Human milk archaeal communities associated with neonatal gut colonization and their co-occurrence with bacteria.

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Abstract

Archaea have been identified as early colonizers of the human intestine, appearing from the first days of life. It is hypothesized that the origin of many of these archaea is through vertical transmission during breastfeeding. In this study, we aimed to characterize the archaeal composition in samples of mother-neonate pairs to observe the potential vertical transmission. We performed a cross-sectional study characterizing the archaeal diversity of 40 human colostrum-neonatal stool samples by next-generation sequencing of V5-V6 16S rDNA libraries. Intra- and inter-sample analyses were carried out to describe the Archaeal diversity in each sample type. Human colostrum and neonatal stool presented similar core microbiota, mainly composed of the methanogens *Methanoculleus* and *Methanosarcina*. Beta diversity and metabolic prediction results suggest homogeneity between sample types. Further, the co-occurrence network analysis showed associations between Archaea and Bacteria, which might be relevant for these organisms' presence in the human milk and neonatal stool ecosystems. According to relative abundance proportions, beta diversity, and co-occurrence analyses, the similarities found imply there is vertical transmission of archaea through breastfeeding. Nonetheless, differential abundances between the sample types suggest other relevant sources for colonizing archaea to the neonatal gut.

1 Introduction

Human milk is composed of essential nutrients and bioactive constituents, including proteins, carbohydrates, fatty acids (Pace et al., 2021), cytokines (Gila-Diaz et al., 2019), oligosaccharides,

immune factors (Le Doare et al., 2018), and microbiota (Fernández et al., 2013) that cater to the evolving needs of the growing infant (Selma-Royo et al., 2022). Human milk can be classified into colostrum, transitional, and mature milk according to the composition and lactation stage (Ballard and Morrow, 2013). For instance, colostrum is the first milk produced during the first days after birth (Thapa, 2005). It is characterized by a high content of growth factors and immunoglobulins, which provide passive immunity to newborns, protecting them against infections (Uruakpa et al., 2002).

Human milk's microbiota includes bacteria, eukaryotes, fungi, and archaea (Stinson et al., 2021). Most studies have focused on the former (Fitzstevens et al., 2017), thus revealing the central bacteriome of human milk and its impact on newborn health (Notarbartolo et al., 2022). The origin of bacteria in human milk is not fully understood. However, two sources have been proposed: the entero-mammary pathway and the retrograde flow (Rodríguez, 2014; Ruiz et al., 2019; Moossavi and Azad, 2020). The first consists of immune cell-mediated bacterial translocation from the maternal gastrointestinal tract to the mammary gland (Fernández et al., 2013; Rodríguez, 2014; Ruiz et al., 2019; Moossavi and Azad, 2020). In more detail, dendritic cells penetrate the gut epithelium and select bacteria, which are then transported to the mammary gland through lymphatic circulation (Rescigno et al., 2001; Fernández et al., 2013; Moubareck, 2021). The second refers to external contamination or the transfer from the mother's skin or infant's mouth to the mammary gland (Moossavi and Azad, 2020; Moubareck, 2021).

In contrast to bacteria, archaea diversity in human milk has long been neglected (Stinson et al., 2021). The first evidence of archaea presence in human milk came from metagenomics studies (Jiménez et al., 2015; Stinson et al., 2021). Nevertheless, a recent study was able to prove archaea viability by cultivating *Methanobrevibacter smithii,* a methanogenic archaeon, from colostrum and mature milk (Togo et al., 2019). Interestingly, archaea are reported to be present in the gastrointestinal tract from the first days of life (Palmer et al., 2007; Grine et al., 2017; Sagheddu et al., 2017; Wampach et al., 2017). Accordingly, *M. smithii* has been identified as an early colonizer, establishing in the gastric mucosa just after birth (Grine et al., 2017).

Methanogenic archaea (MA) are prevalent archaea in the digestive tract of adults, particularly *M. smithii* and *Methanosphaera stadmanae* (Dridi et al., 2009; Borrel et al., 2020; Chibani et al., 2022; Mohammadzadeh et al., 2022). MA play a fundamental role in the human gut, as they are responsible for producing methane through the assimilation of H_2 and CO_2, which are products of polysaccharide fermentation by bacteria (Kim and Whitman, 2014; van de Pol et al., 2017; Buan, 2018; Meier et al., 2024). They use hydrogen as an electron donor, reducing carbon dioxide, acetate, and multiple methyl-containing compounds into methane (Chaudhary et al., 2018; Meier et al., 2024). This metabolic activity facilitates the growth of fermentative bacteria in the gut, thus conforming to a syntropic relationship (Samuel and Gordon, 2006; Chaudhary et al., 2018). MA have been associated with various diseases such as diverticulosis (Weaver et al., 1986; Yazici et al., 2016), inflammatory bowel disease(Lecours et al., 2014; Ghavami et al., 2018), atherosclerosis (Brugère et al., 2014; Ramezani et al., 2018; Sereme et al., 2019), malnutrition(Camara et al., 2021), and obesity (Samuel and Gordon, 2006; Zhang et al., 2009; Maya-Lucas et al., 2019; Amabebe et al., 2020). However, the relationship between MA and illness is not entirely understood and can be contradictory. For example, MA have been associated with obese and normal-weight individuals(Zhang et al., 2009; Chakraborti, 2015; Ignacio et al., 2016; Maya-Lucas et al., 2019; Togo et al., 2019; Amabebe et al., 2020; Djemai et al., 2022). Although the role of archaea in disease is not clear, they seem to be key microbiota components of the human gastrointestinal tract (Lurie-Weinberger and Gophna, 2015; Nkamga et al., 2017).

