Characterization of Vertically transmitted miRNAs during breastfeeding

##En preparación##

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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 characterize the Archaean 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 the colonization of archaea to the neonatal gut.

1 Introduction

Breastfeeding is considered the normative standard for infant feeding and nutrition [@Eidelman2012], since this practice has been associated with multiple infant health and inmune

development benefits, namely, less incidence of gastrointestinal disease, lower risk to develop respiratory and intestinal tract infections, improved cognitive development and lower mortality rates [@Lyons2020; @Koh2017, @Westerfield2018]. Also, several adventages for the lactating mother have been reported, like less incidence of breast and ovary cancer, metabolic syndrome and postpartum depression [@Sattari2019; @Sayres2018]; also breastfeeding helps to reduce postpartum stress produced by preterm labor [@Black2012].

Due to the wide variety of benefits that breastfeeding provides, multiple health care associations, recomend exclusive breastfeeding for at least the first six months and to continue this practice up to two years [@CDC2012; @WHO2021; @Eidelman2012].

It's worth noting that despite the known benefits of breastfeeding, some mothers may face challenges or barriers to breastfeeding, such as difficulty with latching, inadequate milk supply, or returning to work or school. In these cases, alternative feeding methods such as formula may be necessary to ensure adequate infant nutrition. However, healthcare professionals can provide support and resources to help mothers overcome these challenges and continue breastfeeding if they choose to do so [@Academy].

Human milk (HM) is an unique high nutritional value biofluid, resulted of millions of years of an evolutionary process, that composes the optimum feeding regime both for the mother and the neonate/infant [@Christian2021; @Lyons2020]. The composition of HM widely varies along lactation period, nursing process and circadian cycle [@Ballard2013; @Andreas2015; @Italianer2020], but broadly speaking, it consists on macro and micronutrients, responsible for energy and nutritional HM intake, also biological components esential to the correct development of the infant [@Andreas2015].

As mentioned before, HM can be differentiated in three major stages according to lactation period, these are colostrum, transitional milk and mature milk [@Ballard2013].

In addition to macro and micronutrients, human milk also contains various bioactive molecules, including growth factors, hormones, enzymes, immune modulators, and other compounds that have important roles in infant development and protection [@Ballard2013; @Andreas2015]. For example, human milk contains immunoglobulins, lactoferrin, lysozyme, and other proteins that provide passive immunity to the infant and protect against infections [@Hassiotou2013]. HM also contains oligosaccharides that support the growth of beneficial gut bacteria and reduce the risk of infections and other diseases [@Bode2012]. Additionally, human milk is rich in long-chain polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) and arachidonic acid (ARA), which are important for the development of the brain and nervous system [@Andreas2015].

Additionally, HM contains a complex array of bioactive compounds that confer numerous health benefits to the infant, including antimicrobial, anti-inflammatory, and immunomodulatory effects. These bioactive components include lactoferrin, lysozyme, secretory immunoglobulin A (IgA), oligosaccharides, cytokines, growth factors, and nucleotides, among others [@Ballard2013; @Andreas2015]. The specific composition and concentration of these bioactive components also varies throughout lactation, suggesting that HM provides tailored immunological support to the infant during different stages of development.

MicroRNAs (miRNAs), are highly conserved small Ribonucleic Acid (RNA) molecules from 17-25 nucleotides lenght, miRNAs play important roles in biological processes like human development, metabolism, cellular cycle, proliferation and apoptosis, at postranscriptional level by translational

inhibition or mRNA degradation [@Sousa2019; @Tafrihi2018]. MiRNAs can perform their actions either by binding with 3'UTR mRNA transcripts or also by forming complexes with proteins and other miRNAs [@Sousa2019; @Tafrihi2018; @Tome-Carneiro2018].

MiRNAs are first transcribed in the cell nucleus from genomic Desoxyribonucleic Acid (gDNA) by the action of RNA polymerase II, in an initial structure called pri-miRNA, thereafter sliced by a complex formed between Drosha and its cofactor DGCR8 in order to produce a pre-miRNA. This new miRNA precursor in later exported to the cytoplasm by exportin 5 and its cofactor Ran. Finally, exported pre-miRNAs are procesed by Dicer and argonaut proteins to produce mature miRNAs [@He2016].

MicroRNAs (miRNAs) are important regulators of gene expression, and they play crucial roles in many biological processes, including development, differentiation, metabolism, and disease. They are small non-coding RNA molecules that are typically 17-25 nucleotides in length, and they act post-transcriptionally to regulate gene expression by binding to complementary sequences in target messenger RNA (mRNA) transcripts, resulting in translational inhibition or mRNA degradation [@Sousa2019; @Tafrihi2018].

