



Social networks, cooperative breeding, and the human milk microbiome

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Abstract

Objectives: We present the first available data on the human milk microbiome (HMM) from small-scale societies (hunter-gatherers and horticulturalists in the Central African Republic [CAR]) and explore relationships among subsistence type and seasonality on HMM diversity and composition. Additionally, as humans are cooperative breeders and, throughout our evolutionary history and today, we rear offspring within social networks, we examine associations between the social environment and the HMM. Childrearing and breastfeeding exist in a biosocial nexus, which we hypothesize influences the HMM.

Methods: Milk samples from hunter-gatherer and horticultural mothers ($n = 41$) collected over two seasons, were analyzed for their microbial composition. A subsample of these women's infants ($n = 33$) also participated in detailed naturalistic behavioral observations which identified the breadth of infants' social and caregiving networks and the frequency of contact they had with caregivers.

Results: Analyses of milk produced by CAR women indicated that HMM diversity and community composition were related to the size of the mother-infant dyad's social network and frequency of care that infants receive. The abundance of some microbial taxa also varied significantly across populations and seasons. Alpha diversity, however, was not related to subsistence type or seasonality.

Conclusion: While the origins of the HMM are not fully understood, our results provide evidence regarding possible feedback loops among the infant, the mother, and the mother's social network that might influence HMM composition.

1 | INTRODUCTION

1.1 | The human milk microbiome

Milk has evolved over millions of years to provide nutrition, immunological protection, and water to neonates (Ofstedal, 2012). Human milk contains thousands of biologically-active factors, most of which likely evolved to support infant health and development (Ballard & Morrow, 2013; Jensen, 1995). Additionally, human milk provides infants protection from infections and respiratory diseases, resulting in breastfeeding

being one of the best predictors of infant health and success in early life, particularly in high-risk environments (Lamberti, Walker, Noiman, Victora, & Black, 2011; Li, Dee, Li, Hoffman, & Grummer-Strawn, 2014; Lodge et al., 2015).

Despite its extraordinary importance, the pathways by which human milk influences infant health and contributes to successful development are not yet fully articulated. However, technological and methodological advances have recently enhanced explorations into the complexities of human milk (Jiménez, 2017; Jost, Lacroix, Braegger, &



Chassard, 2013). Additionally, human milk researchers are moving toward interdisciplinary studies (McGuire et al., 2017). This shift is predicated by the increasing understanding that lactation and breastfeeding are biosocial phenomena, influenced by maternal environment, physiology, health, structural barriers, infant behavior, and culture (Hinde, 2013; Stuart-Macadam & Dettwyler, 1995).

Here, we focus on one component of human milk—the human milk microbiome (HMM). All human milk contains bacteria and, as such, milk is one of the earliest, most consistent, and likely influential sources of microbes to the infant (Collado et al., 2009; Cabrera-Rubio et al., 2012; Hunt et al., 2011; Palmer, Bik, DiGiulio, Relman, & Brown, 2007; Williams et al., 2017). Although the role of milk microbiota in infant and maternal health has yet to be determined, microbiota may colonize the gastrointestinal (GI) tract and modulate development of the infant's immune system with short- and long-term health consequences (Garcia-Mantrana, Gómez-Gallego, Cabrera-Rubio, & Collado, 2017; Rautava, 2016; Walker & Iyengar, 2015). The HMM, in concert with immune factors in milk and human milk oligosaccharides, provides a link between the mother's mature immune system, possibly honed via environmental exposures, and the infant's developing GI microbiome and immune system (Ruiz et al., 2017).

The origins of the HMM are still debated, but researchers posit that milk is colonized via bacterial translocation from the mother's GI tract to the mammary glands during late pregnancy and lactation, transfer from breast skin, and/or from the infant's oral cavity via flowback of milk into the mammary gland while the infant suckles (Fernández et al., 2013; Jeurink et al., 2013; Ramsay, Kent, Owens, & Hartmann, 2004). Given the interconnectedness of the microbial communities within the human body, it is likely that all of the above, and other sources, are responsible for shaping the milk's microbial communities (Mira & Rodríguez, 2017). Additionally, because complex microbial communities appear to be universally present in milk, these potential routes of bacterial transfer to the mammary glands should not be considered sources of contamination, but rather the natural origins of the microbial community.

While the origins of the HMM remain under investigation, researchers are exploring a host of pre-, peri-, and postnatal factors associated with variation in the HMM diversity and community composition. Among other factors, gestational age, geography, maternal obesity, and antibiotic therapy are thought to be related to variation in the HMM (Cabrera-Rubio et al., 2012; Collado, Laitinen, Salminen, & Isolauri, 2012; Khodayar-Pardo, Mira-Pascual, Collado, & Martínez-Costa, 2014; Kumar et al., 2016; Soto et al., 2014). Studies on the roles of birth mode (Cabrera-Rubio et al., 2012; Kumar et al., 2016; Urbaniak, Angelini, Gloor, & Reid, 2016) and time postpartum (Cabrera-Rubio et al.,

2012; Cabrera-Rubio, Mira-Pascual, Mira, & Collado, 2016; Khodayar-Pardo et al., 2014; Soto et al., 2014) have resulted in inconsistent findings, perhaps the consequence of methodological differences (McGuire & McGuire, 2017). For instance, greater bacterial diversity and richness were found in the milk of mothers who delivered vaginally compared to the milk from mothers who delivered their infants surgically (Cabrera-Rubio et al., 2012). However, bacterial communities in milk from women who had undergone elective cesareans were different from communities found in milk produced by women who underwent nonelective cesareans and vaginal births, suggesting that physiological stress or hormonal signals may be guiding the colonization of the milk (Cabrera-Rubio et al., 2016). Additionally, Urbaniak et al. (2016) found no variation based on birth mode in milk produced by Canadian women, and Williams et al. (2017) found only minor differences in US women. While Kumar et al.'s (2016) study among Chinese, South African, Finnish, and Spanish women noted a role of birth mode, they suggest the impact varies by geography—likely due to differences in birthing practices and procedures. Thus, despite a proliferation of research on the HMM, our understanding of factors influencing bacterial community structure is still in its infancy. Moreover, to our knowledge, the role of the social environments and behavior in shaping the HMM has not been explored.

1.2 | Human cooperative breeding and the social environment

Humans are highly prosocial (Burkart et al., 2014; Hill et al., 2011), relying on group membership and cooperation for reproduction and survival. During human evolution, cooperation and interaction within one's social network in the postnatal period for mothers and across early development for children almost certainly had an enormous influence on biological fitness. So important are others to successful reproduction and child development that humans are classified as cooperative breeders—species who require nonparental investment through caregiving and provisioning to successfully rear their offspring (Hrady, 2005). Cross-culturally, human infants are born into, engage with, and receive care from multiple individuals. The mother-infant dyad often exists within deep social networks comprised of individuals who occupy different generational, gender, familial, and non-familial roles (Meehan, Helfrecht, & Malcom, 2016). Allogrands (nonmaternal caregivers) provide the mother-infant dyad support, assistance, and protection against environmental and social perturbations, offering energetic and nutritional safety nets to the mother-infant dyad during high-risk periods (Meehan, Helfrecht, & Quinlan, 2014; Meehan, Quinlan, & Malcom, 2013; Sear & Mace, 2008). Furthermore, studies that have quantified their contributions demonstrate that



allomothers offer a considerable amount of the direct care that infants and young children receive (for review, see Meehan et al., 2016), indicating frequent interactions and a direct pathway by which allomothers may influence microbial exposure to the mother-infant dyad.

