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Research Letter

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Capturing the diversity of the human milk microbiota through culture-enriched molecular profiling: a feasibility study

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One sentence summary: Culture-enriched molecular profiling of milk microbiota and the effect of storage and milk fractions.

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ABSTRACT

Previous human milk studies have confirmed the existence of a highly diverse bacterial community using culture-independent and targeted culture-dependent techniques. However, culture-enriched molecular profiling of milk microbiota has not been done. Additionally, the impact of storage conditions and milk fractionation on microbiota composition is not understood. In this feasibility study, we optimized and applied culture-enriched molecular profiling to study culturable milk microbiota in eight milk samples collected from mothers of infants admitted to a neonatal intensive care unit. Fresh samples were immediately plated or stored at −80°C for 2 weeks (short-term frozen). Long-term samples were stored at −20°C for >6 months. Samples were cultured using 10 different culture media and incubated both aerobically and anaerobically. We successfully isolated major milk bacteria, including *Streptococcus*, *Staphylococcus* and *Bifidobacterium*, from fresh milk samples, but were unable to culture any bacteria from the long-term frozen samples.

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Short-term freezing shifted the composition of viable milk bacteria from the original composition in fresh samples. Nevertheless, the inter-individual variability of milk microbiota composition was observed even after short-term storage. There was no major difference in the overall milk microbiota composition between milk fractions in this feasibility study. This is among the first studies on culture-enriched molecular profiling of the milk microbiota demonstrating the effect of storage and fractionation on milk microbiota composition.

Keywords: culture-enriched molecular profiling; milk microbiome; microbiota; storage; milk fractions; bacterial viability; culture media; freezing

INTRODUCTION

Culture-independent (DNA sequencing-based) approaches have recovered a high diversity of bacteria in human milk with a high degree of inter-individual variability (Ruiz, Garcia-Carral and Rodriguez 2019). Although culture-independent approaches have considerably expanded our understanding of the human microbiome (Hiergeist *et al.* 2015), inherent limitations of the technology such as lack of access to the bacterial isolates from the samples have resulted in a renewed interest in employing culture-based approaches (Lagier *et al.* 2015; Bilen *et al.* 2018). Human milk has been historically studied using targeted culture approaches to identify potential pathogens or probiotics (Martin *et al.* 2003; Perez *et al.* 2007; Abrahamsson *et al.* 2009; Solis *et al.* 2010; Arboleya *et al.* 2011; Murphy *et al.* 2017). Targeted culture-based methods have been employed to assess diversity of milk bacteria (Jost *et al.* 2013); however, culture-enriched molecular profiling has not been employed to investigate the diversity of the milk microbiota (Ruiz, Garcia-Carral and Rodriguez 2019).

Culture-enriched molecular profiling refers to the method of using multiple culture conditions followed by 16S ribosomal RNA (rRNA) gene sequencing (Lau *et al.* 2016). Using this approach on stool samples, 95% of the abundant taxa (>0.1% relative abundance in culture-independent direct sequencing) were successfully isolated (Lau *et al.* 2016) highlighting the culturability of many of human microbiome members. A similar approach was successfully applied to low-biomass sputum samples from cystic fibrosis patients (Sibley *et al.* 2011). These and other studies (Lau *et al.* 2016) show that culture- and sequencing-based approaches are complementary with some non-overlapping taxa identified by both methods (Wilson and Blitchington 1996; Lagier *et al.* 2012), highlighting the advantage of this dual strategy in studying the human microbiome.

To our knowledge, all sequencing-based human milk microbiota studies to date have been conducted on the cell/casein pellets following removal of the milk fat, which can interfere with polymerase chain reaction (PCR) amplification (Wilson 1997). However, it has previously been shown that different milk fractions have different profiles of bacteria (Anderson 1909; Boix-Amoros, Collado and Mira 2016; Lima, Bicalho and Bicalho 2018; Sun *et al.* 2019). In a dairy milk study, the microbiome profiles of whole milk, fat and pellet were separately assessed, and some unique bacterial families were identified in each fraction, although alpha and beta diversities were not different (Lima, Bicalho and Bicalho 2018), while minimal differences were observed in human sequencing-based diversity and profiles of milk microbiota with or without lipid removal (Ojo-Okunola *et al.* 2020). Additionally, storage temperature and duration are important factors that can influence milk components, including bacteria (Handa *et al.* 2014; Ahrabi *et al.* 2016; Peters, McArthur and Munn 2016). Suboptimal storage of bovine and human milk could lead to shifts in the milk microbiota composition or viability (Ahrabi *et al.* 2016; Peters, McArthur and Munn 2016). Therefore, the objectives of this study were to capture the diversity of milk microbiota using culture-enriched molecular profiling while comparing the effect of storage and different milk fractions on the microbiota composition.

