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RESEARCH ARTICLE

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Diversity and temporal dynamics of primate milk microbiomes

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Abstract

Milk is inhabited by a community of bacteria and is one of the first postnatal sources of microbial exposure for mammalian young. Bacteria in breast milk may enhance immune development, improve intestinal health, and stimulate the gut-brain axis for infants. Variation in milk microbiome structure (e.g., operational taxonomic unit [OTU] diversity, community composition) may lead to different infant developmental outcomes. Milk microbiome structure may depend on evolutionary processes acting at the host species level and ecological processes occurring over lactation time, among others. We quantified milk microbiomes using 16S rRNA high-throughput sequencing for nine primate species and for six primate mothers sampled over lactation. Our data set included humans (*Homo sapiens*, Philippines and USA) and eight nonhuman primate species living in captivity (bonobo [*Pan paniscus*], chimpanzee [*Pan troglodytes*], western lowland gorilla [*Gorilla gorilla gorilla*], Bornean orangutan [*Pongo pygmaeus*], Sumatran orangutan [*Pongo abelii*], rhesus macaque [*Macaca mulatta*], owl monkey [*Aotus nancymaae*]) and in the wild (mantled howler monkey [*Alouatta palliata*]). For a subset of the data, we paired microbiome data with nutrient and hormone assay results to quantify the effect of milk chemistry on milk microbiomes. We detected a core primate milk microbiome of seven bacterial OTUs indicating a robust relationship between these bacteria and primate species. Milk microbiomes differed among primate species with rhesus macaques, humans and mantled howler monkeys having notably distinct milk microbiomes. Gross energy in milk from protein and fat explained some of the variations in microbiome composition among species. Microbiome composition changed in a predictable manner for three primate mothers over lactation time, suggesting that different bacterial communities may be selected for as the infant ages. Our results contribute to understanding ecological and evolutionary relationships between bacteria and primate hosts, which can have applied benefits for humans and endangered primates in our care.

KEYWORDS
bacteria, breast milk, infant, lactation, mammals, microbiota, symbiosis

1 | INTRODUCTION

Milk and lactation are ancient mammalian adaptations. Milk provides nutrition, immune factors, growth factors, hormones, and other bioactive molecules that serve to regulate and guide mammalian infant growth and development. Milk also contains numerous types of bacteria (Hunt et al., 2011), and may be an important source of bacteria for the infant gut (Ascinar et al., 2017; Heikkilä & Saris, 2013; Wang et al., 2017). The mammalian lineage contains 5,488 species (IUCN), in which we have characterized the milk microbiome for humans and 13 nonhuman species, primarily of agricultural importance (Tables S1 and S2). Mammals can nurse their young for as little as 4 days (hooded seals [*Cystophora cristata*]: Bowen, Boness, & Oftedal, 1987) to as long as 8.8 years (orangutans [*Pongo abelii* and *Pongo pygmaeus*]: Smith, Austin, Hinde, Vogel, & Arora, 2017), but only a few studies have examined differences in the bacterial communities in milk over lactation (e.g., Cabrera-Rubio et al., 2012; Chen et al., 2018; Hunt et al., 2011; McInnis, Kalanetra, Mills, & Maga, 2015). Determining how milk microbiomes differ among mammalian species and change as infants age elucidates how relationships between bacteria and host change over evolutionary and ecological time. Bacteria in breast milk can provide health benefits to developing infants (Allen-Blevins, Sela, & Hinde, 2015; Fernández et al., 2013; Martin & Sela, 2013). Understanding bacteria-host relationships in milk can inform strategies to manipulate the milk microbiome or to seed infant formula with beneficial bacteria. Such strategies offer the potential to reduce risks of health disorders for humans, and for captive assurance populations of endangered mammals.

Milk is colonized by a community of bacteria, a milk microbiome (Funkhouser & Bordenstein, 2013; Zivkovic, Lewis, German, & Mills, 2013). Microbes found in milk were previously thought to represent contamination from skin or the environment, or a sign of infection in the mammary gland (West, Hewitt, & Murphy, 1979). However, compelling evidence has shown that colostrum and breast milk contain a microbiome. For instance, milk has a distinct bacterial community from other maternal and infant body sites (Biagi et al., 2017; Pannaraj et al., 2017), diverse bacterial communities can still be recovered when using extreme sterile technique to collect samples (Metzger et al., 2018), and the bacterial community diminishes sharply after weaning (Fernández et al., 2013). Bacteria likely colonize breast milk through retrograde flow bringing infant saliva into the mammary gland (Cabrera-Rubio et al., 2012; Hunt et al., 2011) and/or through entero-mammary trafficking (EMT; Perez et al., 2007; Stagg, Hart, Knight, & Kamm, 2003). Colonization of breast milk through EMT can occur when intestinal bacteria are engulfed by dendritic cells and sent to the mammary gland through systemic circulation (Fernández et al., 2013; LaTuga, Stuebe & Seed, 2014). Milk is a continuous source of bacteria to the infant gut, which may be one of the aspects of milk that influences neonatal, infant and later-life health (Murphy et al., 2017).

The function of bacteria in breast milk may include enhanced immune development, improved intestinal health, and stimulation of the gut-brain axis, which have been identified primarily through research among human populations or in biomedical animal models (Allen-Blevins et al., 2015; LaTuga et al., 2014; Turnbaugh et al., 2006). Breastfed human infants consume approximately 10^5 to 10^7 bacteria daily (Heikkilä & Saris, 2013), and have a gut microbiome that differs from formula-fed infants (Bezirtzoglou, Tsiotsias, & Welling, 2011). Breastfed infants have increased immune response activity compared to formula-fed infants (Carver, Pimentel, Wiener, Lowell, & Barness, 1991, Stephens et al., 1986), which may be mediated by bacterial ligands triggering immune cell proliferation (Spörri & Reis e Sousa, 2005). However, the community of bacteria present in milk can determine their beneficial effect. For instance, allowing mouse pups of lean mothers to nurse from obese mothers results in the lean pups becoming vulnerable to obesity and metabolic disease (Oben et al., 2010), possibly by establishing an obesity-associated gut microbiome (Turnbaugh et al., 2006). Gut microbes affect brain chemistry and behavior (Bercik et al., 2011, Sylvia & Demas, 2018). Bacteria in milk are potential early colonizers of infants' guts, suggesting that the milk microbiome may indirectly modulate offspring physiology and behavior in the short- and long-term. While we are beginning to learn which host and environmental factors influence milk microbes, we know little about how milk microbiomes vary in nonhuman mammals.