2 | STUDY GOALS AND PREDICTIONS

We utilized milk samples and behavioral data collected on mother-infant dyads among hunter-gatherers and horticulturalists in the Central African Republic (CAR) to characterize their HMM and explore the potential influences of population, seasonal, and social factors on the HMM. A focus on rural small-scale populations, particularly hunter-gatherer populations, is essential in microbiome research as these populations provide a window, albeit imperfect, to environments, diet, and caregiving patterns more common throughout our evolutionary history. For example, the two small-scale societies in this study practice on-demand (multiple bouts per hour) and extended breastfeeding, unmedicated childbirth, and reside in dense social environments. Moreover, they are subsistence-level societies who do not consume Western diets. Data from nonindustrial, small-scale populations are critical, as we need to avoid interpreting results from microbiome studies conducted in Western populations as “normal,” when Western diets, social patterns, childrearing practices, and wide-scale antibiotic usage, for example, have likely shifted human microbial composition. No single population or type of populations can fully inform our understanding of the HMM. Thus, data from a wide range of geographic locales and populations is essential to uncovering the human pattern, if it exists.

We broadly hypothesized that, given geographic and environmental differences, the most-abundant bacterial genera in milk produced by women in these subpopulations would differ from previously reported studies on the HMM in, for example, the United States, Spain, Finland, South Africa, and China (Hunt et al., 2011; Kumar et al., 2016; Williams et al., 2017). Additionally, as previous studies have found variation in GI microbiome across subsistence type (Obregon-Tito et al., 2015) and season (Davenport et al., 2014), we investigated whether there was variation in the HMM of hunter-gatherer and horticultural women and whether it varied across season. The hunter-gather and horticultural populations in this study interact and reside in proximity to each other, but are ethnically, culturally, and linguistically distinct populations. Furthermore, they practice different subsistence patterns leading to varied diets and exposure to different environments, thus offering a unique comparison. Thus, we predicted variation in HMM diversity and bacterial community composition would vary between

the hunter-gatherer and horticulturalist samples, despite their geographic proximity to each other. We further broadly predicted seasonal variation in the HMM, given the changes in local environment (wet and dry seasons), possibly associated with corresponding changes in diet and/or environmental bacterial exposure across the year. We also hypothesized that social environments, as measured through the breastfed infant's exposure to others, would be associated with HMM diversity and community composition. We had no *a priori* hypotheses regarding variation of specific genera across populations, seasons, or variation in social networks. All such analyses were exploratory.

3 | MATERIALS AND METHODS

3.1 | Participants and region

The study was conducted among 41 hunter-gatherer and horticulturalist women and infants in the CAR (hunter-gatherer $n = 27$ [mean maternal age = 27.0 ± 5.3 years; mean time postpartum = 14.4 ± 12.2 months]; horticulturalist $n = 14$ [mean maternal age = 29.9 ± 7.5 years; mean time postpartum = 7.8 ± 6.6 months]) in March-April (season 1 [end of dry/beginning of wet season] $n = 18$) and July-August (season 2 [height of wet season] $n = 23$) in the CAR in 2011. The region has two major seasons (wet/dry). The short dry season lasts approximately 3 months from December-February, followed by rains that begin and gradually increase in intensity until the height of the wet season from July-October (Bahuchet, 1988).

Aka hunter-gatherers reside in small, mobile camps of approximately 25–35 individuals in and on the periphery of the Congo Basin rainforest (Hewlett, Lamb, Shannon, Leyendecker, & Schölmerich, 1998). They move back and forth from the forest to camps constructed in the fields of their horticulturalist neighbors. In recent years, these camps have been occupied for longer durations of the year and some are permanent settlements. However, camp composition varies and members make frequent excursions into the forest (Meehan, Hagen, & Hewlett, 2017). Childcare is intimate and intensive. Infants travel in a sling on the side of their mothers and others during the day, are rarely out of physical contact with caregivers, receive substantial care from allomothers, co-sleep with their family, and breastfeed on-demand for approximately 3 years (Hewlett et al., 1998; Meehan & Roulette 2013; Meehan et al., 2013).

The hunter-gatherers in this study, like other extant hunter-gatherers, reside in association with other communities and obtain some food from their horticultural neighbors. Thus, hunter-gatherer diets are not ancestral. Nevertheless, their diet is more “traditional” than Western, industrialized diets. The Aka diet broadly includes wild yams, forest game (e.g., duiker, large birds, and porcupine), mushrooms, nuts,



fruits, honey, caterpillars, and leafy greens (e.g., koko). Particularly when residing close to the horticultural villages, their diet more heavily relies on manioc leaves and roots obtained from their neighbors' fields. Seasonal changes in access to wild food resources occur, with, for example, wild yams available from approximately November–June and caterpillars available in August and September (Bahuchet, 1988).

The horticulturalist population is comprised of women and infants from subsistence-level slash-and-burn farming communities on the edge of the forest. Household size is approximately 6–8 individuals, but families often reside in extended family compounds, enabling the mother–infant dyad frequent contact with others (Meehan & Roulette, 2013). Infant care is less intimate, in comparison to the Aka, in terms of frequency and responsivity (Hewlett et al., 1998), but allomaternal investment is nevertheless very common (Meehan, 2008). Infants are carried in a sling on mothers' backs during travel, but are left home in the care of others when mothers feel their infants are ready, allowing mothers to work in the fields unencumbered (Meehan, 2008). Mothers typically wean their infants around 2 years of age (Meehan & Roulette 2013), and parents transfer children to sibling beds once night time feedings are not required.

The horticulturalists' staple food products include manioc, maize, plantains, and taros. They also grow a variety of vegetables and fruits, including squashes, cucumbers, sweet bananas, papaya, pineapples, mangoes, and oranges. Many families keep a few small livestock such as goats, sheep, and chickens, and occasionally hunt or trade for bush meat and other forest products (e.g., koko and caterpillars) when available. Manioc and their leaves are available throughout the year, but other cultivated foods such as corn is only available starting in approximately July for a short period. There is also seasonal variation in fruits and vegetables.

Given the proximity and interaction between the two populations, there is, of course, contact, shared environments, and overlap in diet. However, as noted by Gómez-Gallego, Garcia-Mantrana, Salminen, & Collado (2016), variation in diet between hunter-gatherer and horticultural populations is a gradient from more traditional to more Western. Thus, these nonindustrialized populations provide a unique opportunity to explore the HMM in groups that are ethnically and culturally distinct in terms of subsistence pattern, culture, use of the environment, and diet, but who both practice cooperative childcare.

3.2 | Sample collection

Recruitment was based on a woman's breastfeeding status and willingness and consent to participate. Additionally, mothers needed to be over 18 years of age and have no reported or visible breast infection. The study protocols were

approved by institutional review boards at Washington State University (#09070 & #11638) and the University of Bangui in the CAR. Informed verbal consent was obtained by the data collection team and local translators who served as witnesses.

