, with diverse functions that go beyond just serving as ‘food for bugs’ (Bode 2012). HMOs are a complex structure of different oligosaccharides, each with a unique structure and potential function. While some HMOs have been studied in detail and their effects on the gut microbiome and the host have been characterised, most HMOs have not been fully investigated. Microorganisms from the human milk can be maximally isolated by culturomics and could be grown with different HMOs to further understand their role.

In addition to living microorganisms, human milk has also been shown to contain phages that can be transmitted to the infant’s gut (Duranti et al. 2017). These phages may have a role in defending against pathogenic bacteria infections, particularly since infants have underdeveloped adaptive immune responses. The ‘kill the winner’ theory, where phages suppress the most dominant bacterial species, allowing other species to thrive, has been observed in the infant gut microbiome (Thingstad 2000). This dynamic relationship between bacteria and phages may explain the highly unstable nature of the early infant gut microbiome. Maternal transmission of phages through human milk is therefore likely a crucial factor in shaping the composition and function of infant gut microbiome. The interaction between phages and bacteria in human milk is still an active research. While phages are generally considered safe for human use, it is possible that they could interact with human milk cells and bacteria unexpectedly, especially if they are used to treat infections in the human body. More research is needed to understand the potential risks and benefits of the presence of phage and phage–bacteria interaction in human milk. We believe culturomics has the potential to significantly increase our capacity to manipulate and isolate gut phages by culturing and isolating bacterial hosts and identifying host–phage pairings in vitro. Therefore, by combining
viral tagging (Důnková et al. 2019) with culturomics, researchers can identify and isolate unknown phages, which can be purified and studied in the laboratory. This has the potential to revolutionise our understanding of the gut virome and its interaction with the bacterial microbiome.

It is believed that the composition of human milk microbiota may play a role in mammary health. Studies have suggested that maternal obesity and gestational diabetes mellitus (GDM) are associated with alterations in the gut microbiota composition and these changes may potentially impact the human milk microbiota composition, such as decreased levels of Bifidobacteria, which are beneficial bacteria for infant health (Crusell et al. 2018). Furthermore, maternal obesity has been associated with changes in human milk microbiota composition, specifically a decrease in Bifidobacterium and an increase in Staphylococcus (Koren et al. 2012). These findings highlight the potential impact of maternal health on the composition of human milk microbiota and the importance of promoting maternal health to ensure optimal breastfeeding outcomes. Understanding the underlying mechanism of specific diseases, especially the involvement of bacteria (upregulation and downregulation) through a targeted culturomics approach, can help the researchers to modulate the gut bacteria of mothers, which in turn would impact the mother’s milk and infant’s gut colonisation.

Concluding remarks

In recent years, there has been a paradigm shift in our understanding of the human milk microbiota. By moving from a pathogen-focussed assessment to one that is more holistic of the benefits associated with commensal microorganisms which are present, studies of the human milk microbiota can move towards greater mechanistic understanding of the complex host–microbiota interactions. As the importance of the human milk microbiota which is passed to the nursing is increasingly understood, mechanistic studies will be essential. To achieve these, however, microorganisms will need to be studied and manipulated in vitro and in vivo. As the field has understandably been reliant on culture-independent sequencing, this is not currently achievable. As this review has highlighted, we believe that culturomics workflows will become a key enabler of future human milk microbiota work. By isolating microorganisms, across the domains of life, from human milk, researchers will have greater scope to understand the complexity of human milk as a living biofluid and how this can impact maternal and infant health and development.

Declaration of interest

SKC and RL declare no conflicts of interest in relation to this work. SJSC is Associate Editor of Microbiota and Host and serves on the journal’s Editorial Board.

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Author contribution statement

SKC conceived the scope of the paper, reviewed literature, and wrote and edited the manuscript. RL conceived the scope of the paper, reviewed literature, and wrote and edited the manuscript. SJSC conceived the scope of the paper, reviewed literature, and wrote and edited the manuscript. SKC and RL contributed equally to the manuscript. All authors approved the manuscript prior to submission.

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