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ORIGINAL ARTICLE

Impact of expression mode and timing of sample collection, relative to milk ejection, on human milk bacterial DNA profiles

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Keywords
breast feeding, expressed breast milk, human milk, human milk microbiome, milk ejection, mode of expression, sample collection.

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Abstract

Aim: To investigate the impact of expression mode: electric breast pump or hand expression, and timing of sample collection: pre- and post-milk ejection on human milk (HM) bacterial DNA profiles.
Methods and results: Three HM samples from the same breast were collected from 30 breastfeeding mothers: a pre-milk ejection pump-expressed sample (pre-pump), a post-milk ejection pump-expressed sample (post-pump) and a post-milk ejection hand-expressed sample (post-hand). Full-length 16S rRNA gene sequencing was used to assess milk bacterial DNA profiles. Bacterial profiles did not differ significantly based on mode of expression nor timing of sample collection. No significant differences were detected in the relative abundance of any OTUs based on expression condition (pre-pump/ post-pump and post-pump/post-hand) with univariate linear mixed-effects regression analyses (all P-values > 0·01; $\alpha = 0{\cdot}01$). Similarly, no difference in richness was observed between sample types (number of observed OTUs: post-pump/post-hand $P = 0{\cdot}13$; pre-pump/post-pump $P = 0.45$).
Conclusion: Bacterial DNA profiles of HM did not differ according to either expression method or timing of sample collection.
Significance and Impact of the Study: Hand or pump expression can be utilized to collect samples for microbiome studies. This has implications for the design of future HM microbiome studies.

Introduction

Human milk (HM) bacteria have been extensively studied using both hand-expressed and pump-expressed samples (Stinson *et al.* 2020). The reported bacterial profile of HM varies significantly between studies with respect to composition, richness and diversity (Li *et al.* 2017, Lackey *et al.* 2019, Moossavi *et al.* 2019). These variations are likely attributed to many factors, such as genetics, geography, mode of delivery, gestational age at delivery, lactation stage and maternal health (Gronlund *et al.* 2007; Cabrera-Rubio *et al.* 2012; Lewis *et al.* 2015; Kunz *et al.* 2017; Lackey *et al.* 2019; Demmelmair *et al.* 2020). Methodological differences also influence the apparent HM microbiome (Cheema *et al.* 2020; Douglas *et al.* 2020; Ojo-Okunola *et al.* 2020). However, little is known about whether mode of expression (pump *vs* hand) influences HM bacterial DNA profiles. Breast pumps rely on vacuum to remove milk from the breast, while hand expression uses positive pressure on individual milk ducts. This may increase the likelihood of pumped milk being drawn from more ducts than hand-expressed milk,

resulting in a more representative sample of the milk in the breast. Therefore, sampling method may impact bacterial DNA profiles in HM microbiome studies.

A small number of studies have reported that different modes of milk expression result in variations in the resulting bacterial composition of HM (Marin *et al.* 2009; Jimenez *et al.* 2017). Jiménez *et al.* reported that compared to hand expression, samples expressed with the mother's own pump had a higher frequency and mean bacterial counts of members of the Enterobacteriaceae family and other Gram-negative bacteria (*Pseudomonas* sp. and *Stenotrophomonas* sp.), as well as *Candida albicans*. Importantly, these microbes were also detected in swabs taken of the internal surfaces of the pumps before sampling, suggesting pump-derived contamination (Jimenez *et al.* 2017). Similarly, Marin *et al.* (2009) reported that compared to hand expression, pump-expressed HM samples contained significantly higher bacterial counts across all culture media tested, potentially reflecting pump-introduced contamination. However, these authors did not report the cleaning procedures used for the pump nor the breast prior to sampling. Therefore, it is unclear whether such differences are due to non-sterile pumping equipment-introduced contamination, or whether use of a pump can extract HM from more ducts, providing a more representative sample of the full HM bacterial profile.