Women were asked to choose a breast from which to express milk. Participants used water and soap to cleanse the breast to remove any obvious soil or dirt contaminants. The participating women used hand sanitizer to clean their hands prior to collection. The nipple and surrounding area were cleaned twice with alcohol wipes. Participants hand expressed milk (~15 mL) into sterile containers, and a representative aliquot of milk (~2 mL) was chemically preserved with Milk Preservation Solution (Norgen Biotek Corporation, Thorold, ON) in a 1:1 ratio. Previous work has shown that this is an appropriate method to preserve the bacterial community structure of milk samples for 2–4 weeks (Lackey et al., 2017). Preserved milk samples remained in ambient temperature (regional mean temperature = 25°C [Bahuchet, 1988]; ranging from approximately 16–35°C) for up to 4 weeks, after which they were shipped on dry ice to Washington State University, and then stored at –20°C upon arrival. Between the time of arrival and analysis, the samples were moved to a –80°C freezer where they remained until thawed for DNA extraction.

3.3 | DNA extraction and PCR amplification

Briefly, DNA from 0.5 mL of preserved milk was extracted using the Milk DNA Preservation and Isolation Kit (Norgen Biotek Corporation), including an additional 2 h lysozyme lysis (20 mg/mL) targeting Gram-positive genera as described previously for preserved samples (Lackey et al., 2017). We also included a negative control of nuclease-free water with each set of samples that were extracted. Bacterial DNA was amplified using a two-step PCR procedure previously described (Williams et al., 2017). The first PCR used sevenfold degenerate primers for the V1–V3 region of the bacterial 16S rRNA gene (27F and 534R). The second PCR used primers with dual-index barcodes to identify individual samples and Illumina sequencing adapters. Negative extraction controls, a PCR negative of nuclease-free water, and a PCR positive of extracted *E. coli* DNA were also run alongside each PCR. However, since no band was observed when the PCR product was electrophoresed on a 1% agarose gel and no band was observed via a QIAxcel DNA screening evaluation, we did not sequence any of the PCR product from these negatives.

Barcoded PCR amplicons generated from each sample were pooled to create a composite with equal mass of each sample (50 ng DNA) and were multiplexed for sequencing at the University of Idaho Institute for Bioinformatics and Evolutionary Studies Genomics Core facility using an Illumina

MiSeq v3 protocol for 600 cycles and paired end 300-bp reads. Sequences were demultiplexed and assigned to individual samples, trimmed, merged, and classified using default parameters in the custom python application dbcAmplicons (https://github.com/msettles/dbcAmplicons/blob/master/docs/manual/DBC_ampliconsUserManual.pdf) as described and used previously by our group and others (Carruthers et al., 2015; Dai, Gliniewicz, Settles, Coats, & McDonald, 2015; Lackey et al., 2017; Liang et al., 2015; Ma et al., 2015; Williams et al., 2017). Briefly, during preprocessing barcodes were allowed ≤ 1 mismatch (hamming distance) and primers ≤ 4 mismatches (Levenshtein distance) if the final 4 bases of the primer perfectly matched the target sequence. Reads were trimmed of their primer sequence and merged into a single amplicon sequence using a modified version of FLASH (<https://github.com/dstreett/FLASH2>; Magoč & Salzberg, 2011). The Ribosomal Database Project (RDP) Bayesian classifier (Wang, Garrity, Tiedje, & Cole, 2007) was used to assign sequences to phylotypes. Reads were assigned to the first RDP taxonomic level with a bootstrap score ≥ 50 .

3.4 | Naturalistic behavioral observations

Thirty-four mothers represented in the HMM analysis had infants who participated in the naturalistic behavioral observation study of infant and caregiver behavior. (The milk sample of one mother had a low read count and was eliminated from further analysis, resulting in a final subsample size of $n = 33$; see “Section 4”.) This subsample was the focus of our analysis on the role of social and caregiving networks on HMM. Observations spanned all daylight hours (6 am–6 pm), but were split into 4-h observational segments (6–10 am; 10 am–2 pm; 2–6 pm) and conducted over several days. Fifteen-minute breaks following every 45 min of observations, resulted in observations covering 9 out of the approximately 12 daylight hours in the region. Infant behavior and infant-caregiver interactions were recorded every 30 s or 1,080 times per child, totaling $>35,000$ observations on the 33 mother-infant dyads represented in this portion of the study. Observers recorded infant and caregiver behavior on handheld datasheets and wore an earphone to cue observation and recording times. Observers followed the focal infant during the entire observation period, and thus observed the infant in their natural day-to-day activities (e.g., in the compound/camp, travel in fields/forest, wake, and sleep periods) (see Hewlett et al., 1998 and Meehan et al., 2013 for additional descriptions of observational methodology). Household, camp, and compound demographic data were collected prior to commencing the observations, providing detailed data on kin relations to the focal infant. All potential caregivers, identified in the demographic surveys, were pre-assigned unique identifiers. Novel interactors (e.g., a women

from another camp who interacted with the focal infant) were assigned unique identifiers on the spot during observations. The identification of the allomothers, their relationship to the focal infant, and their caregiving category (e.g., sister, brother, grandmother) allowed for analysis of the social and caregiving networks.

Holding was defined as a caregiver supporting the infant’s weight in their arms, lap, or in a sling. Physical contact was defined as a caregiver holding and/or touching the focal child. Proximity included all individuals who were in physical contact or came within a forearm’s distance of the focal child. While proximity does not always necessitate physical contact and thus direct microbial exposure, individuals who were in proximity are those most likely to have been in contact with the infant at some point.

3.5 | Statistical analysis

All data were analyzed with R version 3.3.2 (2016-10-31) using phyloseq (McMurdie & Holmes, 2013), DESeq2 (Love, Huber, & Anders, 2014), and tidyverse (Wickham, 2017). Read counts and taxonomic assignments exported from the dbcAmplicons pipeline in BIOM format were imported into R using phyloseq. Two subsets of abundance data were used in some analyses: (1) counts of taxa identified at the genus level only and (2) samples with complete social data only. Distributions of total read counts per sample between subsistence types and seasons were compared visually with a histogram and a density plot, and formally by the Kolmogorov-Smirnov test, which is sensitive to differences in both location and distribution shape.

We used phyloseq to compute the following alpha diversity indices on all samples using counts of all taxa: number of observed taxa (richness), Shannon evenness index, Simpson evenness index, Shannon index, Simpson’s index, inverse Simpson’s index, the α parameter of Fisher’s logarithmic series, the Chao1 index, and abundance-based coverage estimators (ACE). We then, by visual inspection of cumulative distribution plots and formally by Kolmogorov-Smirnov tests, compared the distributions of alpha diversity indices in hunter-gatherers vs. horticulturalists and season.

With respect to beta diversity, which estimates the degree that two samples are different, both analysis of similarity (ANOSIM) and nonparametric permutation MANOVA (ADONIS) using Bray-Curtis dissimilarity distances were performed in R using all taxa identified at any level. We also compared dissimilarities via detrended correspondence analysis (DCA), redundancy analysis (RDA), nonmetric multidimensional scaling (NMDS), multidimensional scaling (MDS), and principal coordinate analysis (PCoA) in phyloseq using log-transformed counts [$\log(x + 1)$] and the Bray-Curtis dissimilarity matrix. Clustering of hunter-gatherers

and horticulturalists as well as season was assessed by visual inspection of the scatterplots.

Principal components analysis (PCA) was used to identify any underlying structure of social and caregiving environmental variables. We tested the hypothesis that sample alpha diversity is correlated with infant social interactions by computing Spearman's rank correlations between each alpha diversity index and social PC1 and PC2; because Simpson's and inverse Simpson's metrics have a rank correlation = 1, we omitted the latter.