Nonhuman primates offer an exceptional opportunity to investigate milk microbiomes across taxa because many can be trained to provide milk samples in captive populations. Approximately 60% of nonhuman primates are now threatened with extinction (Estrada et al., 2017), with many species having representatives in zoos. As primates diverged so did the nutritional composition of their milk (Goto et al., 2010; Hinde & Milligan, 2011) and the bacterial community composition of their skin (Council et al., 2016) and guts (Amato et al., 2015, Yildirim et al., 2010). These differences among species in (a) milk nutrient content and (b) potential sources of bacteria to seed breast milk may relate to variation in bacterial communities found in primate milk. Collectively, we might predict differences in the milk microbiomes among primate hosts, including humans, which could reflect both the recent shifts in human hygiene and diet and more ancient divergences in the biology of milk over evolutionary time.

We quantified the roles of host species, infant age, and nutrient/hormone content on determining milk microbiome diversity and composition (i.e., microbiome structure) in nine species from seven genera of primarily captive primates. We had three main objectives. Our first objective was to compare milk microbiomes of nine primate species to identify: (a) bacterial taxa that are shared among all primate species (the core primate milk microbiome) and (b) microbiome patterns that are unique to each species. Our second objective was to characterize the milk microbiomes of six primate mothers from two primate species longitudinally (western lowland gorillas [*Gorilla gorilla gorilla*] and Sumatran orangutan [*Pongo abelii*]) to determine the effect of time on bacterial community turnover. Our third objective was to determine the relationship between nutrient content or hormone profiles in milk and microbiome structure.

Quantifying primate milk microbiomes contributes to our understanding of coevolutionary relationships between bacteria and host, which can also have applied benefits for humans and mammals in our care.

2 | METHODS

2.1 | Sample collection

We used primate milk samples archived in the mammalian Milk Repository at the Smithsonian National Zoological Park's (NZP) Nutrition Department. We focused on nine species of primates that span catarrhines to platyrrhines primates to characterize milk microbiome structure of mature milk among the nine species (Table 1) and over lactation for two species (Table 2). We define mature milk as milk from established lactation, which is after the colostrum/transitional milk stage and before the weaning stage. The amount of milk collected from a female ranged from 1 ml in owl monkeys (*Aotus nancymaae*) to 40+ ml in rhesus macaques (*Macaca mulatta*; details below). Our research abided by the ASP Principles for Ethical Treatments of Nonhuman Primates and all of the laws of the relevant countries.

Milk samples for nonhuman primates were collected either voluntarily or while under anesthesia. Samples were collected with approved protocols from NZP IACUC and Zoo Atlanta Scientific Review Committee for western lowland gorillas, Bornean orangutans (*Pongo pygmaeus*), and Sumatran orangutans (Garcia, Power, & Moyes, 2017; Power et al., 2017) and UCLA and UC Davis IACUCs for rhesus macaques (*Macaca mulatta*; Hinde, Power, & Oftedal, 2009). Bonobo (*Pan paniscus*), chimpanzee (*Pan troglodytes*), and owl monkey (*Aotus nancymaae*) samples were collected as part of standard management procedures in accordance with institutional guidelines for care and use of animals at their respective facilities (see Table 1). Rhesus macaque milk samples were collected midday (11:30-13:00) between 3 and 4 months postpartum after a standardized 3.5 to 4 hr period of milk accumulation using manual expression and full mammary evacuation (range 4–40+ ml). For all other captive nonhuman primates, milk samples were collected from individuals that (a) allowed keepers to collect milk via hand stripping the nipple or by breast pump or (b) were administered exogenous oxytocin and hand stripped while under anesthesia for another procedure. Mantled howler monkeys (*Alouatta palliate*) were sampled in the wild as outlined in Glander (1992) in Hacienda La Pacifica, Guanacaste Province, Costa Rica, and the date of parturition was estimated based on infant size by field researchers. Nonhuman primate samples were typically stored on ice immediately following collection, then remained frozen until they were shipped on dry ice to NZP's Nutrition Lab where they were aliquoted into cryovials and stored at −80°C until microbial analyses.

Human milk samples were collected from female participants in the Cebu Longitudinal Health and Nutrition Study, with IRB approval from Northwestern University and the University of San Carlos (USC), Philippines (Miller et al., 2013; Quinn, Largado, Power, & Kuzawa, 2012). Milk samples were collected between 6 a.m. and 10 a.m. by manual expression of 10 ml of milk after 2 minutes of active suckling by the infant following protocols described elsewhere (Miller et al., 2013; Quinn et al., 2012). Samples were transported on cold packs to the laboratory at USC where they were frozen at −20°C until they could be shipped back to the United States for analysis. The samples from the USA human subjects were donated to the Smithsonian National Zoological Park's Milk Repository by the individuals.

2.2 | Molecular methods

We extracted DNA from 100 µl of milk using the Qiagen BioSprint 96 One-For-All Vet kit following the manufacturer's instructions (sample $n = 175$), and included a negative extraction control with each set of sample extractions. We prepared 16S rRNA meta-barcoding libraries for each sample and for negative extraction and negative PCR

TABLE 1 Nine species comparison data set

Common name	Scientific name	Family	Sample size	Infant age range (DPP)	Location
Human	*Homo sapiens*	Hominidae	28	9–328	Cebu, Philippines; Maryland & New York, USA
Bonobo	*Pan paniscus*	Hominidae	2	126–162	Milwaukee Zoo
Chimpanzee	*Pan troglodytes*	Hominidae	2	1558–1755	St. Louis Zoo; Kansas City Zoo
Western lowland gorilla*	*Gorilla gorilla gorilla*	Hominidae	9	26–993	National Zoo; Zoo Atlanta; Columbus Zoo; Philadelphia Zoo; Buffalo Zoo
Bornean orangutan*	*Pongo pygmaeus*	Hominidae	5	7–1182	Zoo Atlanta; Toledo Zoo; Brookfield Zoo
Sumatran orangutan*	*Pongo abelii*	Hominidae	2	153–175	Zoo Atlanta; Fresno Chaffee Zoo
Rhesus macaque*	*Macaca mulatta*	Cercopithecidae	32	91–123	California National Primate Research Center
Mantled howler monkey*	*Alouatta palliata*	Atelidae	6	30–180	Costa Rica (wild)
Owl monkey*	*Aotus nancymaae*	Atelidae	2	45–85	Keeling Center for Comparative Medicine and Research, TX

Note: Species for which we had nutritional metadata for are indicated with a *. For humans, we had hormone data for the Philippines population.

