



# Human Milk Microbiota in an Indigenous Population Is Associated with Maternal Factors, Stage of Lactation, and Breastfeeding Practices

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## ABSTRACT

**Background:** Human milk contains a diverse community of bacteria that are modified by maternal factors, but whether these or other factors are similar in developing countries has not been explored. Our objective was to determine whether the milk microbiota was modified by maternal age, BMI, parity, lactation stage, subclinical mastitis (SCM), and breastfeeding practices in the first 6 mo of lactation in an indigenous population from Guatemala.

**Methods:** For this cross-sectional study, Mam-Mayan indigenous mothers nursing infants aged <6 mo were recruited. Unilateral human milk samples were collected ( $n = 86$ ) and processed for 16S rRNA sequencing at the genus level. Microbial diversity and relative abundance were compared with maternal factors [age, BMI, parity, stage of lactation, SCM, and 3 breastfeeding practices (exclusive, predominant, mixed)] obtained through questionnaires.

**Results:** *Streptococcus* was the most abundant genus (33.8%), followed by *Pseudomonas* (18.7%) and *Shingobium* (10.7%) but relative abundance was associated with maternal factors. First, *Lactobacillus* and *Streptococcus* were more abundant in early lactation whereas the common oral (*Leptotrichia*) and environmental (*Comamonas*) bacteria were more abundant in established lactation. Second, *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Micrococcus* had a higher abundance in multiparous mothers compared with primiparous mothers. Third, a more diverse microbiota characterized by a higher abundance of lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, and *Lactococcus*), *Leucobacter*, and *Micrococcus* was found in mothers with a healthy BMI. Finally, distinct microbial communities differed by stage of lactation and by exclusive, predominant, or mixed breastfeeding practices.

**Conclusion:** Milk bacterial communities in an indigenous community were associated with maternal factors. Higher microbial diversity was supported by having a healthy BMI, the absence of SCM, and by breastfeeding. Interestingly, breastfeeding practices when assessed by lactation stage were associated with distinct microbiota profiles. *Curr Dev Nutr* 2021;5:nzab013.

**Keywords:** indigenous, Mam-Mayan, milk microbiota, 16S rRNA sequencing, microbial diversity, exclusive breastfeeding, predominant breastfeeding

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Supplemental Table 1 and Supplemental Figures 1–3 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/cdn/>.

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Abbreviations used: CAP, constrained analysis of principal coordinates; db-RDA, distance-based redundancy analysis; EBF, exclusive breastfeeding; FC, fold change; FDR, false discovery rate; PBF, predominant breastfeeding; SCM, subclinical mastitis.

## Introduction

Human milk has long been acknowledged as the ideal food for infants to meet energy and nutrient needs and for its immune properties (1). Human milk is also recognized as an important source of bacteria for the infant (2, 3). In fact, the human milk microbiota has been described

as possibly the most influential postnatal factor in the metabolic and immunological programming of infant growth (4).

Multi-country studies reveal that the most common genera present in the human milk microbiota differ among countries (5, 6). For example, although *Lactobacillus* is relatively common in Spain (7–11), most of the studies conducted in Africa, Asia, and Latin-America

do not report *Lactobacillus* as a predominant taxon (12–16). There is also evidence that the microbiota differs within a country based on social characteristics including socioeconomic status (14, 17) and rural versus urban setting (4–6, 13, 16, 18), where a more diverse microbiota have been reported in rural agrarian populations. There is evidence that some bacterial taxa in milk vary between hunter-gatherers versus horticulturalist women (19). For instance, *Enhydrobacter*, *Renibacterium*, and *Lactobacillus* were more abundant in horticulturalist women whereas *Peptoniphilus*, *Salinococcus*, *Planobacterium*, *Anaerococcus*, *Granulicatella*, *Riemerella*, *Kocuria*, and *Luteimonas* were more abundant in hunter-gatherers. Both populations showed unique bacteria although  $\alpha$ -diversity did not vary (19). A study in India reported high Alphaproteobacteria, Betaproteobacteria, Actinobacteria, Clostridia, and Firmicutes in agrarian communities, whereas human milk from urban women had more Proteobacteria and Gammaproteobacteria (16). The rural Ethiopian community differed from urban populations in abundance of the following genera in descending order, *Rhizobium*, *Achromobacter*, *Streptococcus*, *Staphylococcus*, *Propionibacterium*, and *Rothia* (6).

Specific maternal factors including maternal BMI, delivery mode, and lactation stage are known to modify the human milk microbiota (7, 8, 11, 14, 18, 20–24). With regard to BMI, some studies report no association (20, 25) but others report that higher maternal BMI and weight gain during pregnancy impact the diversity of the bacterial community in milk (8, 26). Other studies have correlated human milk microbial composition with lactation stage (27–29). It has been reported that colostrum and transitional milk only have 50% of bacterial genera and 42% of bacterial species in common (12).

In contrast, several important factors that could be associated with the human milk microbiota have not been widely explored; these include maternal age, parity, SCM, and breastfeeding practices. Maternal age, in particular adolescent pregnancies, is associated with increased nutrient requirements (30). Moreover, it is also known that adolescence can impact the development and maturity of the mammary gland and has been associated with changes in immune factors in human milk as well as its microbiota (31). Parity is another factor that could be related to age and might influence the milk microbiota. Assuming retrograde flow, the milk from a multiparous mother will have been previously inoculated by the bacteria transferred by her previous infant(s) during earlier pregnancies (32). The other 2 factors that have been overlooked in the study of the milk microbiota include SCM and breastfeeding practices. The limited research available focuses on mastitis (33) rather than SCM and in relation to breastfeeding practices, most of the literature compares human milk and formula rather than exclusive, predominant, and mixed feeding (27, 29, 34–37).

These overlooked factors may be particularly important in developing countries. Worldwide, 16 M (million) adolescents aged between 15 and 19 y and another 2.5 M under the age of 16 y give birth; moreover, adolescent pregnancies are 3× more frequent in rural and indigenous populations (38). In relation to parity, Central America, Guatemala, Nicaragua, and Panama have the highest adolescent fertility rates (39). Finally, for SCM and breastfeeding practices, previous reports have shown that SCM is common in Guatemala and differs by stage of lactation (40, 41).

On the other hand, developing countries have higher rates of exclusive breastfeeding, whereas mothers in developed countries have

lower breastfeeding rates and routinely do not exclusively or predominantly breastfeed for 6 mo (42). Current WHO guidelines recommend either exclusive or predominant breastfeeding for 6 mo (43), and according to recent UNICEF data, rural populations in developing countries are more likely to achieve these goals than other sectors (42). Guatemala reportedly has one of the highest exclusive breastfeeding rates, approaching 76.8%, from which 78.6% of the sample were indigenous women (44). The Mam-Mayan community of Guatemala also has the tradition of introducing ritual fluids called “*agüitas*” during breastfeeding feeding in order to promote infant health and treat gastrointestinal or other infections (45). The consumption of *agüitas* in Guatemala has been reported to be 22% during the first 6 mo (46).