MATERIALS AND METHODS

Sample collection

Milk samples were collected from mothers of premature infants admitted to the Neonatal Intensive Care Unit (NICU), McMaster Children's Hospital, McMaster University. Milk samples were not handled or stored aseptically or anaerobically. Anonymous long-term frozen milk samples ($N = 3$) previously collected at ~60 days postpartum were obtained from the Department of Pediatrics, McMaster University. These samples were stored for >6 months at −20°C prior to analysis. In addition, fresh anonymous leftover milk samples ($N = 5$) were collected from the NICU. Fresh milk samples were collected by pump expression, fortified with Enfamil® human milk fortifier powder (Mead Johnson Nutrition, Chicago IL, USA) per standard NICU protocol and stored for up to 24 h in the fridge before feeding the infant. Subsequently, the leftover samples were transferred to the lab for immediate processing or to a −80°C freezer for 2 weeks (Fig. 1A). The study was considered quality assurance activity and thus exempt from ethics approval by the Ethics Review Board at McMaster University.

Culturing of milk samples

One hundred microliters of milk sample was diluted in 900 μL of brain heart infusion (BHI) broth (BD, Sparks, MD, USA) with 0.05% L-cysteine hydrochloride hydrate as previously described (Lau *et al.* 2016). One hundred microliters of 10^{-1} dilution was plated on pre-reduced 100-mm agar plates made from different culture media (Table 1). The choice of culture conditions was modified from Lau *et al.* (2016) based on anticipated bacterial profile of the human milk according to our previously published 16S rRNA gene sequencing-based study (Moossavi *et al.* 2019).

Sample culture was performed both aerobically and anaerobically as previously described (Lau *et al.* 2016). Anaerobic culture was performed at 37°C for 5 days in an anaerobic chamber and aerobic culture was incubated at 37°C in 5% CO_2 for 3 days. In a subset of fresh samples ($N = 2$), the fat and pellet fractions were separated by centrifugation at 12 500 × *g* for 10 min. Each fraction was resuspended in 1 mL of BHI, plated and incubated aerobically as described. For the comparison of fresh and short-term frozen samples, one aliquot was immediately cultured and another was frozen at −80°C for 2 weeks after the initial plating ($N = 3$). One hundred microliters of the short-term frozen samples were cultured both aerobically and anaerobically as described above. After incubation, the colonies on each plate were pooled and collected by adding 1 mL BHI broth and scraping the surface of the plate with a cell scraper. The harvested