TABLE 2 The two primate species longitudinal data set

Common name	Scientific name	Name	Sample size	Infant age range (DPP)	Location
Western lowland gorilla	*Gorilla gorilla gorilla*	Mandara	33	26–1702	National Zoo
Western lowland gorilla	*Gorilla gorilla gorilla*	Kuchi	15	993–1412	Zoo Atlanta
Western lowland gorilla	*Gorilla gorilla gorilla*	Lulu	14	36–396	Zoo Atlanta
Western lowland gorilla	*Gorilla gorilla gorilla*	Sukari	6	49–139	Zoo Atlanta
Sumatran orangutan	*Pongo abelii*	Sara	8	175–309	Fresno Chaffee Zoo
Sumatran orangutan	*Pongo abelii*	Blaze	5	199–430	Zoo Atlanta

controls with fusion primers (515F and 939R: V3–V5 region) and pooled cleaned libraries in equimolar ratios following the methods outlined in Muletz Wolz, Yarwood, Campbell Grant, Fleischer, and Lips (2017). We sequenced libraries on two Roche 454 FLX+runs at the Smithsonian Conservation Biology Institute-Center for Conservation Genomics. We used MacQIIME 1.9.1 (Caporaso et al., 2010) and UPARSE (Edgar, 2013) to quality-filter and process the 454 reads following Muletz Wolz et al. (2017).

We define operational taxonomic units (OTUs) as taxa whose DNA sequences match at ≥97% similarity. We removed OTUs that were present in all extraction and PCR controls ($n = 11$). We removed samples that had fewer than 600 reads (sample $n = 12$), with a final data set consisting of 163 samples.

2.3 | Data overview

We provide a general descriptive analysis of the bacterial OTUs identified from the nine primate species, including a description of a core primate milk microbiome (present in >80% of samples, $n = 163$). Then, we examined milk microbiome structure (alpha and beta diversity) in two sections of the data: (a) a primate species comparison data set and, (b) a longitudinal data set. The species comparison data set consisted of nine primate species ($n = 88$), including eight species with captive representatives and one species with wild representatives (Table 1). Maggie and Miri, two Bornean orangutans, were represented twice as we considered their two separate pregnancies as statistically independent (lactation samples collected in 1977 and 1989 [Maggie] and 2003 and in 2013 [Miri]). We verified that even if we exclude the earlier replicate samples of Miri and Maggie from our analyses, the results remain the same as reported below. For a subset of these samples (Table 1), we had additional metadata: nutritional for 45 individuals from five of the species and hormonal for 26 individuals from two human populations (more information in next subsection). The longitudinal data set consisted of four captive western lowland gorilla mothers and two captive Sumatran orangutan mothers that were sampled at least five times over the course of lactation (Table 2). In this data set, samples for some mothers represent shorter time periods of lactation (shortest = 90 days for Sukari), while others span the entire range of lactation during the mature milk stage (longest = 1,702 days for Mandara; Table 2). For a subset of this longitudinal data set, we had nutrient assay results for two western lowland gorilla mothers, Mandara ($n = 15$) and Sarah ($n = 8$; more information in next subsection). Gorilla and Sumatran orangutan samples were included in both datasets; we randomly selected a time point from the longitudinal data set to use in the species comparison data set to avoid pseudoreplication.

2.4 | Nutrient and hormone characterization of milk

We leveraged existing metadata of milk nutrient composition, energetic density, and hormone concentration from previous research (Anderson et al., 2016; Garcia et al., 2017; Hinde et al., 2009; Power et al., 2017; Quinn et al., 2012). We previously assayed a subset of samples using standard nutrient composition methods to calculate % crude protein, % total sugar, and % crude fat (Garcia et al., 2017; Hinde et al., 2009; Power et al., 2017). For the species comparison data set, we had nutrient assay data available for five species (sample $n = 45$; Table 1). For the data derived from longitudinal milk sampling (Table 2), we had nutrient assay results for two mothers, Mandara ($n = 15$) and Sarah ($n = 8$; Garcia et al., 2017; Power et al., 2017). Gross energy (GE) was calculated for each milk sample using the formula: GE = (9.11 kcal/g * % fat + 5.86 kcal/g * % crude protein + 3.95 kcal/g * % sugar)/100 (Hinde et al., 2009; Petzinger et al., 2014; Power, Watts, Murtough, & Knight, 2018). We determined the GE from each of the three nutrients (crude protein, total sugar, and crude fat) by dividing the percent of that nutrient by GE and then multiplying by 1,000 to calculate GE in mg/kcal. We hereafter refer to the GE of each nutrient in mg/kcal as protein GE, sugar GE, and fat GE. We previously characterized hormone concentrations in breast milk from 26 human mothers from rural and urban Cebu, Philippines (Anderson et al., 2016, Quinn, Largado, Borja, & Kuzawa, 2014). Two hormones (leptin, adiponectin) were assayed using standard procedures as outlined in Quinn et al. (2014) and Anderson et al. (2016); secretory immunoglobulin A (sIgA) data were also available from unpublished research.

2.5 | Statistical analyses

All statistical analyses were performed in R version 3.4.1 (R Core Team, 2018). We performed variance-stabilizing normalization (Muletz Wolz et al., 2017) on the raw sequence counts, which corrects for biases associated with uneven sequencing depth for

alpha and beta diversity analyses (McMurdie & Holmes, 2014; Paulson, Stine, Bravo, & Pop, 2013; Weiss et al., 2017).

We quantified the milk microbiome structure for alpha and beta diversity among nine primate species (Table 1). For alpha diversity, we used the Kruskal–Wallis test (the data were not normally distributed) to determine if OTU richness differed among species. We performed post hoc analyses with a Dunn Test using Bonferroni corrections for multiple comparisons in the package "FSA" (Ogle, 2018). For beta diversity, we computed Jaccard and Bray–Curtis distances and used a PERMANOVA to determine if community composition differed among species using the function *procD.lm* and then using the function *advanced.procD.lm* for post hoc analyses in the package "geomorph" (Adams, Collyer, & Kaliontzopoulou, 2018). We used principal coordinate analysis to visualize beta diversity patterns using "phyloSeq" and "ggplot2" packages (McMurdie & Holmes, 2013, Wickham, 2016). Because human cultural/hygiene practices can influence microbiomes (Flerer, Hamady, Lauber, & Knight, 2008; Ramanan et al., 2016), we first compared microbiome structure among the three human population samples (urban Philippines [Cebu City], rural Philippines [Cebu], and urban USA [MD/NY]).

We quantified the milk microbiome structure among six primate mothers sampled over time (Table 2). For alpha diversity, we used a linear regression model for each primate mother to determine if OTU richness was correlated with time (i.e., infant age in days). For beta diversity, we computed Mantel correlations between compositional dissimilarity matrices (Jaccard and Bray–Curtis) and a time distance matrix of Euclidean distances using 10,000 permutations in the package "vegan" (Okasanen et al., 2018) for each mother. We determined if community composition (beta diversity) differed between the two primate species using a PERMANOVA (function *procD.lm* in the package "geomorph" [Adams et al., 2018) and corrected for pseudoreplication by specifying individual ID a random effect.