Differential abundance analyses were performed to compare microbial community membership in milk produced by hunter-gatherers versus horticulturalists on all taxa identified to the genus level on all samples using the DESeq2 package. False discovery rate (FDR, Benjamini-Hochberg) was set at 0.1. We used phyloseq to convert our data to DESeq2 format, and then, using a "parametric" fit and Wald test, computed \log_2 -fold differences and adjusted P-values for each taxon (Callahan, Sankaran, Fukuyama, McMurdie, & Holmes, 2016; Love et al., 2014; McMurdie & Holmes, 2014)

A reproducible record of all statistical analyses and the corresponding R code is provided in the Supporting Information Materials as Supplemental_R_analysis.html.

4 | RESULTS

4.1 | Sequencing summary

Forty-one unique milk samples were analyzed in this study. A total of 1,943,048 high-quality reads were obtained from sequencing the 41 milk samples, with a range of 122 to 355,864 reads per sample (mean reads = 47,391; SD = 93,076). One sample was removed based on low total read counts, that is, having a read count of <500 reads, resulting in analysis of microbiome data garnered from a total of 40 samples. Taxa assigned to "unknown" at the domain level ($n = 1$) and those appearing in only one sample and with a read count ≤ 10 ($n = 198$) were considered noise and removed prior to analyses (Callahan et al., 2016; McMurdie & Holmes, 2013), resulting in 718 unique taxa identified. Of the 718 taxa identified in these milk samples, 576 were classifiable to the genus level.

4.2 | Associations among population, season, and HMM diversity

Most taxa (98%) present in the overall dataset were present in the hunter-gatherer samples, and a majority of taxa (81%) were present in the horticulturalist samples. However, 139 (19%) of the taxa were unique to hunter-gatherers, and 11 (2%) were unique to horticulturalists. With respect to field season, most taxa were present in samples from both

seasons: 676/718 (94%) in season 1 and 665/718 (93%) in season 2. However, 53/718 (7%) and 42/718 (6%) of the taxa were unique to seasons 1 and 2, respectively. Cumulative distribution plots were constructed for the total number of reads per sample by subsistence type and field season. Kolmogorov-Smirnov tests found no significant difference between subsistence types, but a significant difference between field seasons ($P = .043$; see Supporting Information for cumulative distribution plots).

Alpha and beta diversity indices were calculated in R using all 718 identified taxa. Cumulative distribution plots (Supporting Information Figures S1 and S2) indicate minimal differences in the shape of the distributions of alpha diversity indices of the HMM of hunter-gatherers and horticulturalists and between seasons. In addition, two-sample Kolmogorov-Smirnov tests indicated neither significant differences between populations (all $P > .05$) nor seasons (all $P > .1$) for all diversity measures computed. Using a variety of analyses (see "Section 3"), we failed to detect any meaningful differences in beta diversity between subsistence types or seasons (see Supplemental_R_analysis.html).

4.3 | HMM membership and associations with population or season

The top 10 most abundant genera present in the milk samples of the women overall were *Streptococcus* (21.8%), *Staphylococcus* (17.4%), *Veillonella* (5.0%), *Corynebacterium* (4.5%), *Rhodococcus* (3.0%), *Dyella* (2.5%), *Lactobacillus* (2.3%), *Prevotella* (2.2%), *Micrococcus* (1.9%), and *Hafnia* (1.7%). A visual depiction of the relative abundances of the top 25 genera overall, grouped by subsistence type, is provided in Figure 1. It is noteworthy that many genera were abundant in a few samples but rare in most; these genera, therefore, do not appear in Figure 1. To help overcome this potential bias, we compiled a list containing all genera characterized as being one of the 10 most-abundant genera in at least one sample, resulting in a total of 61 unique genera (Supporting Information Figure S3). This suggests substantial variation in the microbial communities of each sample. Additionally, Supporting Information Figure S3 suggests that this variation is not patterned by subsistence type or field season. Supporting Information Figures S4 and S5 provide the individual and average relative abundance values for the 30 most abundant bacterial genera by subsistence pattern and season, respectively.

Differential bacterial abundance by subsistence type and season of all taxa identified to the genus level ($n = 576$) was investigated using DESeq2 (Love et al., 2014; McMurdie & Holmes, 2014) with a false discovery rate (FDR) of 0.1. This analysis indicated associations between subsistence type or season and abundance of several genera (Figure 2A,B). The relative abundance of 15 genera were different between

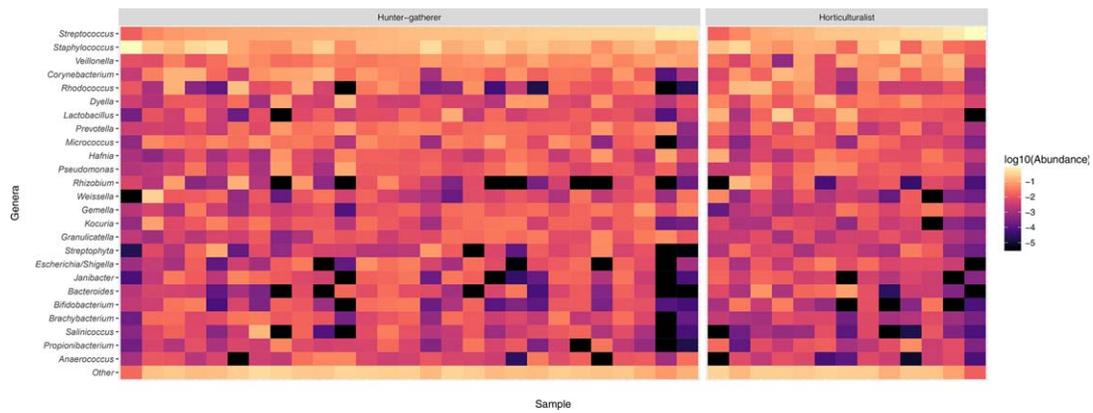


FIGURE 1 The contribution of the 25 most-abundant genera and an aggregation of all other genera identified to the genus level (“other”) in the overall dataset to the HMM of each sample by subsistence type. Relative abundances are on a log 10 scale, with lighter colors indicating higher relative abundances. Samples (columns) are sorted by the relative abundance of *Streptococcus*

subsistence groups (adjusted $P < .1$). Of these, *Enhydrobacter*, *Renibacterium*, and *Lactobacillus* were more abundant in milk produced by horticulturalists as compared to that produced by hunter-gatherers. *Enhydrobacter* was nine

times more abundant in milk produced by horticulturalists compared to that produced by hunter-gatherers, while *Renibacterium* and *Lactobacillus* were nearly four times more abundant. Conversely, *Luteimonas* was 14 times more

