

Variation of the human milk bacterial diversity during the time of the day [†]

Karina Corona-Cervantes ¹, Norma Gabriela Zavala-Torres ¹, Rodrigo García- Gutiérrez ¹, Alberto Piña- Escobedo ¹, Fernando Hernández-Quiroz ¹ and Jaime García- Mena ^{1, *}

¹ Departamento de Genética y Biología Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (Cinvestav), Av. Instituto Politécnico Nacional 2508, Ciudad de México 07360, México.

* Correspondence: jgmena@cinvestav.mx.

[†] Presented at the 1st International Electronic Conference on Microbiology, 2–30 November 2020; Available online: <https://ecm2020.sciforum.net/>

Published: 2 November 2020

Abstract: Many bacterial communities display oscillations throughout the day; it has been shown that in human, the healthy gut microbiome also shows fluctuations in abundance of bacterial community, related with feeding times and sleep cycles. Likewise, in the human milk there are beneficial bacteria that undergo changes in the same time interval and are transferred to the newborn by breastfeeding colonizing the gastrointestinal tract. The aim of this work was to identify changes in the bacterial diversity on the human milk along the day. Human milk samples were collected from a single donor three times during the day (morning, afternoon, night) from 5 consecutive days, and bacterial DNA was extracted. Bacterial diversity was characterized by high-throughput DNA sequencing of 16S rDNA libraries, and taxonomy was assigned by comparison of sequences against a database, finally the significant differences in relative abundance and alpha and beta diversity was determined. The analysis of the human milk displays changes in the bacterial diversity during the day, with significant changes in the Shannon diversity index. Our data show that the human milk seems to be affected by the daytime change and these changes could influence the infant gut microbiota.

Keywords: human milk; bacterial oscillation; circadian clock; microbiota

1. Introduction

Circadian rhythms are present in almost every organism in respond to continuous change in light for the rotation of the planet around its axis [1]. Some reports describe the presence of photosynthetic bacteria in this rhythmic process, however the circadian clock operate in non-photosynthetic bacteria too, such as those of the intestinal microbiota [2]. The gut microbiota composition and diversity are sensitive to changes in the environment of the host, as the circadian cycle [3]. Many of these bacteria display oscillations throughout the day regulated by the host feeding times and sleep cycles, contributing to homeostasis and participate on a several physiological processes [4, 5]. Likewise, in the human milk there are beneficial bacteria that are transferred to the newborn by breastfeeding, colonizing the gastrointestinal tract [6]. In addition, other studies suggest that some components of the human milk such as proteins, nucleotides, vitamins among others undergo diurnal variations in the same way the gut microbiota does in both mice and humans [4, 7]. We believe in the human milk; these oscillations enhance the well-being of the breast-fed infant.

Besides the knowledge of the gut microbiota and circadian rhythm, it is not well understood the relationship of the variation of the bacterial diversity in the human milk at a different hour of the day, for this reason the aim of this work is to characterize the bacterial community in the human milk over

the course of the day, and to identify the possible changes in the bacterial composition and diversity in the human milk microbiota.

2. Materials and Methods

2.1. Study design

A longitudinal case report study was performed on a healthy 35 years-old multiparous, volunteer Mexican woman, four months after a vaginal delivery of a male neonate without complications and no fetal distress.

2.2. Sample collection

Human milk samples were collected from the donor three times a day, approximately every 8 hours: morning (7:00), afternoon (15:00) and night (22:00) over five consecutive days period. The samples were manually collected in aseptic conditions and immediately stored at -20°C until processing for DNA extraction.

2.3. DNA extraction and Preparation of V3 16S rDNA libraries

Bacterial DNA was extracted using the FavorPrep™ Stool DNA Isolation Mini Kit (Cat No. FASTI 001-1) following the manufacturer instructions. For libraries preparation ~281 bp amplicon containing the V3 hypervariable region of the 16S RNA gene in each sample was amplified and equimolar concentrations of amplicons were pooled. After that, bacterial communities were characterized by high-throughput sequencing of V3-16S rDNA libraries in Ion Torrent PGM System as previously reported [6].