Given the maintenance of exclusive and predominant breastfeeding and the ease of obtaining human milk from Mam-Mayan mothers from the Western Highlands of Guatemala, our study provided us with a unique opportunity to measure the milk microbiota in our biobanked human milk samples. The purpose of this cross-sectional study was to explore if genera in the human milk microbiota were associated with maternal age, BMI, parity, lactation stage, the presence of SCM, and various breastfeeding practices.

## Methods

### Study site and participants

This cross-sectional study was part of a collaboration between McGill University and the Center for Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM) in the Republic of Guatemala. Field studies were conducted from June 2012 through January 2013 in 8 rural Mam-speaking communities of the San Juan Ostuncalco region in Guatemala (47). Lactating mothers of infants aged from 5 to 46 d or 4 to 6 mo postpartum were identified and invited to participate by community health workers. Inclusion criteria included mothers who breastfed for 6 mo, had healthy BMIs, and had vaginal deliveries. Individuals who had been treated with antibiotics during the postpartum period were excluded as the use of antibiotics has been demonstrated to decrease the rate of SCM (48). Ethical approval was obtained from McGill Institutional Review Board and Center for Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM) Human Subjects Committee. All participating mothers provided written informed consent for participation.

### Study design

We compared human milk bacterial communities by maternal age (adolescents aged:  $\leq 19$  y versus adults:  $> 19$  y), weight (healthy: BMI = 18.5–24.9 kg/m<sup>2</sup> versus overweight: BMI  $\geq 25$ ), parity (primiparous versus multiparous), stage of lactation [early (5–46 d) versus established (4–6 mo)], SCM (yes: Na/K  $> 0.6$  or no: Na/K  $\leq 0.6$ ), and breastfeeding (exclusive or predominant versus mixed). Exclusive breastfeeding (EBF) was defined as providing only human milk to the infant and predominant breastfeeding (PBF) was defined as providing water or *agüitas* in addition to human milk. *Agüitas* are ritual fluids; the infusions more commonly used are boiled water, sugar water, chamomile tea, corn paste water, anise water, orange leaf water, mint water, or sage water. They are given for perceived insufficient milk, for irritability and crying and to

maintain infant's health, or as herbal medicine to treat gastrointestinal or other infections (45, 47, 49). Breastfeeding was categorized as mixed if mothers provided complementary foods to the infant.

### Human milk sample collection

Milk samples from early lactation (5–46 d postpartum) and established lactation (4–6 mo postpartum) were collected. These ranges were chosen to be consistent with our previous studies that measured infant growth (40, 41, 50). Prior to collection, the nipple and areola of the breast were cleaned with 70% ethyl alcohol. Human milk samples were collected during a 3-h time window in the morning from the breast not recently used for breastfeeding via full manual expression by a trained midwife, who used hand sanitizer before and after collection. Milk was collected into 60 mL plastic trace-element-free vials and immediately stored on ice. Samples were partitioned into 15 mL trace-element-free vials at the field laboratory (−30°C) prior to transfer on dry ice to McGill University where they were stored at −80°C until analysis was performed.

### 16S rRNA pyrosequencing using Illumina MiSeq

DNA extraction was done using 1 mL of whole milk (direct aliquots from the stock) with the DNeasy Blood and Tissue mini kit from Qiagen according to the manufacturer's protocol. A centrifuge was used to spin down the sample and only the pellet was kept for further extraction steps; all the supernatant, including the fat, were discarded. For PCR, a region of ~526 bp in the 16S rRNA gene, covering V1–V3 was amplified with the universal eubacterial primers 27F (AGAGTTTGATCCTGGCTCAG) and 533R (TTACCGCGGCTGCTGGCAC) (8). The subsequent 16S rRNA sequencing was performed using Illumina MiSeq at McGill University and Genome Quebec Innovation Centre.

### Microbial data processing

Using Mothur's MiSeq protocol (51), raw paired-end reads (13.0 M) were assembled into contigs that were aligned together. Unique representative sequences were then extracted and their abundance evaluated. Raw contig counts were used to remove sequences with low abundance (contig count cut-off of 10); 14,742 sequences representing possible unique amplicons were extracted, comprising 10.5 M counts in total (accounting for 97% of initial raw reads).

Metagenomic sequences were analyzed via BLASTn against the nonredundant nucleotide database (nt) with an Expect (E) Value threshold of  $1.10^{-10}$  and an identity cut-off of 97%. The metatranscriptomic (unconstrained) annotation strategy (52) was employed together with a previously reported annotation selection method used to help select the primary annotation from ambiguous BLASTn returns from metatranscriptomic/metagenomic data (53).

Sequences were identified as a potential genus when their identity cut-off was  $\geq 99\%$ , the BLASTn alignment coverage was  $\geq 95\%$ , and no other competing genera label was present. Taxonomic units ( $n = 125$ ) were detected at 99% identity (from genus up to phylum level) and were used to bin the remaining sequences at 97%, capturing 5.0 M sequences in total. Sparse taxonomic units (a high proportion of zero counts across samples) and samples with low diversity were removed manually.

The  $\alpha$ -diversity of human milk samples, describing the within-community diversity of the microbiota within a milk sample, was measured using 2 different metrics (Shannon and Inverse Simpson) within

**TABLE 1** Characteristics of the indigenous population of Mam-Mayan mothers ( $n = 86$ )

Maternal factors	Mean $\pm$ SD or %
BMI, kg/m <sup>2</sup>	23.3 $\pm$ 3.2
Healthy weight (18.5–24.9), %	78
Overweight (>25), %	22
Stage of lactation	
Early 5–46 d, %	45
Early, x days	20.2 $\pm$ 10.6
Established 4–6 mo, %	55
Established, x days	144.1 $\pm$ 20.1
Parity	
Primiparous, %	48
Multiparous, %	52
Age, y	23.5 $\pm$ 6.4
Adolescent $\leq 19$ , %	23
Adult >19, %	77
Subclinical mastitis	
Yes Na/K >0.6, %	12
No Na/K $\leq 0.6$ , %	88
Breastfeeding practice	
Exclusive and predominant, %	86
Mixed, %	14

the Phyloseq R package (54). The  $\alpha$ -diversity was compared between groups of samples using a *t*-test. The  $\beta$ -diversity, describing the microbiota diversity among samples, was estimated based on variation stabilization (VS) normalized counts (DESeq) using Bray–Curtis dissimilarity and the Constrained Analysis of Principal Coordinates (CAP) ordination method. Dispersion ellipses were drawn using the *vegan*-*CovEllipse* function in the *Vegan* package in R (55). Significant distance was evaluated between groups using nonparametric analysis of similarities (ANOSIM) on normalized counts based on Bray distances (*Vegan* package). The species richness of samples was also assessed with rarefaction curves.

Diversity relative abundance was represented with Krona (56). Taxonomic profiles were plotted with GraPhlAn (57). Parametric models developed in transcriptomics to characterize differentially abundant taxonomic units between groups of samples perform well when applied to microbiota biomarker data with uneven library sizes, sparsity, and sample representativity (58). From the several programs available, DESeq2 (59) has been found to have the best overall performance (60). The DESeq2 univariate procedure was selected, as implemented in *Phyloseq*, to calculate differentially abundant taxonomic units. To account for the power to identify differentially abundant genera, the group size was fixed so none of the groups compared had <10 samples and the FC effect size threshold was set at 5 (60). Taxonomic units tested with a Benjamini–Hochberg adjusted *P* value < 0.05 were considered significant.