For quantitative measurements (nutrient and hormone content), we examined associations of these factors with bacterial community composition using distance-based linear modeling (function *capscale* in the package "vegan") with stepwise AIC (Akaike information criterion, function *step* in the package "stats"; Kueneman et al., 2014, Muletz Wolz et al., 2017). We built four separate models: two models for nutrient analysis (protein GE, sugar GE, and fat GE as explanatory variables) comparing differences in bacterial composition (Jaccard or Bray–Curtis as the response variable in two separate models) among five species of primates, and two models for hormone analysis (sIgA, leptin, adiponectin as explanatory variables) comparing differences in bacterial composition (Jaccard and Bray–Curtis as the response variable in two separate models) among two populations of humans from rural and urban Cebu, Philippines. For quantitative variables that were significant, we determined their effect on OTU relative abundance. We used the package "DAtest" to first filter low abundance OTUs (present in <10 samples) using the function *preDA* and then rank various statistical methods used to test for differential abundance (Russel et al., 2018). We input raw sequence counts and each statistical method performed its default transformation of the data using the function *testDA*. We used the differential abundance test that had the highest DAtest score following guidelines by Russel et al. (2018). The DAtest score ranks how well each differential abundance test performs on your data based on the area under the curve, false positive rate, and false discovery rate.

3 | RESULTS

After quality filtering, we had 589,876 high-quality bacterial sequences (314 bp average length) from 165 primate milk samples representing 1,752 OTUs from 27 described bacterial phyla and one archaeal phyla (Table S3). Five phyla were the most abundant across samples (Figure 1; Table S3) and were represented by multiple OTUs (Firmicutes: 42.2% mean relative abundance, $n = 611$ OTUs; Proteobacteria: 31.6%, $n = 282$ OTUs; Bacteroidetes: 11.6%, $n = 311$ OTUs, Actinobacteria: 10%, $n = 215$ OTUs, and Cyanobacteria: 2.3%, $n = 39$ OTUs). Common genera (>1% relative abundance) detected in the milk of each primate species are listed in Table S4. Seven OTUs made up the core primate milk microbiome (found in 80% of samples; Table 3) and included four OTUs in the Firmicutes phylum (*Staphylococcus*, *Streptococcus*, and Granulicatella *spp.*), two OTUs in the Proteobacteria phylum (*Acinetobacter lwoffii* and *Acinetobacter johnsonii*) and one Actinobacteria OTU in the *Kocuria* genus. All of these bacterial genera have been detected in milk microbiomes from at least one other mammalian species (Table S2).

3.1 | Primate species differ in milk microbiomes

Milk microbiomes differed among primate species (Figures 2,3), with rhesus macaques, mantled howler monkeys, and humans having notably distinct milk microbiomes. Bacterial OTU richness differed among primate species (Figure 2; Kruskal–Wallis $X^2 = 64.5$, $df = 8$; $p < .001$), with rhesus macaques, chimpanzees, and gorillas having a higher number of bacterial OTUs in their milk compared with humans (Dunn test, pairwise $p < .05$). Rhesus macaques also had higher OTU richness than mantled howler monkeys (pairwise $p = .013$). Bacterial community composition differed among primate species (Figure 3, Jaccard: PERMANOVA, Pseudo $F = 5.21$, $df = 8$, $R^2 = 34.5\%$; $p = 0.001$; Bray–Curtis PERMANOVA, Pseudo $F = 6.31$, $df = 8$, $R^2 = 39.0\%$; $p = .001$). Rhesus macaques, mantled howler monkeys, and humans differed from one another and all other species (pairwise $p < .05$ for both Jaccard and Bray–Curtis distance), except humans and mantled howler monkeys did not differ from Sumatran orangutans (which likely reflects a low sample size [$n = 2$] for Sumatran orangutans). Human populations (urban Philippines, rural Philippines, and urban USA) did not differ in OTU richness (ANOVA, $p > .05$) or in community composition (Jaccard PERMANOVA, $p > .05$; Bray–Curtis PERMANOVA, $p > .05$), and were pooled together to increase statistical power in the above analyses. Hormone or sIgA content in milk did not predict bacterial community composition in human milk (distance-based linear model, $p > .05$).

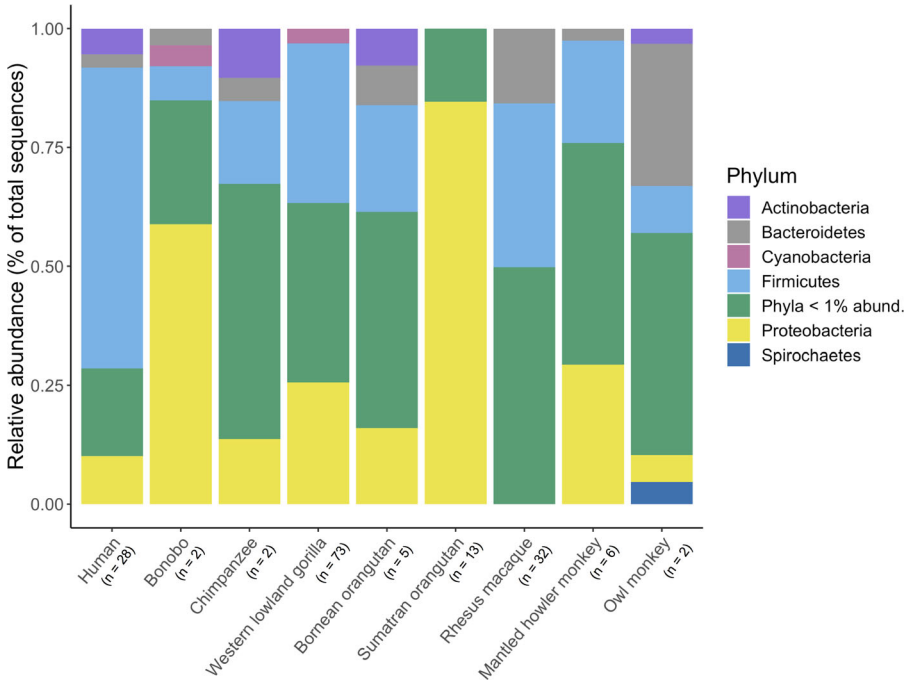

FIGURE 1 Stacked bar plot of the relative abundance of dominant bacterial phyla across primate species. Phyla that were represented by <1% average relative abundance per species were pooled together and shown as one bar. Sample size are shown under each primate species. Western lowland gorillas and Bornean and Sumatran orangutans include replicate sampling over time for some individuals

TABLE 3 Core microbiome present in 80% of primate milk samples (nine primate species; sample $n = 163$)

OTU ID	Phylum	Order	Genus	Species	Avg. RA	SD RA
OTU_1	Firmicutes	Bacillales	*Staphylococcus*	–	31.5%	25.0%
OTU_1117	Firmicutes	Lactobacillales	*Streptococcus*	–	23.2%	21.3%
OTU_31	Firmicutes	Lactobacillales	*Streptococcus*	–	14.0%	15.5%
OTU_41	Proteobacteria	Pseudomonadales	*Acinetobacter*	*johnsonii*	13.1%	19.8%
OTU_21	Proteobacteria	Pseudomonadales	*Acinetobacter*	*lwoffii*	9.8%	13.6%
OTU_58	Actinobacteria	Actinomycetales	*Kocuria*	–	5.1%	7.3%
OTU_43	Firmicutes	Lactobacillales	*Granulicatella*	–	3.3%	3.6%

Note: Average relative abundance (RA) per individual and standard deviation are reported.
Abbreviations: OTU, operational taxonomic unit; RA, relative abundance.

