Received: 12 November 2018 | Revised: 10 April 2019 | Accepted: 14 April 2019

DOI: 10.1002/ajp.22982

RESEARCH ARTICLE

AMERICAN JOURNAL OF PRIMATOLOGY WILEY

Composition and stability of the vervet monkey milk microbiome

Lauren Petrullo[1] | Matthew J. Jorgensen[2] | Noah Snyder-Mackler[3,4,5] | Amy Lu[6]

[1]Interdepartmental Doctoral Program in Anthropological Sciences, Stony Brook University, Stony Brook, NY

[2]Department of Pathology, Section on Comparative Medicine, Wake Forest School of Medicine, Winston-Salem, NC

[3]Department of Psychology, University of Washington, Seattle, WA

[4]Center for Studies in Demography & Ecology, University of Washington, Seattle, WA

[5]Washington National Primate Research Center, University of Washington, Seattle, WA

[6]Department of Anthropology, Stony Brook University, Stony Brook, NY

Correspondence
Lauren Petrullo, Stony Brook University, Social and Behavioral Sciences Bldg. Room S-501, Stony Brook, NY, 11794-4364.
Email: lauren.petrullo@stonybrook.edu

Funding information
Stony Brook University, Grant/Award Number: Turner Fellowship program; University of Washington; National Institutes of Health CTSA pilot, Grant/Award Number: UL1-TR001420; a P40, Grant/Award Number: OD010965

Abstract

The human milk microbiome is vertically transmitted to offspring during the postnatal period and has emerged as a critical driver of infant immune and metabolic development. Despite this importance in humans, the milk microbiome of nonhuman primates remains largely unexplored. This dearth of comparative work precludes our ability to understand how species-specific differences in the milk microbiome may differentially drive maternal effects and limits how translational models can be used to understand the role of vertically transmitted milk microbes in human development. Here, we present the first culture-independent data on the milk microbiome of a nonhuman primate. We collected milk and matched fecal microbiome samples at early and late lactation from a cohort of captive lactating vervet monkeys ($N = 15$). We found that, similar to humans, the vervet monkey milk microbiome comprises a shared community of taxa that are universally present across individuals. However, unlike in humans, this shared community is dominated by the genera *Lactobacillus*, *Bacteroides*, and *Prevotella*. We also found that, in contrast to previous culture-dependent studies in humans, the vervet milk microbiome exhibits greater alpha-diversity than the gut microbiome across lactation. Finally, we did not find support for the translocation of microbes from the gut to the mammary gland within females (i.e., "entero-mammary pathway"). Taken together, our results show that the vervet monkey milk microbiome is taxonomically diverse, distinct from the gut microbiome, and largely stable. These findings demonstrate that the milk microbiome is a unique substrate that may selectively favor the establishment and persistence of particular microbes across lactation and highlights the need for future experimental studies on the origin of microbes in milk.

KEYWORDS
gut, microbiome, milk, vervet monkey

1 | INTRODUCTION

Maternal microbes are vertically transmitted to offspring during early life and establish the infant gut microbiome (Mueller, Bakacs, Combellick, Grigoryan, & Dominguez-Bello, 2015). This vertical transmission occurs first at birth when maternal vaginal microbes colonize the offspring gut as neonates pass through the birth canal (Mueller et al., 2015), and then during postnatal life as females transmit milk microbes to offspring during lactation (McDermott & Huffnagle, 2014). Studies on humans and other model species suggest that perturbations to perinatal vertical transmission can alter microbial succession in the developing infant gut, leading to changes in microbiome composition and other aspects of phenotype that may persist into adult life (Dominguez-Bello et al., 2010;

Jašarević, Howerton, Howard, & Bale, 2015; Madan et al., 2016). Determining the composition and function of vertically transmitted microbiota is therefore of critical importance to understanding how maternal microbiota may drive variation in offspring development during early life.

While microbiome research has made great strides in understanding the dynamics and function of the gut microbiome, comparatively little work has been done on vertically transmitted microbial communities, especially the milk microbiome. Milk microbiota are of both clinical and evolutionary interest because they are vertically transmitted to offspring across the entire period of lactation and thus have the potential to influence offspring across an extended period of time. This sets the milk microbiome apart from the maternal vaginal microbiome, which is vertically transmitted to offspring only once during vaginal birth. Early researchers posited that the milk microbiome forms when microbes in the skin and oral cavity contaminate the mammary gland (West, Hewitt, & Murphy, 1979). More recently, researchers have proposed an entero-mammary pathway, where dendritic cells in the immune system translocate commensal microbes (Macpherson & Uhr, 2004; Rescigno et al., 2001) from the gut to the mammary gland (Rodríguez, 2014). In support of this pathway, oral supplements of a known strain of *Lactobacillus* given to lactating women were found to result in increases in the same strain in milk (Arroyo et al., 2010). However, others have argued against the existence of an entero-mammary pathway, citing evidence that the human milk microbiome is compositionally distinct from the gut microbiome (Cabrera-Rubio et al., 2012).

Further, a handful of studies in humans using culture-dependent methods have concluded that the milk microbiome is lower in diversity compared to the gut microbiome (Heikkilä & Saris, 2003; Martín et al., 2003). Yet, recent culture-independent studies have found divergent patterns, revealing a human milk microbiome that is richer and more diverse than culture-dependent research had previously reported. Such studies have found that human milk microbial communities are dominated by *Staphylococcus*, *Streptococcus*, and other lactic acid bacteria (Cabrera-Rubio et al., 2012; Gomez-Gallego et al., 2016; Hunt et al., 2011) which, in a sample of 16 females, together constitute a "core" community that is conserved across individuals (Hunt et al., 2011). Intriguingly, the composition of the human milk microbiome is not uniform across lactation: milk produced just after birth is more abundant in lactic acid bacteria and greater in microbial diversity than milk produced later during lactation (Cabrera-Rubio et al., 2012). Late lactation is also characterized by an increase in *Prevotella*, a microbial genus common to the gut microbiome and heavily involved in host metabolism (Cabrera-Rubio et al., 2012; Gomez-Gallego et al., 2016). These patterns suggest that the milk microbiome is a dynamic and potentially shifting community that may exert differential influence on offspring development depending on the stage of lactation.

Outside of humans, there remains a dearth of comparative data on the milk microbiome of nonhuman primates. At present, the only published data on the milk microbiome of a nonhuman primate comes from a single study that cultured lactic acid bacteria from rhesus macaque milk (Jin, Hinde, & Tao, 2011). Although the researchers identified 19 species from five microbial genera (*Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, and *Streptococcus)*—many of which are shared with humans—the presence and relative abundance of other taxa known to play critical roles in the human infant gut (e.g., *Bacteroides*: Koleva, Bridgman, & Kozyrskyj, 2015) remains unknown. Thus, it is unclear whether phylogenetic parallels exist between the microbes present in human milk and those of other closely related host species (Amato, 2013; Amato et al., 2015; Stumpf et al., 2013; Yildirim et al., 2014). This gap in knowledge is particularly problematic because nonhuman primates serve as ideal models for investigating the causal role of milk microbiota on early infant development.

In this study, we present the first culture-independent data on the milk microbiome of a nonhuman primate. We use a cohort of captive lactating female vervet monkeys (*Chlorocebus aethiops sabaeus)* to achieve three main goals: First, we explore the composition and diversity of the milk microbiome to determine whether, similar to a prior study in humans (Hunt et al., 2011), there will be a shared community of microbes that are present in the milk of all of our subjects (i.e., "core"), with relatively low interindividual variation in community composition. We predict that vervet monkey samples will be dominated by lactic acid bacteria, but expect to find that nonlactic acid bacteria are abundant as well. In line with the majority of prior studies, we also predict that the diversity of the milk microbiome will be lower than the diversity of the maternal gut microbiome. Second, we investigate whether, like humans, the composition and diversity of the milk microbiome changes from the earliest period of lactation (colostrum), to 4 months postpartum (late lactation milk). In line with previous studies on humans, we predict that the milk microbiome at 2–5 days postpartum will exhibit greater taxonomic diversity than at 4 months postpartum. Finally, we test whether the milk microbiome results from an entero-mammary pathway via two approaches: a broad, compositional approach utilizing dissimilarity indices, and a variant-level approach that examines the proportion of sequence variants shared between the two communities. If an entero-mammary pathway exists, we predict to find that a female's milk microbiome is more similar to her own gut microbiome than the gut microbiome of other females and that the proportion of sequence variants shared between her own milk and gut is greater than between her milk and another female's gut.