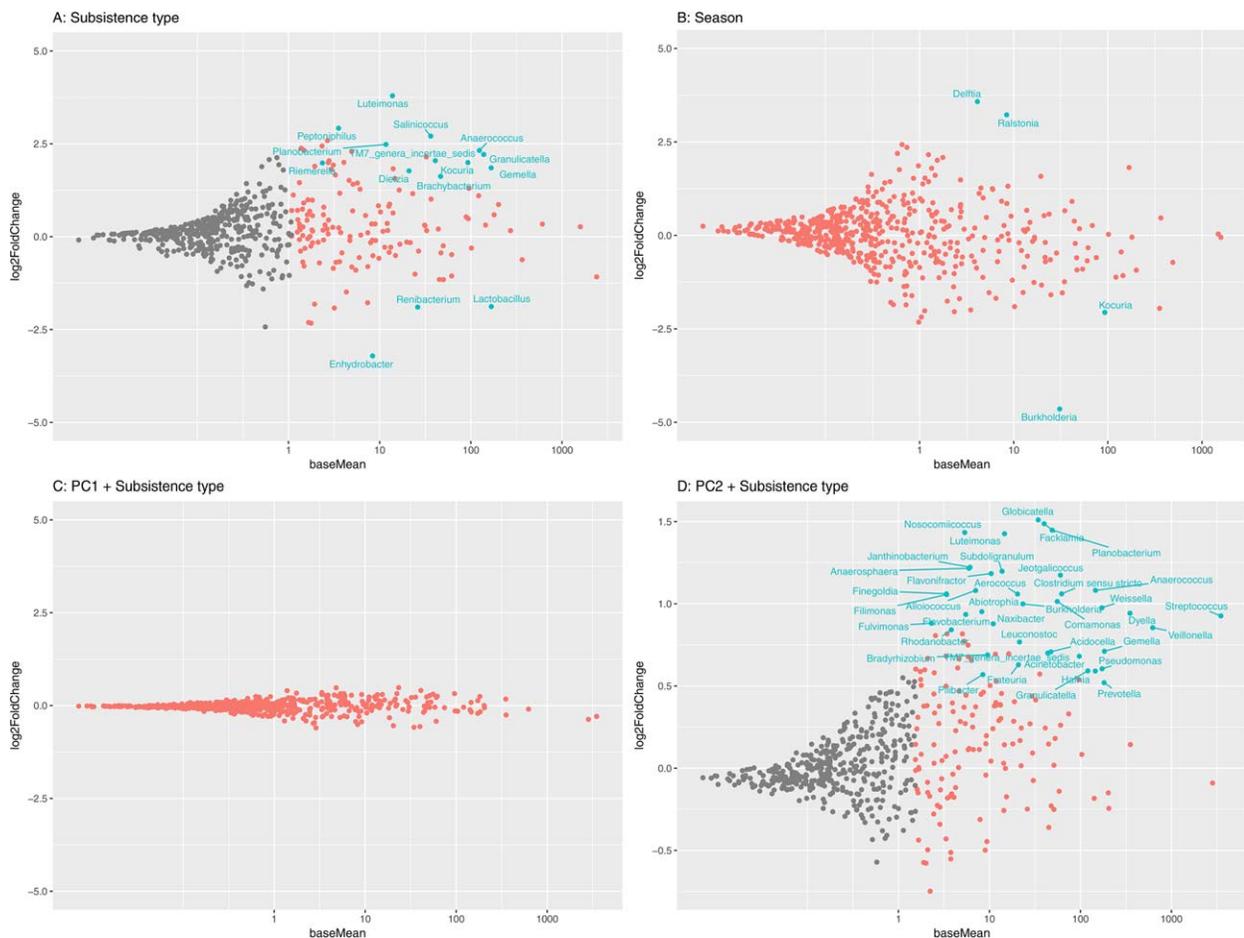


FIGURE 2 Differential abundance analysis using DESeq2 (v 1.14.1). Each dot is one genus. The baseMean is the mean of normalized counts of all samples. Log2foldChange is the log2 ratio of abundances in the two conditions—Values above 0 indicate an increase in abundance, and values below 0 indicate a decrease in abundance. A, Hunter-gatherers relative to horticulturalists. B, season 2 relative to season 1. C, a one-unit increase in PC1. D, a one-unit increase in PC2. Blue dots: adjusted P value $< .1$. Red dots: adjusted P value $\geq .1$. Gray dots: adjusted P value could not be computed. Note different y axis scale in panel D; see text for details on subsistence groups, seasons, and PC1 and PC2

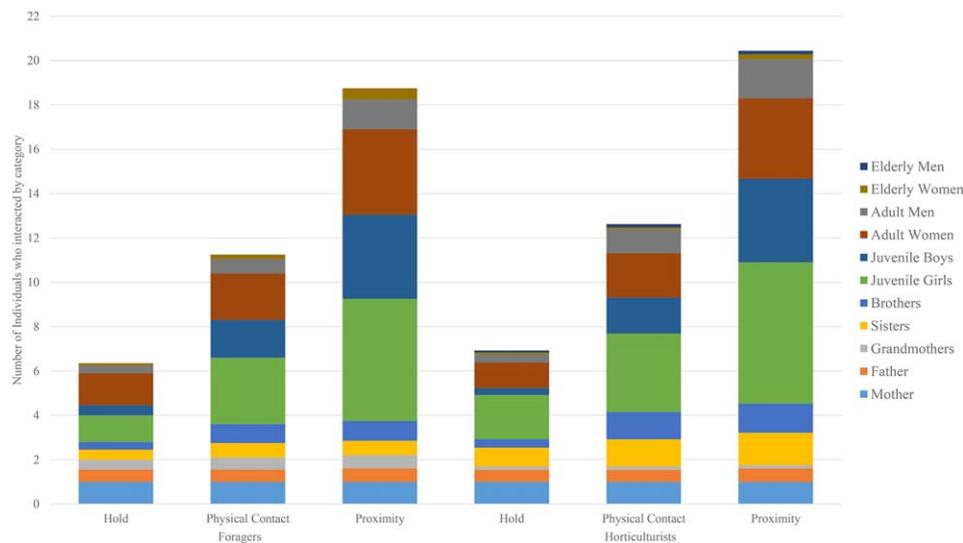


FIGURE 3 Mean number of individuals who held, were in physical contact with, or came into proximity of the focal infant by subsistence strategy and caregiver category ($n = 33$). Each stacked bar represents the mean number of caregivers, by subsistence and care type, who interacted with the focal infants. The colored stacks represent the mean number of caregivers that engaged with the focal infant via holding, physical and proximal contact. Categories that comprise only one individual (e.g., mother) should be interpreted as the proportion of individuals who engaged in these behaviors. For example, all children were held, came into physical and proximal contact with their mothers across the observation period. Thus, the mean number of mothers equal 1.0. Colored bars representing a caregiver category that is not limited to only one individual (e.g., sisters, juvenile girls, etc.), presents the mean number of individuals in that category that engage with the infant over the course to the observation period. Thus, it is possible to have a mean of <1 (e.g., mean number of hunter-gatherer juvenile boys holding) as not all of the focal infants were held by juvenile boys. All categories of caregivers are represented, with the exception of elderly men among hunter-gatherers, illustrating that childcare is not limited to one or two caregivers and children have the potential to engage with a wide variety of caregivers across early development. See Supporting Information Table S1 for description of inter- and intra-cultural variation in these patterns

abundant; *Peptoniphilus* and *Salinococcus* more than 6 times more abundant; and *Planobacterium*, *Anaerococcus*, *Granulicatella*, *TM7_genera_incertainae_sedis*, *Riemerella*, and *Kocuria* at least 4-times more abundant in hunter-gatherers' milk than horticulturalists' milk (Figure 2A). Relative abundances of *Kocuria* and *Burkholderia* were 4- and 25-times more abundant in milk produced in season 1 (end of the dry season/beginning of the wet season), than in milk produced in season 2 (height of the wet season; Figure 2B). The relative abundances of *Delftia* and *Ralstonia* were 12- and 9-times more abundant in season 2 samples compared to season 1 samples, respectively.