In contrast, the results of one small study suggest that mode of expression does not impact the HM microbiome when sterile pumps are used. Rodriguez-Cruz *et al.* (2020) compared HM samples from eight mothers: the first sample was collected by hand expression on the first day, and a second sample was collected from the same breast by a single-use sterile pump kit on the second day. In contrast to the findings of previous studies, these authors did not find any significant differences between the bacterial DNA profiles of hand-expressed HM and pump-expressed HM. However, the fact that the breast was not cleaned prior to sample collection, and that sampling occurred over 2 days, may have confounded the results of this study.

Given the above conflicting results between studies which employed non-sterile pumps to those which employed single-use sterile pumps, and the very small sample sizes associated with these, further work is needed to investigate the influence of mode of expression on bacterial DNA profiles in HM.

Another consideration for HM microbiome study sampling protocols is the timing of sample collection. Milk ejection occurs when the myoepithelial cells that surround the breast alveoli are stimulated by oxytocin and contract, causing milk to be ejected into the milk ducts towards the nipple for removal by the infant or a breast pump (Gardner *et al.* 2015). The vacuum of a breast pump can express small volumes of milk (approximately 0–37·5 ml) prior to milk ejection (Kent *et al.* 2003). This sample type (pre-milk ejection) may represent residual HM present in the ducts which can be expressed before the milk ejection propels milk from the alveoli through the milk ducts to exit the nipple Kent *et al.* (2003). Given that the microenvironments of the milk ducts and the alveoli may vary, the residual milk expressed pre-milk ejection may vary in bacterial composition to milk ejected from the alveoli (post-milk ejection). To date, the bacterial DNA profiles generated from pre- and post-milk ejection samples have not been compared.

Given these outstanding questions relating to the method and timing of HM sampling for microbiome studies, the aim of this study was to assess the impact of expression mode (hand and electric breast pump) and timing of sample collection with respect to milk ejection on HM bacterial DNA profiles.

Materials and methods

Participants and sample collection

Human milk samples were collected from predominantly breastfeeding mothers (n = 30), 3–83 weeks postpartum, who self-reported being healthy with no nipple infection or pain. Participant characteristics are presented in Table 1. Mothers provided written informed consent for participation in the study, which was approved by the Human Research Ethics Committee at the University of Western Australia (RA/4/1/2369).

Participants elected one breast from which to donate milk samples and were asked not to breastfeed or express milk from that breast for at least 2 h prior to sample collection. The sample collection equipment was sterilized by autoclave prior to collection and participants and investigators thoroughly washed their hands with soap/

Table 1 Maternal and infant characteristics

Characteristics (n = 30)	Mean ± SD (range), or n (%)
Maternal age (years)	32·9 ± 4 (24–40)
Mode of delivery (vaginal)	20 (66·7%)
Right breast sampled	12 (40·0%)
Parity	1·7 ± 0·8 (1–3)
Male infant	17 (56·7%)
Infant birth weight (grams)	3556·1 ± 380·8 (2435–4345)
Infant gestational age (weeks)	39·5 ± 1·3 (35·1–41·4)
Infant age at HM sample collection (weeks)	23·4 ± 18·2 (3·6–83·4)

water, alcohol-free hand sanitizer and sterile saline. Gloves were worn during the collection process. To reduce contamination from skin microbiota, the nipple and areola of the expressing breast were cleaned with alcohol & chlorhexidine prep pads (70% isopropyl alcohol and 2% chlorhexidine digluconate), followed by rinsing with sterile saline solution and drying with sterile gauze swabs. Two HM samples, a pre-milk ejection pump-expressed sample (pre-pump, 1–3 ml) and a post-milk ejection pump-expressed sample (post-pump, 10 ml) were expressed using a Symphony electric breast pump (Medela AG, Baar, Switzerland) with a sterile pump kit and were collected directly into a sterile falcon tube. Immediately following this, the nipple and areola were cleaned again, and a third sample (post-milk ejection) was collected by hand-expression (post-hand) into a sterile tube. No bottles were used for collecting or transferring the samples. Samples were immediately transported to the laboratory on ice, where they were aliquoted into certified DNA-free tubes in a level two biosafety cabinet and stored at −80°C until further analysis.