## Results

### Characteristics of the population

Population characteristics are summarized in Table 1 and individual characteristics of participating mothers in Supplemental Table 1. They had a mean age of 23.5  $\pm$  6.4 y and mean maternal BMI was healthy

(23.3 ± 3.2); 48% were primiparous and 52% were multiparous. In relation to the breastfeeding practices, 86% exclusively or predominantly breastfed, and 14% breastfed and also provided complementary foods (mixed breastfeeding); 45% were in the early stage of lactation (5–46 d), 55% in established lactation (4–6 mo), and 12% had SCM and 88% did not (non-SCM).

### Human milk microbiota

The 16S rRNA analysis of 86 milk samples resulted in 5,053,154 sequences and successfully identified 47 different taxonomic units (from phylum to genera). The total pool of sequences was classified to 3 known phyla, Proteobacteria (52.8%), Firmicutes (40.3%), and Actinobacteria (2.4%). *Streptococcus* was the most abundant genus making up 33.8% of the sequences, followed by *Pseudomonas* at 18.7% and *Sphingobium* at 10.7%. *Lactobacillus* only accounted for 0.60% of the milk bacterial community (Figure 1). Rarefaction curves are shown in Supplemental Figure 1.

### Maternal factors

The distance-based redundancy analysis (db-RDA) plots (Figure 2) showed interesting separations in bacterial communities for 3 conditions: maternal BMI, parity, and lactation stage. A lesser separation was observed among communities for maternal age, feeding practices, and SCM. No differences in  $\alpha$ -diversities (within-sample diversity) were detected for maternal age, BMI, lactation stage, feeding practices, and SCM, but multiparous mothers had higher richness than primiparous mothers (Shannon,  $P = 0.01$ , Inv Simpson,  $P = 0.03$ ) (Supplemental Figure 2). The  $\beta$ -diversity (between-sample diversity) differed between early and established lactation only ( $P = 0.001$ ).

Differential abundance analyses showed that 5 bacterial taxonomic units significantly differed between the milk of adult and adolescent mothers. Adult mothers had a higher abundance of *Streptococcus* [false discovery rate (FDR) =  $3.74 \times 10^{-15}$ ; fold change (FC) = 130], *Neisseria* (FDR =  $7.09 \times 10^{-15}$ ; FC = 48), and *Micrococcus* (FDR =  $3.40 \times 10^{-9}$ ; FC = 17), whereas *Roseomonas* (FDR =  $6.77 \times 10^{-11}$ ; FC = 10) and *Afipia* (FDR =  $1.09 \times 10^{-8}$ ; FC = 8) were more abundant in adolescent mothers. Compared with adult mothers, adolescent mothers had low mean counts of *Streptococcus* (Figure 3, Table 2). The relative abundance values of the top 20 genera for maternal age are shown in Supplemental Figure 3.

For maternal BMI, 6 differentially abundant taxonomic units also emerged. Healthy weight mothers had a higher abundance of *Comamonas* (FDR =  $3.47 \times 10^{-14}$ ; FC = 142), *Streptococcus* (FDR =  $8.47 \times 10^{-13}$ ; FC = 134), *Sphingobium* (FDR =  $8.14 \times 10^{-14}$ ; FC = 120), *Lactobacillus* (FDR =  $1.37 \times 10^{-7}$ ; FC = 23), and *Dietzia* (FDR =  $1.31 \times 10^{-5}$ ; FC = 13) than overweight mothers. The mean counts of *Comamonas* and *Streptococcus* of overweight mothers were very low. Only *Roseomonas* (FDR =  $1.93 \times 10^{-9}$ ; FC = 8) was more abundant in the milk of overweight mothers than healthy weight mothers (Figure 3, Table 2). The relative abundance values of the top 20 genera for maternal BMI are shown in Supplemental Figure 3.

For parity, 8 bacterial taxonomic units significantly differed between the milk of primiparous and multiparous mothers. Multiparous mothers had a higher abundance of *Streptococcus* (FDR =  $3.92 \times 10^{-21}$ ; FC = 127), *Lactobacillus* (FDR =  $1.29 \times 10^{-9}$ ; FC = 26), *Lactococ-*

*cus* (FDR =  $1.73 \times 10^{-14}$ ; FC = 24), *Leuconostoc* (FDR =  $5.81 \times 10^{-7}$ ; FC = 7), and *Micrococcus* (FDR =  $1.49 \times 10^{-7}$ ; FC = 7) in milk than primiparous mothers, whereas *Klebsiella* (FDR =  $1.49 \times 10^{-7}$ ; FC = 11), *Comamonas* (FDR =  $3.03 \times 10^{-4}$ ; FC = 11), and *Roseomonas* (FDR =  $1.49 \times 10^{-7}$ ; FC = 7) were more abundant in the milk of primiparous mothers (Figure 3, Table 2). The relative abundance values of the top 20 genera for parity are shown in Supplemental Figure 3.

### Lactation characteristics

Differential abundance analyses showed that 4 bacterial taxonomic units significantly differed between early and established lactation. During early lactation, milk had a higher abundance of *Lactobacillus* (FDR =  $3.73 \times 10^{-40}$ ; FC = 378) and *Streptococcus* (FDR =  $4.61 \times 10^{-28}$ ; FC = 178), whereas during established lactation, milk had a higher abundance of *Comamonas* (FDR =  $1.47 \times 10^{-9}$ ; FC = 37) and *Leptotrichia* (FDR =  $3.18 \times 10^{-6}$ ; FC = 9). *Lactobacillus* spp. were not detected in most milk samples collected during established lactation (Figure 3, Table 2). The relative abundance values of the top 20 genera for stage of lactation are shown in Supplemental Figure 3.