4.4 | Cooperative breeding, social networks, and the HMM

The relationship between social network size and caregiving patterns and the HMM was explored via a subsample of women ($n = 33$) who both provided a milk sample and whose infants were observed during naturalistic behavioral observations. We centered our analysis on nine observed dimensions of the social and caregiving environment, including the number of individuals who had contact with the mother's infant [number of individuals who held, were in

physical contact with or came into proximity (a forearm's distance) to the focal infant] and the frequency of observed care (number of observed occurrences of maternal/allomatern holding, physical contact, and proximity across the standardized observation period).

We examined the characteristics of hunter-gatherers' and horticulturalists' social networks and caregiving patterns (Supporting Information Table S1). In this sample, hunter-gatherer infants were held by an average 6 different caregivers, were in physical contact with 11 people, and were in proximal contact with 19 individuals across the day. Horticulturalist infants were held by an average of 7 caregivers, in physical contact with 13 people, and in proximal contact with 20 individuals across the day. Network composition and caregiving were widely distributed across caregiver relational, age, and sex categories (Figure 3). All 11 possible categories of caregivers were represented among the horticulturalists and 10 of the 11 were represented among the hunter-gatherers. The one exception in the hunter-gatherer sample was elderly men. This may simply be an artifact of the absence of elderly men in the represented hunter-gatherer camps. The wide distribution of caregivers indicates that care and, in turn, exposure to others was not limited to a particular age, sex, or category of caregivers. Intra-population variation, however, was substantial. The number of people

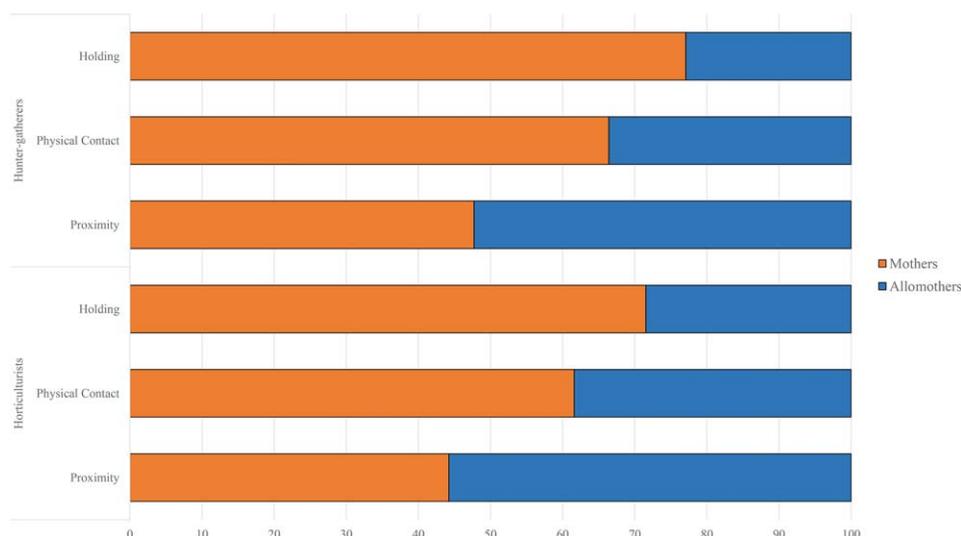


FIGURE 4 Mean percent of holding, physical and proximal contact by mothers and allomothers across the observation period, by subsistence strategy ($n = 33$)

engaging in physical contact with the focal infants across the standardized observation period among the hunter-gatherers and horticulturalists ranged from 3 to 18 and 2 to 26 individuals, respectively. Results also indicated that while mothers are primary caregivers (Supporting Information Table S1), allomaternal care represents 23–56% of all interactions infants had with caregivers (Figure 4). However, again, there was substantial intra- and intra-cultural variation; some infants received little allomaternal investment and hunter-gatherer infants received more care and physical contact than horticulturalist infants. Caregiving patterns and inter-cultural variation among these populations have been described elsewhere (Hewlett et al., 1998; Meehan, 2005, 2008) in more detail.

We centered, scaled, and subjected these 9 inter-correlated social variables (mean absolute value of correlation coefficients = 0.44) to a PCA to determine if there was a lower-dimensional representation of these data (Figure 5; Supporting Information Table S2). PC1 and PC2 together accounted for 76% of the variance. We, therefore, used participant scores on the first and second principal components (PC1 and PC2) for all further analysis of these variables. The maternal care and allocare variables both loaded on PC1 (54% of the variance) with approximately equal magnitudes but opposite signs. PC1 appears to represent a tradeoff in caregiving between mothers and allomothers, with more positive values indicating more allomaternal care and less maternal care, and more negative values indicating more maternal care and less allomaternal care. The majority, but not all, of the maternal care and allocare variables also both loaded positively (ie, with the *same* sign) on PC2 (22% of the variance). PC2 appears to represent variation in the care that comes from both mothers and allomothers, but is primarily driven by maternal care and can be thought of as the intensity of care that infants receive (with larger positive values indicating more care).

We examined whether PC1 and PC2 were associated with alpha diversity indices (Figure 6). Spearman's rank correlations demonstrated a positive significant relationship between the infant's allomaternal environment (PC1) and the Pielou ($r_s = 0.42$; $P = .014$), Shannon ($r_s = 0.39$; $P = .024$), and Simpson ($r_s = 0.45$; $P = .0083$) evenness measures of the bacterial community present in milk produced by the infant's mother. Additionally, PC1 was positively and significantly associated with the Shannon ($r_s = 0.46$; $P = .0077$) and Simpson ($r_s = 0.49$; $P = .0041$) diversity indices, which account for both richness and evenness of the taxa present. In other words, an increase in the infant's allomaternal environment and corresponding decrease in maternal interactions were positively associated with HMM diversity, in terms of evenness and of indices taking into account both richness and evenness, with similar effect sizes. In contrast, PC2, or the intensity of the infant's caregiving environment, was not associated with HMM evenness. However, there was a nonsignificant trend for 3 of the 4 [richness ($P = .06$), ACE ($P = .07$), and Chao1 ($P = .08$)] richness estimators with PC2, with similar effect sizes ($r_s \approx 0.3$), indicating the possible relationship between the intensity of the infant's caregiving environment, primarily driven by mothers, and the richness of the mother's HMM.