DNA extraction

DNA was extracted from 1 ml of HM using the QIAGEN MagAttract Microbial DNA Isolation Kit (Qiagen, Chadstone, Australia) on the Kingfisher Flex platform following the manufacturer's instructions. Prior to DNA extraction, HM samples were centrifuged at 40 000 ***g*** for 5 min at 4°C, the supernatant and lipid fraction were removed, and the pellet was resuspended in lysis buffer. Two negative extraction controls consisting of 1 ml of sterile DNA-free water (Integrated DNA Technologies, Queenstown, Singapore) were included at the centre of the 96-well extraction plate.

16S rRNA gene amplification and barcoding

PCR amplification was used to generate PacBio sequencing-ready amplicons as previously described (Stinson *et al.* 2020). The full-length 16S rRNA gene was amplified using the primer pair 27F (5′-gcagtcgaacatgtagctgactcaggtcacAGRGTTYGATYMTGGCTCAG-3′) and 1492R (5′-tggatcacttgtgcaagcatcacatcgtagRGYTACCTTGTTACGACTT-3′), with a universal UNITAG sequence (provided in lower case) and amine block attached to the 5′ ends of each primer ($'NH_4$-C_6). The primary PCR was carried out in 20 µl reactions containing 1X UCP Multiplex PCR Master Mix (Qiagen), 0·3 µmol l^{-1} each of the forward and reverse primers, 0·5 µl each of dsDNase and DTT (ArcticZymes PCR decontamination kit), and 5 µl of template or nuclease-free water (negative template control). The PCR cycling conditions consisted of an initial heating step at 95°C for 1 min; 40 cycles of 95°C for 10 s, 52°C for 30 s and 72°C for 2 min; and a final extension step of 72°C for 7 min. Primary PCR products were purified using NucleoMag NGS magnetic beads (Macherey-Nagel, Duren, Germany), normalized to 1 ng μl^{-1}, and used as template for secondary PCR. The secondary PCR reaction was carried out in 20 µl reactions containing 1X HotStarTaq Plus Master Mix (Qiagen), 0·3 µmol l^{-1} each of the forward and reverse barcoded primers, and 2 µl of template or nuclease-free water (negative template control). The PCR cycling conditions consisted of an initial heating step at 95°C for 5 min; 20 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 2 min; and a final extension step of 72°C for 10 min. Barcoded PCR amplicons were purified using NucleoMag NGS magnetic beads and pooled in equimolar concentrations. Amplicon pools were gel purified in-house using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol. Five hundred nanogram of DNA (pooled purified amplicons) was used for library preparation for sequencing.

PacBio sequencing

Purified pools were sequenced at the Ramaciotti Centre for Genomics, NSW, Australia. SMRTbell adapters were ligated onto barcoded PCR products and Pacific Biosciences (PacBio, Menlo Park, CA, USA) single molecule real-time (SMRT) circular consensus sequencing was performed on a single SMRT cell using ver. 3.0 sequencing chemistry and a 10-h movie. Raw data were processed at the Ramaciotti Centre using PacBio SMRTLink analysis software ver. 6.0 to generate demultiplexed .fastq files.

Sequence data processing

Full-length 16S rRNA gene sequence data was processed using MOTHUR ver. 1.39.5 (Schloss *et al.* 2009) on a web-based computational workbench, Galaxy (Jalili *et al.* 2020). Demultiplexed .fastq files were converted to .fasta files, which were then length filtered (1336–1743 bp). Sequences containing homopolymers of >9 bases were removed. Sequences were aligned to the SILVA reference alignment v138 using the align.seqs command, pre-clustered using pre.cluster and chimeric sequences were subsequently removed using chimera.vsearch. Sequence taxonomy was determined using classify.seqs with the SILVA taxonomy database (v138) and a confidence threshold of 80. After classification, non-bacterial sequences were removed from the dataset using remove.lineage. Classified sequences were clustered into operational taxonomic units (OTUs) by calculating the