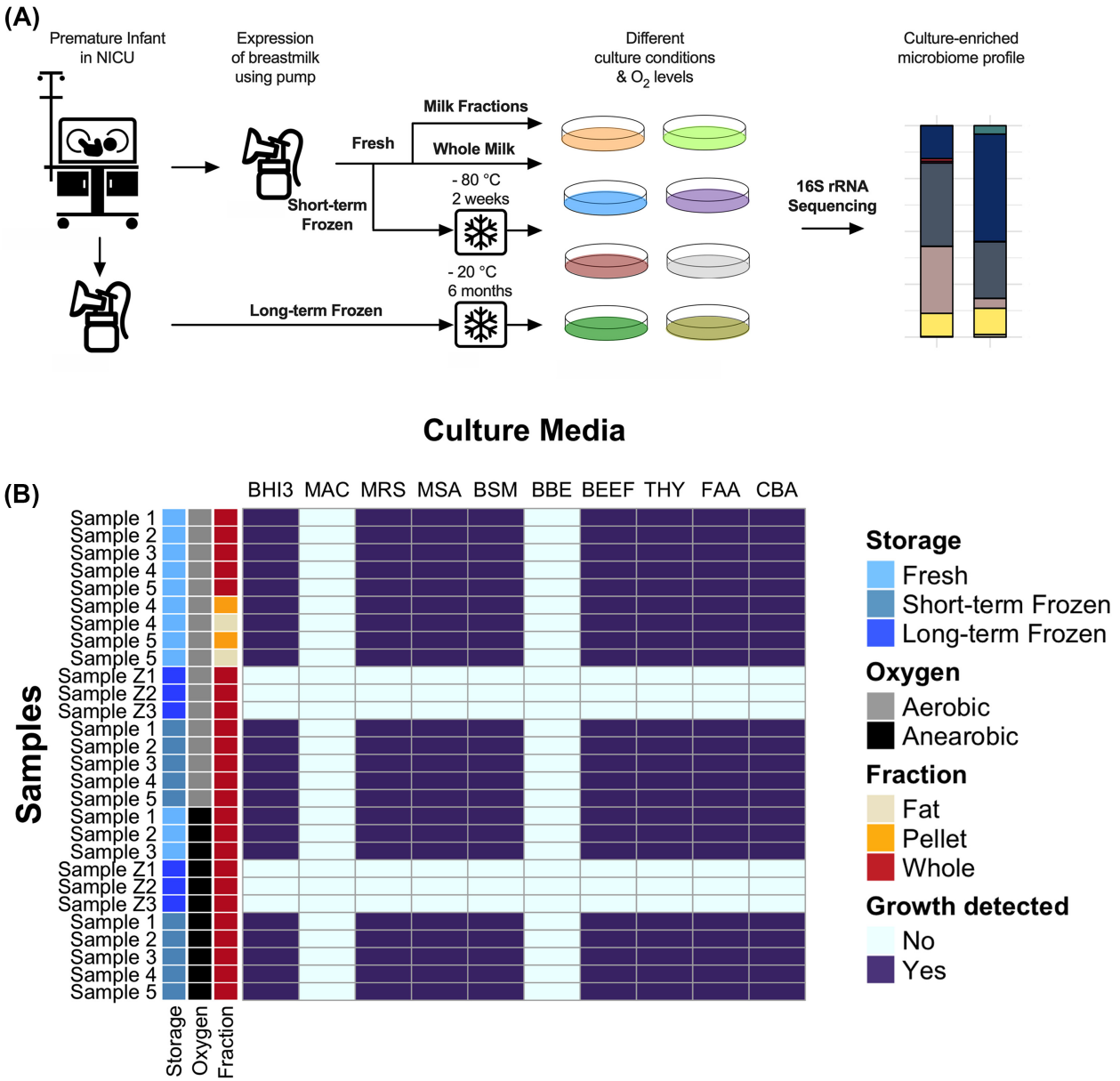

Figure 1. Schematic overview of milk microbiota culture-enriched molecular profiling in human milk samples. **(A)** Milk samples were obtained from the NICU, McMaster Children's Hospital, and were cultured on 10 different media under both aerobic and anaerobic conditions. **(B)** Summary of bacterial isolation results (present or absent) for all the samples studied. BHI, brain heart infusion; BEEF, cooked meat agar; BBE, *Bacteroides* bile esculin agar; BSM, *Bifidobacterium* selective media; CBA, Columbia blood agar; FAA, fastidious anaerobe agar; MAC, MacConkey agar; MRS, de Man Rogosa Sharpe agar; MSA, mannitol salt agar; THY, tryptic soy agar. Long-term frozen milk samples are designated as sample Z.

colonies were frozen in 50% glycerol at −80°C and 300 μL was used for DNA extraction and sequencing.

DNA extraction and 16S rRNA gene sequencing

DNA extraction and purification were performed as previously described (Whelan *et al.* 2014). Briefly, 500 μL of plate pools was mechanically and enzymatically homogenized. Subsequently, DNA was extracted from the supernatant with phenol:chloroform:isoamyl alcohol (25:24:1; Sigma, St Louis, MO, USA) and further purified using DNA Clean and Concentrator-25 columns (Zymo, Irvine, CA, USA). Isolated DNA was stored at −20°C until further processing. Samples were sequenced following amplification of V3 hypervariable region of the 16S rRNA gene with F341/R518 primers (Bartram *et al.* 2011) on a MiSeq platform (Illumina, San Diego, CA, USA) at the McMaster Genome Facility (Hamilton, ON, Canada). Sterile DNA-free water was used as negative controls in sequencing library preparation.

Microbial data pre-processing and reagent contaminant removal

Demultiplexed forward and reverse sequencing reads were imported in R (R Core Team 2017). Primers and their reverse complements were identified using Biostrings package (Pagès *et al.* 2019) and subsequently removed from the sequencing data using cutadapt (Martin 2011). Overlapping paired-end reads were processed using DADA2 (Callahan *et al.* 2016) in R. Unique

Table 1. Culture media used in the culture-enriched molecular profiling of human milk samples.

Culture media	Company	Supplement	Target bacterial groups
Brain heart infusion (BHI) 3	BD[a]	0.5 g/L L-cysteine 10 mg/L hemin 1 mg/L vitamin K	Anaerobes
Bacteroides bile esculin agar (BBE)[b]	NA	None	*Bacteroides*
Cooked meat agar (BEEF)	Fluka[c]	None	Strict anaerobes
Bifidobacterium selective media (BSM)	Fluka	None	*Bifidobacterium*
Columbia blood agar (CBA)	BD	5% Sheep blood	Non-selective
Fastidious anaerobe agar (FAA)	Neogen[d]	None	Fastidious anaerobe
MacConkey agar (MAC)	BD	None	Gram-negative
de Man Rogosa Sharpe agar (MRS)	BD	None	*Lactobacillus*
Mannitol salt agar (MSA)	BD	None	Gram-positive; *Staphylococcus*
Tryptic soy agar (THY)	BD	5 g/L yeast extract	Fastidious bacteria

[a]Sparks, MD, USA.
[b]Prepared according to the protocol in Atlas (2004).
[c]St Louis, MO, USA.
[d]Lansing, MI, USA.

amplicon sequence variants (ASVs) were assigned taxonomy and aligned to the SILVA reference database version 132 at 99% sequence similarity (Yilmaz *et al*. 2014). Data analysis was conducted in R using the Phyloseq package (McMurdie and Holmes 2013). ASVs with zero abundance across the samples and non-bacterial taxa (belonging to phylum Cyanobacteria, family of mitochondria and class of chloroplast) were removed. The data underlying this article are available in Sequencing Read Archive of NCBI at https://www.ncbi.nlm.nih.gov/sra, and can be accessed with accession number PRJNA613840.