2 | METHODS

2.1 | Study population

The research presented in this manuscript adhered to the American Society of Primatologists Principles for the Ethical Treatment of Nonhuman Primates. All animal use procedures and sample collection methods were approved by the Institutional Care and Use Committee at Wake Forest School of Medicine. Subjects for this study were 15 captive lactating female vervet monkeys housed as part of the Vervet Research Colony (VRC) at Wake Forest School of Medicine. Vervet monkeys are Old World Monkeys that are highly social and breed annually, giving birth to single offspring (Else, Eley, Wangula, Worthman, & Lequin, 1986). Subjects were housed at the

VRC in matrilineal social groups ($N = 8$) that resemble vervet social groups in the wild, with females remaining in their natal groups with other female kin for life. Animals were housed with indoor-outdoor access and 11 of the animals were provisioned daily with commercial monkey chow (Purina Monkey Chow, LabDiet 5038). Four animals were fed a western-style lab diet (LabDiet 5L3K) as part of a different study. All animals received supplementary fresh fruits and vegetables. Subjects ranged in parity from one to nine prior pregnancies and had recently given birth after typical and unmanipulated gestations.

2.2 | Sample collection

Milk samples were collected at two postnatal timepoints (Timepoint 0 = 2–5 days postpartum; Timepoint 4 = 4 months postpartum) from each female, with the exception of one female that did not provide a Timepoint 4 sample because she was dropped from the study for logistic reasons. Milk samples were collected following a previously established protocol for the collection of milk from rhesus monkeys (Hinde, Power, & Oftedal, 2009). Briefly, females were coaxed into a capture tunnel with their infants, lightly sedated with 5–10 mg/kg ketamine hydrochloride administered intramuscularly, and temporarily moved to a separate laboratory area. Females were then placed in mesh primate jackets (Lomir Inc., Malone, NY) and moved, along with their infants, to individual cages for a 3-hr standardized period of milk accumulation. The mesh jackets allowed for mother-infant contact while preventing nursing. Mother-infant pairs were monitored at 30-min intervals throughout the accumulation period. At the end of accumulation, females were sedated a second time with 5–10 mg/kg of ketamine hydrochloride administered intramuscularly and given a 0.1 ml/kg dose of oxytocin to stimulate milk letdown. Mammary glands were cleaned with alcohol-soaked gauze to prevent contamination of the sample by maternal skin and environmental bacteria. Both mammary glands were then fully evacuated via manual expression into a single sample tube. Samples were placed immediately on ice, briefly vortexed, aliquoted into cryovials, and frozen at −80°C until shipment to the University of Washington for analyses.

Matched fecal samples were collected at both timepoints immediately before jacketing. Samples were collected by briefly inserting flocked nylon swabs (FLOQSwabs, COPAN Diagnostics, Murrieta, CA) into the anal canal. Swabs were snapped off into polypropylene tubes and immediately frozen at −80°C until shipment to the University of Washington.

2.3 | DNA extraction, amplification, and library preparation

Microbial DNA was extracted from milk using the PowerFood Microbial kit (QIAGEN, Valencia, CA) following the manufacturer's kit protocols with the addition of the following front-end processing steps to increase overall yield: milk was first thawed to room temperature and briefly vortexed. Up to 1 ml of whole milk was then transferred to a 2 ml sample tube and centrifuged at 5,000 g for 30 min to separate bacterial cells from lipids and aqueous supernatant. After removing the latter, the remaining pelleted bacterial cells were resuspended in 450 ul of kit MBL solution. The resuspension was then subjected to a heat treatment at 70°C for 10 min (Quigley et al., 2012) on a heating block before being transferred to a bead tube (garnet, 0.7 mm) for mechanical lysing at 30 Hz for 2 min. From here, extractions followed kit protocols, with a modification to the last step: 30 µl of elution buffer was added to the spin column, incubated for 3 min, centrifuged at 13,000 g for 1 min, and the same 30 µl was rerun through the column and repeated a second time. Microbial DNA was extracted from fecal swabs using the Qiagen PowerLyzer PowerSoil kit. Briefly, samples were thawed to room temperature and swab tips were cut off directly into bead tubes (glass, 0.1 mm), and mechanically lysed at 30 Hz for 2 min. From here, fecal DNA extractions followed the manufacturer's protocol.

The hypervariable V4 region of the 16S rRNA gene was amplified using PCR primer set 515F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA) and 806R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT) from The Human Microbiome Project and a dual-indexing approach. The V4 region is highly reliable and has been widely used to characterize microbial communities at numerous body locations and was previously used in the Earth Microbiome Project (Gilbert, Jansson, & Knight, 2014; Yang, Wang, & Qian, 2016). For milk samples, each 12.5 µl PCR reaction was comprised of 6 µl of Nebnext Ultra II Q5 mastermix, 0.25 µl of each primer, and 6 µl total of DNA template and water (25 ng template). PCR was performed in an Eppendorf thermocycler with a 100°C heated lid using the following cycling steps: an initial denaturing for 5 min at 95°C, followed by 20 cycles of 20 s at 98°C, 15 s at 62°C, 60 s at 72°C with a final hold at 4°C. After a 1:1 bead clean-up, a second indexing PCR was run to attach individual barcodes to each sample. The 12 µl PCR reaction consisted of 4 µl of product from the first PCR, 6 µl of Nebnext Ultra II Q5 mastermix, and 1 µl each of Illumina Nextera n5 and n7 primers, with each sample being assigned a unique n5/n7 primer combination. In an Eppendorf thermocycler with a 100°C heated lid, an initial denaturing for 5 min at 95°C was followed by 10 cycles of 20 s at 98°C, 15 s at 55°C, and 60 s at 72°C with a final hold at 4°C, for a total of 30 PCR cycles (20 PCR 1 and 10 PCR 2). Fecal samples were amplified following the same procedure, but with 13 µl total PCR 1 reaction volume (0.5 µl of each primer rather than 0.25 µl) and 15 PCR 1 cycles rather than 20. After a 2:1 bead clean-up, amplification of the V4 region was confirmed using a fragment analyzer and samples were quantified using a qubit fluorometer.

2.4 | Sequencing and bioinformatics

Amplicon libraries were balanced, pooled together, spiked with PhiX to increase library complexity, and sequenced together on a single Illumina MiSeq flow cell using 301 bp paired-end sequences, resulting in 1,456,972 reads for milk samples and 2,491,940 reads for fecal samples. We analyzed the resulting data using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) platform (Caporaso et al., 2010; Hall & Beiko, 2018). We denoised the sequencing data by filtering and correcting Illumina amplicon sequencing errors using the QIIME2 plugin

Divisive Amplicon Denoising Algorithm 2 (DADA2: Callahan et al., 2016). In contrast to clustering sequencing reads based on a fixed dissimilarity threshold (e.g., the assignment of Operational Taxonomic Units [OTUs]), which can conflate sequencing errors with biological variation, DADA2 infers sequences exactly (resulting in amplicon sequence variants, hereafter referred to as ASVs) for higher resolution than OTUs (Callahan et al., 2016). This approach is crucial for assessing changes in microbial composition (Tikhonov, Leach, & Wingreen, 2015) as well as overall diversity (Rosen, Davison, Bhaya, & Fisher, 2015), particularly in microbial communities presumed to be low in diversity based on prior OTU clustering methods (e.g., the human vaginal microbiome, Callahan et al., 2016). Briefly, forward and reverse reads were trimmed to 240 bases long to remove the low-quality portion of the sequences. Next, the forward and reverse reads were merged and chimeric sequences removed. After filtering, trimming, merging, and chimera removal, 811,225 16S rRNA sequences from 28 milk samples (28,972 ± 17,165 reads per sample, $N = 28$) and 1,547,442 sequences from 29 fecal samples (48,357 ± 15,100 reads per sample, $N = 28$) remained. We aligned ASVs using MAFFT (Katoh, Misawa, Kuma, & Miyata, 2002) and constructed a phylogenetic tree using fasttree2 (Price, Dehal, & Arkin, 2010). ASVs were taxonomically assigned using the q2-feature-classifier in QIIME2 against the 13_8 version of the GreenGenes database (McDonald et al., 2012) based on 100% identity.