pairwise distances between sequences with a 0·03 similarity cut-off value using dist.seqs and grouped into OTUs using cluster. The clustered OTUs were assigned taxonomy using classify.otu. Subsampling was performed at 1303 reads based on an average Good's coverage value of 94·2%. This eliminated three samples that had fewer than 1303 sequencing reads: subject 2 post-hand (450 reads), subject 11 post-pump (790 reads) and subject 22 post-hand (254 reads). Additionally, we were unable to generate an amplicon from one sample (subject 5 post-pump). The sequencing reads from the negative extraction controls and negative PCR controls are provided in Table S1.

Richness (number of observed OTUs) and Shannon diversity was generated using mothur and analysed using a linear mixed effects model with alpha diversity as the response variable, sample as the explanatory variable and mother as the random factor in RStudio ver. 1.3.1073 (Team R, 2020). Principal co-ordinates analysis (PCoA) was conducted based on the Bray-Curtis dissimilarity measure (generated using MOTHUR) and visualised using RStudio. AMOVA analysis was performed on Bray–Curtis dissimilarity distances using MOTHUR.

To analyse whether any OTUs were differentially abundant based on mode of expression or timing of sampling, univariate linear mixed-effects regressions were fit for the OTUs with ≥0·05% relative abundance under at least one condition. We implemented a random effects modelling structure to account for the hierarchical dependencies resulting from the repeated measurements observed for each mother. Here, each OTU was a response variable with sample and mother as fixed and random explanatory variables respectively. To control for the inflated Type 1 error rate induced by multiple comparisons (as there are many regression analyses), but to still identify relationships of interest, we considered *P*-values <0·01 as significant.

Microsoft Excel was used to plot the relative abundance of bacterial genera, while RStudio v1·3.1073 was used to plot alpha diversity and PCoAs. For all tests, excluding the univariate linear mixed-effects regression analyses, *P*-values <0·05 were considered significant.

Results

Mode of expression does not influence HM bacterial profiles

The bacterial DNA profile of HM samples expressed by pump (post-pump) did not differ significantly from those expressed by hand (post-hand) (Fig. 1). Univariate linear mixed-effects regression analysis showed that no OTUs differed in their relative abundance between samples expressed by hand or pump (all *P* values >0·01). A total of 65 bacterial genera were recovered from post-pump samples and 71 from post-hand samples (Fig. 1). While most genera (52·8%) were shared between the two conditions, 18 genera were recovered from post-pump samples only and 24 from post-hand samples only. However, these genera made up a very small fraction of the overall bacterial profile (total relative abundance <0·65%). Alpha diversity analysis showed no significant differences between the two conditions (OTU richness $P = 0{\cdot}13$, Shannon diversity $P = 0{\cdot}6$) (Fig. 2a). Similarly, no significant differences were observed in beta diversity (AMOVA $P = 0{\cdot}59$) (Fig. 2b).

Timing of sample collection (with respect to milk ejection) does not influence HM bacterial profiles

The bacterial DNA profiles of HM samples expressed prior to and after milk ejection did not differ significantly in their composition (Fig. 1). No OTUs were found to be significantly differentially abundant between pre- and post-milk ejection samples following univariate linear mixed-effects analyses (all *P* values >0·01). We recovered 70 bacterial genera from pre-pump samples and 65 from post-pump samples (Fig. 1), with 47 shared genera (53·4% of total shared genera). Twenty-three genera were detected in pre-pump samples only, while 18 were detected in post-pump samples only. However, these genera were of very low abundance (total relative abundance <0·46%). Alpha diversity analysis did not show any significant differences between the two conditions (OTU richness $P = 0{\cdot}45$, Shannon diversity $P = 0{\cdot}53$) (Fig. 3a). Beta diversity also did not show any difference between two sample types (AMOVA $P = 0{\cdot}97$) (Fig. 3b).