Data analysis

Data analysis and visualization were performed in R. Alpha diversity was assessed by the observed ASVs (richness) and was reported per sample combining the results from all culture conditions. Association of richness with factors of interest was assessed by paired *t*-test.

RESULTS

Bacteria were isolated from fresh and short-term frozen but not long-term frozen milk samples

We could not grow any bacterial isolates from long-term frozen samples plated with (10^{-1}) or without dilution on any of the culture conditions. In contrast, fresh and short-term frozen samples yielded detectable colonies on all culture media incubated aerobically and anaerobically except *Bacteroides* bile esculin and MacConkey (enriching Gram-negative bacteria) (Fig. 1B). In two samples where milk fractions were studied separately, we detected viable bacteria from both pellet and the fat layer (Fig. 1).

Overall, from 183 plate pools (generated from 8 milk samples, each cultured under 20 conditions), we obtained a mean (SD) of 10 730 (12 969) and median (IQR) of 5172 (2–56 467) high-quality sequencing reads per plate pool, compared with 263 reads in the negative control. Overall, 251 unique ASVs were detected. There was no difference in the sequencing depth of fresh vs short-term frozen samples (mean ± SD: 10 694 ± 12 831 vs 11 908 ± 13 402, $P > 0.05$), while plate pools of long-term frozen samples without visible colonies had depth of sequencing of just 385 ± 763, which was not different from the negative control. Therefore, long-term frozen samples were considered negative for bacteria growth and subsequently excluded from downstream analysis.

Overall milk microbiota richness is not impacted by short-term freezing and milk fraction

Overall, 196 ± 81 (mean ± SD) unique ASVs were identified from fresh samples. Bacterial richness was similar in fresh and frozen samples after combining both the aerobic and anaerobic results (Fig. 2A). On average, the number of detected ASVs was slightly lower after short-term freezing (Fig. 2B) and was slightly higher in anaerobic vs aerobic incubation, albeit not statistically significant (Fig. 2B). On the individual level, different patterns of change were observed based on freezing and oxygen level. For example, while richness was decreased after short-term freezing in samples 3–5 in aerobic incubation, there was a sharp increase in the number of bacteria isolated from sample 1 (Fig. 2C). In addition, while short-term freezing increased the number of isolated aerobic bacteria in samples 1 and 2, it reduced the number of anaerobic bacterial isolates (Fig. 2C). This opposing direction of change has resulted in an overall consistent bacterial richness in fresh vs short-term frozen samples (Fig. 2A). In the two samples where milk fractions were analyzed separately, there were no prominent differences in richness between the pellet and fat layer (Fig. 2D).

The composition of cultured milk microbiota is influenced by short-term freezing and contains both aerobic and anaerobic bacteria

Overall, 18 families constituted 219 out of 251 detected ASVs. These families constituted the majority (99%) of the sequencing reads per sample. High inter-individual variability of milk microbiota was observed demonstrating different dominant bacterial families in each sample. For example, samples 1–3 were, respectively, dominated by *Bifidobacteriaceae*, *Moraxellaceae* and Clostridiales family XI (Fig. 3A).

The composition of culturable milk bacteria was influenced by short-term storage (Fig. 3). The compositional shifts were observed on an individual level. For example, *Propionibacteriaceae* were present and their relative abundance in the cultured plate pools was lower after short-term freezing in all samples, while