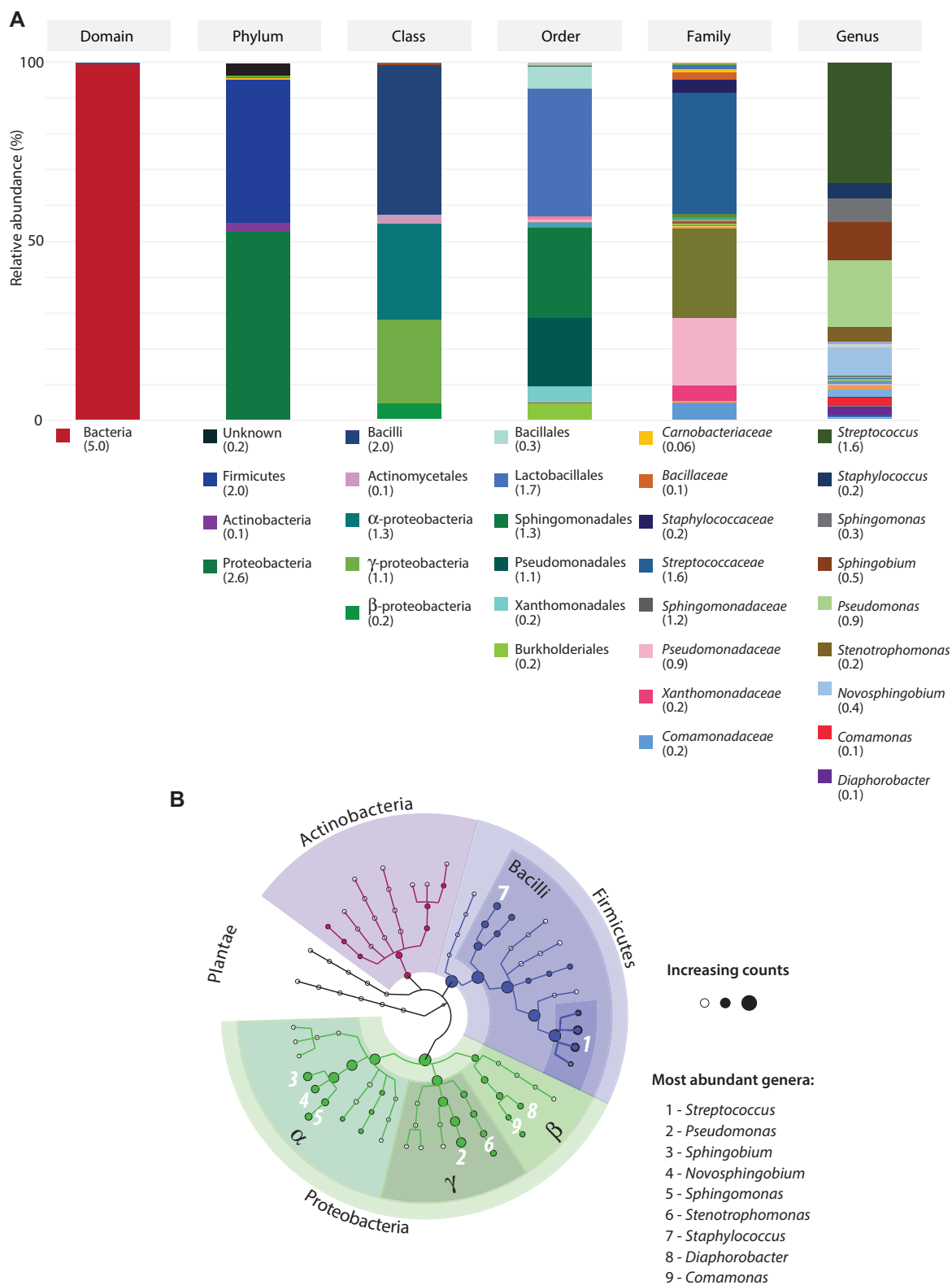
For breastfeeding practices, 10 bacterial taxonomic units significantly differed between EBF/PBF and mixed feeding mothers. EBF/PBF mothers had a higher abundance of *Brevundimonas* (FDR =  $2.11 \times 10^{-7}$ ; FC = 51), *Paracoccus* (FDR =  $4.84 \times 10^{-7}$ ; FC = 32), *Corynebacterium* (FDR =  $3.13 \times 10^{-5}$ ; FC = 20), *Lactobacillus* (FDR =  $2.82 \times 10^{-4}$ ; FC = 17), *Neisseria* (FDR =  $1.72 \times 10^{-4}$ ; FC = 17), *Lactococcus* (FDR =  $5.05 \times 10^{-4}$ ; FC = 12), *Micrococcus* (FDR =  $5.05 \times 10^{-4}$ ; FC = 11), *Leucobacter* (FDR =  $9.81 \times 10^{-4}$ ; FC = 11), and *Afipia* (FDR =  $8.24 \times 10^{-4}$ ; FC = 10) in milk. Only *Leptotrichia* (FDR =  $1.34 \times 10^{-4}$ ; FC = 13) was more abundant in the milk of mixed feeding mothers (Figure 3, Table 2). The relative abundance values of the top 20 genera for breastfeeding practices are shown in Supplemental Figure 3.

When we further categorized breastfeeding practices (exclusive, predominant, and mixed) by stage of lactation (early and established), distinct separations in microbiota among each of these groups were observed. The microbiota of exclusive differed from predominant by stage of lactation and both differed compared with mixed feeding (Figure 4).

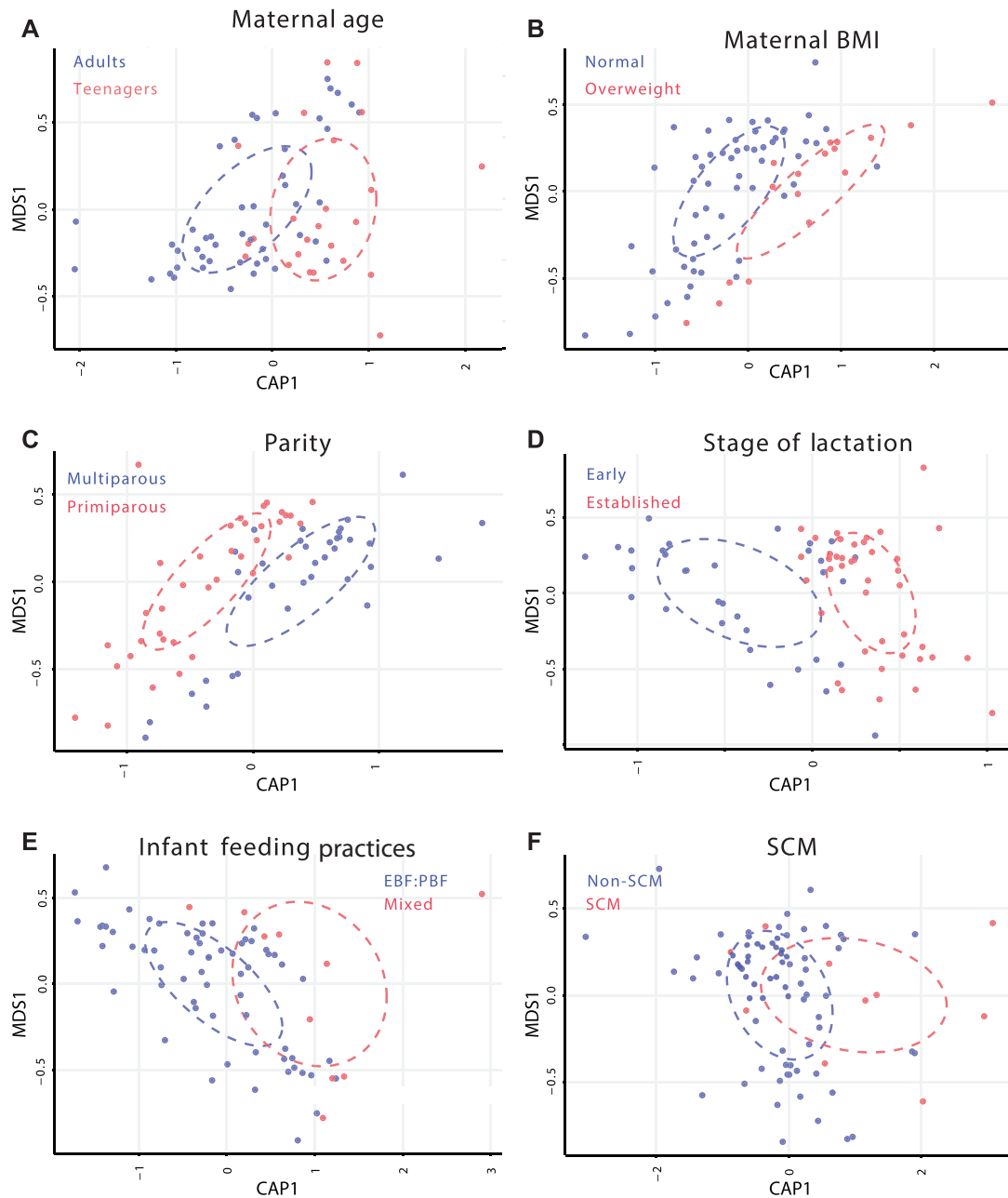
With regards to SCM, 7 bacterial taxonomic units were significantly more abundant in the milk of mothers without SCM than mothers with SCM. Mothers without SCM had a higher abundance of *Lactobacillus* (FDR =  $4.53 \times 10^{-5}$ ; FC = 32), *Bosea* (FDR =  $7.37 \times 10^{-5}$ ; FC = 24), *Leuconostoc* (FDR =  $1.63 \times 10^{-4}$ ; FC = 18), *Lactococcus* (FDR =  $2.27 \times 10^{-4}$ ; FC = 17), *Micrococcus* (FDR =  $2.27 \times 10^{-4}$ ; FC = 16), *Leucobacter* (FDR =  $8.73 \times 10^{-4}$ ; FC = 13), and *Klebsiella* (FDR =  $9.67 \times 10^{-4}$ ; FC = 9). *Bosea*, *Lactococcus*, and *Leucobacter* were absent in all SCM samples and *Lactobacillus* was only detected in 1 SCM sample (Figure 3, Table 2). The relative abundance values of the top 20 genera for SCM are shown in Supplemental Figure 3.