Differential abundance of all taxa identified to the genus level versus PC1 and PC2 was investigated using DESeq2 (Figure 2C,D). Because PC2 was associated with subsistence type but not season (t tests and permutation tests not reported; see also Figure 5 biplot), we controlled for subsistence type in DESeq2 analyses of both PC1 and PC2. The differential abundance analysis results indicate no significant associations (all adj $P > .1$) between PC1 and the microbial composition of milk. However, the intensity of the infant's caregiving environment (PC2) was positively associated with

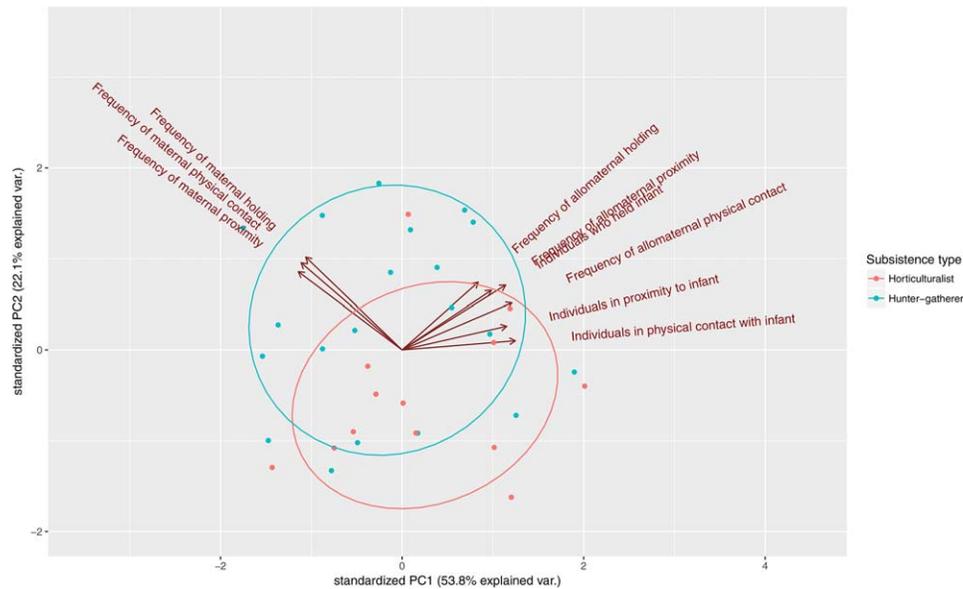


FIGURE 5 Biplot map of PC1 and PC2. Each dot is one infant. Arrows represent loadings of PC1 and PC2 on each social variable. Ellipses are 68% data ellipses

the relative abundance of 39 HMM genera. For example, for each unit increase in PC2, the abundances of *Globicatella*, *Facklamia*, *Planobacterium*, *Nosocomiicoccus*, and *Luteimonas* increased by a factor of more than 2.5 (adj $P < .1$). The other 34 significant taxa in this analysis increased by a factor of between 1.4 and 2.3.

5 | DISCUSSION

This is the first study of the HMM in hunter-gatherer and horticulturalist women. The most-abundant genera present in milk produced by both hunter-gatherers and horticulturalists were, in general, consistent with other reports of the milk microbiome in other populations (Cabrera-Rubio et al., 2012; Hunt et al., 2011; Khodayar-Pardo et al., 2014; McGuire & McGuire, 2017; Williams et al., 2017). For instance, *Streptococcus* and *Staphylococcus* are commonly reported in the milk of Western and non-Western women (Murphy et al., 2017; Urbaniak et al., 2016; Vaidya et al., 2017). However, several genera in the milk produced by CAR women were not identified in other studies or were more abundant than that in the milk of women in other populations. For instance, *Rhizobium* was characterized in CAR women's milk but was not reported in milk collected in the US (Hunt et al., 2011; Williams et al., 2017). Additionally, one of the first reports of the human milk microbiome found *Serratia* to be one of the most predominant genera in US women (Hunt et al., 2011), while *Propionibacterium*, *Bifidobacterium*, and *Veillonella* were among the most predominant in Swiss women (Jost et al., 2013). *Veillonella* was the third most abundant genera in the CAR women's milk. *Bifidobacterium*, while present, was not highly abundant in the milk of either the

hunter-gatherers or horticulturalists, and *Serratia* was not identified as a major contributor to the community composition of milk in either community of Central African women. Currently, it is unknown whether variation are biological differences, due to environmental, genetic, or other factors, or if these differences are due to other causes, such as methodology.

Comparisons among results from other studies should be made with caution though, as methodological differences exist (e.g., differences in DNA extraction method, culture-dependent versus culture-independent methods, region of 16S rRNA targeted during PCR, downstream bioinformatic methods) and could contribute to variation in results. It is

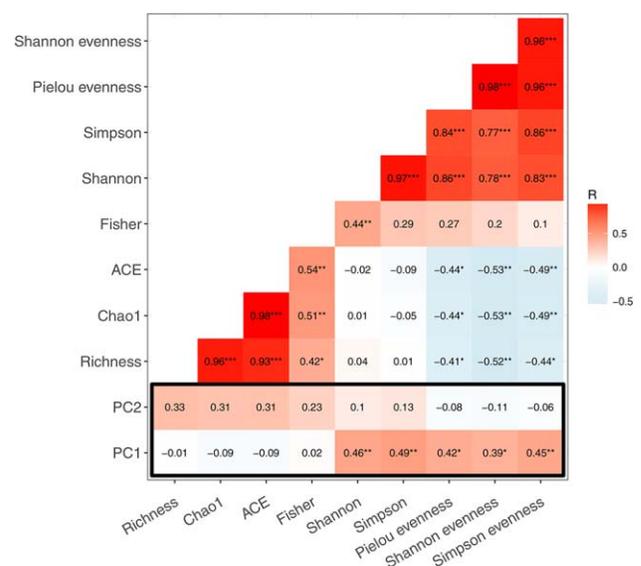


FIGURE 6 Spearman's correlation coefficient (r_s) of alpha diversity indices with social network and caregiving variables (PC1 and PC2). ** $P < .01$; * $P < .05$

noteworthy, however, that the milk samples from the study by Williams and colleagues (2017) used primers targeting the same 16S rRNA region and similar PCR methods as well as the same sequencing technology and bioinformatics. Comparison of the Williams et al. study and our study indicate that, of the 10 most abundant genera in American women's milk, 5 genera (*Streptococcus*, *Staphylococcus*, *Veillonella*, *Corynebacterium*, and *Lactobacillus*) were also members of the 10 most abundant taxa in CAR women's milk. However, *Gemella*, *Rothia*, *Propionibacterium*, *Granulicatella*, and *Pseudomonas* were among the 10 most abundant taxa in U.S. women's milk but not in the 10 most abundant taxa in CAR women's milk, while *Rhodococcus*, *Dyella*, *Prevotella*, *Micrococcus*, and *Hafnia*, were members of the 10 most abundant taxa in the CAR women's samples, but not in the 10 most abundant taxa in US women's samples. Clearly, there are broad similarities, but substantial variation in membership of the HMM exist. Given the paucity of global HMM data, these data contribute to our understanding of core similarities and differences in the microbial content of human milk. Additional cross-cultural studies, utilizing identical collection, analysis, and bioinformatics methods, are needed to begin to identify the possible causes of differences in populations around the world and to possibly identify a core "normal" milk microbiome.

Comparison of the hunter-gatherer and horticulturalist women's milk in this study indicates variation in Central African women's HMM composition. While both genetics and geography are suggested drivers of differences in the milk microbiome (for review of this topic see Gómez-Gallego et al., 2016), little evidence exists as to whether these differences are real or a result of methodological differences. Our results provide evidence that community composition differs between the two populations, despite their proximity to each other. For example, the horticulturalists have a greater relative abundance of *Lactobacillus* in their milk than the hunter-gatherers. Whether such variation is biologically relevant is not currently known.