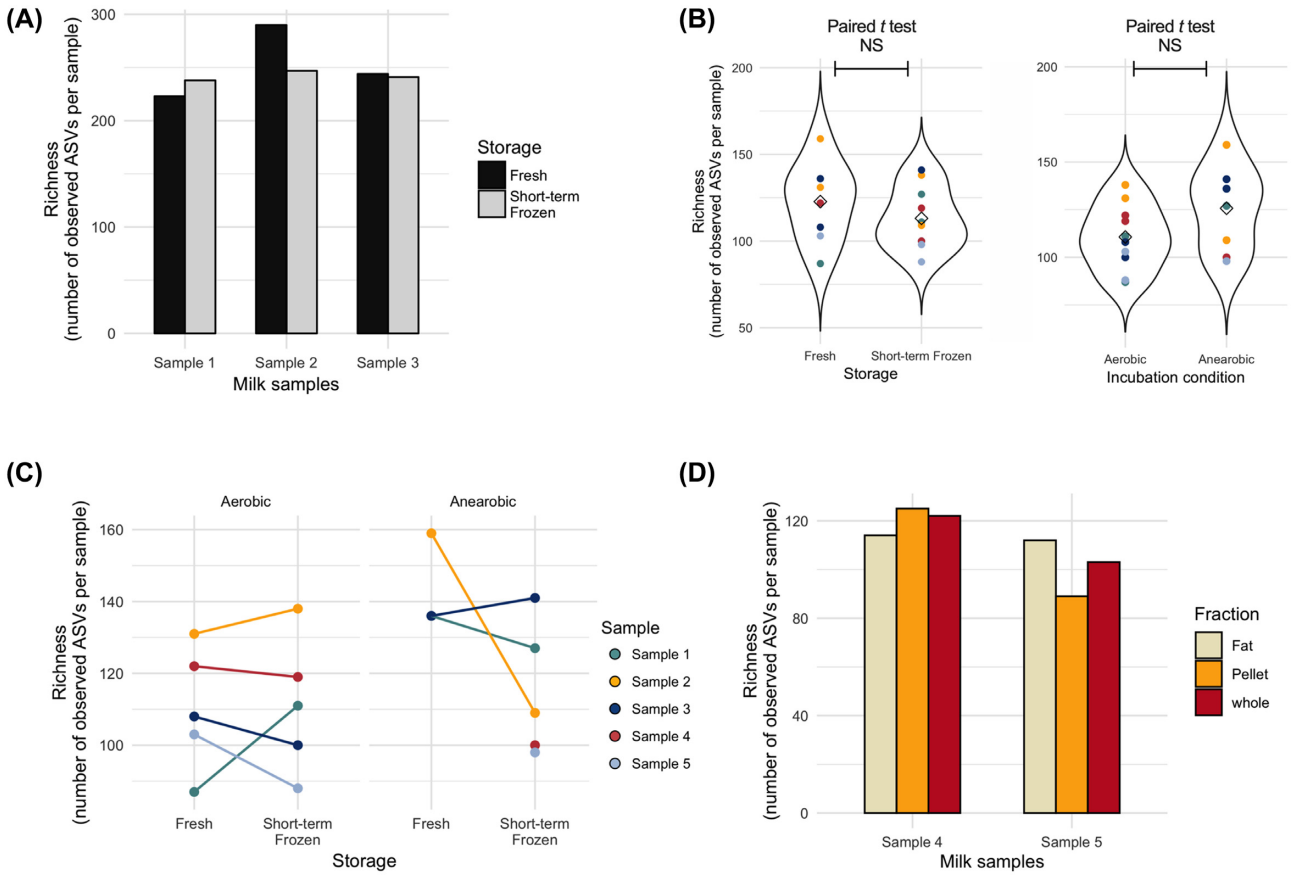

Figure 2. Comparison of the number of detected ASVs in five human milk samples using culture-enriched molecular profiling. **(A)** Overall comparison of isolated bacterial richness in samples with both aerobic and anaerobic culture ($N = 3$). Fresh samples 4 and 5 were cultured only aerobically. **(B)** Individual-level comparison of isolated bacterial richness in all samples in aerobic and anaerobic culture ($N = 5$). **(C)** Comparison of average richness in fresh vs short-term frozen samples ($N = 5$) and aerobic vs anaerobic culture conditions; paired *t*-test analysis *P*-value is reported. **(D)** Bacterial richness of different milk fractions ($N = 2$). NS, not significant.

the relative abundances of *Bifidobacteriaceae*, *Moraxellaceae* and Clostridiales family XI increased in samples 1–3, respectively (Fig. 3A and B). In contrast, the relative abundance of *Staphylococcaceae* was relatively unchanged after short-term freezing.

Overall, *Staphylococcaceae*, Clostridiales family XI and *Corynebacteriaceae* were the most frequently isolated bacterial families in our feasibility study with *Staphylococcaceae*, Clostridiales family XI and *Streptococcaceae* being the most abundant bacteria (Fig. 3B), in accordance with previous studies (Ward *et al.* 2013; Jimenez *et al.* 2015; Sam Ma *et al.* 2015; Kumar *et al.* 2016; Sakwinska *et al.* 2016; Drago *et al.* 2017; Li *et al.* 2017; Murphy *et al.* 2017; Patel *et al.* 2017; Williams *et al.* 2017). Of note, each milk sample was dominated by one or two isolates regardless of the storage conditions (Fig. 3B). Anaerobic bacteria belonging to *Bifidobacteriaceae*, Clostridiales family XI, *Propionibacteriaceae* and *Veillonellaceae* were more commonly isolated in anaerobic culture, while aerobic bacteria belonging to *Corynebacteriaceae* and *Moraxellaceae* were more abundant in aerobic culture (Fig. 3B), confirming that expressed human milk contains both aerobic and anaerobic bacteria.

Milk microbiota composition is similar in different milk fractions

Almost all sequencing-based milk microbiome studies are conducted on the pellet following the removal of the milk fat through centrifugation (Ward *et al.* 2013; Jimenez *et al.* 2015; Sam Ma *et al.* 2015; Kumar *et al.* 2016; Sakwinska *et al.* 2016; Drago *et al.* 2017; Li *et al.* 2017; Murphy *et al.* 2017; Patel *et al.* 2017; Williams *et al.* 2017). Acknowledging the small sample size ($N = 2$), we observed that milk fractions preserve the inter-individual variability of milk microbiota composition while demonstrating slight variations in the relative abundances of the dominant bacterial families (Fig. 4).