Considering its importance in human health, its presence in human milk, and the gastrointestinal tract from the early days of life, this work aimed to characterize the archaeal composition of human

colostrum-neonatal stool samples by Ion torrent semiconductor DNA sequencing of V5-V6- 16S rRNA gene libraries. We considered that mothers' human milk is a main source of archaea to the neonate's gut, therefore there is a vertical transmission of archaea during breastfeeding.

2 Materials and Methods

Study design and selection of subjects

The cross-sectional descriptive study consisted of 40 mother-neonatal pairs of patients from the "Dr. José María Rodríguez" General Hospital, located in Ecatepec de Morelos, State of Mexico (19°36'35'' N, 99° 3'36'' W). The samples were obtained from healthy lactating women and exclusively breastfed newborns. Colostrum and neonatal stool samples were collected from 0 to 3 days after birth, up to 2 h after the newborn was breastfed, from November 2017 to January 2018. The inclusion criteria were as follows: (1) Mexican origin with at least two generations of ancestry, (2) gestational age between 37 and 41 weeks, (3) birth weight between 2500 - 4500g, (4) Apgar score greater than 7 at 5 min after birth. Exclusion criteria: (1) Maternal probiotic and alcohol consumption, (2) smoking, (3) diabetes before or during pregnancy, (4) antibiotic use during the last trimester of pregnancy and before sampling. The participants were given a survey where sociodemographic and clinical data was recorded (maternal age, gestational age at delivery, type of delivery; newborn sex, and age). Written informed consent was obtained from all participants before the study, following the 2013 Declaration of Helsinki. The protocol was approved by the Ethics Committee of the General Hospital "Dr José María Rodríguez" (Project identification code: 217B560002018006).

Sample collection

The colostrum-neonatal stool sample pairs were collected on the same day up to 2 h after the newborn was breastfed. Human colostrum (HC) was collected manually into a sterile polypropylene tube (about 5 to 10 mL). Breast sanitation was not employed to better represent the microorganisms transferred during breastfeeding. The neonatal stool (NS) was recovered from diapers into sterile containers using sterile tongue depressors. The samples were sent to the laboratory in a cold environment; distributed in aliquots of 1 mL (HC) or 200 mg (NS) and stored at -20° C until DNA was extracted within 24 h of arrival.

DNA extraction

First, 1 mL of HC was centrifuged at 4° C, 10,000 g for 15 min in a refrigerated centrifuge (Eppendorf 5415R) and the fat was removed with a roll of sterile dental cotton. The aqueous supernatant was removed by decantation, the pellet was resuspended in 1 ml of sterile PBS pH 7.4 and then centrifuged again at 10,000 g for 15 min. The obtained pellet was resuspended in 300 µL of PBS pH 7.4 and processed for DNA extraction using FavorPrep Milk bacterial DNA extraction kit (Cat: FAMBD001, Favorgen, Biotech Corp, Taiwan) following the manufacturer's instructions. Fecal DNA was extracted from 200 mg NS samples using a QIAamp DNA Stool Mini Kit (Cat.: 12830-50, Qiagen, The Netherlands), following the manufacturer's instructions. In the two cases, 300 µL of PBS pH 7.4 was used as a negative control for DNA extraction. The DNA concentration in samples was measured at 260/280 absorbance using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), no absorbance was detected for negative controls. DNA integrity was assessed by electrophoretic fractionation of 5 µL of DNA sample in 0.5% agarose gel stained with 0.80 µL of Midori Green advanced dye (1:15) using TBE buffer. DNA was visualized using the MolecularImager® Gel DocTM XR System program (Bio-Rad Laboratories, Chicago, IL, USA). Extracted DNA was stored at -20° C.