2.4. Data Analysis

The sequencing data were analyzed using QIIME pipeline v1.9.0. [8]. and taxonomy was assigned by comparison of the sequences against the Greengenes reference database. To detect significant differences in the relative abundance of bacterial taxa among groups, the linear discriminant analysis effect size program (LEfSe v1.0) was used. Finally, the alpha diversity was determined using Phyloseq and ggplot2 packages in R environment (v3.4.4). Beta diversity, the dissimilarity was estimated using Bray Curtis analysis. A two-dimensional scatter plot was generated using Multidimensional scaling (MDS) in the R environment.

2.5. Statistical analyses

The statistical comparison of groups was calculated with the non-parametric Friedman Test for Repeated-Measures using the Social Science Statistic software. $p < 0.05$ was considered statistically significant. ANOSIM was used for category comparisons of phylogenetic distances metrics.

3. Results

3.1. Bacterial diversity changes according the sampling time in the day.

The characterization of microbiota in the human milk at different sampling times of the day, showed significant changes in the bacterial alpha diversity according to the Shannon diversity index ($p = 0.022$), however no significant changes were detected in the bacterial richness evaluated by observed number of species ($p = 0.861$), Chao 1 (0.846) or in the dominance by Simpson index (0.449) (Fig 1a). This difference in the diversity was detected between the morning sampling time and the night. Conversely, no differences were observed in the bacterial composition or diversity (alpha and beta diversity) in the afternoon group compared with the morning and night sampling time groups (Fig 1a and 1b).