Discussion

These results suggest that neither mode of expression nor collection of HM before or after milk ejection substantially impact downstream bacterial DNA profiles. This provides reassurance that hand- or sterile pump-expressed samples can be used for HM microbiome studies.

The finding that mode of expression does not have a significant impact on the resulting HM bacterial DNA profile is in contrast with previous studies which suggested that pump-expressed HM samples differed from hand-expressed samples in terms of bacterial composition and diversity (Marin *et al.* 2009; Jimenez *et al.* 2017). However, these studies compared hand-expressed samples to samples expressed with non-sterile breast pumps. The use of a non-sterile pump may introduce contamination into the sample. Indeed, when Rodriguez-Cruz *et al.* compared HM samples expressed using sterile single-use pumps with hand-expressed samples, no significant

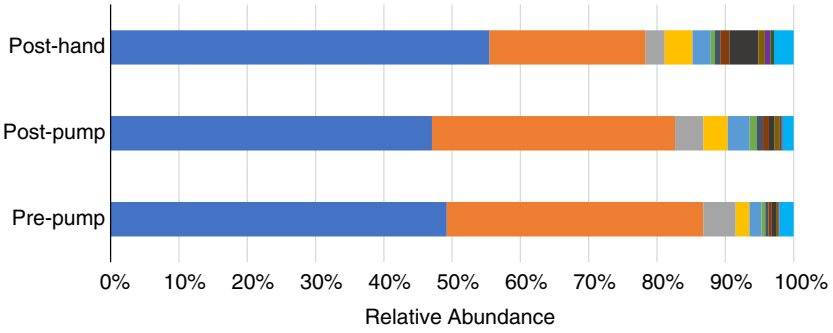

Figure 1 Relative abundance of bacterial genera detected in human milk samples expressed under different conditions. Pre-pump: pre-milk ejection pump-expressed sample (n = 30). Post-pump: post-milk ejection pump-expressed sample (n = 28). Post-hand: post-milk ejection hand-expressed sample (n = 28). 'Others' represents genera accounting for <1% relative abundance. (■) *Streptococcus*, (■) *Staphylococcus*, (■) *Cutibacterium*, (■) *Haemophilus*, (■) *Veillonella*, (■) *Neisseria*, (■) *Gemella*, (■) *Granulicatella*, (■) *Pelomonas*, (■) *Bifidobacterium*, (■) *Rothia*, (■) *Escherichia-Shigella*, (■) Others.

impact on microbial profiles were observed. However, this study also had its limitations; a small sample size (n = 8), collection of samples from mothers on different days, and use of partial 16S rRNA gene sequencing (Rodriguez-Cruz *et al.* 2020). In comparison, our sample size, although still small, is three times larger, and our use of consecutively obtained samples allowed us to confirm that any similarity/difference in the results was due to the different expression methods applied and not due to variation in the HM microbiome over time. Finally, the current study used full-length 16S rRNA gene sequencing, which provided significant analytical advantages over partial amplicon sequencing in relation to both taxonomic bias and depth (Johnson *et al.* 2019). Despite these differences in methodology, the data presented here agree with the results of Rodriguez-Cruz *et al.* Together, these results suggest that mode of expression does not influence the resulting HM bacterial DNA profile when sterile pumps are used. This has obvious consequences for sampling protocols for microbiome research, and it may also have implications for infant feeding practices.