2.5 | Statistical analysis

To assess whether a community of microbial taxa was shared across all individuals, we identified which genera were present in every female (i.e., every sample) at both timepoints and at any abundance threshold. To determine which of these shared taxa were most abundant, we then identified taxa that contributed more than 3% to the total abundance of the sample in at least one female in our data set. We then used DESeq2 (Love, Huber, & Anders, 2014) to determine differential abundance of taxa at the genus level across timepoints using an alternative size factor estimator (type = "iterate"), corrected for multiple testing (Benjamini–Hochberg correction) and alpha set to 0.05.

The R package phyloseq (McMurdie & Holmes, 2013) was used to calculate the Shannon Index of alpha-diversity for both milk and fecal samples. The Shannon Index is a common ecological measure of community diversity and accounts for both the richness and the evenness of species (Li, Bihan, Yooseph, & Methé, 2012). In contrast to other diversity indices that place greater weight on taxa commonness and dominance (e.g., Simpson Index), the Shannon Index better captures species richness and the contributions of rarer taxa (Jost, 2007) and is thus more appropriate for examining a microbial community where overall bacteria load is relatively low and rare taxa may be important (e.g., milk). Calculated Shannon diversity indices were then transformed using Tukey transformation to achieve normality in the residuals and linear mixed models were used to test whether sample type (milk/fecal) and timepoint (0/4) significantly predicted differences in alpha-diversity, controlling for a diet with individual ID as a random effect.

To examine the degree of interindividual differences among females in milk and fecals at both timepoints, we measured beta-diversity by generating weighted UniFrac distance matrices using QIIME2, which account for presence/absence, relative abundance, and phylogeny of taxa (Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011). Although both weighted and unweighted UniFrac distances take into account the phylogenetic relatedness of taxa within a community, weighted UniFrac distances better reflect overall community structure because branch lengths are weighted based on the relative abundance of lineages within the community (Lozupone et al., 2011). Average weighted UniFrac distances were then compared across sample type and timepoint using nonparametric Kruskal–Wallis tests of medians. To compare the community composition of the milk microbiome to that of the gut microbiome, we used permutational multivariate analysis of variance (PERMANOVA) on QIIME2-generated weighted UniFrac distance matrices and the adonis() function in the vegan package in R.

To test whether there may be an entero-mammary pathway linking the milk and gut microbiomes, we employed two approaches (broad dissimilarity approach and sequence variant approach): In the first approach, we calculated the beta-diversity (dissimilarity) of all samples using weighted UniFrac in QIIME2. We then used linear mixed models to test the hypothesis that a female's milk microbiome would be more similar (i.e., lower dissimilarity index) to her own gut microbiome than to the gut microbiome of other individuals. Using dissimilarity indices as a dependent variable, we tested whether category (self vs. other) significantly predicted dissimilarity between two samples while including the individual ID of the first member of a dyad (ID1) and individual ID of the second member of the dyad (ID2) as random factors, and controlling for diet. We tested for these similarity effects separately in each timepoint.

In the second approach, we identified the proportion of ASVs at any threshold within a female's milk microbiome that were also found in that same female's gut microbiome. Similar to the above, we then calculated the proportion of ASVs within a female's milk microbiome that were also found the gut microbiome of other females. We used linear mixed models to test the hypothesis that the proportion of a female's milk ASVs also present in her own gut is greater than the proportion of her milk ASVs present in the gut of other females. Proportions were arc-sine square root transformed to achieve normality in residuals and were used as the dependent variable in the model, with comparison category (self vs. different) as a predictor variable and the IDs of both individuals within the dyad as random effects, controlling for diet. All analyses were performed in R (R Core Developmental Team, 2014).

3 | RESULTS

3.1 | Shared community of taxa

Milk samples were comprised of 2714 unique ASVs (mean = 475 ASVs per sample) of bacteria from 186 different genera and 23 phyla. Of these 186 genera, 21 were present in every milk sample: *Anaerovibrio*, *Bacteroides*, *Blautia*, *Clostridium*, *Collinsella*, *Coprococcus*, *Dialister*, *Dorea*,

Eubacterium, *Faecalibacterium*, *Gemmiger*, *Lactobacillus*, *Megasphaera*, *Oscillospira*, *Phascolarctobacterium*, *Prevotella*, *Ruminococcus*, *Sarcina*, *Staphylococcus*, *Streptococcus*, and *Veillonella*. Together, these 21 genera were highly stable across lactation, comprising 87.4% of the total microbial abundance in milk at Timepoint 0% and 86.86% of the total abundance at Timepoint 4 (Figure 1a; Figure 2). By contrast, maternal fecal samples were comprised of fewer unique ASVs: the maternal gut microbiome was comprised of 1834 ASVs (mean = 400 ASVs per sample) from 125 genera (19 phyla). At the genus level, only 14 genera were present in every fecal sample, suggesting a smaller community of shared taxa (Figure 1b). The shared community of taxa in the gut comprised 73% of the total abundance in the maternal gut microbiome at Timepoint 0 and 82% of the total abundance at Timepoint 4. Of these genera, seven were present in both milk and fecal samples, whereas more than half of the remaining shared taxa differed between the two sample types (Figure 1a,b).

3.2 | Abundant taxa

Of the 21 taxa present in all milk samples, nine genera ($N = 9$) were highly abundant in at least one female in our data set and represented more than 3% of the sample. These genera included *Anaerovibrio*, *Bacteroides*, *Blautia*, *Faecalibaterium*, *Lactobacillus*, *Prevotella*, *Staphylococcus*, *Streptococcus*, and *Veillonella*. Among these genera, *Lactobacillus*, *Prevotella*, *Bacteroides* were the most abundant at both timepoints and each contributed >3% to the overall abundance in 25 of the 28 samples. However, these genera were largely stable across lactation and did not significantly differ in their relative abundance in the milk microbiome between Timepoint 0 to Timepoint 4 ($p > .05$; Figure 1a). By contrast, at Timepoint 0, milk samples exhibited an abundance of *Staphylococcus* (6.1%), which decreased significantly to 0.7% by Timepoint 4 (log 2 FC: −3.31, $P_{adj} < 0.01$; Figure 1a), and *Veillonella* decreased significantly from 1.4% to 0.7% (log 2 FC: −2.44, $P_{adj} < 0.001$; Figure 1a).

By comparison, the maternal gut contained far more genera ($N = 23$) contributing more than 3% to the total abundance. In particular, *Bacteroides*–a bacteria found in all milk samples–was not universally present across all samples (absent in 2 samples) and did not contribute > 3% to the gut microbiome at either timepoint, despite a much larger abundance of this genus in matched milk samples. In addition, there was a shift in *Prevotella* in the maternal gut microbiome across lactation: at Timepoint 0, *Prevotella* comprised 17.6% of the maternal gut, but by Timepoint 4, this had significantly increased to 48.2% ($p < .001$; Figure 1b). Further, despite nonsignificant changes in *Lactobacillus* in milk across timepoints, maternal fecal samples exhibited a significant decrease in *Lactobacillus* from Timepoint 0 (14%) to Timepoint 4 (2.3%) ($p < .001$; Figure 1b).