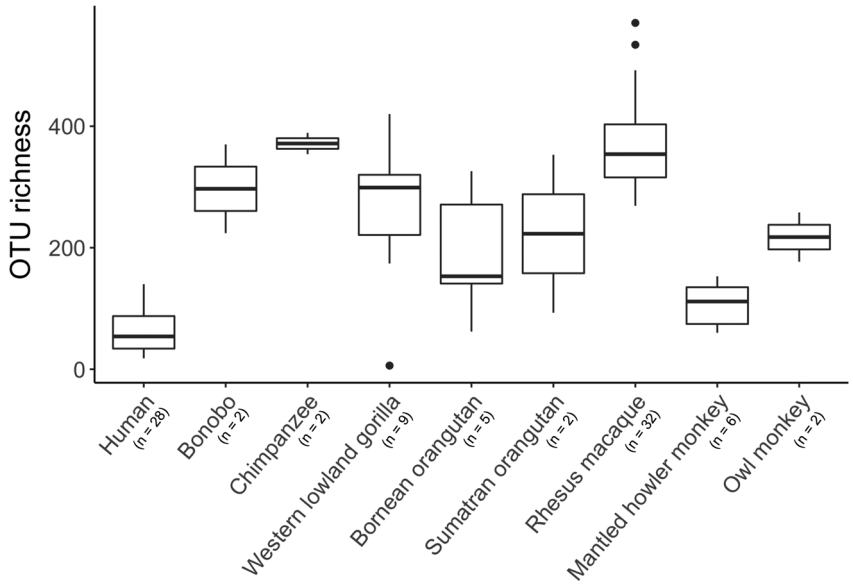

FIGURE 2 Boxplot of the number of operational taxonomic units (OTUs) found in milk among nine species of primates. Rhesus macaques, chimpanzees and gorillas had greater OTU richness compared with humans (pairwise $p < .05$). And rhesus macaques had greater OTU richness than mantled howler monkeys (pairwise $p = .011$). Sample size is shown under each primate species

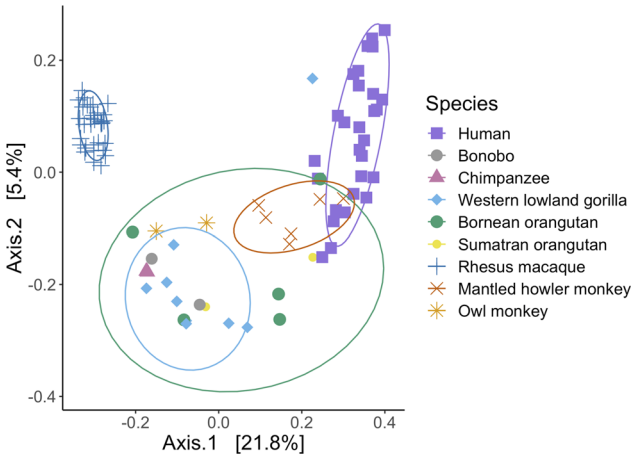

FIGURE 3 Principal coordinate analysis of bacterial community composition (beta diversity, Jaccard distances) in milk from nine species of primates. Rhesus macaques, mantled howler monkeys, and humans differed from all other species (pairwise $p < .05$), except humans and mantled howler monkeys did not differ from Sumatran orangutans. 95% confidence ellipses are shown for species with >2 samples

Nutrient content of milk explained some of the variation in milk microbiomes among primate species (Figure 4). We found that protein GE and fat GE, but not sugar GE, were significant predictors of bacterial community composition for both presence–absence composition (Jaccard: distance-based linear model, AIC 109.97, $p < .02$), and for abundance-weighted composition (Bray–Curtis: distance-based linear model, AIC $p < .02$) explaining 17.3% and 24.7% of overall variation, respectively. Rhesus macaques had higher fat GE and lower protein GE, while mantled howler monkeys had lower fat GE and higher protein GE, which were associated with variation in bacterial community composition (Figure 4). For fat GE, 10 OTUs decreased in relative abundance as fat GE increased, whereas 144 OTUs increased with increasing fat GE (Table S5; log LIMMA 2, $p < .05$). For protein, 19 OTUs decreased in relative abundance as protein GE increased, whereas 6 OTUs increased with increasing protein GE (Table S6; log LIMMA 2, $p < .05$). Notable were (a) increases in nine *Lactobacillus* OTUs with increasing fat, but decreases in four of those OTUs with increasing protein and (b) increases in 19 *Prevotella* OTUs with increasing fat, but decreases in six of those OTUs with increasing protein.

3.2 | Milk microbiomes change over time

Milk microbiomes showed variable patterns in the number of bacterial taxa in milk over time but showed a more consistent pattern of bacterial community composition changing over time. OTU richness did not show a predictable pattern for any primate mother measured over time (Figure S1). Three of six primate mothers (all western lowland gorillas) showed a predictable change in bacterial community composition in their milk as their infants aged (Figure 5). For Mandara, Kuchi, and Lulu, bacterial composition became increasingly dissimilar with time (Jaccard Mantel: $p = .009, .08, .001$, $R^2 = 29.7\%, 24\%, 57.5\%$; Bray Mantel: $p = .019, .10, .001$, $R^2 = 24.4\%, 19.9\%, 57.4\%$, presented in respective order). Figure 6 illustrates the change in the relative abundance of dominant bacterial phyla in milk microbiome over time for the six mothers. Changes in nutrient content over time did not explain the turnover in the milk microbiome for Mandara (distance-based linear model, $p > .05$; the sole mother that showed a relationship and for which we also had nutrient content metadata). Even with variation over time, bacterial community composition still differed between primate species (comparing western lowland gorillas and Sumatran orangutans), after correcting for pseudoreplication of individuals (Jaccard:

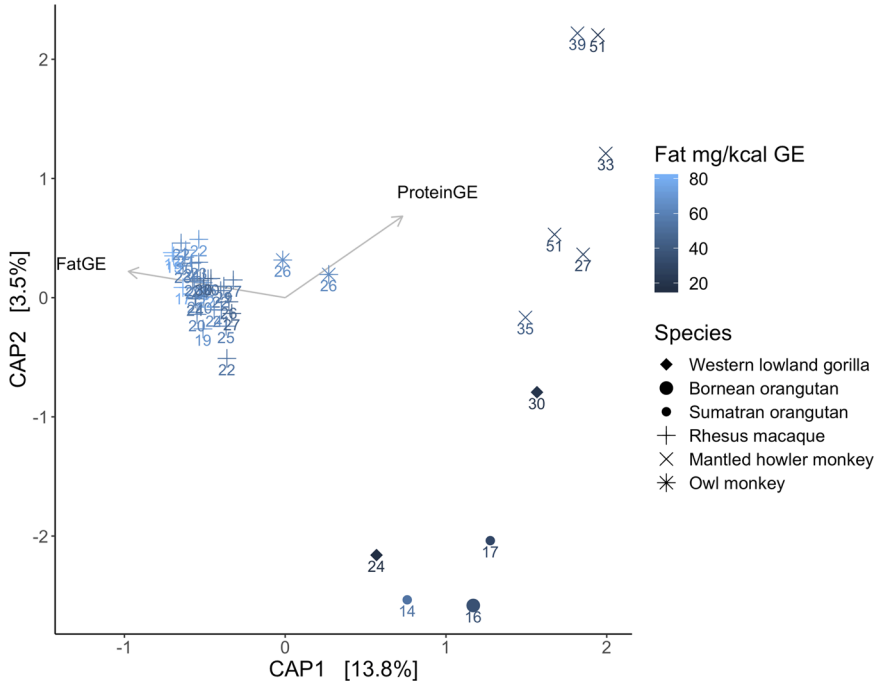

FIGURE 4 Constrained analysis of principal coordinates showing the relationship between the nutrient content of milk and microbiome composition. Protein GE and fat GE explained 17.3% of the variation in differences of microbiome composition among 45 individuals from six species (Jaccard distance). Sugar GE was not significant. Protein GE is labeled under each point. GE, gross energy

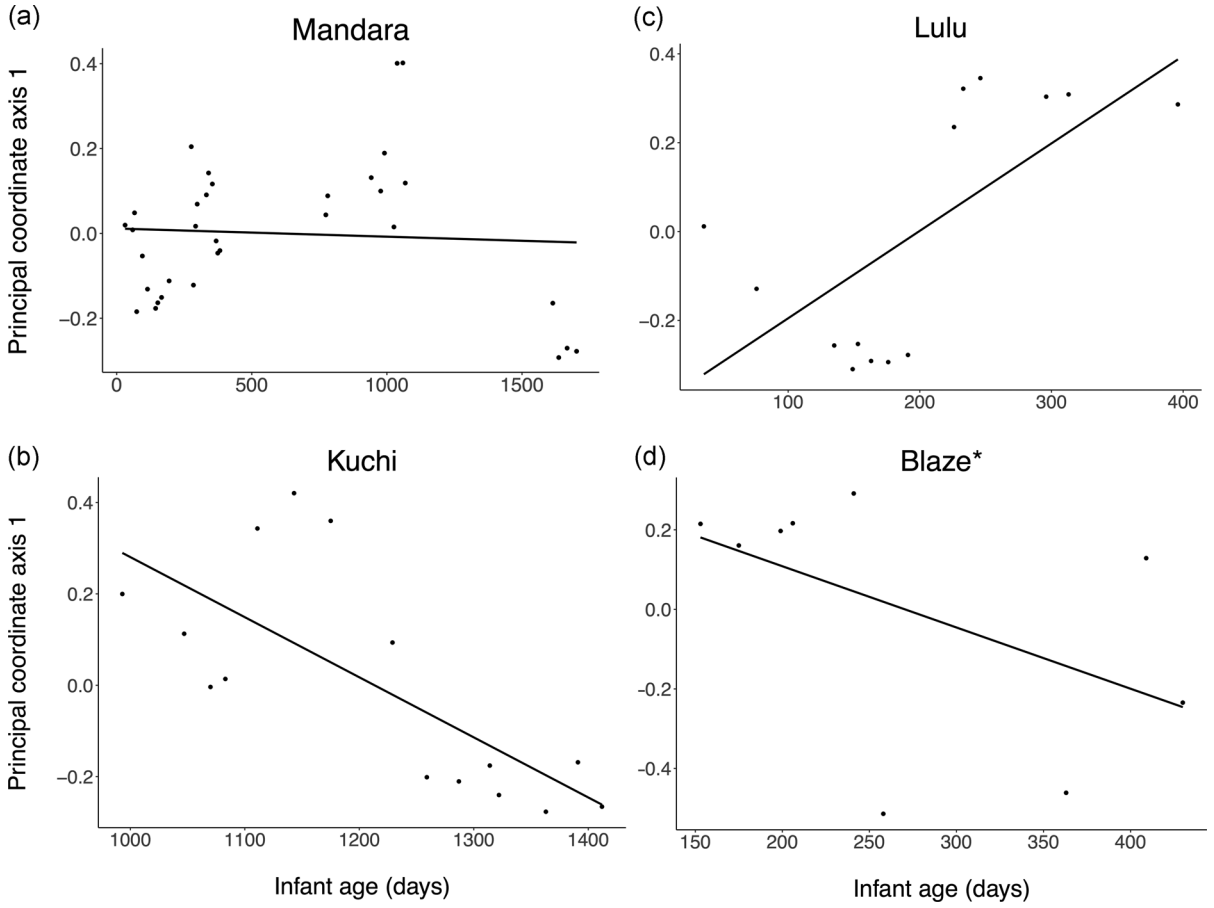

FIGURE 5 Relationship between infant age and bacterial community composition (represented with principal coordinate axis 1 from Jaccard distances). Three of six primate mothers Mandara, Kuchi, and Lulu (western lowland gorillas) showed an increasingly greater change in bacterial community composition in their milk as their infant aged (Mantel, $p < .05$), while Blaze, a Sumatran orangutan showed a trend (Mantel, $p = .07$). Samples collected in temporal proximity were generally more similar than those collected between more distant time points

PERMANOVA, Pseudo $F = 5.06$, $df = 1$, $R^2 = 5.5\%$, $p = .001$; Bray–Curtis PERMANOVA, Pseudo $F = 5.6$, $df = 1$, $R^2 = 5.8\%$, $p = .001$).

4 | DISCUSSION

Milk is the sole source of nutrition for mammalian neonates and represents one mechanism for bacterial inoculation of the infant gut (Ascinar et al., 2017; Funkhouser & Bordenstein, 2013; Wang et al., 2017). Bacteria in milk may aid in establishing a healthy infant gut microbiome from an early age, which may subsequently influence immune system development, intestinal health, and maturation of the gut-brain axis (Allen-Blevins et al., 2015; LaTuga et al., 2014; Turnbaugh et al., 2006). Host-bacterial relationships in milk are evolutionary ancient, likely predating the emergence of the mammalian lineage (Oftedal, 2012). In the ancestral mammalian lineage, milk was the earliest mechanism by which mothers interacted biochemically with their offspring, predating the placenta by more than 100 million years (Power & Schulkin, 2009). Our study shows a diverse and dynamic community of bacteria present in primate milk. We found that host biology and lactation timepoint are associated with the number of bacterial taxa and their composition in milk of primates. Our study serves as a foundational study on the relationships between bacteria and primate milk, which can guide future studies on primate milk microbiome ecology, evolution and the potential for applied use.