Initially, we predicted that mode of expression may influence the bacterial DNA profile of the sample as pump expression is distinct from hand expression in that it relies on vacuum rather than manual pressure. Each breast contains 4–20 lobes of glandular tissue. These lobes are comprised of alveoli from which milk drains into small ducts that merge and eventually culminate in a single main milk duct which then exits the nipple (Going and Moffat 2004; Ramsay *et al.* 2005). Typically, under vacuum milk is extracted from more ducts compared to hand expression, thus the pumped milk may be more representative of the microbiome of the milk in the breast. If the HM microbiome varies between lobes within the mammary gland then differences may be seen based on mode of expression. Using sterile pumps, we have been able to remove the possibility of pump-derived contamination, allowing a comparison of pump-expressed milk (drawn from five to eight ducts) (Love and Barsky 2004) and hand-expressed milk (drawn from 1 to 3 ducts) (Murase *et al.* 2009). Our results suggest that this difference in the number of lobes from which the milk is drawn does not impact the resulting bacterial DNA profiles. This may also suggest that bacterial profiles do not differ greatly between individual lobes; however, animal studies are required to more directly address this question. Importantly, our findings provide evidence that either mode of expression can be used to collect HM samples for microbiome research, provided the pump used is sterile. This is in line with previous work which has reported no association between mode of expression and other HM components, including fat, protein and viable cells (Hassiotou *et al.* 2013).

We also found that the timing of sampling with respect to milk ejection did not impact the HM bacterial DNA profiles. The majority of the milk in the breast is stored within the alveoli until it is made available to the infant or pump via milk ejection. Stimulation of the nipple triggers milk ejection during breastfeeding and pumping. Thus, between milk removal sessions some milk is retained in the ducts for a substantial period of time (Dewey *et al.* 1991). Variable volumes of residual HM can be removed prior to milk ejection using the vacuum of a milk pump (0–37·5 ml) (Kent *et al.* 2003); In this study, women did not feed/express for 2 h prior to sampling. Given that the microenvironment of the HM ducts varies from that of the mammary alveoli, the bacterial composition of HM remaining in the distal ducts may differ from HM in the alveoli and proximal ductal structures as time passes between feeds and more milk is

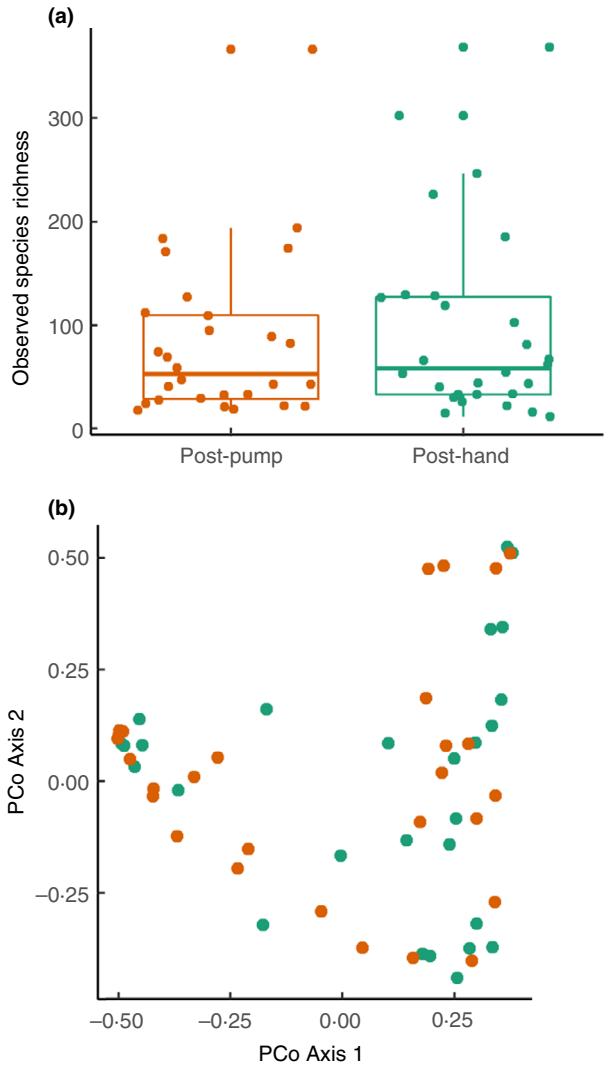

Figure 2 (a) Richness (number of observed OTUs) of human milk samples expressed by hand or pump. (b) PCoA of Bray-Curtis distances between human milk samples expressed by hand or pump. (●) Post-pump: post-milk ejection pump-expressed sample (n = 28). (●) Post-hand: post-milk ejection hand-expressed sample (n = 28).