### Discussion

Our study is the first to investigate variations of bacterial communities in the human milk of the Mam-Mayan indigenous community in Guatemala, where mothers routinely comply with WHO recommendations to exclusively or predominantly breastfeed for 6 mo. In addition



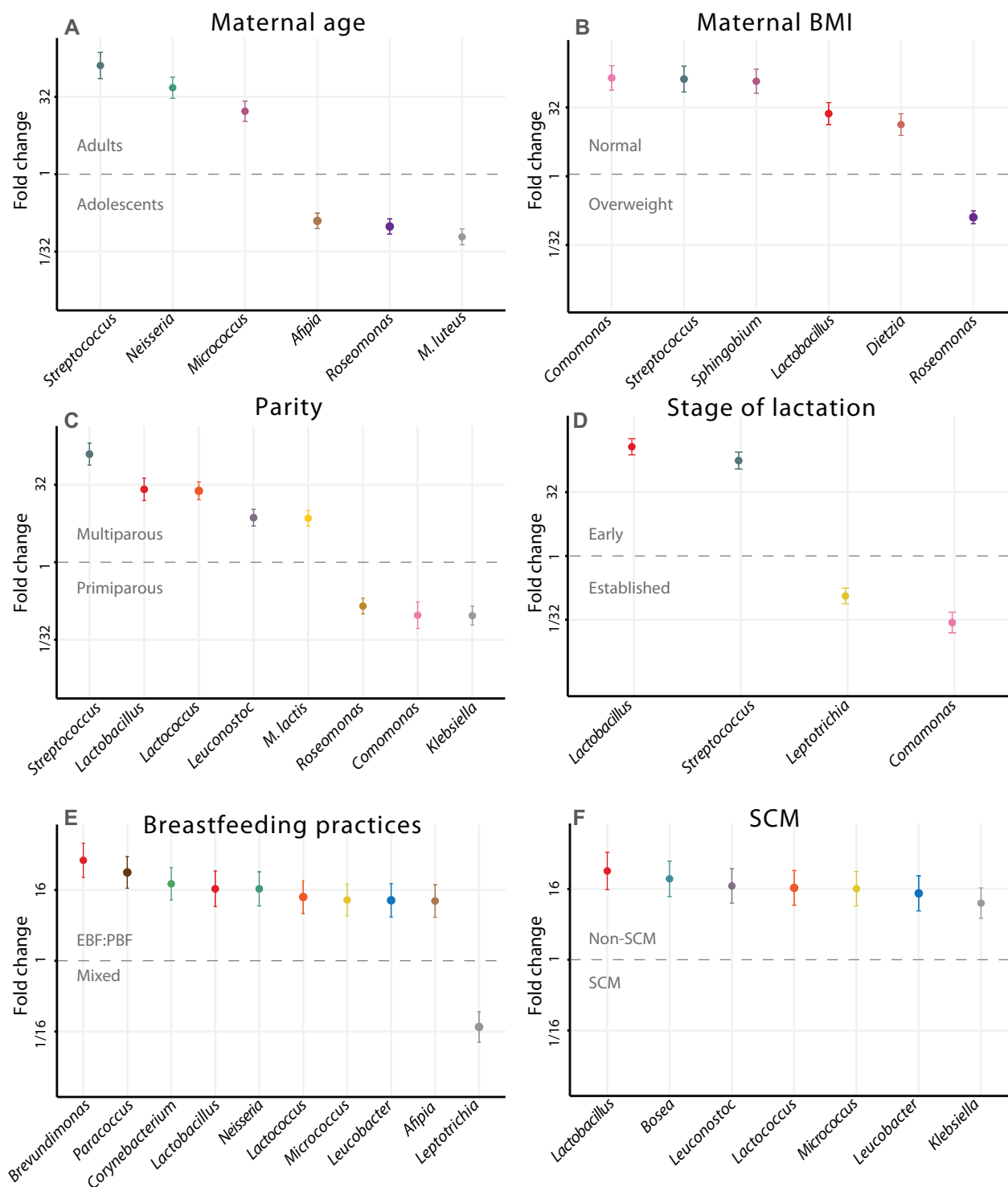
**FIGURE 1** A) Human milk microbiota diversity based on pooled samples from all mothers, illustrated as relative abundance along major taxonomic levels. Total counts for each taxonomic unit are specified between parentheses (in million). Only taxonomic units with a total count >100,000 are accounted for in the legends. B) Major clades including weighted nodes (genera >100,000 counts) as analyzed in the human milk microbiota. The 9 most abundant genera are identified.