Preparation of the 16S rRNA gene library and next-generation sequencing

The forward Arc787F (5′-ATT-AgA-TAC-CCG-BgT-AgT-CC-3′) and reverse primer Arc1059R (5′-gCC-ATg-CAC-CWC-CTC-T-3′) were used for the polymerase chain reactions (PCR) (Yu et al., 2005). The PCR reactions were performed using the Phusion High-Fidelity PCR Kit (Cat F-530S), ThermoFisher Scientific, Waltham, MA, USA). The reaction mixture consisted of 4.0 µL of 1X HF buffer, 0.4 µL of dNTPs (200 uM), 0.2 µL of Phusion polymerase (0.02 U/ µL), 1.0 µL of each Forward and Reverse primer (10 uM), and 0.2 uL of $MgCl_2$ (0.5 mM**)**. The DNA template volume was adjusted to 13.2 µL with nuclease-free water for a final concentration of 8.0 ng in 20.0 µL. The reactions were programmed in 2720 Thermal Cycler (Applied Biosystems™, ThermoFisher Scientific, Waltham, MA, USA) with 5 min 95° C hot start, followed by 5 min initial denaturation at 95° C, 25 X (94° C, 15s denaturation, 56° C, 15s annealing, 72° C for 15s extension) and final 7 min extension at 72° C. Archaeal DNA from a bioreactor (Gállego-Bravo et al., 2023) was used as positive control. Blank reactions (PCR products with no DNA template from the DNA extraction pipeline) were used as negative controls. The 358 bp amplicons were fractionated in 1.5% agarose gel dyed with Midori Green (Nippon Genetics®, Dueren, Germany) in 0.5X TBE using GeneRuler™100 bp DNA Ladder (Cat. 15628019, ThermoFisher Scientific, Waltham, MA, USA). Electrophoresis lasted 45 min at 80 V. The DNA was visualized using the Molecular Imager® Gel DocTM XR System program (Bio-Rad Laboratories, Chicago, IL, USA). The library was purified using 2% E-Gel™ EX stained with SYBR GOLD DNA (Cat. G401002, Thermo Scientific, Waltham, MA, USA). The library size and concentration were assessed with the 2100 Bioanalyzer equipment and High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA). PCR emulsion was carried out with Ion One Touch™ (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Amplicon enrichment with ionic spheres was carried out using Ion OneTouch ES (Life Technologies, Carlsbad, CA, USA). Sequencing was performed using the Ion 318 Kit V2 Chip (Cat. 4488146, Life Technologies, Carlsbad, CA, USA). Ion Torrent PGM software v4.0.2 was used to demultiplex the sequence data based on their barcodes, reads were filtered to exclude low-quality (quality score ≤ 20), polyclonal sequences (homopolymers > 6) and the adapters were trimmed. The datasets generated for this study can be found in the NCBI BioProject ID PRJNA1018680.

ASV Determination and Taxonomic Annotation

The FASTQ files were further processed and analyzed with QIIME 2022.2 (Bolyen et al., 2019). With this software, the ASVs (Amplicon Sequence Variants) were determined as well as the taxonomic assignations. The first was performed with the QIIME dada2 denoise-single plugin, with sequence truncation at 238 nt. The latter was done with the feature-classifier classify-consensus-blast plugin, with a 97% percentage identity. The Silva 138 database was used for analysis with BLAST (Robeson et al., 2020).

Archaeal Relative Abundance, Diversity, DESeq2, PICRUSt2, and Co-occurrence analyses

R 4.2.0(R Core Team, 2021) in Rstudio (RStudio Team, 2022) was used for the relative abundance, diversity, DESeq2, and co-occurrence analyses. For that purpose, the following packages were used: to import the qiime artifacts, qiime2R (Bisanz, 2018), for alpha and beta diversity analyses, phyloseq 1.4.0(McMurdie and Holmes, 2013), for the DESEQ2 analysis, DESEq2 1.3.6 (Love et al., 2023), for the heatmap elaboration, ComplexHeatmap 2.12.0 (Gu, 2022), for the ANOSIM, vegan 2.6-2 (Oksanen et al., 2022), and for figures, tidyverse 1.3.1 (Wickham et al., 2019), dplyr 1.09 (data frame manipulation), ggplot2 3.3.6, scales 1.2.0, ggpubr 0.4.4 and, gridExtra 2.3 (Auguie, 2017). PICRUSt2 was executed with the MetaCyc metabolic pathway database option following a published pipeline

tutorial(Douglas et al., 2020). Co-occurrence network analysis was carried out with microeco (Liu et al., 2021) and meconetcomp (Liu et al., 2023) packages using 0.6 Spearman's rank correlation coefficient with a 99% confidence level. All analyses were filtered to the Archaeal Domain except for the co-occurrence analysis.