MiRNAs can interact with mRNA transcripts by binding to their 3' untranslated region (UTR), but they can also form complexes with proteins and other miRNAs to regulate gene expression [@Sousa2019; @Tafrihi2018; @Tome-Carneiro2018].

MiRNAs are initially transcribed from genomic DNA by RNA polymerase II into a primary transcript (pri-miRNA), which is processed in the nucleus by the Drosha-DGCR8 complex to generate a precursor miRNA (pre-miRNA). The pre-miRNA is then transported to the cytoplasm by Exportin 5 and its cofactor Ran, where it is further processed by Dicer and Argonaut proteins to generate mature miRNAs that can then bind to their target mRNA transcripts [@He2016].

miRBase is the primary public repository and online resource for microRNA sequences and annotation, is responsible for microRNA gene nomenclature and provides a wide-range of information on published microRNAs, including their sequences, their biogenesis precursors, genome coordinates and context, literature references, deep sequencing expression data and community-driven annotation [@Kozomara2019].

miRBase is a database that collects, curates and provides comprehensive information on microRNAs. It was established in 2002 and has since become the primary source for microRNA nomenclature, sequences and functional annotation. The database is managed and maintained by a team of curators, who collect and organize data on new and existing microRNAs from published literature and experimental datasets.

In addition to providing information on microRNA sequences and annotation, miRBase also includes tools for sequence search, target prediction, and expression analysis. Users can search for microRNAs by name, sequence, or genome location, and can also browse and download datasets of microRNA expression profiles from a variety of tissues and conditions.

Despite previous works had studied and documented miRNAs it human milk, yet little is known about miRNome at population level. Moreover, investigation regarding human milk components, both in mother and neonate intestine, could help to clarify the influence of human milk in the infant gut miRNome and the possible similarities with the mother.

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 each mother-infant pair 1 to 6 days after delivery, 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) 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, and 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

he 60 dyad samples will be collected from mothers who gave their informed consent according to the Declaration of Helsinki, 2013, the protocol was approved by the "Instituto National de Perinatología" Bioethics Committee. After collection, samples will be stored at -20°C until processing at "Laboratorio de Referencia y apoyo para la Caracterización de Genomas, Transcriptomas y Microbiomas" Genetics and Molecular Biology, Cinvestav Zacatenco. Inclusion criteria for participants are samples from healthy women, residing in Mexico, full-term delivery, at first breastfeeding week (0 to 7 postpartum days) and who had previously breastfed their infant. Neonate stool sample will correspond to mother offspring which human milk sample was collected and had breastfed. In addition, exclusion criteria will be alcohol, cigarettes or other drugs consumption during pregnancy or sample collection, mothers who do not breastfed and mothers suffering metabolic syndrome, chronic or neurodegenerative diseases.

RNA extraction

One (1) mL of colostrum will be separated in Neofuge 12R centrifuge (HealForce®) using fresh 1.5 mL polypropylene tubes at 13 800 rpm, 5 min, 4° C, repeat this procces until 3 distinc phases are clearly distinguishible. Discart intermediate (proteic) phase with 100 µL filter pippete tips and corresponding micropippete; keep lipidic and celular fraction. Wash two times with 1 mL cold PBS, ensuring to disrupt the pellet in vortex, centrifuge under the same conditions after each wash. Spin 10 seconds. For the stool samples, weigh 200 mg and wash with cold PBS in a 0.4 µm filter, collect in 15 mL Falcon tube. Transfer 1 mL to a fresh centrifuge tube. For total RNA extraction, use mirVana™ miRNA Isolation Kit (Invitrogen™, Cat. AM1560) following manufacturer's intructions. Check RNA integrity in 2% low melting point agarose gel. Concentration was assessed in Qubit with Qubit™ RNA High Sensitivity kit (Cat. Q32852).

Preparation of the 16S rDNA library and next generation sequencing

The small RNA (sRNA) Library with TruSeq® Small RNA Library Prep (Cat., RS-200-0012) were prepared following manufacturer's instructions as follows. Starting total RNA should be at least at