**FIGURE 2** Distance-based redundancy analysis (db-RDA or CAP) ordination representations (Bray distance) for A) maternal age; B) maternal BMI; C) parity; D) stage of lactation; E) Infant feeding practices; and F) SCM. Significant clustering of groups was found for maternal BMI, parity, and stage of lactation (ANOVA),  $P < 0.05$ . EBF, exclusive breastfeeding; PBF, predominant breastfeeding; SCM, subclinical mastitis.

to BMI, the impact of understudied maternal factors including adolescent pregnancy, parity, late-stage lactation, the presence of SCM, and breastfeeding practices on the human milk microbiota were also explored. Six findings emerged. First, the human milk microbiota in our study population differed between early (<6 wk) and established lactation (4–6 mo). *Lactobacillus* and *Streptococcus* were more abundant in early lactation, whereas the common oral (*Leptotrichia*) and environmental (*Comamonas*) bacteria were more abundant in established lactation. Second, the milk microbiota differed between adolescent and

adult mothers. *Streptococcus*, *Neisseria*, and *Micrococcus* were higher in adult milk whereas, *Roseomonas* and *Afipia* were predominant in the milk of adolescent mothers. Third, a significantly higher abundance of *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Micrococcus* were observed in multiparous mothers, whereas the milk of primiparous mothers had a higher abundance of *Comamonas*, *Klebsiella*, and *Roseomonas*. Fourth, the milk of mothers with a healthy BMI was higher in *Comamonas*, *Streptococcus*, *Sphingobium*, *Lactobacillus*, and *Dietzia*, whereas in mothers with obesity only *Roseomonas* was higher. Fifth, in



**FIGURE 3** Differential abundance plots for each maternal factor: A) maternal age (adult versus adolescent); B) maternal BMI (healthy weight versus overweight); C) parity (primiparous versus multiparous); D) stage of lactation (early versus established); E) breastfeeding practices (exclusively/predominant breastfeeding versus mixed feeding); and F) SCM (yes or no). Each plot shows fold change for the differentially abundant taxonomic units (group size fixed to a minimum of 10 and effect size to a fold change of 5). Error bars represent the SE on the estimated fold change. EBF, exclusive breastfeeding; PBF, predominant breastfeeding; SCM, subclinical mastitis.

the absence of SCM, mothers had a more diverse microbiota that was characterized by a higher abundance of lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, and *Lactococcus*), *Leucobacter*, and *Micrococcus* compared with mothers with SCM, suggesting that the absence of lactic acid-producing bacteria may be associated with human milk dysbiosis and the emergence of SCM. Finally, greater microbial diversity was associated with exclusive and predominant breastfeeding practices compared with mixed feeding. Moreover, the microbiota differed between exclusive and predominant at each stage of lactation. Collectively, these findings showed that indigenous communities have several distinct maternal factors that shift the milk microbiota. Based on our findings, we propose that evidence from developed countries regarding the milk microbiota may not be transferable to developing countries due to differing environments and population characteristics.

### Characteristics of predominant phyla and genera

Our identification of 3 phyla in human milk, Proteobacteria (52.8%), Firmicutes (40.3%), and Actinobacteria (2.4%), aligns with the latest systematic review that described Firmicutes and Proteobacteria as the most predominant phyla with Actinobacteria present at lower relative abundances (24). The most abundant genera were *Streptococcus* (33.8%), *Pseudomonas* (18.7%), and *Sphingobium* (10.7%). On one hand, *Streptococcus* spp. have been commonly reported as one of the most abundant genera in human milk (15, 18–20, 23, 25, 61–63). *Pseudomonas* is also a commonly detected genus in a range of relative abundance from <1 to 17% (24). On the other hand, *Sphingobium* spp. have been previously observed in the human milk microbiota (10, 64); however, it is not generally considered as an abundant or frequent genus.

### Maternal factors

#### Stage of lactation.

It is well-established that human milk volume and nutritional immunological composition progressively change over the lactation period (36, 65). There is now evidence that the milk microbiota also differs by stage of lactation (8, 11, 37, 66), although not all studies report differences (18, 20, 23). However, none of these aforementioned studies were conducted in agrarian indigenous communities. In our study, we were able to take advantage of the fact that all of the indigenous mothers complied with WHO guidelines to breastfeed for 6 mo. Under these conditions *Streptococcus* and *Lactobacillus* were higher in early lactation. *Lactobacillus* has been reported to be more abundant in the early stage of lactation (8) and *Streptococcus* is considered to be part of the “core” microbiota in human milk (6, 8, 22, 62). Additionally, *Streptococcus*, the most abundant genus observed in our samples, has been reported as a putative organism (67) that is capable of suppressing the growth of pathogenic bacteria such as *Staphylococcus aureus*. Both *Lactobacillus* and *Streptococcus*, which were more abundant in early lactation in our study, have immunomodulatory and anti-inflammatory properties and functions that contribute to the stability of bacterial populations (68–70). On the other hand, *Lactobacillus* was absent in 55% of milk samples collected during established lactation, which contrasts with previous research that reports an increasing prevalence of *Lactobacillus* as lactation progresses (8, 11). However, these studies were conducted in developed countries and other factors could be intervening as well. We observed that both *Comamonas* and *Leptotrichia* were higher in established lactation. *Leptotrichia* is a common oral bacteria previously reported in

established lactation, whereas *Comamonas* is a common environmental bacteria that has been associated on occasion with human disease (71), suggesting dynamic differences in the milk microbiota during lactation associated with other factors that also differed by stage of lactation in our study.

#### Maternal age.

To date, the only study to consider whether maternal age was associated with the human milk microbiota reported no association between adult maternal age ( $33.0 \pm 4.2$  y) and the human milk microbiota; all participants were adults aged between 20 and 40 y (22). Our study is the first to perform a differential abundance analysis of the human milk bacterial taxonomy between adolescent and adult mothers. Our findings show that *Streptococcus*, *Neisseria*, and *Micrococcus* had a higher abundance in milk samples of adult mothers. *Streptococcus* and *Neisseria* are normally present in the infant oral cavity and saliva (72, 73). *Micrococcus*, which is a common human colonizer that rarely causes infections, is found in the skin, mucosa, and oropharynx and is likely transmitted through retrograde flow (66). In contrast, the genera *Roseomonas* and *Afipia*, of which some species are known to be opportunistic bacteria, had a higher abundance in the milk of adolescents (74, 75).

It is possible that these differences in the human milk microbiota of adolescent and adult mothers could be linked to the development of breast milk immunity, which occurs during adolescence (76). The immature mammary gland undergoes 3 age-related developmental phases; embryonic, adolescent, and adult, with the majority of its alveolar branching taking place during adolescence (31). The alveolar epithelium is responsible for secreting immune modulators including IL-6, TNF, and IgA (77), and these concentrations in milk can be affected during adolescence (78). Perhaps the immaturity of the adolescent breast and its associations with lower concentrations of immune factors might underscore a higher relative abundance of opportunistic bacteria in adolescents, but this requires further investigation.

In addition to the immune system, adolescence is a period characterized by its own requirements for growth and physical development. In the developing country context where the prevalence of adolescence pregnancy is high (39) and where nutrient deficiency and malnutrition are common (79), human milk nutrient composition might modulate the human milk microbiota. There is evidence that adolescents' milk is lower in copper, zinc, retinol, and  $\alpha$ -tocopherol compared with adult milk (80–84). In these Mam-Mayan mothers, we have evidence that several human milk minerals and trace elements associated with human SCM were altered (40). Thus, variation in the nutrient composition of human milk might contribute to further alterations in the human milk microbiota.