Additionally, it appears from these results that seasonal variation and/or differences in dietary intake may lead to some differences in milk microbial community composition. Only a limited number of studies have explored seasonality as a driver of microbial community structure in the GI tract (Dubois, Girard, Lapointe, & Shapiro, 2017; Hisada, Endoh, & Kuriki, 2015) and there is variation in results. To our knowledge, this is the first exploration of seasonality of the HMM. Results provide initial evidence suggesting seasonality may be influencing the relative abundance of specific taxa in the HMM. For example, *Kocuria* was one of the most abundant genera in milk produced by hunter-gatherer and horticultural women and *Burkholderia* were more prevalent in the dry season than in the wet season. Dietary (e.g., food recalls) and environmental exposure (e.g., when the

forager women were last residing in the forest and for how long) data were not collected, inhibiting our ability to parse apart the influence of these factors on the HMM. Thus, it is not possible to identify whether the variation in milk microbial community composition was due to differences in seasonal environmental exposure and/or seasonal variation in diet. However, it is reasonable to assume that seasonal changes to the environment and dietary influences are intertwined. For example, the higher abundance of *Burkholderia*, a common soil bacterium (Mahenthalingam, Urban, & Goldberg, 2005), during the dry season may relate to environmental and dietary exposure during that time. The Aka frequently assist their horticulturalist neighbors in their fields during this time period with land clearing and coffee-cropping (Bahuchet & Guillaume 1982), possibly increasing environmental and dietary exposure, via increased reliance on manioc, while residing on the periphery of the village. Although speculative, we suggest this pattern offers avenues for future research. It is also important to note that we observed a difference in the total read counts per sample between seasons. Yet, even with this difference, there were minimal or no differences in the alpha diversity measures between seasons. Future studies should explore seasonal and dietary variation in the HMM across match-paired samples.

Notably, *Streptophyta* was present in the 25 most-abundant genera in our dataset. In some databases used for classification, *Streptophyta* is assigned as a genus within the phylum Cyanobacteria. The basis for this classification is that Cyanobacteria are dominated by chloroplast sequences (Leliaert et al., 2012; Raven & Allen, 2003). While some researchers remove these sequences from their datasets because they consider them as contaminants, others choose to keep them as there is no solid explanation for the presence of *Streptophyta*, in the same way that we currently have no explanation for the majority of other taxa present in the HMM identified through culture-dependent methods (Fernández et al., 2013). For this reason, in this exploratory analysis we elected to retain this genus. Additionally, healthy human milk samples would also likely be considered as samples with low bacterial biomass, and as such would potentially be impacted more with the presence of "contaminating DNA" stemming from DNA isolation kits, PCR master mixes, and other laboratory supplies (Glassing, Dowd, Galandiuk, Davis, & Chiodini, 2016; Salter et al., 2014). It is noteworthy that we implemented negative controls at each step, from DNA isolation through evaluation of PCR amplicons and found no evidence of "contaminating DNA". Although bacterial DNA emanating from various solutions and kits used during the extraction and PCR of samples might have been present, the negative controls suggest that the impact on bacterial composition of the milk samples would have been negligible or very minimal.

We found numerous indications that the structure of the HMM is associated with social and caregiving environments.



Milk produced by mothers of infants with larger caregiving networks and higher frequency of allocare had higher microbial evenness than milk produced by mothers whose infants had few caregivers and spent the majority of their time in contact with their mother, as opposed to allomothers; however, these social factors were not associated with microbial richness. In contrast, although only a trend, there were indications that the intensity of an infant's caregiving environment was associated with greater microbial richness in the milk produced by his/her mother, but not with evenness. These patterns suggest that different dimensions of the social environment impact different dimensions of microbial diversity. Although this is the first study, to our knowledge, that has examined the potential role of the social environment in shaping the HMM, these results are consistent with the few human and nonhuman primate studies examining the influence of social interactions on GI microbiome diversity and composition (Grieneisen, Livermore, Alberts, Tung, & Archie, 2017; Moeller et al., 2016; Raulo et al., 2017; Schnorr et al., 2014; Tung et al., 2015).

The relationship between milk microbial biodiversity, that is, the richness and evenness of the bacterial community, and the impact on maternal and infant health are unclear. Yet, previous studies on human gastrointestinal microbial communities have shown that differences in bacterial community biodiversity are associated with different health states (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). Because humans are a cooperative breeding species, these results suggest a possible alternative pathway for mothers to prime infants' immature immune systems for their environment. Further research is needed to better understand the implications of differences in evenness and biodiversity of the HMM on health of the mother-infant dyad.

That social interactions are associated with variation in the HMM offers additional evidence that the maternal-infant dyad's exchange of microorganisms, should not be considered unidirectional (mother-to-infant), but rather bidirectional (mother-to-infant and infant-to-mother). Such transfers have been noted previously by Ramsay et al. (2004) through ultrasound imaging and by Cabrera-Rubio et al. (2012) who identified *Veillonella*, *Leptotrichia*, and *Prevotella*, common oral bacteria, as more common in mature milk samples, suggesting infant-to-mother transfer. Our findings provide an additional line of evidence that the microbial composition of milk may be influenced via a feedback loop from the infant to the mother. However, as noted above, it is unlikely that any one pathway is solely responsible for the microbial colonization of the mammary gland.

Beyond increasing our understanding of variation in the HMM, these results expand our understanding of the evolutionary role of cooperative breeding in humans. Although we know that the presence of and investment by others is influential, with health and fitness consequences (Sear & Mace,

2008), the pathways of influence are rather elusive. Understanding such pathways is essential, as cooperative breeding is ubiquitous across human populations. Around the world, social networks are not supplementary in the postpartum period; rather, they frequently serve as the foundation to the mother-infant relationship (Meehan et al., 2016). Thus, mother-infant dyads should not be studied in isolation. Here we begin to consider possible pathways by which others influence the health and well-being of the mother-infant dyad. For example, it is thought that bacteria and other milk components stimulate development of the infant's immune system by providing antigens to prime existing immune defenses (Walker & Iyengar, 2015); although, this may be contextually dependent, in that immune profiles of human milk vary based on geographic location (Ruiz et al., 2017). It is also possible that the milk microbiome protects the infant from infection, though these mechanisms have yet to be fully elucidated (Fernández et al., 2013). If the role of the HMM on infant health is to be fully understood, particularly in the instance of disease states or dysbiosis, the normal microbial composition of human milk must be understood within the context in which it is produced. As such, connections between the HMM and social environment generated from this study could contribute to a better understanding of what is influencing infant health in high-risk environments and, more importantly, how their immune systems are primed for the social and physical worlds in which they reside.

This study also highlights the essential nature of interdisciplinary microbiome research. As argued by Benezra, DeStefano, and Gordon (2012), collaboration between biologists and anthropologists will create "an anthropology of microbes" that will transform our understanding of the bacterial communities that colonize humans. Microbiome research sits at the intersection of evolution, biology, ecology, culture, behavior, and health, and as such requires transdisciplinary collaborations to address its coevolution with host species and its role in health and wellbeing.

In summary, our results provide further evidence for common bacterial components in human milk and contribute to previous research highlighting broad regional and geographic influences on the HMM (Kumar et al., 2016). Additionally, our results highlight that although HMM alpha diversity does not vary greatly among women residing in a similar ecology or across seasons, the bacterial community composition of milk does vary across these populations and seasons, suggesting possible genetic and/or microenvironmental influences. Moreover, we found evidence for an influence of social environments on milk microbiota composition. This study was the first step in our attempt to understand how environment, behavior, and social networks interact to affect human milk composition. Future studies should identify whether the social transmission of microorganisms is related to and/or influences mammary and infant health.

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AUTHOR CONTRIBUTIONS

CLM, JEW, MAM, and MKM designed the project. JR, CH, CLM collected the samples and data. JEW and KAL processed the samples. CLM, KAL, JEW, EHH analyzed the data and wrote the paper. All authors discussed the results and commented on the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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