Statistical methods

Archaeal diversity within samples was estimated with alpha diversity, determining Observed ASVs, Shannon, Simpson, and Fisher indexes. The Shapiro-Wilk preliminary test was applied to test if the data followed a normal distribution; then, the Kruskal-Walli's test was applied. The differences in beta diversity between samples were evaluated by ANOSIM (Analyses of similarities). A differential abundance analysis (DESeq2) for dependent samples was performed to identify relevant taxa in the distinct sample types and pairs, and was evaluated with a Wald test, *p*-values were adjusted with Holm-Bonferroni method statistics. *p* or *q*-values < 0.05 were considered significant.

3 Results

Most participant mother-neonate pairs were from urban areas

Forty colostrum and neonatal stool samples were collected during the days after birth (1.28 ± 0.65). Most participants came from the State of Mexico (19.4969° N, 99.7233° W) and Mexico City (19.4326° N, 99.1332° W) low-income areas (Table 1). Almost all mothers work at home (95%), and close to 50% of them have a high school education. Most of them had normal weight and more than half of the deliveries were vaginal (Table 1). Regarding the neonate's anthropometric data, 60% were females and 35% were males, for two of the samples we had no information.

Methanoculleus spp. was the most abundant genus in the mother-neonate pairs

We examined the archaeal diversity in the colostrum and neonatal stool collected from mother-neonate pairs using high-throughput semiconductor DNA sequencing of a V5-V6 16S rRNA gene library. In summary, we obtained 4,531,448 raw sequences, 2,269,715 for colostrum, and 2,261,733 for neonatal stool with a Phred median value of 27 (Supplementary Table 1). A length of 238 nt was selected for the analyses. We observed four phyla in colostrum and neonatal stool: Halobacterota, Euryarchaeota, Crenarchaeota, and Thermoplasmatota (Figure 1B). The phylum Halobacterota was the most dominant in the two sample types, being more abundant in the neonatal stool (88.36 ± 5.17) than in colostrum (54.2 ± 27.88), this difference was statistically significant ($p = 1.38X10^{-14}$, $q = 5.52X10^{-14}$). The phylums Euryarchaeota and Crenarchaeota were more abundant in colostrum (10.52 ± 10.64, 0.66 ± 3.29) than in neonatal stool (4.7 ± 4.61, 0.16 ± 0.54) ($p = 0.175$, $q = 0.355$; $p = 0.212$, $q = 0.355$), while Thermoplasmatota was more abundant in neonatal stool (0.71± 2.56) than in colostrum (0.09 ± 0.4) ($p = 0.118$, $q = 0.355$), however these differences were not statistically significant (Supplementary Table 2). Concerning genera, we observed four: two from the phylum Halobacterota *Methanoculleus* and *Methanosarcina*, and two from the phylum Euryarchaeota *Methanothermobacter* and *Methanobrevibacter*. The dominant genus was *Methanoculleus* accounting for 37.35% of the abundance in colostrum and 46.47% in neonatal stool, followed by *Methanosarcina* (27.57% colostrum, 30.90% neonatal stool). *Methanothermobacter* accounted for 5.55% of colostrum and 4.68% in neonatal stool, while *Methanobrevibacter* was the least abundant in the sample types (3.15% colostrum, 1.33% neonatal stool). The remaining percentage is the "Others" group (genera with less than 1% relative abundance) and unassigned sequences (Supplementary Figure 1). We further compared the archaeal communities by characterizing the core microbiota. Our criteria were genera with ≥10 % of prevalence among samples and ≥1 % abundance of reads in each sample. In the two

samples, we found members of the Euryarchaetoa, Halobacterota and Thermoplasmatota phyla, including the genera *Methanoculleus* followed by *Methanosarcina* (Figure 1A). These results suggest similar taxa composition in the core archaeal community of colostrum and neonatal stool.

Colostrum archaea diversity was higher than the diversity in neonatal stool

The alpha diversity analysis was carried out to characterize the number of ASVs, richness, and homogeneity within samples and to compare between sample types (Figure 2A). We found that in colostrum there were 33.2 ± 18.04 observed taxa, while in neonatal stool there were 24.52 ± 15.62, this difference is statistically significant (*Cohen's d* = 0.727, p = 0.04), showing that colostrum is richer than neonatal stool. The Shannon index value indicates that colostrum is the most diverse of the two sample types, and the difference is significant (*Cohen's d*= 0.819, p = 0.015), this is also the case with the Fisher index (*Cohen's d* = 0.825, p = 0.042). Regarding the dominance, Simpson (*Cohen's d* = 0.653, p = 0.009) revealed that both sample types have high dominance, with colostrum having more taxa dominance than neonatal stool (Supplementary Table 3, for numerical data of indexes). Next, the unweighted and weighted UniFrac beta diversity analyses showed that sample types overlap (Figure 2B-2C), depicting that the samples are quite similar (ANOSIM, unweighted R = 0.345, p = 0.001; weighted R = 0.071, p = 0.001). This also was confirmed by the Adonis test (R^2 = 0.0313, p = 0.009).