DISCUSSION

To our knowledge, this is among the first studies to apply culture-enriched molecular profiling to profile human milk microbiota. In our feasibility study, we successfully isolated major milk bacteria, including *Streptococcus*, *Staphylococcus* and *Bifidobacterium*, from fresh milk samples in accordance with a recently published report isolating *Streptococcus* spp. and *Enterobacteriaceae* using anaerobic culture (Jost *et al.* 2013; Schwab *et al.* 2019). Bacteria were isolated from different milk fractions (pellet and fat layer) and there was no major difference in overall richness or composition between the fractions. Short-term freezing (2 weeks at −80 °C) shifted the composition of viable milk bacteria from the original composition in fresh samples, although the inter-individual variability of milk microbiota composition was largely conserved. We could not isolate any bacteria from the long-term frozen samples (>6 months at −20°C) in this feasibility study.

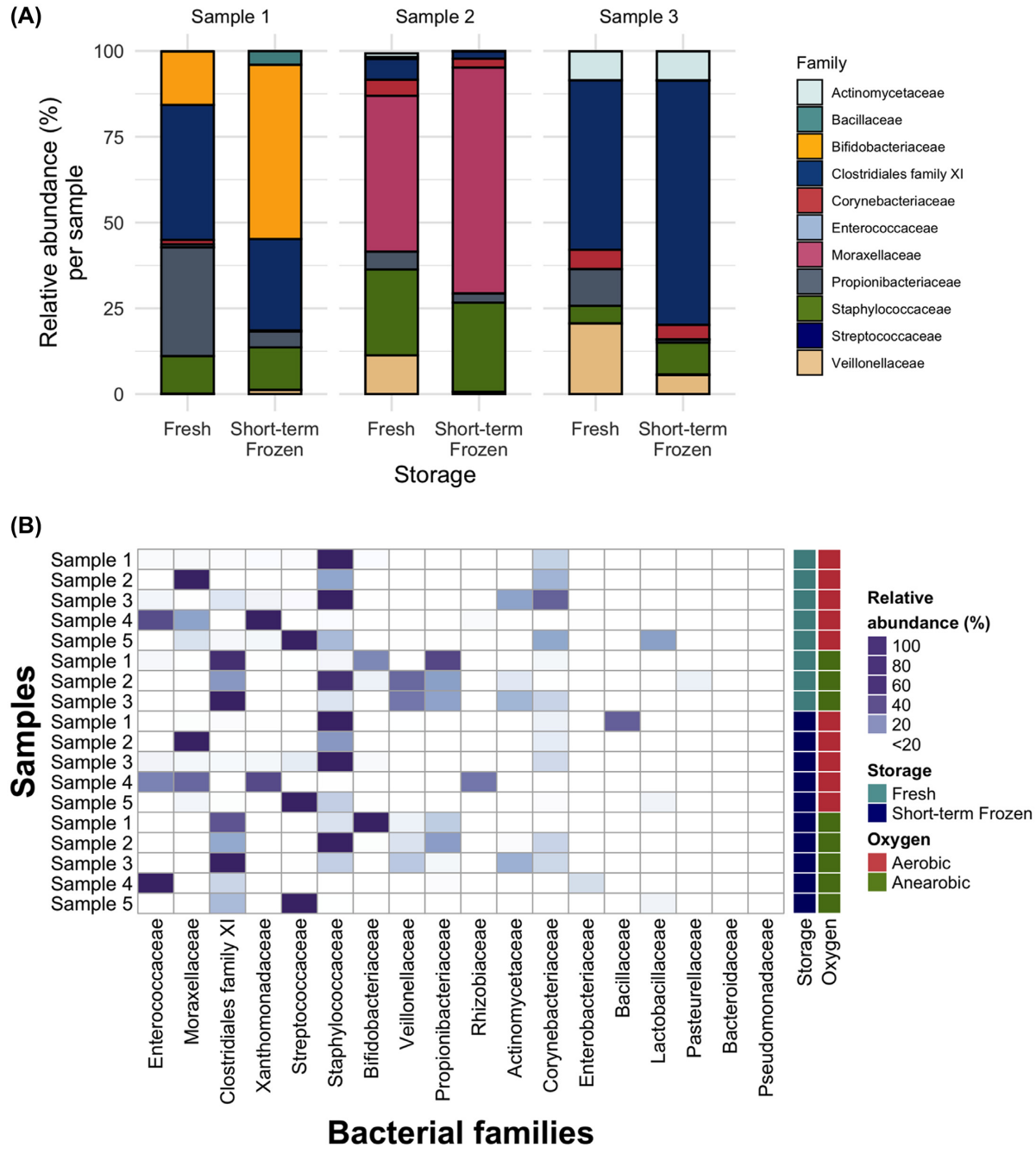

Figure 3. Milk microbiota composition in human milk samples using culture-enriched molecular profiling at the family level. **(A)** Comparison of milk microbiota composition of individual samples in fresh vs short-term frozen samples, $N = 3$). **(B)** Isolated bacterial composition in different growth conditions.