3.3 | Alpha-diversity

The milk microbiome exhibited significantly greater alpha-diversity (richness and evenness) than maternal fecal samples (Figure 3; estimate ± *SEM* = 2961.5 ± 632.1; $t = 4.7$; $P < .0001$). There were also significant temporal differences in alpha-diversity of the milk microbiome across lactation: diversity was lower at 2–5 days postpartum than at 4-month postpartum (Figure 3; estimate ± *SEM* = 2386.4 ± 715.8; $t = 3.3$; $P = .005$). By contrast, alpha-diversity of the gut microbiome did not significantly differ between the two lactional timepoints (estimate ± *SEM* = 673.74 ± 854.3; $t = 0.79$; $P = .44$).

3.4 | Beta-diversity

Despite a relatively smaller community of shared taxa in the gut, the gut microbiome exhibited higher dissimilarity indices (weighted UniFrac) across females compared with the milk microbiome, which exhibited significantly less interindividual variation (Figure 4; Kruskal–Wallis $p = 2.593 \times 10^{-14}$). Within milk samples, samples were significantly more diverse at Timepoint 0 compared at Timepoint 4 (Figure 4; Kruskal–Wallis; $p < 2.2 \times 10^{-16}$).

3.5 | Milk and fecal comparisons

Nonmetric multidimensional scaling based on weighted UniFrac distances showed that samples clustered by sample type at both timepoints and PERMANOVA showed that microbial community composition differed based on sample type ($F = 5.98$, $R^2 = 0.09$, $p < 0.0001$), but not timepoint (Figure 5). Further, weighted UniFrac distances were not significantly different between milk and fecal samples from the same female compared to inter-female milk-fecal sample pairs at either timepoint (Timepoint 0: estimate ± *SEM* = $3.55 \times 10^{-3} \pm 1.03 \times 10^{-2}$, $t = 0.34$, $P = 0.73$; Timepoint 4: estimate ± *SEM* = $3.67 \times 10^{-3} \pm 1.03 \times 10^{-2}$, $t = 0.36$, $P = .72$).

Sequence variant analyses revealed that at Timepoint 0, an average of 45.3% of the ASVs present in a female's milk microbiome were also present in her gut microbiome. At Timepoint 4, slightly less (37.8% on average) of a female's milk ASVs were also present in her gut. Of these shared milk ASVs, approximately 10% were shared between the milk and gut microbiome of every female in our data set, such that approximately 90% of ASVs shared between the milk and gut microbiome of a single female were unique to that female. However, the proportion of ASVs shared between a milk and fecal sample was not significantly predicted by whether the samples were from the same female or different females (estimate ± *SEM* = $1.8 \times 10^{-3} \pm 1.6 \times 10^{-2}$; $t = -0.11$; $P = .91$).

4 | DISCUSSION

The present study extends prior work on the primate milk microbiome by using culture-independent techniques via high throughput sequencing to more thoroughly capture the microbes present in milk. These techniques allowed us to capture a broader range of taxa, many of which cannot be cultured in a laboratory setting (Turnbaugh et al., 2007), allowing us to characterize the full taxonomic breadth of the nonhuman primate milk microbiome. Further, because we assigned reads based on 100% similarity to the reference sequences, we were able to identify microbial taxa with

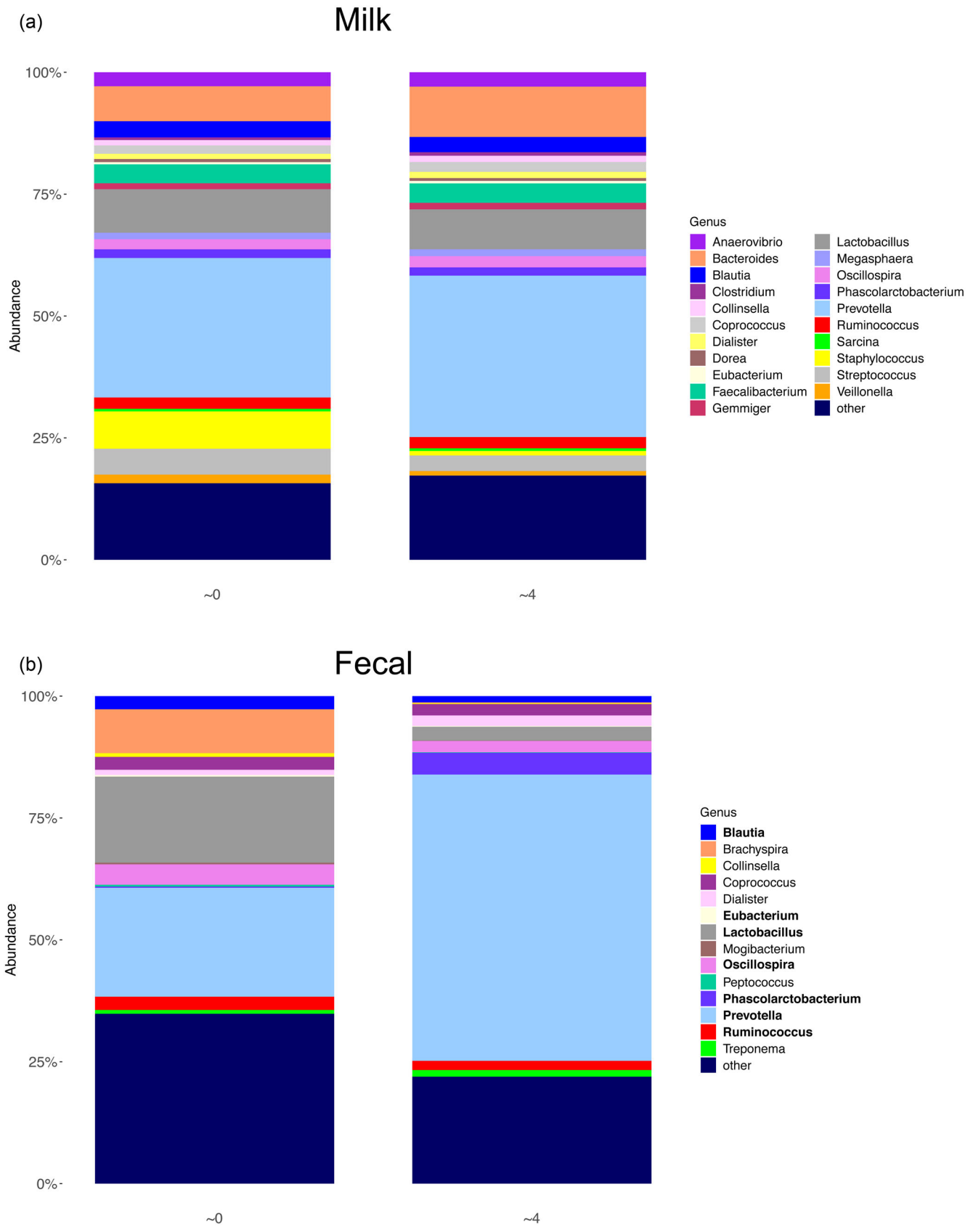

FIGURE 1 (a) Barplot depicting the average abundance of the 21 genera present in every milk sample across two timepoints (i.e., "milk core"); "other" indicates an agglomeration of taxa not present in every milk sample (i.e., not core). (b) Barplot depicting the average abundance of the 14 core genera present in fecal samples; "other" indicates an agglomeration of taxa not present in every sample. Boldface genera indicate fecal genera that are also part of the core vervet milk microbiome