There were differentially abundant taxa between human colostrum and neonatal stool

Differential abundance analysis for paired samples based on the negative binomial distribution (DESeq2) was carried out to look for statistically significantly differentially abundant taxa in each sample (Figure 3). We found that three ASVs were predominant in colostrum and six in neonatal stool. The archaeal diversity in neonatal stool was characterized by an increased abundance of *Nitrososphaeraceae*, *Methanosarcina*, three different ASVs of the *Methanoculleus* genus, and one possibly belonging to *Methanoplasma*. Meanwhile, colostrum had an increased abundance of *Methanothermobacter*, *Methanobrevibacter*, and *Methanoculleus* (Supplementary Table 4).

The predicted functional metagenome shows pathways associated with methanogens

We identified the predicted functional metabolic pathways in colostrum and neonatal stool microbiota by PICRUSt2 analysis using the ASV table. We did not find any differentially abundant pathway between the two sample types. We observed that the main pathways present in at least 10% of the samples were associated with methanogens, such as the incomplete reductive TCA cycle pathway and the L-isoleucine biosynthesis IV & II pathways, while others were directly related to methanogenesis, like the factor 420 biosynthesis pathway and the methanogenesis from H_2 to CO_2 (Figure 4).

Human colostrum and neonatal stool co-occurrence networks

A co-occurrence network analysis was performed to observe the topology and the microbial interactions of each sample type. We found 114 (39.6%) edges in common in the co-occurrence networks between human colostrum and neonatal stool (Figure 5A). There were similar associations between HC and NS, common Bacteria-Bacteria associations were *Gemella-Gemella*, *Enterococcus-Lactococcus*, *Hungatella-Hungatella*, *Lactobacillus-Lactobacillus*, *Lactococcus-Lactococcus*, *Staphylococcus-Staphylococcus*, *Enterococcus-Enterococcus*, *Cutibacterium-Cutibacterium*, *Streptococcus-Streptococcus*, *Bifidobacterium-Bifidobacterium*, and *Clostridium_sensu_stricto_1-Clostridium_sensu_stricto_1*. Regarding Archaea-Archaea associations we observed four: *Methanosarcina-Methanosarcina*, *Methanosarcina-Methanothermobacter*, *Methanoculleus-Methanosarcina* and *Methanoculleus- Methanoculleus*. Finally, accounting for Archaea-Bacteria

associations there were: *Enterococcus-Methanothermobacter* and *Enterococcus-Methanosarcina* (Figure 5B). The HC network showed a large hub comprising Archaea-Bacteria associations, particularly between the Firmicutes and Halobacterota phyla, which included *Streptococcus* (Firmicutes), *Methanoculleus* (Halobacterota), and *Methanosarcina* (Halobacterota) to mention a few (Figure 5C, for more detail see Supplementary Figure 2). Meanwhile, the NS network showed a large hub comprised of archaeal associations (*Methanocullleus*, *Methanothermobacter* and *Methanosarcina*) related to Actinobacteriota (*Cutibacterium* and *Corynebacterium*) and to Firmicutes (*Agathobacter* and *Streptococcus*) (Figure 5D, for more detail see Supplementary Figure 3).

4 Discussion

In this study, the archaeal composition of colostrum and neonatal stool from Mexican mother-neonate pairs was characterized, being to our knowledge, the first report in Mexican individuals. We found a high abundance of Halobacterota phylum in both colostrum and neonatal stool (65% and 77%, respectively). Accordingly, the main genera were members of this phylum (*Methanoculleus* and *Methanosarcina*). The *Methanoculleus* genus had already been found in the human intestinal mucosa by sequence analysis of the archaeal methyl coenzyme-M reductase (*mcr*A) gene present in colonic biopsies(Nava et al., 2012). However, apart from said study, little is known about its presence in the human gut. Similarly, for the *Methanosarcina* genus, there are no direct reports (Hugon et al., 2017; Sereme et al., 2019). Although recent studies have not found this genus (Chibani et al., 2022; Mohammadzadeh et al., 2022), there is one report on human gut methanogens where it is reported a *mcr*A gene phylotype, named Mx-01, which was later associated with the Methanosarcinales order (Mihajlovski et al., 2008, 2010; Scanlan et al., 2008; Gaci et al., 2014). Nevertheless, this phylotype was also suggested to belong to a new order of methanogens (Mihajlovski et al., 2008). However, it is important to mention that *Methanosarcina* has been found in the gastrointestinal tract of different animals like goats, termites, and pigs (Mukhopadhyay et al., 1991; Gomathi et al., 2009; Xiong et al., 2022). Alternatively, *Methanosarcina* spp. is part of humans' oral and skin microbiota (Robichaux et al., 2003; Matarazzo et al., 2011; Nguyen-Hieu et al., 2013; Probst et al., 2013; Huynh et al., 2015; Deng et al., 2017; Weiland-Bräuer, 2023). Particularly, the species *Methanosarcina mazeii* and *Methanosarcina vacuolata* have been observed in subjects affected by periodontitis and healthy subjects (Matarazzo et al., 2011; Deng et al., 2017). It could be the case that the presence of this genus in the neonate's gut was due to oral or skin microbiota. Another hypothesis would be that it has not been well characterized in the human intestine, its abundance being underestimated in infant populations.