We detected a core milk microbiome (present in >80% of samples and in all nine primate species) of seven bacterial OTUs indicating a robust relationship between these bacteria and primate species. Core microbiome bacteria were present in samples that represented both ecological time (i.e., lactation time) and evolutionary time (i.e., different primate species). Five OTUs belonged to bacterial genera (*Acinetobacter*, *Staphylococcus*, *Streptococcus*) that have been commonly reported to occur in milk from diverse mammalian lineages, suggesting that these bacteria-host relationships may be robust across mammals at least at the bacterial genus level. New molecular methods have been developed to improve the resolution of bacterial species- and strain-level variation, which can be used to recover fine-level diversity within microbiomes (Ascinar et al., 2017; Caro-Quintero & Ochman, 2015). Future work could use these methods to identify how these specific genera associate with different primate species, and if there is a cospeciation pattern similar to primate gut microbiomes (Moeller et al., 2016).

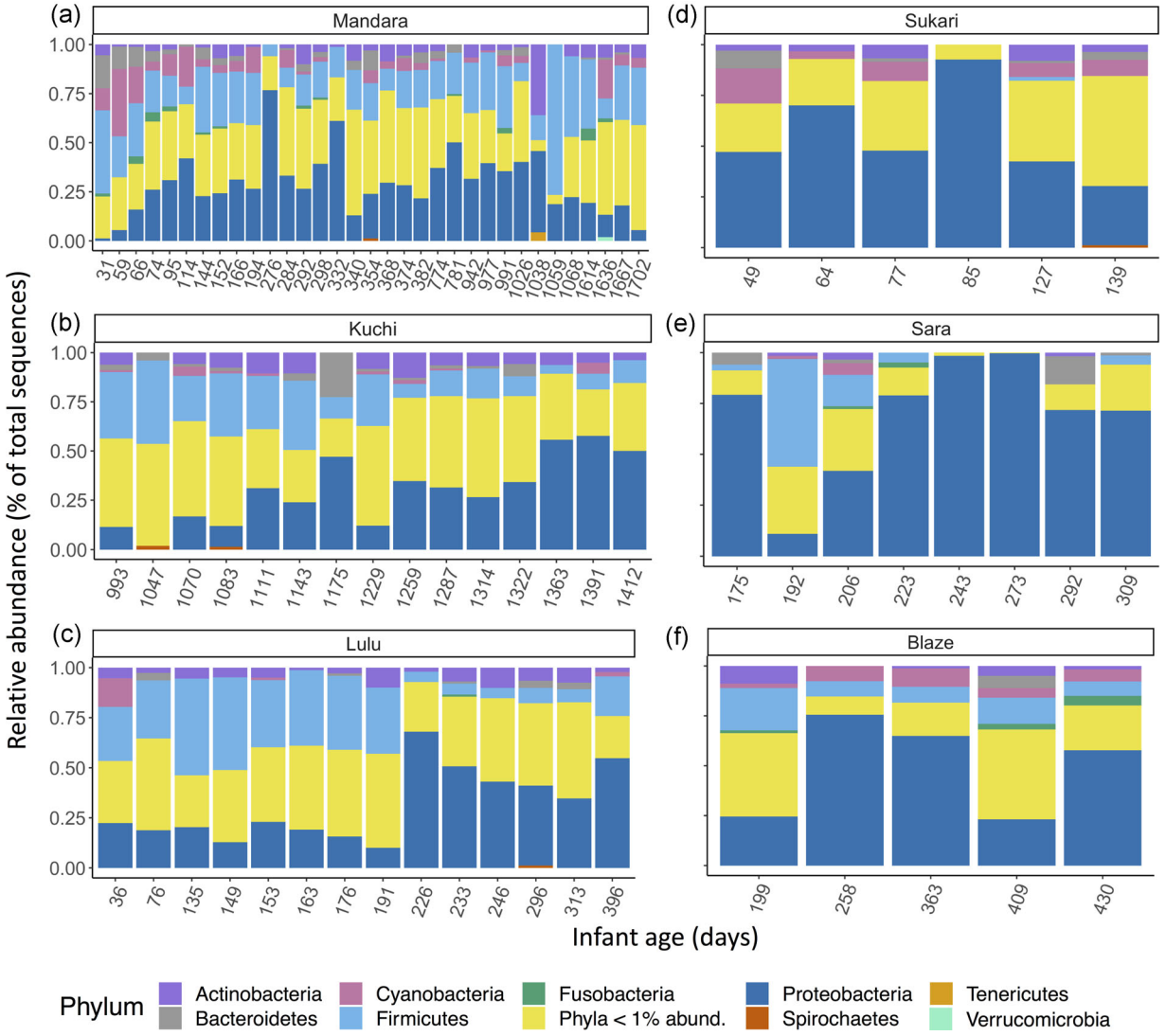

FIGURE 6 Stacked bar plot of the relative abundance of bacterial phyla for each primate mother. The relative abundance of major bacterial phyla changed over time, but not in a linear manner. (a–d) are four western lowland gorillas and (e,f) are two Sumatran orangutans. Phyla that were represented by <1% average relative abundance per sample were pooled together and shown as one bar

Across nine primate species, we found that some species, but not all, differed from one another in milk microbiome structure. Captive individuals of different species often consume a more similar diet to one another than their wild representatives, which may explain the similarity in milk microbiome composition that we observed for bonobos, chimpanzees, western lowland gorillas, Bornean orangutans, and owl monkeys. Yet, we found that bacterial community composition was distinct in humans, mantled howler monkeys and rhesus macaques from each other and all other primate species (except from Sumatran orangutans). Sumatran orangutans likely did not differ from mantled howler monkeys and humans due to small sample size, in which one of the two individuals sampled were similar to humans and mantled howler monkeys. With greater sampling, we hypothesize that Sumatran orangutans would differ from those three primate species. Humans also had the lowest bacterial richness in their milk compared to nonhuman primates. Humans likely differ from nonhuman primate in bacterial richness and composition given distinct hygiene and cultural practices that have changed our microbiomes from our ancestral state (Clemente et al., 2015; Schnorr et al., 2014). Mantled howler monkeys were the one wild primate species that we sampled; the pressures of living in the wild through variable seasons and habitats (Amato et al., 2015) may explain their unique microbiome. Rhesus macaques were the only captive primate species for which human contact is highly limited, given the concern of disease transmission (Gardner & Luciw, 2008), and this limited contact with humans may relate to the stark difference in milk microbiome composition we observed. Microbiome structure is affected by a myriad of ecological and evolutionary processes (e.g., Groussin et al., 2017), that can lead to certain host species differing from one another, while others do not. Since diet, social context, and sample size may all be playing a role—or having a synthetic effect—we are cautious about speculating too greatly.