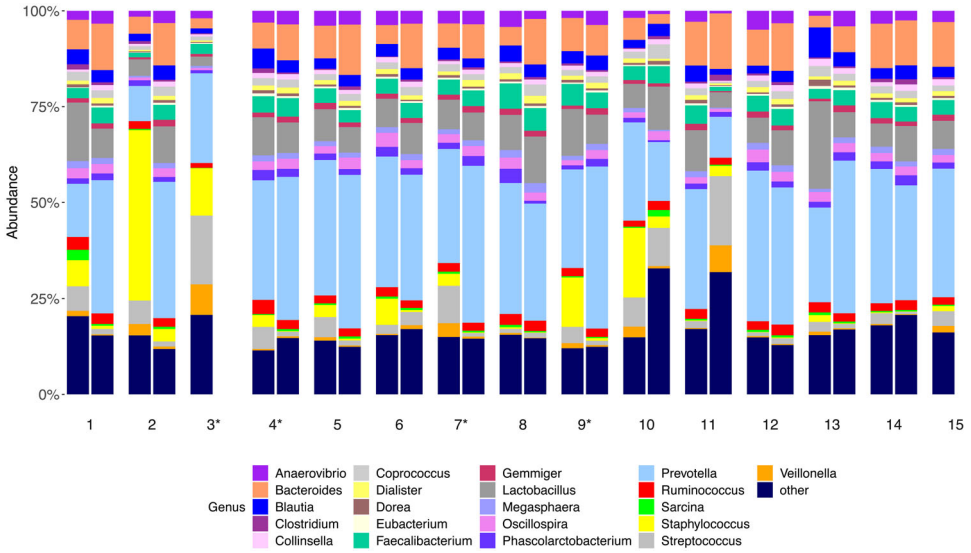

FIGURE 2 Barplot depicting individual and temporal variation in the relative abundance of the 21 core taxa present in vervet monkey milk. Each individual is represented by a pair of adjacent bars—within each pair, the first bar is Timepoint 0, the second is Timepoint 4. The four individuals denoted with an asterisk ("*") were fed a Western-style commercial diet. Unmarked individuals were fed the standard commercial monkey lab diet

higher resolution than previous studies—which commonly assign reads to OTUs—and thus capture the richness and diversity of milk microbial communities more completely than OTU-based studies (Callahan, McMurdie, & Holmes, 2017).

We found that 21 bacterial genera were present in every milk sample and that overall, milk samples showed little interindividual variation. In line with previous studies on humans (Hunt et al., 2011), these results suggest that vervet monkeys may have a "core" milk microbiome that is consistently shared across individuals. However, both the present study and that by Hunt et al., 2011 had small sample sizes ($N = 15$ and $N = 16$ females, respectively), thus whether a "core" milk microbiome exists across a larger dataset of individuals remains to be tested. Nonetheless, a high prevalence of shared taxa may reflect evolutionary pressures to conserve these taxa, possibly because they are critical for developing offspring. Indeed, 9 of these shared taxa were highly abundant genera that contributed > 3% to the total abundance in at least one female in our data set. This grouping of 9 highly abundant genera was dominated by *Lactobacillus*, *Prevotella*, and *Bacteroides*, taxa that are involved in infant immune development, metabolism, and the establishment of the infant gut microbiome (Dominguez-Bello et al., 2010; Forsberg, 2016; Koleva et al., 2015). Alternatively, our finding of a shared community of taxa across all individuals may reflect the shared living conditions of our study subjects, however, we did not have the statistical power to test the effect of a social group on microbiome composition in this study. Future comparative work on the milk microbiome is needed to establish whether shared communities of taxa within the milk microbiome are a result of evolutionary pressures, living conditions, or both.

In contrast to prior studies on human milk that have found a very low abundance of *Lactobacillus* (Cabrera-Rubio et al., 2012; Collado, Delgado, Maldonado, & Rodríguez, 2009; Hunt et al., 2011),

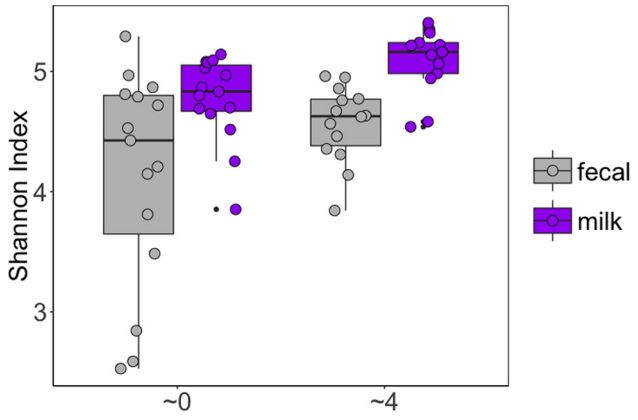

FIGURE 3 Boxplot (line = median) depicting alpha-diversity of fecal versus milk samples at two timepoints measured as the Shannon Index of alpha-diversity

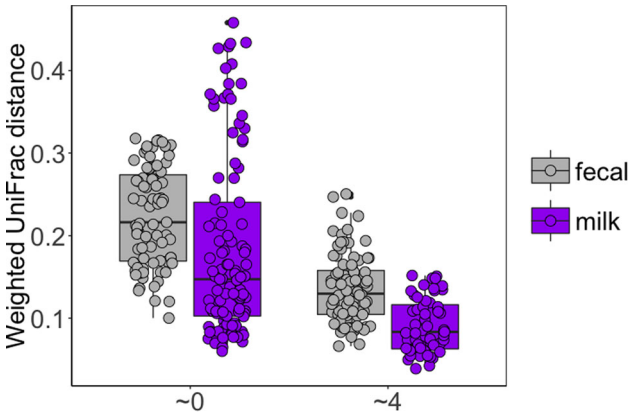

FIGURE 4 Boxplot (line = median) depicting the degree of difference in microbial community composition among individuals by sample type and lactation timepoint. Points represent weighted UniFrac distances measures

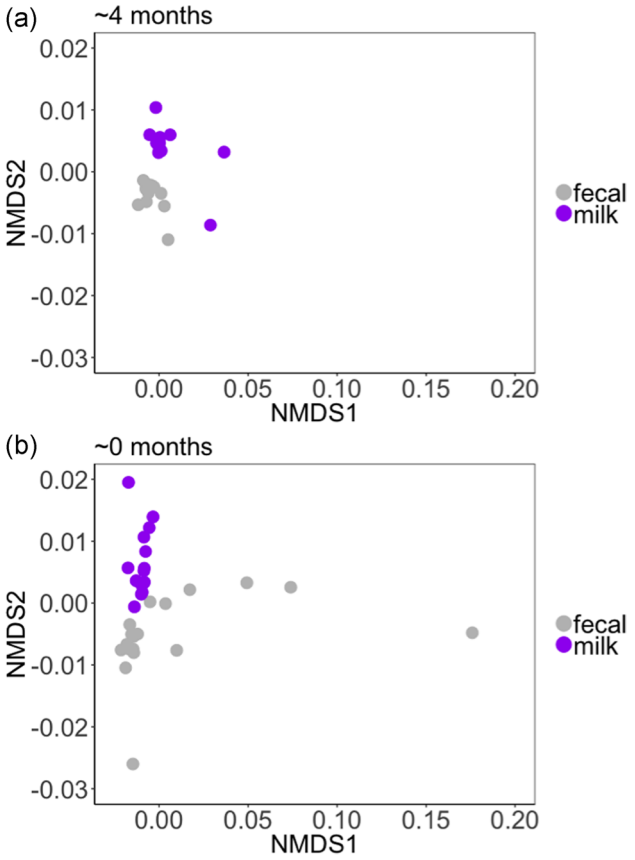

FIGURE 5 (a,b) Visualization of differences in milk and gut microbiome composition using nonmetric multidimensional scaling based on weighted UniFrac distance dissimilarity matrix. Points represent individual samples

Lactobacillus was one of the most dominant genera (~6% of total abundance at both timepoints) in vervet monkey milk (Figure 1a). *Lactobacillus* are lactic acid bacteria that aid in host digestion of milk and milk components in the gut and are critical for offspring development during the postnatal period (Ahrné et al., 2005; Martín, Heilig, Zoetendal, Smidt, & Rodríguez, 2007). Similar to other commensal microbes, infant gut *Lactobacillus* protect against invading pathogens through competitive exclusion and the production of antimicrobials, such as hydrogen peroxide (Heikkilä & Saris, 2003). In addition, gut *Lactobacillus* plays a key role in the priming and maturation of the infant innate immune system in humans (Galdeano & Perdigón, 2006). Given these functions, the relatively large abundance of *Lactobacillus* in vervet monkey milk samples compared with humans may suggest similar and perhaps species-specific roles for this taxon in vervet infant development.