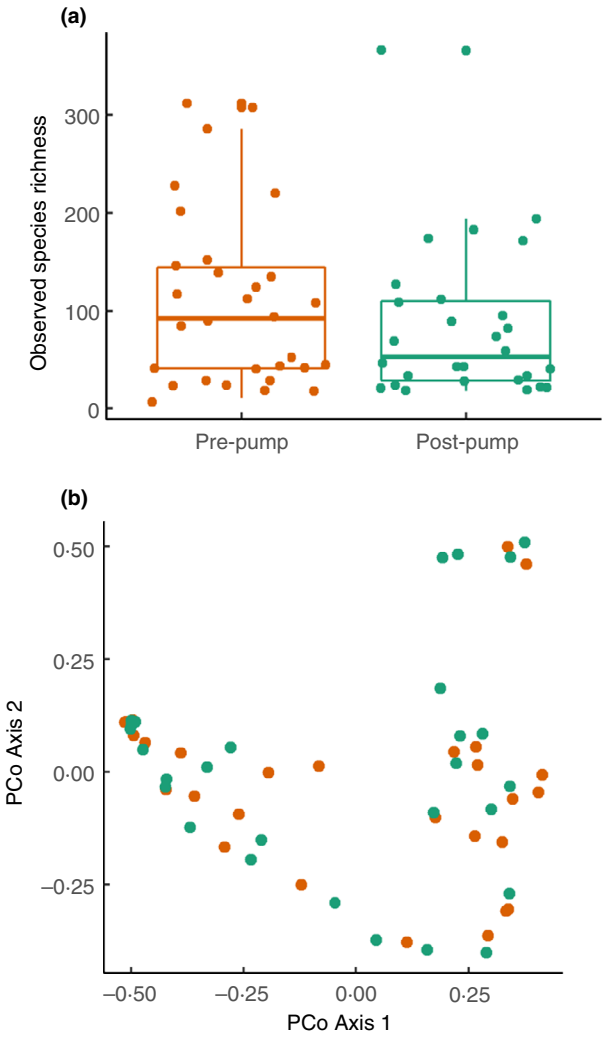

Figure 3 (a) Richness (number of observed OTUs) of human milk samples expressed pre- and post-milk ejection. (b) PCoA of Bray-Curtis distances between human milk samples expressed pre- and post-milk ejection. (●) Pre-pump: pre-milk ejection pump-expressed sample (n = 30). (●) Post-pump: post-milk ejection pump-expressed sample (n = 28).

secreted. Despite these speculations, no differences in the composition of pre-milk ejection samples (residual milk in the ducts) and post-milk ejection samples (milk from the alveoli) were detected. These results are reassuring for HM microbiome studies which rely on pump-expressed milk, as they suggest timing of sample collection with respect to milk ejection does not significantly alter the resulting bacterial DNA profiles.

In conclusion, this study has shown that mode of expression and timing of sample collection prior to and after milk ejection does not influence the resultant HM bacterial DNA profile. Therefore, either expression method can be utilized to collect samples for microbiome studies. Furthermore, if pump expression is used, pre- or post-milk ejection samples are both suitable. However, it is important to note that sterilised breast pump kits and appropriate sterile technique should be used at all times in order to limit the potential for pump-introduced bacterial contaminants to influence HM bacterial DNA profiles.

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Conflict of Interest

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Author contributions

A.S.C. collected samples and performed the experiments under the supervision of M.S.P. M.S.P. and D.T.G. designed the experiments. A.S.C. performed data analysis under the supervision of L.F.S., with addition analyses carried out by A.R. and M.D. A.S.C. wrote the manuscript and all authors critically edited it. All authors read and approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Number of sequencing reads of bacterial genera detected in negative extraction controls ($n = 2$) and negative PCR control ($n = 2$).