#### Parity.

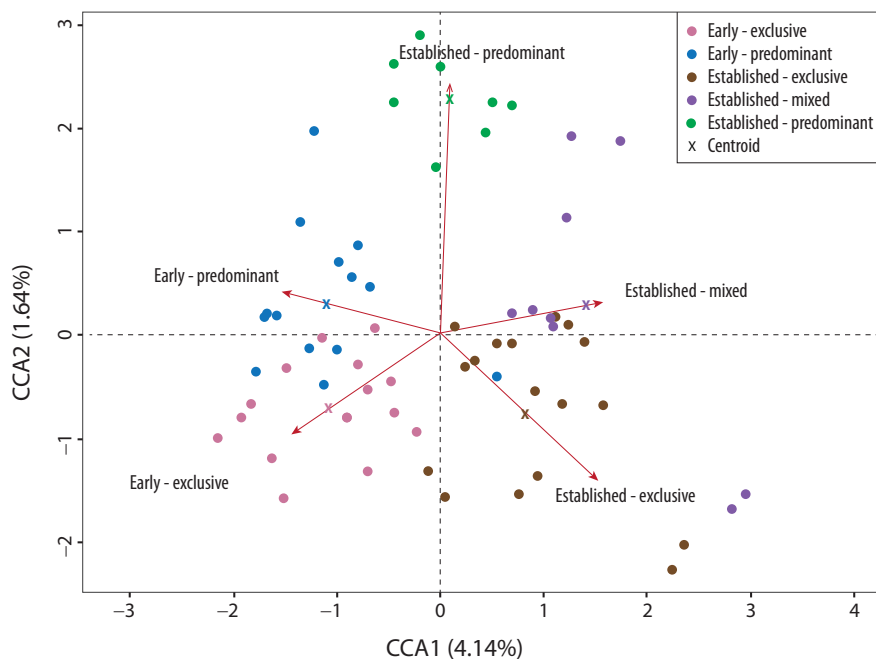
Parity is another underexplored maternal factor. To date, only 1 study has reported an association of milk microbiota composition with multiparity but did not characterize the microbiota (22). In our study, a significantly higher abundance of *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Micrococcus* were observed in our multiparous mothers. By contrast, primiparity was associated with a significant abundance of *Comamonas*, *Klebsiella*, and *Roseomonas*. Interestingly, *Roseomonas* is an example of bacteria that showed a higher relative abundance in both adolescent and primiparous mothers. Presumably primiparous



**TABLE 2** Comparative summary of differentially abundant bacterial genera by maternal factors and infant feeding practices<sup>1</sup>

	Adult mothers	Healthy BMI	Multiparous > 1 childbirth	Early lactation	EBF:PBF	Non-SCM
Actinobacteria	—	↑	—	—	—	—
Actinobacteria; Actinomycetales; Dietziaceae; Dietzia	—	↑	—	—	—	—
Actinobacteria; Actinomycetales; Micrococaceae; Micrococcus	↑	—	—	—	↑	↑
Actinobacteria; Corynebacteriales; Corynebacteriaceae; Corynebacteriumhis	—	—	—	—	↑	—
Actinobacteria; Micrococineae; Microbacteriaceae; Leucobacter	—	—	—	—	↑	↑
Firmicutes	—	↑	↑	↑	↑	↑
Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	—	↑	↑	↑	↑	↑
Bacilli; Lactobacillales; Leuconostocaceae; Leuconostoc	—	—	↑	—	—	↑
Bacilli; Lactobacillales; Streptococcaceae; Lactococcus	—	—	↑	—	↑	↑
Bacilli; Lactobacillales; Streptococcaceae; Streptococcus	↑	↑	↑	↑	—	—
Fusobacteria	—	—	—	↓	—	—
Fusobacteria; Fusobacteriales; Fusobacteriaceae; Leptotrichia	—	—	—	↓	↓	—
Proteobacteria	—	—	—	—	—	—
Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Brevundimonas	—	—	—	—	↑	—
Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Alfipia	↓	—	—	—	↑	—
Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bosea	—	—	—	—	—	↑
Alphaproteobacteria; Rhodobacteriales; Rhodobacteraceae; Paracoccus	—	—	—	—	↑	—
Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Roseomonas	↓	↓	↓	—	—	—
Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingobium	—	↑	—	—	—	—
Betaproteobacteria; Burkholderiales; Comamonadaceae; Comamonas	—	↑	↓	↓	—	—
Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria	↑	—	—	—	↑	—
Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Klebsiella	—	—	↓	—	—	↑

<sup>1</sup>Group size fixed to a minimum of 10 and effect size to a fold change of 5. Each arrow reflects the higher or lower differential abundance in comparison to the contrasting group (adults versus adolescents, healthy BMI versus overweight, multiparous versus primiparous, early lactation versus established lactation, exclusive and predominant breastfeeding versus mixed feeding and Non-SCM versus SCM). The symbol (—) represents "not found".



**FIGURE 4** Comparison at genera level by lactation stage and breastfeeding practices. Centroids crosses represent the shift of each group and their distinct cluster. Early lactation clusters differently than established; predominant and mixed feeding cluster higher on the y-axis scale than exclusive breastfeeding.

mothers were more likely to be adolescents, further supporting a link with immaturity of the human milk immune system during adolescence, as previously reported (76).

#### Maternal BMI.

Most studies that have considered the impact of BMI on the human milk microbiota have been conducted in urban populations and developed countries, and the results are contradictory. Some studies do not find any association (20, 25), whereas according to other studies a high maternal BMI impacts the bacterial composition in human milk (85); in some cases the diversity is less, with higher amounts of *Staphylococcus* and *Akkermansia* and lower numbers of *Bifidobacterium* in mature milk at 6 mo observed in women of a higher maternal BMI (8, 26). Among those conducted in developing countries, some have also reported no association (14, 20). In our study, only *Roseomonas* was more abundant in the milk of obese than healthy weight mothers. *Roseomonas*, which is considered an opportunistic bacteria to humans, and although it is a lactic acid producer, it has been isolated from aquatic environments and has been found in human blood (86). It has not been reported in previous studies in human milk but due to its environmental origin it might colonize milk after the infant's consumption of *agüitas* or directly via consumption by the mother. Further research is needed to understand its higher abundance in obese mothers.

#### SCM.

SCM is a common, asymptomatic, inflammatory condition of the lactating mammary gland (87, 88). Due to the lack of symptoms, SCM is commonly underdiagnosed and it has been suggested that human

milk bacterial dysbiosis may underscore SCM (48) and lead to impaired growth (40, 41). Previous reports describing the microbiota composition in both acute and subacute mastitis have identified *Aeromonas*, *Staphylococcus*, *Ralstonia*, *Klebsiella*, *Serratia*, *Enterococcus*, and *Pseudomonas* (33). Specifically, in subacute mastitis, human milk is reportedly dominated by the genus *Staphylococcus* (33, 89, 90). *Erwinia*, *Bacillus*, *Pantoea*, *Cronobacter*, and *Pseudomonas* may be present but *Acinetobacter*, *Ruminococcus*, *Clostridium*, *Faecalibacterium*, and *Eubacterium* are reportedly scarce (91). In general, previous studies have associated subacute (different from subclinical) mastitis with a lower microbial diversity, a greater abundance of pathogenic bacteria, and a depletion of commensal bacteria in human milk (33, 63, 91). In our SCM mothers, a lower microbial diversity was also found. Of note, *Bosea*, *Lactococcus*, and *Leucobacter* were absent in all our SCM samples and *Lactobacillus* was only rarely detected. In contrast, mothers who did not have SCM had a much higher abundance of lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, *Lactococcus*), *Leucobacter* and *Micrococcus* compared with mothers with SCM. Lactic acid bacteria isolated from human milk have previously been shown to prevent proliferation of pathogenic bacteria such as *Staphylococcus aureus* (92) and more recently, against a wider range of human pathogens such as *Salmonella enterica*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Vibrio cholerae*, and *Shigella flexneri* (69). In addition, *Lactobacillus gasseri*, *Lactobacillus salivarius*, and *Lactobacillus fermentum* have been used to treat mastitis with positive results; a lower recurrence of mastitis (93), lower staphylococcal count, and absence of symptoms at 14 d posttreatment (94) have been reported in mothers in Spain. In our study, the presence of *Lactobacillus* and *Lactococcus* in non-SCM milk and the complete absence of these bacteria in

SCM milk would suggest that the lack of lactic-acid-producing bacteria is associated with human milk dysbiosis and the emergence of SCM.