The vervet monkey milk microbiome was also abundant in *Prevotella* and *Bacteroides*, two nonlactic acid bacterial genera that are also prominent in the human microbiome. In particular, *Prevotella* was the most abundant (~24%) genus across all milk samples in this study. In the infant's gut, *Prevotella* likely aids in host digestion of carbohydrate-rich foods. It is largely absent from the infant gut microbiome in European children, but prevalent in the guts of West African children (Yatsunenko et al., 2012). This difference likely reflects the importance of fiber-rich foods in West African, but not in Western diets, the latter of which are often richer in protein and animal fat (Yatsunenko et al., 2012). Given that the vervet monkeys in our colony are provisioned with high fiber diets, the transfer of large amounts of maternal-origin milk *Prevotella* to infants may prepare infants for the eventual transition to solid foods. Interestingly, however, a prior study on captive and wild vervet monkeys found that gut *Prevotella* abundance actually increased in response to provisioning with a Western diet (Amato et al., 2015). In our dataset, four individuals were fed a Western diet rather than the standard commercial laboratory diet received by the remainder of our study subjects. However, these individuals did not exhibit larger than average abundances of *Prevotella* in their milk samples at either timepoint. More research is therefore needed to clarify the functional role of *Prevotella*, which is common in both the gut and milk microbiome of humans and vervet monkeys.

Although *Prevotella* is abundant in the milk of both vervet monkeys and humans, the abundance of *Bacteroides* (~5–6% of total abundance) in vervet monkey milk far outstrips its representation in human milk, where *Bacteroides* is not even one of the 15 most abundant genera (Hunt et al., 2011). *Bacteroides* are mutualistic obligate anaerobes that—although largely absent from human milk—are abundant in the human gut and are responsible for the processing of complex molecules. *Bacteroides* are generally commensal and can provide host protection through the ecological exclusion of pathogenic bacteria (Costello, Stagaman, Dethlefsen, Bohannan, & Relman, 2012). In humans, the species *Bacteroides fragilis* has been particularly well-studied, initially as being involved in neonatal infection when displaced from the gut (Myers et al., 1987; Okubadejo, Green, & Payne, 1973; Sears et al., 2008), but more recently as a commensal microbe that breaks down milk oligosaccharides, which are complex sugars that help establish the infant gut microbiome (Marcobal et al., 2011). In the present study, descriptive statistics showed that *B. fragilis* was one of the three most dominant *Bacteroides* species in vervet monkey milk. Similar to humans, the abundance of *Bacteroides* and *B. fragilis* specifically in vervet milk may aid offspring in the assimilation of milk oligosaccharides. Future research on nonhuman primate milk should aim to incorporate an analysis of milk oligosaccharides alongside microbiota to determine how the interplay between the two ultimately shapes the infant gut microbiome.

Importantly, all three dominant genera (*Lactobacillus*, *Prevotella*, and *Bacteroides*) did not significantly change in abundance from Timepoint 0 to Timepoint 4, reflecting high temporal stability of these genera and suggesting that their functional role in the infant gut likely remains important across lactation. However, another dominant genera, *Staphylococcus*, was significantly greater in abundance at Timepoint 0 than at Timepoint 4. *Staphylococcus* are common in human skin and the gut (David et al., 2014), and its abundance in the milk microbiome of both humans and nonhuman primates may suggest a primate-specific role for this genus during early lactation. In contrast to the apparent stability of the vervet milk microbiome, we found a significant decrease in the relative abundance of *Lactobacillus* from Timepoint 0 (14%) to Timepoint 4 (2.3%), coupled with an

increase in Prevotella from 17.6% to 48.2% in the maternal gut microbiome. While a large abundance of *Lactobacillus* at Timepoint 0 may serve to promote the transfer of *Lactobacillus* to the mammary gland via an entero-mammary pathway and ultimately to the infant gut, a decrease at 4-month postpartum—perhaps to accommodate an increase in *Prevotella*—may reflect a maternal metabolic shift related to the resumption of cycling. This is purely speculative, however, as the dearth of data on changes to the maternal gut microbiome during lactation makes this finding difficult to interpret. Thus we encourage future studies on reproductive state and the maternal microbiome to investigate the relationship between microbial changes and maternal energy balance during lactation.

In terms of diversity, we found that the vervet milk microbiome was comprised of more unique ASVs (2714) than the gut microbiome (1834) and exhibited higher alpha-diversity, suggesting that, counter to our predictions, the milk microbiome is a richer and more diverse community than the gut. Given the incredible diversity of the human gut microbiome and its bidirectional relationship with multiple aspects of host physiology (Human Microbiome Project Consortium, 2012; Yatsunenko et al., 2012), the large taxonomic diversity of the milk microbiome may suggest that it is a similarly impactful aspect of host physiology. However, although we utilized sterile collection methods and low-cycle PCR for amplification of the V4 region in milk samples, our study was limited by not including negative controls on our sequencing run, which would serve to increase the certainty that the high taxonomic diversity of the milk samples in this study is not a result of contamination.

Further, while a previous study in humans found that colostrum was significantly more diverse than milk produced at 1- and 6-month postpartum (Cabrera-Rubio et al., 2012), we found that milk produced at 2–5 days postpartum (Timepoint 0) was actually less diverse than milk produced at 4 months postpartum (Timepoint 4). While our Timepoint 0 is likely comparable to early lactation in humans, estimates of weaning age in our population suggests that Timepoint 4 may have represented late lactation and/or weaning (Fairbanks, 1988), making comparisons to 6-month postpartum samples from humans—which likely reflect "peak" milk production—complicated (Wambach & Riordan, 2014). To better assess whether the temporal change in alpha-diversity we found in this study truly diverges from humans, future studies should aim to collect milk samples that capture peak production (i.e., at more frequent, shorter time intervals closer to parturition), particularly in animals where the period of lactation is shorter than in humans (e.g., nonhuman primates).

Despite a general consensus that the milk microbiome plays a crucial role in early offspring development, the precise origin of milk microbes remains a longstanding debate. One major hypothesis proposes an entero-mammary pathway whereby microbes from the gut are translocated via dendritic cells to the mammary gland (Rodriguez, 2014). In this study, we found that neither a broad dissimilarity-based approach nor a sequence variant approach provided support for an entero-mammary pathway. While this may suggest that there is no microbial pathway linking the gut and mammary gland, another interpretation of these results is that broad compositional and sequence variant approaches are not sensitive enough to capture an entero-mammary pathway. Although dissimilarity indices have proven useful for detecting how a single microbial community type (e.g., the gut) clusters based on social and ecological predictors (Tung et al., 2015), they may not be robust enough to detect relationships between different microbial communities (e.g., milk and gut) that may exhibit large compositional differences that can swamp small patterns of shared characteristics. Further, the mammary gland and gut are vastly different ecological substrates with different biochemical environments that can bias the types of microbes capable of proliferating in each community (Coyte, Schluter, & Foster, 2015; Fernández et al., 2013; Russo & Russo, 1987). Thus, a selective pathway whereby dendritic cells transport only certain taxa from the gut to the mammary gland may exist, similar to the process of perinatal vertical transmission and selective seeding of maternal microbes to the infant gut (Korpela et al., 2018). This may further decrease the likelihood of detecting shared taxa across the communities using broad compositional metrics such as dissimilarity indices. Thus, the best test of an entero-mammary pathway is likely through experimental studies that move past ASV-sharing analyses and are capable of directly tracing ASVs from the gut to the mammary gland. Importantly, a second potential route of microbial colonization of the mammary gland is through infant saliva (i.e., "retrograde inoculation"; Ramsay, Kent, Owens, & Hartmann, 2004). Testing the retrograde inoculation hypothesis would similarly require an experimental framework, as comparing infant saliva samples with maternal milk samples is likely to be plagued with the same methodological issues as comparing milk to fecal samples.