Since milk bacteria are not highly abundant and most previous studies have relied on culture-independent methods, it is unclear whether milk bacteria are viable and/or metabolically active. Our current results build on prior studies that have successfully cultured milk bacteria (either through targeted or high-throughput approaches) (Heikkila and Saris 2003; Schanler *et al.* 2011; Gonzalez *et al.* 2013; Jost *et al.* 2013; Tušar *et al.* 2014; Kozak *et al.* 2015; Damaceno *et al.* 2017; Jimenez *et al.* 2017; Schwab *et al.* 2019) and confirm that at least a subset of bacteria present in expressed milk is viable. However, it is less clear whether milk bacteria are actively proliferating in the milk within the mammary gland. Additionally, the growth dynamics of milk bacteria following pumping and storage is not clear and cannot be addressed in our current study.

The impact of storage on the viability and composition of milk microbiota is not understood. We and others have successfully profiled milk microbiota in long-term frozen samples by sequencing (Moossavi *et al.* 2019). However, in our small culture-dependent study of pumped milk, we could not isolate viable bacteria from long-term frozen samples. In a previous targeted culture-dependent study, ~5% of fresh milk samples did not yield any bacterial isolates (Schanler *et al.* 2011) suggesting that some milk samples may not contain detectable bacteria or have low bacterial cell concentrations in accordance with other

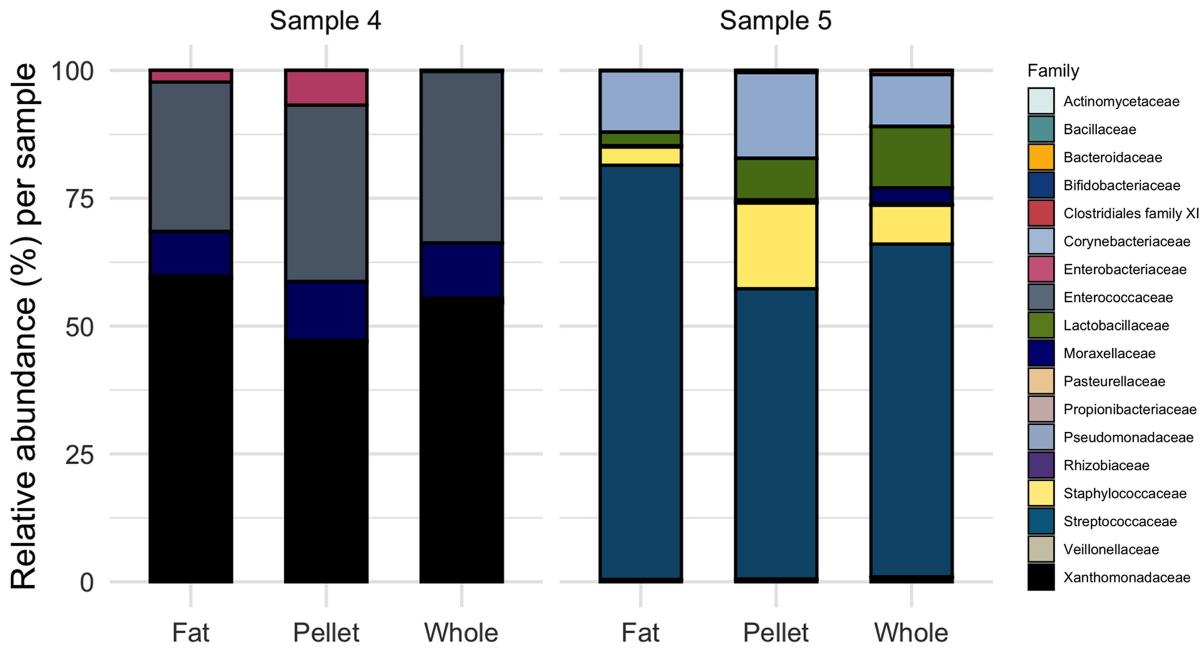

Figure 4. Milk microbiota composition in different fractions of human milk samples using culture-enriched molecular profiling in individual milk samples.

previous studies (Davidson, Poll and Roberts 1979). While we could not isolate any bacteria from samples frozen for long term at −20°C, short-term storage at −80°C did not impact the culturability of milk bacteria. In accordance, it has previously been found that total milk bacterial load declines rapidly in the first month and reaches undetectable levels after 3 months of storage at −20°C (Ahrabi *et al.* 2016). Notably, in our study, the composition of milk bacteria was influenced by the short-term storage, suggesting that some members of the milk microbiota are more vulnerable to freezing. While skim milk is frequently used as a cryoprotectant, the low density and proliferation rate of bacteria in human milk could underlie our inability to isolate bacteria from long-term frozen samples (De Paoli 2005). Additionally, it is currently not clear how the length or temperature of storage affects the culturability of milk bacteria. Researchers commonly store milk samples for months to years before analysis, especially in large longitudinal cohort studies. Therefore, the potential impact of length of storage should be further investigated and taken into account in the design of milk microbiome studies in the future. In addition, more research is needed to understand the impact of short-term freezing at −20°C, which is highly relevant to mothers who store and feed expressed milk to their infants and donor milk banking practices.