Milk microbiomes might differ among primate hosts as a function of their evolutionary dissimilarity, whether as a result of drift or

selection on host traits that influence which bacteria colonize (Clayton et al., 2018; Council et al., 2016). However, we found no indication of differences in microbiome composition paralleling evolutionary changes in the host, which is often observed for gut microbiomes of both wild and captive animal populations, including wild primates (Brooks, Kohl, Brucker, van Opstal, & Bordenstein, 2016; Ochman et al., 2010). One hypothesis for this trend could be that host species traits are selecting for certain bacteria to colonize the milk, but that the selective host trait(s) are not diverging in a similar way as neutral gene markers (Perelman et al., 2011). Our study is the first characterization of primate milk microbiomes among host species. With greater sampling within and among primate species, we can improve our understanding of the impact primate evolutionary history has on milk microbiome evolution.

Within primate species, we found consistent species-level signatures of milk microbiome structure. For instance, we found that individuals of the same nonhuman primate species, even if they were sampled at different facilities or at different time points (e.g., western lowland gorillas), were generally more similar to one another in microbiome composition than to other primate species. Similarly, the three populations of humans (rural Phillipines, urban Phillipines, urban US) did not differ from one another in bacterial richness or composition. This is in contrast to human gut microbiomes, which often differ between geographic regions (Fujio-Vejar et al., 2017; Gupta, Paul, & Dutta, 2017; Pasolli et al., 2019; Suzuki & Worobey, 2014; Yatsunenko et al., 2012). Milk may be a more specific niche than the gut, as it appears that milk is enriched in particular bacterial taxa from the gut and suppressed in others (Ascinar et al., 2017; Jin, Hinde, & Tao, 2011). Only specific bacteria may be trafficked to the mammary glands by EMT or colonize milk from infant retrograde flow, which may be a conserved evolutionary pathway regardless of population origin (Klein et al., 2017; 2018).

Milk nutrient content can vary tremendously among species (Oftedal & Iverson, 1995) and is largely a function of evolutionary history, maternal diet and duration of milk production (Skibiel, Downing, Orr, & Hodd, 2013). We found that fat GE and protein GE, but not sugar GE in milk explained some of the variation among individuals of five primate species. Notably, rhesus macaques and mantled howler monkeys had more dissimilar fat and protein content as well as more dissimilar microbiomes. Nutrient content in milk likely favors colonization and/or proliferation of certain bacteria. We found similar patterns in certain bacterial genera changing with nutrient content as in suid milk (Chen et al., 2018); *Prevotella* and *Lactobacillus* spp. were positively correlated with fat content, but negatively correlated with protein content. Interestingly, *Prevotella* has been found to decrease in abundance in people who shift diets from vegetarian to solely animal-based foods (David et al., 2014), highlighting a strong relationship between *Prevotella* abundance and protein in diverse microbial habitats (e.g, milk and guts). Identifying the relationships between environmental characteristics and OTU abundance is especially useful for the development of probiotics as certain microbes will be ineffective in habitats that do not meet their nutritional requirements (Bashan et al., 2016).

We provide one of the most comprehensive views of milk microbiome change over time, particularly in three mothers (western lowland gorillas: Mandara, Kuchi, and Lulu). Other studies have examined change over lactation in microbiome composition, but with either fewer sampling time points or in a more narrow window of time (Cabrera-Rubio et al., 2012; Chen et al., 2018; Hunt et al., 2011; McInnis et al., 2015). Those studies as a whole have revealed the microbiome change is reasonably complex (Cabrera-Rubio et al., 2012; Hunt et al., 2011), with the most predictable and pronounced changes occurring during the transition from colostrum to mature milk (Chen et al., 2018), and the weaning lactation (McInnis et al., 2015). We sampled western lowland gorillas and Sumatran orangutans during the mature milk stage and found a bacterial community that changed in the number of bacterial taxa and composition over time. Our comprehensive sampling demonstrates that even during nontransitional time periods, the mature milk microbiome is dynamically variable. Bacterial samples taken more closely in time were more similar than those from more distant time points. This indicates that the bacterial community was gradually turning over with time. Nutrient content is largely stable in mature milk intra-individually (Garcia et al., 2017; Hinde et al., 2009; Power et al., 2017), and we found no association of nutrient content predicting changes in the microbiome over time. Instead these changes over time may reflect EMT moving different bacteria from the mother's gut to the mammary gland and/or changes in the infant oral microbiome over time (Dzidic et al., 2018) that changes which bacteria colonize milk through retrograde flow during suction (Ascinar et al., 2017; Biagi et al., 2017).

With increasing threats on primates globally, more and more primates may need human assistance to survive (Estrada et al., 2017). Living in human care (e.g., in zoos) as opposed to in the wild can change primate gut microbiomes, which may impact their health (McKenzie et al., 2017). For milk, we do not know the effect of living in human care on primate milk microbiomes. Most of the primate individuals we sampled were from zoo populations, and we still detected hundreds of OTUs in the milk suggesting that zoo animals still harbor a diverse milk microbiome. Humans and the one wild nonhuman primate species, surprisingly had some of the lowest OTU richness, indicating that living in the wild is not necessarily a predictor of bacterial diversity in milk. It may be difficult to fully quantify changes in milk microbiomes from wild populations to those in zoo populations given the need to sedate wild animals to collect milk samples. Our study working with primate mothers in mostly zoo populations provided foundational data on the ecology of primate milk microbiomes and demonstrates the contribution of zoo populations to scientific knowledge.

Milk microbiomes may protect mammalian young against infections, contribute to immune system development and influence later-life health and behavior. Manipulating the milk microbiome through diet changes in the mother or by seeding infant formula for humans and endangered primates in our care are potential strategies for future consideration. We found that host species and nutrient content affected the microbiome, indicating that manipulations

should take into account host species and nutrient differences to achieve predictable and stable results. Nonetheless, we did recover a core primate milk microbiome, which means thes particular bacteria could be used as probiotics more broadly at the primate level if they are found to have a positive effect on health. Future studies should examine these core bacteria at a finer scale to resolve strain level variation among primate species and their association with health. Therapies designed to improve health through manipulations of microbiomes deserve careful study, and have great potential for improving health and well-being.

DATA ACCESSIBILITY

Demultiplexed pyrosequencing run sequence data and associated metadata has been deposited in the National Center for Biotechnology Information Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under BioProject ID: PRJNA518076.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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