One surprising finding in our study was that only approximately 10% of the milk ASVs also present in an individual's gut microbiome were similarly shared by the milk/gut microbiomes of other females. Thus, approximately 90% of the ASVs shared between a female's milk and gut microbiomes were uniquely shared within that individual. If microbes are in fact translocated from the gut to the mammary gland, this finding may suggest that the majority of translocated bacteria are "private" and not universally transferred by all females. While the contributions of these "private" bacteria to the overall stability and succession of the milk microbiome and the subsequent implications for maternal and infant health remain unknown, their presence may suggest that the milk microbial community is highly individualized.

In sum, this study is the first to describe the full taxonomic breadth of the milk microbiome of a nonhuman primate using culture-independent methods. We show that the milk microbiome is compositionally distinct from the gut microbiome and taxonomically diverse, with a number of genera shared across individuals and many taxa unique to females at the level of sequence variants. Although our statistical approaches found no support for the entero-mammary pathway, we encourage future studies to directly test this pathway and the precise role of milk microbiota by utilizing experimental frameworks and combining high-resolution bioinformatic techniques capable of ASV-level differentiation.

ACKNOWLEDGMENTS

The authors would like to thank the veterinary and technical staff of the Vervet Research Colony, especially Edison Floyd and Chrissy Long for their role in sample collections, and Katie Hinde for providing expertise and protocol guidance for milk sample collection. We would also like to thank Sierra Sams at the University of Washington for assistance with extractions and sequencing, and two anonymous reviewers for their helpful input on earlier versions of this manuscript.

FUNDING

This work was supported by the National Institutes of Health CTSA pilot grant [UL1-TR001420, Donald Mcclain PI], a P40 grant [OD010965, Matthew Jorgensen PI], Stony Brook University, the University of Washington, and the Turner Fellowship program at Stony Brook University (to L.P.).

ORCID

Lauren Petrullo http://orcid.org/0000-0001-6464-3177

REFERENCES

Ahrné, S., Lönnermark, E., Wold, A. E., Aberg, N., Hesselmar, B., Saalman, R., … Adlerberth, I. (2005). Lactobacilli in the intestinal microbiota of Swedish infants. *Microbes and Infection*, *7*(11-12), 1256–1262. https://doi.org/10.1016/j.micinf.2005.04.011

Amato, K. R. (2013). Co-evolution in context: The importance of studying gut microbiomes in wild animals. *Microbiome Sci. Med.*, *1*, 1.

Amato, K. R., Yeoman, C. J., Cerda, G., Schmitt, C. A., Cramer, J. D., Miller, M. E. B., … Leigh, S. R. (2015). Variable responses of human and non-human primate gut microbiomes to a Western diet. *Microbiome*, *3*, 53. https://doi.org/10.1186/s40168-015-0120-7

Arroyo, R., Martín, V., Maldonado, A., Jiménez, E., Fernández, L., & Rodríguez, J. M. (2010). Treatment of infectious mastitis during lactation: Antibiotics versus oral administration of Lactobacilli isolated from breast milk. *Clinical Infectious Diseases*, *50*(12), 1551–1558. https://doi.org/10.1086/652763

Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E., & Mira, A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *The American Journal of Clinical Nutrition*, *96*(3), 544–551.

Callahan, B. J., McMurdie, P. J., & Holmes, S. P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME journal*, *11*(12), 2639–2643. https://doi.org/10.1038/ismej.2017.119

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Collado, M. C., Delgado, S., Maldonado, A., & Rodríguez, J. M. (2009). Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. *Letters in Applied Microbiology*, *48*(5), 523–528. https://doi.org/10.1111/j.1472-765X.2009.02567.x

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M., & Relman, D. A. (2012). The application of ecological theory toward an understanding of the human microbiome. *Science*, *336*(6086), 1255–1262. https://doi.org/10.1126/science.1224203

Coyte, K. Z., Schluter, J., & Foster, K. R. (2015). The ecology of the microbiome: Networks, competition, and stability. *Science*, *350*(6261), 663–666. https://doi.org/10.1126/science.aad2602

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., … Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559–563. https://doi.org/10.1038/nature12820

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., & Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(26), 11971–11975. https://doi.org/10.1073/pnas.1002601107

Else, J. G., Eley, R. M., Wangula, C., Worthman, C., & Lequin, R. M. (1986). Reproduction in the vervet monkey (Cercopithecus aethiops): II. Annual menstrual patterns and seasonality. *American Journal of Primatology*, *11*(4), 333–342. https://doi.org/10.1002/ajp.1350110404

Fairbanks, L. A. (1988). Mother-infant behavior in vervet monkeys. *Behavioral Ecology and Sociobiology*, *23*(3), 157–165. https://doi.org/10.1007/BF00300350

Fernández, L., Langa, S., Martín, V., Maldonado, A., Jiménez, E., Martín, R., & Rodríguez, J. M. (2013). The human milk microbiota: Origin and potential roles in health and disease. *Pharmacological Research*, *69*(1), 1–10. https://doi.org/10.1016/j.phrs.2012.09.001

Forsberg, A. (2016). Lactobacillus reuteri, infant allergy prevention and childhood immune maturation. In V. Rao, & L. G. Rao (Eds.), *Probiotics and Prebiotics in Human Nutrition and Health* (pp. 59–82). InTech. https://doi.org/10.5772/63120.

Galdeano, C. M., & Perdigón, G. (2006). The probiotic bacterium Lactobacillus casei induces activation of the gut mucosal immune system through innate immunity. *Clinical and Vaccine Immunology*, *13*(2), 219–226. https://doi.org/10.1128/CVI.13.2.219-226.2006

Gilbert, J. A., Jansson, J. K., & Knight, R. (2014). The Earth Microbiome project: Successes and aspirations. *BMC Biology*, *12*(1), 69. https://doi.org/10:1186/s12915-014-0069-1

Gomez-Gallego, C., Garcia-Mantrana, I., Salminen, S., & Collado, M. C. (2016). The human milk microbiome and factors influencing its composition and activity. *Seminars in fetal & neonatal medicine*, *21*(6), 400–405. https://doi.org/10.1016/j.siny.2016.05.003

Hall, M., & Beiko, R. G. (2018). 16S rRNA gene analysis with QIIME2. In R. Beiko, W. Hsiao, & J. Parkinson (Eds.), *Microbiome Analysis* 1849, pp. 113–129). New York, NY: Humana Press. https://doi.org/10.1007/978-1-4939-8728-3_8

Heikkilä, M. P., & Saris, P. E. J. (2003). Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. *Journal of Applied Microbiology*, *95*(3), 471–478.

Hinde, K., Power, M. L., & Oftedal, O. T. (2009). Rhesus macaque milk: Magnitude, sources, and consequences of individual variation over lactation. *American Journal of Physical Anthropology*, *138*(2), 148–157. https://doi.org/10.1002/ajpa.20911

Human Microbiome Project Consortium (2012). Structure, function, and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214. https://doi.org/10.1038/nature11234

Hunt, K. M., Foster, J. A., Forney, L. J., Schütte, U. M. E., Beck, D. L., Abdo, Z., … McGuire, M. A. (2011). Characterization of the diversity and temporal stability of bacterial communities in human milk. *PLoS One*, *6*(6), e21313. https://doi.org/10.1371/journal.pone.0021313

Jašarević, E., Howerton, C. L., Howard, C. D., & Bale, T. L. (2015). Alterations in the vaginal microbiome by maternal stress are associated with metabolic reprogramming of the offspring gut and

brain. *Endocrinology*, *156*(9), 3265–3276. https://doi.org/10.1210/en.2015-1177

Jin, L., Hinde, K., & Tao, L. (2011). Species diversity and relative abundance of lactic acid bacteria in the milk of rhesus monkeys (*Macaca mulatta*). *Journal of Medical Primatology*, *40*(1), 52–58. https://doi.org/10.1111/j.1600-0684.2010.00450.x

Jost, L. (2007). Partitioning diversity into independent alpha and beta components. *Ecology*, *88*(10), 2427–2439.