200 ng and sRNA 10-50 ng/µL. Use DNAse treatement after extraction. Positive control is Human Brain Total RNA (Cat., # AM7962). For 3' adapter ligation, add 1 µL RA3 and 1 µL RNA in 5 µL water, mix and incubate 70 °C for 2 minutes. In a fresh PCR tube, mix the following reagents for each sample: 2 µL HML, 1 µL RNAse inhibitor and 1 µL T4 RNA ligase, Deletion Mutant. Add 4 µL to each sample, incubate at 28° C for 1 hour, add 1 µL STP, mix and incubate for 15 min more, then place on ice. For 5' adpater ligation, take a fresh PCR tube and for each sample: add 1.1 µL RA5, incubate 70°C, 2 min, add 1.1 µL ATP 10 mM, mix and add 1.1 µL T4 RNA ligase. Add 3 µL to each 3' adapter ligated sample. Incubate at 28° C for 1 hour, the place on ice. From 6 µL ligated RNA, add 1 µL RNA RT Primer, mix and incubate 70° C, 2 min, then place on ice. In a fresh PCR tube, mix for each sample: 2 µL First Strand Buffer, 0.5 dNTP Mix 12.5 mM, 1 µL DDT 100 mM, 1 µL RNAse inhibitor and 1 µL SuperScript II Reverse Transcriptase. Add 5.5 to each library and incubate at 50° C for 1 hour. For each index, mix in a fresh PCR tube: 8.5 µL ultrapure water, 25 µL PML, 2 µL RP1 and 2 µL RPIx. Add 37.5 µL to each library, incubate on thermal cycler with the following program: 98° C, 30 s; 11 cycle of 98° C for 10 s, 60° C for 30 s and 72° C for 15 s; then 72° C, 10 min, and hold at 4° C. Place each library in High Sensitivity DNA chip (Agilennt ,Cat. 5067-4626) to verify the precense of clear bands corresponding to sRNA region. This a safe stop point, cDNA can be stored at -25° C up to 7 days. cDNA construct purification will be conducted with 7% polyacrylamide gel (Novex TBE Gels, Cat., EC6265BOX). Load 1 µL CRL with 1 µL DNA loading dye in wells 1 and 2; 1 µL HRL with 1 µL DNA loading dye in well 3; and 25 µL cDNA library with 25 µL DNA loading dye in wells 4 and 5. Run the gel for 60 min, 145 V. Stain the gel with ethidium bromide for 2-3 min, cut the bands between 147-157 nt, corresponding to sRNA of 22-30 nt, respectively. Disrupt the bands in 0.5 mL gel breaker tube, centrifuge 20 000 x g for 2 min, add 200 µL ultrapure water and mix for two hours to elute DNA. Place in 5 µm filter and centrifuge at 600 x g for 10 seconds.

Data analysis

All bioinformatic analyses were conducted using R 4.3.3 in RStudio, version 2024.04.2.0, along with additional packages including ComplexHeatmap version 2.18.0, DESeq2 version 1.42.1 (Love et al., 2014), ALDEx2 version 1.34 (Fernandes et al., 2013), and edgeR version 4.0.16 (Robinson et al., 2010). A heatmap, a statistical visualization technique used to reveal similar patterns shared by subsets of rows and columns in matrix-type data (Gu, 2022), was generated using the ComplexHeatmap package. For this, we included 100% of the miRNAs with at least 50% abundance. Additionally, a chart was created to highlight the most representative miRNAs found in the two groups of milk samples used in this study by calculating the mean and standard deviation for miRNA abundance in the Before and After milk samples.

For differential analysis, three methods available in R were applied. The first, DESeq2 version 1.42.1, employs shrinkage estimation for dispersions and fold changes to improve the stability and interpretability of the estimates (Love et al., 2014). This method generated a volcano plot, a scatter plot displaying the statistical significance (P-value) against the magnitude of change (fold change), allowing for the visual identification of genes with significantly large fold changes. In the volcano plot, the most statistically significant genes are located at the top; upregulated genes are to the right, while downregulated genes appear on the left (Doyle). For the volcano plot, we used log2FoldChange and P-value variables, and for DESeq2 bar plots, we selected genes with P-value < 0.05 and log2FoldChange > 1.5. The second method, ALDEx2, takes as input the sequencing count per gene for each replicate and a list indicating which replicate belongs to each condition. It produces a table for each gene that includes individual mean expression per gene for each sample, mean expression per gene for each condition, overall mean expression, average absolute fold difference,

mean effect size, median within-condition difference, and zero quantile (Fernandes et al., 2013). A volcano plot was also created to determine variable cutoffs, utilizing mean difference values between the log-ratio (clr) of the groups (diff.btw) and the expected P-value from the Wilcoxon rank-sum test (wi.ep). For ALDEx bar plots, diff.btw and miRNA data were included.

Finally, edgeR version 4.0.16 (Robinson et al., 2010) was employed to estimate gene-specific dispersions using conditional maximum likelihood on total counts for each gene. An empirical Bayes procedure shrinks these dispersions toward a consensus value, and differential expression for each gene is then evaluated using a test for overdispersed data. Here, a volcano plot was also generated, using Log Fold Change (logFC) and P-value variables. For ALDEx bar plots, diff.btw and the miRNAs found in the samples were included.

3 Results

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4 Discussion

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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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11 Supplementary Material

Supplementary Material can be found at

Data Availability Statement

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