The genus *Methanobrevibacter* was present in the two sample types but at low abundance (~ 3%), and the genus *Methanosphaera* was found to have less than 1% abundance. In contrast, *Methanobrevibacter smithii* and *Methanosphaera stadmanae* are considered the most prevalent archaea in the adult human gut (Dridi et al., 2009; Bang et al., 2014; Chibani et al., 2022). Comparing against studies on neonates' gastrointestinal tract, we also found *Methanobrevibacter* and *Methanosphaera*, as well as one uncultured phylotype (Palmer et al., 2007; Grine et al., 2017; Sagheddu et al., 2017; Wampach et al., 2017). These studies consisted of 16S rRNA gene sequencing (Wampach et al., 2017), multispacer sequence typing (Grine et al., 2017), clone library sequencing(Palmer et al., 2007), and qPCR targeting (Sagheddu et al., 2017).

The disparities in the archaeal genera proportions of this work against previously reported research could be attributed to lifestyle differences since no previous reports of archaeal composition in Mexican women exist. It is known that hormonal changes during pregnancy affect the microbiota (Di Simone et al., 2020; Hussain et al., 2021; Yoon and Kim, 2021). Therefore, the archaeal population of a

pregnant woman might be distinct. Moreover, the microbiota is also influenced by diet (David et al., 2014; Merra et al., 2021; Ramos and Martín, 2021; Nova et al., 2022), geographical location, and urban or rural lifestyle (Hasan and Yang, 2019; Cheng et al., 2022). Considering the foregoing, we hypothesized that the differences found in the proportions of archaeal genera might be due to a combination of diet, geographic location, and the pregnancy stage.

The alpha diversity analysis showed that human colostrum had higher archaeal diversity than the neonatal stool. Accordingly, human milk bacteria in a similar cohort also showed higher diversity when compared to neonatal stool (Corona-Cervantes et al., 2020). We believe this is due to the neonate's age (<4 days), which indicates that colonization of their gastrointestinal tract was just beginning, explaining the lower diversity (Palmer et al., 2007; Mihajlovski et al., 2010; Wampach et al., 2017) Apart from this, the significant difference between colostrum's and neonate stool's diversity can also be explained by their ecological niches, which might favor the presence of specific archaea (Koskinen et al., 2017). Interestingly, some authors have concluded that alpha diversity measures might underestimate microbiota and more robust statistical methods might be necessary to assess the differences (Willis, 2019; Kers and Saccenti, 2022).

Colostrum and neonatal stool were found to have a highly similar core archaeal microbiota, consisting of members of Euryarchaeota, Halobacterota and Thermoplasmota phyla, which included the genera *Methanoculleus*, *Methanosarcina*, *Methanofollis*, *Methanomassiliicoccus*, *Methanosphaera*, *Methanobrevibacter*, and *Methanothermobacter*. The beta diversity analysis, both weighted and unweighted, further explained the similarity between colostrum and neonatal stool samples, the NMDS ordination showing that the sample types overlapped. The similarity between the two sample types implies that shared taxa are possibly transmitted via breastfeeding. Moreover, the metagenomic prediction analysis suggested no differentially abundant pathways between the sample types, strengthening our previous results. The low abundance of the predicted metabolic routes detected could be due to the combination of the natural low abundance of archaea in the sample types and the lack of archaeal metabolic information in the MetaCyc database. Observing the most prevalent pathways among the samples, we found that all of them were associated with methanogens. The incomplete reductive TCA pathway was present in almost 90% of the samples. This route is characteristic of methanogens and allows for the synthesis of intermediates needed for amino acid production (Ekiel et al., 1985; Goodchild et al., 2004). The methanogenesis from H_2 and CO_2 was also prevalent although less abundant, thus revealing the predominance of hydrogenotrophic archaea, i.e., methanogens that utilize H_2, formate, or simple alcohols as electron donors and CO_2 as electron acceptor (Lyu et al., 2018). This pathway starts with CO_2 activation and is followed by numerous transformations, including one aided by the factor 420, which was also prevalent in our study samples, and another methanogenesis indicator (Shima et al., 2002). Finally, the L-isoleucine biosynthesis pathways II and IV have been associated with methanogens such as *Methanocaldococcus jannaschii* (Drevland et al., 2007), *Methanothermobacter thermautotrophicus*, and *Methanobrevibacter arboriphilus* (Eikmanns et al., 1983). Together, these results suggest that methanogens are vertically transmitted through lactation.