Research comparing the microbiome of different milk fractions is limited. We isolated bacteria from different milk fractions using culture-enriched molecular profiling, and found that the overall composition of the milk sample was preserved in both fractions, in accordance with recent studies, where bacterial load and composition were not different in human milk with or without fat removal (Ojo-Okunola *et al.* 2020; Rodriguez-Cruz *et al.* 2020). In a study on the microbiome profile of different milk fractions in 54 dairy milk samples, overall, 31 bacterial families (including very rare taxa) were shared between the fractions, while there were 6, 11 and 24 unique families present in fat, pellet and whole milk, respectively (Lima, Bicalho and Bicalho 2018). Despite taxonomic differences, alpha and beta diversities were not different between different fractions (Lima, Bicalho and Bicalho 2018). Milk bacteria potentially exist in both planktonic and cell-associated states. In mature milk, the majority of bacterial cells were found to be associated with human cells (Boix-Amoros, Collado and Mira 2016) suggesting that the majority of the bacteria could be pelleted following centrifugation (Anderson 1909). Previous research indicates that while most milk bacteria aggregate with the casein micelles (Hickey *et al.* 2015), certain bacteria such as *Mycobacterium avium* subsp. *paratuberculosis* and *Staphylococcus* show affinity to the fat globule (Paape and Guidry 1977; Ali-Vehmas *et al.* 1997; O'Flaherty *et al.* 2005; Herthnek *et al.* 2008; Vidanarachchi *et al.* 2015; Beaver *et al.* 2016). However, *Staphylococcus* was not differentially abundant in different fractions in our study. Further studies with larger sample size are required to explore the variability of milk microbiota composition and activity in different milk fractions.

Culture-based microbiome analysis offers several advantages over culture-independent methods. The main advantage is the ability to isolate and bank bacterial species from samples (niches) of interest. These bacterial isolates can then be used for various downstream investigations including in-depth genetic and phenotypic characterizations of the bacteria (Lebeis 2014; Almeida *et al.* 2019). There is a keen interest in exploring probiotic properties of human milk bacteria (McGuire and McGuire 2015) and our successful culture-enriched molecular profiling can potentially accelerate these efforts. Another advantage of culture-based studies is that they can complement and potentially inform the identification of potential reagent contaminants in culture-independent studies (de Goffau *et al.* 2018), further advancing the field of milk microbiota research.

Our feasibility study has several limitations, including the small number of samples studied, the non-standardized collection method, the use of different samples to assess short- and long-term storage, and the lack of information about the length of long-term storage. In addition, the samples contained non-sterile human milk fortifiers as per NICU clinical protocols. Fortifiers are commonly added to the milk consumed by infants in NICU to meet various nutritional needs (Arslanoglu *et al.* 2019). Although the impact of fortifiers on the milk microbiota composition has not been studied, the non-sterile fortifiers

could provide a source of bacteria and/or provide higher nutrient availability in the milk that could conceivably affect bacterial metabolic activity (Steele 2018). However, as the impact of powder fortifier on total milk bacteria has been previously found to be negligible (Telang *et al.* 2005), we postulate that any potential effect on the milk microbiota will also likely be minimal. Further studies are required to investigate the impact of fortifiers on the milk and infant gut microbiota composition. Culture-enriched molecular profiling could identify taxa with >0.1% relative abundance in stool (Lau *et al.* 2016); however, the limit of detection for milk microbiota is not known. Additionally, different growth patterns of bacterial species could potentially result in different relative compositions in comparison to sequencing-based microbiome profiling.

CONCLUSION AND FUTURE DIRECTION

New approaches are needed to advance knowledge about the milk microbiome (Azad *et al.* 2020). Culture-enriched molecular profiling provides an opportunity to isolate diverse members of milk microbiota, which is an important step for investigating their causal role in maternal and infant health (Moossavi and Azad 2020). These culture-dependent approaches will be essential not only for understanding the effect and importance of microbes in donor milk and mother's own milk in the NICU but also for more broadly studying the impact of pumping and storage on human milk microbiota. While we have shown the feasibility of culture-enriched molecular profiling of human milk, further research with larger sample sizes is required to uncover the role of the milk microbiome in health and disease, and potentially develop microbiome-targeted strategies for health promotion and disease prevention.

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Conflict of Interest. None declared.

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