Katoh, K., Misawa, K., Kuma, K. I., & Miyata, T. (2002). MAFFT: A novel method for rapid multiple 328 sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, *30*(14), 3059–3066. https://doi.org/10.1093/nar/gkf436

Koleva, P. T., Bridgman, S. L., & Kozyrskyj, A. L. (2015). The infant gut microbiome: Evidence for obesity risk and dietary intervention. *Nutrients*, *7*(4), 2237–2260. https://doi.org/10.3390/nu7042237

Korpela, K., Costea, P., Coelho, L. P., Kandels-Lewis, S., Willemsen, G., Boomsma, D. I., … Bork, P. (2018). Selective maternal seeding and environment shape the human gut microbiome. *Genome Research*, *28*(4), 561–568. https://doi.org/10.1101/gr.233940.117

Li, K., Bihan, M., Yooseph, S., & Methé, B. A. (2012). Analyses of the microbial diversity across the human microbiome. *PLoS One*, *7*(6), e32118. https://doi.org/10.1371/journal.pone.0032118

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change dispersion for RNA-seq data with DESeq. 2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J., & Knight, R. (2011). UniFrac: An effective distance metric for microbial community comparison. *The ISME journal*, *5*(2), 169–172. https://doi.org/10.1038/ismej.2010.133

Macpherson, A. J., & Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science*, *303*(5664), 1662–1665. https://doi.org/10.1126/science.1091334

Madan, J. C., Hoen, A. G., Lundgren, S. N., Farzan, S. F., Cottingham, K. L., Morrison, H. G., … Karagas, M. R. (2016). Association of cesarean delivery and formula supplementation with the intestinal microbiome of 6-week-old infants. *JAMA Pediatrics*, *170*(3), 212–219. https://doi.org/10.1001/jamapediatrics.2015.3732

Marcobal, A., Barboza, M., Sonnenburg, E. D., Pudlo, N., Martens, E. C., Desai, P., … Sonnenburg, J. L. (2011). Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. *Cell host & microbe*, *10*(5), 507–514. https://doi.org/10.1016/j.chom.2011.10.007

Martín, R., Heilig, G. H. J., Zoetendal, E. G., Smidt, H., & Rodríguez, J. M. (2007). Diversity of the Lactobacillus group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. *Journal of Applied Microbiology*, *103*(6), 2638–2644. https://doi.org/10.1111/j.1365-2672.2007.03497.x

Martín, R., Langa, S., Reviriego, C., Jimínez, E., Marín, M. L., Xaus, J., … Rodríguez, J. M. (2003). Human milk is a source of lactic acid bacteria for the infant gut. *The Journal of Pediatrics*, *143*(6), 754–758. https://doi.org/10.1016/j.jpeds.2003.09.028

McDermott, A. J., & Huffnagle, G. B. (2014). The microbiome and regulation of mucosal immunity. *Immunology*, *142*(1), 24–31. https://doi.org/10.1111/imm.12231

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., … Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME journal*, *6*(3), 610–618. https://doi.org/10.1038/ismej.2011.139

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, *8*(4), e61217.

Mueller, N. T., Bakacs, E., Combellick, J., Grigoryan, Z., & Dominguez-Bello, M. G. (2015). The infant microbiome development: Mom matters. *Trends in Molecular Medicine*, *21*(2), 109–117. https://doi.org/10.1016/j.molmed.2014.12.002

Myers, L. L., Shoop, D. S., Stackhouse, L. L., Newman, F. S., Flaherty, R. J., Letson, G. W., & Sack, R. B. (1987). Isolation of enterotoxigenic *Bacteroides fragilis* from humans with diarrhea. *Journal of Clinical Microbiology*, *25*(12), 2330–2333.

Okubadejo, O. A., Green, P. J., & Payne, D. J. (1973). Bacteroides infection among hospital patients. *British Medical Journal*, *2*(5860), 212–214.

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2 – approximately maximum—likelihood trees for large alignments. *PLoS One*, *5*(3), e9490. https://doi.org/10.1371/journal.pone.0009490

Quigley, L., O'Sullivan, O., Beresford, T. P., Paul Ross, R., Fitzgerald, G. F., & Cotter, P. D. (2012). A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese. *Journal of Applied Microbiology*, *113*(1), 96–105. https://doi.org/10.1111/j.1365-2672.2012.05294.x

R Core Developmental Team (2014). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Ramsay, D. T., Kent, J. C., Owens, R. A., & Hartmann, P. E. (2004). Ultrasound imaging of milk ejection in the breast of lactating women. *Pediatrics*, *113*(2), 361–367.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., … Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology*, *2*, 361–367. https://doi.org/10.1038/86373

Rodríguez, J. M. (2014). The origin of human milk bacteria: Is there a bacterial entero-mammary pathway during late pregnancy and lactation? *Advances in Nutrition*, *5*(6), 779–784. https://doi.org/10.3945/an.114.007229

Rosen, M. J., Davison, M., Bhaya, D., & Fisher, D. S. (2015). Microbial diversity. Fine-scale diversity and extensive recombination in a quasisexual bacterial population occupying a broad niche. *Science*, *348*(6238), 1019–1023. https://doi.org/10.1126/science.aaa4456

Russo, J., & Russo, I. H. (1987). Development of the human mammary gland. In M. C. Neville, & C. W. Daniel (Eds.), *The Mammary Gland* (pp. 67–93). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4899-5043-7_3

Sears, C. L., Islam, S., Saha, A., Arjumand, M., Alam, N. H., Faruque, A. S. G., … Qadri, F. (2008). Association of enterotoxigenic *Bacteroides fragilis* infection with inflammatory diarrhea. *Clinical Infectious Disease*, *47*(6), 797–803. https://doi.org/10.1086/591130

Stumpf, R. M., Wilson, B. A., Rivera, A., Yildirim, S., Yeoman, C. J., Polk, J. D., … Leigh, S. R. (2013). The primate vaginal microbiome: Comparative context and implications for human health and disease. *American Journal of Physical Anthropology*, *152*(Suppl. 57), 119–134. https://doi.org/10.1002/ajpa.22395

Tikhonov, M., Leach, R. W., & Wingreen, N. S. (2015). Interpreting 16S metagenomic data without clustering to achieve sub-OTU resolution. *The ISME journal*, *9*(1), 68–80. https://doi.org/10.1038/ismej.2014.117

Tung, J., Barreiro, L. B., Burns, M. B., Grenier, J. -C., Lynch, J., Grieneisen, L. E., … Archie, E. A. (2015). Social networks predict gut microbiome composition in wild baboons. *eLife*, *4*, e05224. https://doi.org/10.7554/eLife.05224

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The human microbiome project. *Nature*, *449*(7164), 804–810. https://doi.org/10.1038/nature06244

Wambach, K., & Riordan, J. (2014). *Breastfeeding and Human Lactation*. Jones & Bartlett Learning.

West, P. A., Hewitt, J. H., & Murphy, O. M. (1979). The influence of methods of collection and storage on the bacteriology of human milk. *The Journal of Applied Bacteriology*, *46*(2), 269–277. https://doi.org/10.1111/j.1365-2672.1979.tb00820.x

Yang, B., Wang, Y., & Qian, P. -Y. (2016). Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analyses. *BMC Bioinformatics*, *17*(1), 135. https://doi.org/10.1186/s12859-016-0992-y

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227. https://doi.org/10.1038/nature11053

Yildirim, S., Yeoman, C. J., Janga, S. C., Thomas, S. M., Ho, M., Leigh, S. R., … Stumpf. (2014). Primate vaginal microbiomes exhibit species specificity without universal Lactobacillus dominance. *The ISME journal*, *8*(12), 2431–2444. https://doi.org/10.1038/ismej.2014.90

How to cite this article: Petrullo L, Jorgensen MJ, Snyder-Mackler N, Lu A. Composition and stability of the vervet monkey milk microbiome. *Am J Primatol*. 2019;81:e22982. https://doi.org/10.1002/ajp.22982