According to the DESeq2 for paired samples, nine taxa had differential abundance. On the one hand, colostrum had three taxa corresponding to *Methanothermobacter*, *Methanobrevibacter*, and *Methanoculleus*. On the other hand, neonatal stool showed six, including three different ASVs belonging to *Methanoculleus*, in addition to *Nitrososphaeraceae*, *Methanosarcina*, and *Candidatus* Methanoplasma. For colostrum, it could be the case that for some mother-neonate pairs, there was no vertical transmission of archaea. The latter is particularly notable in the few samples that presented the *Methanobrevibacter* genus. The differential taxa in the neonatal stool could indicate archaea's contribution to the newborn from other sources like the environment and the mother's oral, skin, and

vaginal microbiota. Interestingly, *Methanoculleus bourgensis* and *Candidatus* Nitrososphaera evergladensis have been reported on ancient dental calculus of individuals recovered from archaeological sites dating from the 14th to the 19th centuries AD (Huynh et al., 2015). Further, as mentioned before, *Methanosarcina* has been reported as part of the human oral and skin microbiota (Robichaux et al., 2003; Matarazzo et al., 2011; Nguyen-Hieu et al., 2013; Probst et al., 2013; Huynh et al., 2015; Deng et al., 2017). Thus, it is possible that the origin of these archaea in neonatal stool was their mother's oral and skin microbiota as well as the environment. Another plausible explanation for the increased abundance of *Methanothermobacter*, *Methanobrevibacter*, and *Methanoculleus* observed in colostrum, is that these methanogens are more sensitive to oxidation, thus having a slower growth rate, resulting in their lower proportion relative to other methanogens and taxa. In addition, *Methanosarcina* whose abundance is increased in the neonatal gut, may help consume oxygen, thereby enhancing anaerobic conditions for other anaerobic archaea. It is remarkable to mention that the *Methanosarcinales* order has been associated with higher tolerance to oxygen in comparison with the orders *Methanobacteriales* (which include *Methanothermobacter* and *Methanobrevibacter*), *Methanococcales* and *Methanopyrales* (Lyu and Lu, 2018). In the same sense, it has been observed that lactate oxidation in a species of *Methanosarcina* (*M. acetivorans)* is associated with energy conservation and oxygen (Feregrino-Mondragón et al., 2023).

Lastly, the co-occurrence network revealed there were 114 edges or connections (accounting for 39.6%) in common between the human colostrum and neonatal stool networks, supporting the idea of microbial similarity among the sample types. Methanogens and bacteria are known to form syntrophic interactions(Borrel et al., 2020; Djemai et al., 2022; Weiland-Bräuer, 2023; Duller and Moissl-Eichinger, 2024), therefore we sought to see possible Archaea-Bacteria associations. We observed *Enterococcus* associated with two methanogens: *Methanothermobacter* and *Methanosarcina*. Previous studies had only related *Enterococcus* with Archaea in pathogenesis, i.e., abscesses and urinary tract infections (Djemai et al., 2022). *Enterococcus* is capable of producing CO_2, H_2, formate, and acetate (Djemai et al., 2022), all of which are utilized in methanogenesis. Thus, an interaction between these bacteria and the archaea is plausible. In the human colostrum network, we observed *Streptococcus* associated with methanogens. *Streptococcus* co-occurrence with archaea had only been seen in febrile patients' blood (Drancourt et al., 2021; Djemai et al., 2022). These results showed that some associations found in pathogenesis might also be common in healthy individuals. In the neonatal stool network, we found co-occurring archaea with *Cutibacterium*, *Corynebacterium* (Actinobacteria phylum), *Agathobacter*, and *Streptococcus* (Firmicutes phylum). All these genera, except for *Agathobacter*, have been previously associated with methanogenic archaea(Djemai et al., 2022). Moreover, *Cutibacterium* and *Streptococcus* produce CO_2, hydrogen, and formate, seemingly being inclined toward hydrogenotrophic methanogens (Djemai et al., 2022).