*Bosea* and *Klebsiella*, were identified in samples from non-SCM mothers in our study. *Klebsiella* normally colonizes the gut although it may also be found on the skin and nasopharynx and transmission is probably fecal-oral or via direct contact (95). Both *Klebsiella* and *Bosea* have also been isolated from soil and water (96, 97). Recent studies highlight the potential for environmental factors to modify the human milk microbiome (98). These observations may suggest that lactating women in our study acquired such microbes from the environment or from the infant through the retrograde flow of bacteria from *agüitas* to the mammary gland and would suggest that this linkage might be an important contributor to the microbial diversity in human milk (98).

Interestingly, although *Bifidobacteria* is normally found in human milk (61, 99, 100), we did not detect it in our milk samples. Other studies have also reported its absence in human milk samples (8, 25, 62, 101). Several other studies also report low concentrations ranging from 2 to 11% (100, 102–104). Others have reported low concentrations at birth that substantially increase within the first month (105). Remarkably, a longitudinal study concluded that *Bifidobacteria* first appears in infant feces rather than in human milk and suggests that *Bifidobacteria* is more likely to be transferred from the infant gut to human milk, contrary to what was previously believed (102). There is also some limited evidence that the absence of *Bifidobacterium* in the mouse gut has been associated with micronutrient deficiencies (106). We have previously reported a range of mineral and trace element concentrations in breast that fail to meet nutrient requirements for breastfeeding infants (88). Thus, the absence of *Bifidobacteria* in our population may be associated with selection of the primer (98) but could also be associated with low concentrations of micronutrients in the milk; however, this requires further investigation.

#### **Breastfeeding practices.**

Breastfeeding provides the infant with optimal nutrition and protects against morbidity and mortality associated with infection especially in developing countries (107). Furthermore, exclusive breastfeeding has demonstrated protection against infant deaths attributable to respiratory infections and diarrhea in developing countries (108, 109), and exclusive breastfeeding is associated with healthier bacterial communities in the infant's gut (110). It has been suggested that this protection is related to the colonization of the infant gastrointestinal tract with hundreds of bacterial phylotypes transmitted through human milk (62, 111).

When we compared EBF/PBF and mixed feeding mothers, 10 bacterial taxonomic units significantly differed. Comparing our findings with others, previous reports show the presence of similar bacterial genera in the exclusively breastfed group including *Corynebacterium*, *Lactobacillus*, and *Micrococcus* (36). *Corynebacterium* has been previously identified in human milk (6, 25, 62) and has been reported in infant gut and feces, as well as maternal feces, which might suggest that *Corynebacterium* colonizes the infant gut through human milk (100, 112, 113). On the other hand, *Brevundimonas*, *Afipia*, and *Paracoccus*, which were also found in our milk samples, are Gram-negative bacteria that have been associated with human infections (74, 97). *Brevundimonas* have previously been identified in human milk and in the maternal gut. *Lep-*

*totrichia*, which was the only bacteria predominantly abundant in the mixed feeding group, is an oral bacteria where certain species have been associated with clinical diseases (114). Because it has been reported previously in mature milk (8), these observations highlight the importance of promoting further research at species level.

In our study, we used CAP plots to further differentiate the milk microbiota by breastfeeding practices, noting distinct differences among those who exclusively breastfed in early or established lactation, those that were classified as predominantly breastfeeding because of the added *agüitas* during early or established lactation, and those who added solid foods while continuing to breastfeed during established lactation (mixed breastfeeding). The introduction of solid foods is known to be associated with the development of an adult-like microbiota (115). However, there are no reports in the scientific literature describing the addition of ritual fluids or herbal teas, commonly used in this population (49), on the microbiota. In this population, we were interested to know even though prepared with boiled water, if shifts in the composition of the milk microbiota might occur with the consumption of *agüitas*. Our analysis of exclusive and predominant breastfeeding by stage of lactation and by breastfeeding practice revealed variation of the milk microbiota due to the introduction of *agüitas* in predominantly breastfed infants. Together, our identification of subtle changes in the milk microbiota is novel but does require further investigation.

#### **Strengths and limitations**

The strengths of our study include the observation that the majority of Mam-Mayan mothers complied with WHO recommendations to exclusively breastfeed for 6 mo (43, 49). We are among the first studies to explore the influence of parity, maternal age, and SCM on human milk microbiota composition in an indigenous community, where there is evidence that growth faltering occurs within the first 6 mo postpartum (50). The method of extraction is also a strength, by doing it manually we avoided the use of a pump that could be a source of contamination (22).

We recognize our study had some limitations. In this study we used the 27F/533R primer, which can amplify the core human milk genus *Cutibacterium* (10, 62, 100), but cannot amplify species from the genus *Bifidobacterium* (116), whereas the other commonly used primer 515F/806R amplifies *Bifidobacterium* but not *Cutibacterium* (formerly *Propriobacterium*) (98). Yet, *Bifidobacterium* spp. are often (22) but not always identified in human milk studies using such primers (14, 117) if *Bifidobacterium* abundance and prevalence is low (105).

#### **Conclusion**

The WHO recommends EBF for the first 6 mo of life, followed by breastfeeding in combination with the introduction of complementary foods until at least the age of 24 mo (118). Our data supports this global recommendation as we showed that 9 bacterial genera including *Lactobacillus* and *Lactococcus*, had a higher abundance in the milk of mothers who exclusively and/or predominantly breastfed, had a healthy BMI, and who did not have SCM. The current study provides new insights into the composition of microbiota in human milk by establishing those maternal factors that impact the human milk microbiota in the Mam-Mayan indigenous community in Guatemala. Breastfeeding practices

can shift the composition of the milk microbiota during early and in established lactation. These findings highlight the importance of promoting exclusive and predominant breastfeeding to enhance bacterial diversity during lactation. Even though we were able to identify similarities in the major phyla and genera with studies from developed countries, our differential abundance analyses revealed that understudied maternal factors, including adolescent pregnancies, parity, and SCM, which are common characteristics of developing countries and rural populations, modified the differential abundance of the human milk microbiota. Finally, we caution against the direct application of findings in developed countries to mothers living in remote agrarian settings in developing countries. More evidence is needed from developing countries and rural populations in order to confirm our findings.

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### Data Availability

The data for this article may be shared following a written request to the corresponding author.

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