In summary, in this study we characterized the archaeal composition of human colostrum-neonatal stool paired samples, finding *Methanoculleus* and *Methanosarcina* to be the main genera in both sample types. Moreover, the similarities between the sample types suggest there is vertical transmission of archaea during breastfeeding. However, differential abundances between colostrum and neonatal stool imply there are other important sources for the colonization of archaea to the neonatal gut. Finally, the co-occurrence network analysis showed associations between Archaea and Bacteria which might be relevant for these organisms' presence in the human milk and neonatal stool ecosystems. Future studies should aim to characterize other potential sources of archaea in the neonatal stool as well as their associations with Bacteria. All in all, this study represents a first step in understanding the origin of archaea in the gut from the beginning of life and remarks on the importance to continue studying these often-overlooked microorganisms.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

Salas-López M: Conceptualization, Investigation, Methodology, Visualization, Writing-original draft, reviewing & editing. Velez-Ixta J-M: Data curation, Formal analysis, Visualization, Software. Rojas-Guerrero D-L: Investigation, Methodology, Visualization. Piña-Escobedo A: Methodology, Supervision. Hernández-Hernández J-M: Validation, Writing- reviewing & editing. Rangel-Calvillo M-N: Conceptualization, Methodology, Funding Acquisition. Pérez-Cruz C: Conceptualization, Validation, Writing- reviewing & editing. Corona-Cervantes K: Data curation, Methodology, Writing – reviewing & editing, Investigation. Juarez-Castelán C-J: Methodology, Investigation, Supervision, García-Mena J: Writing – original draft, reviewing & editing, Resources, Project administration and Funding acquisition.

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10 Figure captions

Fig. 1 Characterization of the archaeal diversity of the sample types. (A) Core microbiota heatmap among samples. Columns show the abundance of core microbiota members with a prevalence of at least 10% in the samples and an abundance ≥1%. The color scale from blue (−2) to red (2) indicates the relative abundance normalized. Color keys for phyla are shown on the left side of the figure. The sample type is indicated at the bottom of the figure. (B) Archaeal phylum relative abundance stacked bar plots. Color sectors indicate taxa as shown by tags at the right side of the figure; abundances are shown as percentages on the Y-axis.

Fig. 2 Characterization of the archaeal diversity in the sample types. (A) Alpha diversity box plots. The Y-axes indicate the values for the species richness indexes (Observed), and diversity indexes (Shannon, Simpson, and Fisher). The sample type is shown at the bottom (Supplementary Table 3, for numerical data of indexes) (B) Beta diversity Non-Metric Multidimensional Scaling (NMDS) scatter plots. The graphics show archaeal beta diversity calculated by NMDS ordination based on the UniFrac distance matrix (left, unweighted UniFrac; right, weighted UniFrac). The sample types (colostrum and neonatal stool) are similar according to ANOSIM ($p = 0.001$).

Fig. 3 Differential abundance analysis of archaea genera between colostrum and neonatal stool with DESeq2. Horizontal bars represent the effect size (log2 Fold Change). Archaeal taxa with *q* values <0.05 are written alongside the Y-axis. See Supplementary Table 4 for full taxon description, log2FoldChange, *p* and *q* values.

Fig. 4 Heatmap of functional microbial metabolic pathways using PICRUSt2 analysis with MetaCyc database. Columns show the abundance of main metabolic pathways with a prevalence of at least 10% in the samples and an abundance >1%. Sample names are shown in the X axis. The color scale from black (-2) to white (2) indicates the relative abundance of the predicted metabolic pathways.

Fig. 5 Microbial co-occurrence network comparison between human milk and neonatal stool. (A) Venn diagram of edges between the networks of neonatal stool (NS) and human colostrum (HC). (B) Number distribution of taxa associated to the linked nodes of positive edges in networks of NS and HC. The number in the plot indicates the ratio of edges against all the positive edges in the network. (C) Microbial co-occurrence network of human colostrum. A connection between nodes stands for a strong (Spearman's ρ>0.6) and significant (p>0.01) correlation. (D) Microbial co-occurrence network of neonatal stool. A connection stands for a strong (Spearman's ρ>0.6) and significant (p>0.01) correlation.

11 Tables

Table 1. Sociodemographic and clinical characteristics of the study population.

Maternal Data		n (%)
Age (years)[a]		22.7 ± 6.7
IMC[b]		24.2 ± 4.22
Birthplace		
	State-of-Mexico	29 (72.5)
	Mexico City	6 (15)
	Other (Puebla, Veracruz, etc.)	5 (12.5)
Main activity		
	Housewife	38 (95)
	Student	1 (2.5)
	General employee	1 (2.5)
Educational level		
	Elementary school	20 (50)
	High school	18 (45)
	College	2 (5)
Parity		
	Uniparous	18 (45)
	Multiparous	22 (55)
Delivery mode		

	Vaginal	27 (67.5)
	C-Section (non-elective)	13 (32.5)
Neonatal data		**n (%)**
Age at sample collection, days		
	< 4	40 (100)
Sex[c]		
	Female	24 (60)
	Male	14 (35)

a, b Expressed as mean ± standard deviation.
c, no available information for two samples.
n- sample number, values in parenthesis represent the percentage.

12 Supplementary Material

Supplementary Material can be found at

12 Data Availability Statement

The datasets generated for this study can be found in the NCBI BioProject ID PRJNA1018680 Link https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1018680.