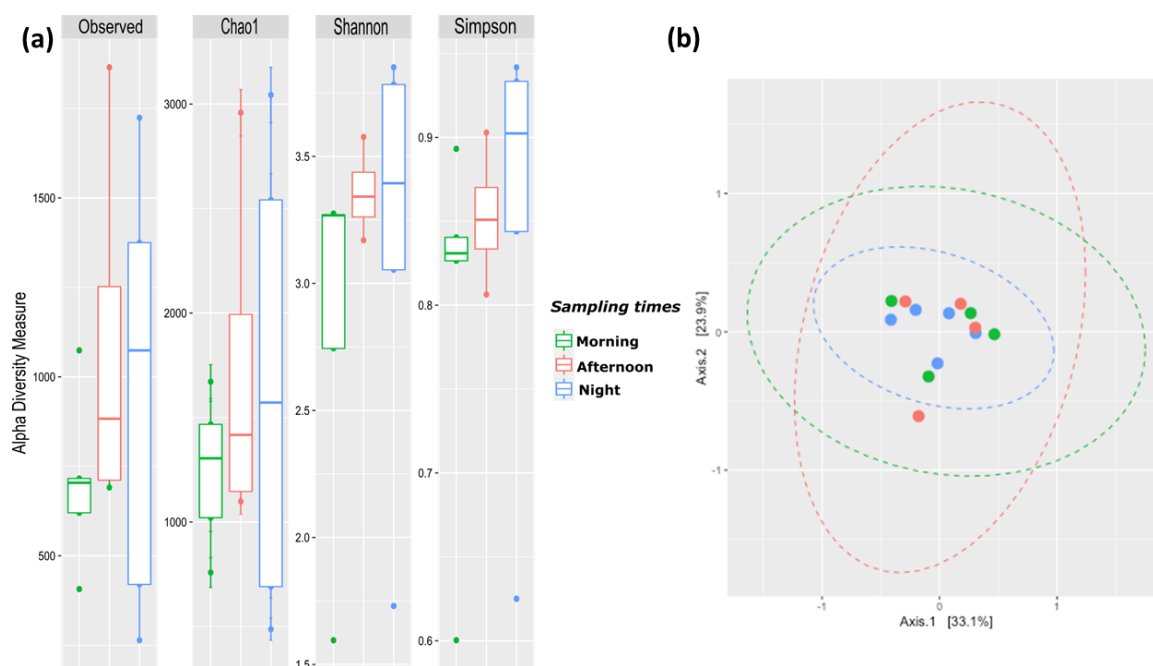


Figure 1. Human milk diversity according of sampling time in the day. **(a)** Alpha diversity indexes based on observed number species, Chao1, Shannon and Simpson. **(b)** Beta diversity analysis two-dimensional plot calculated with Bray Curtis test for the human milk samples. Human milk morning samples are plotted as green dots, Afternoon samples are represented as red dots and Night samples are denoted as blue dots.

3.2. Different bacterial taxa are found between morning and night sampling.

Likewise, variations in the relative abundance of some bacterial taxa were identified between the night and morning samples using linear discriminant effect size. Three proteobacteria, two actinobacteria taxa and the genus lactobacillus were more represented in the night milk samples set. On the other hand, the family Micrococcaceae was more abundant in the samples collected in the morning (Fig 2).

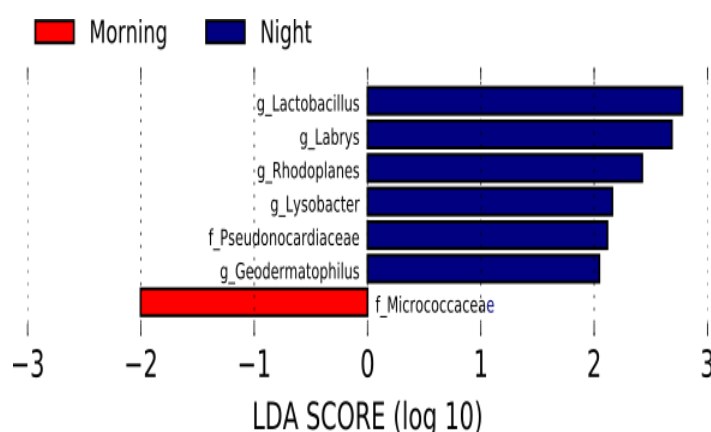


Figure 2. Represented significant bacterial taxa for each time of the day. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) comparison of bacterial taxa between human milk morning samples and night samples. Horizontal bars represent the effect size for each taxon: red indicates taxa enriched in Morning group and blue indicates taxa enriched Night group. LDA score cutoff of 2.0.

4. Discussion

Circadian fluctuations in some bioactive components have been previously reported, these changes are suggested to transfer chronobiological information from mother to child to assist the

development of the biological clock [7]. Our results on bacterial abundance, showed that the members of the phyla Proteobacteria, Actinobacteria and Firmicutes are more abundant at night. This result agrees with other studies about changes in the abundance of members of the microbiota in some organisms. For instance, during the analysis of bacterial abundance in saliva in humans, a night-time predominance of the phylum Actinobacteria was observed in healthy volunteers [9]. In other studies, in mice, it has been determined that members of the phylum Proteobacteria, presented oscillations in its abundance through the day, with a greater abundance of these bacteria at night [1]. Also, *Lactobacillus* (Firmicutes) has exhibited rhythmic oscillations in a 24 h cycle, associated with the feeding time, showing in this manner phase shifts between dark and light in mice [4]. Finally, as previously has been reported for fecal microbiota, these changes could influence the composition of the infant gut microbiota, since breastfeeding is considered the most optimal mode of feeding for neonates.

5. Conclusions

As some of the human milk components, the human milk microbiota seems to be affected by circadian changes. The results presented in this work suggest that the milk microbiota exhibits diurnal fluctuation resulting in specific bacterial arrangement depending on the time of day (Fig.3), at least in the studied case. However, since conclusions from a case study are limited, it is necessary to study a larger number of samples to obtain more conclusive results.

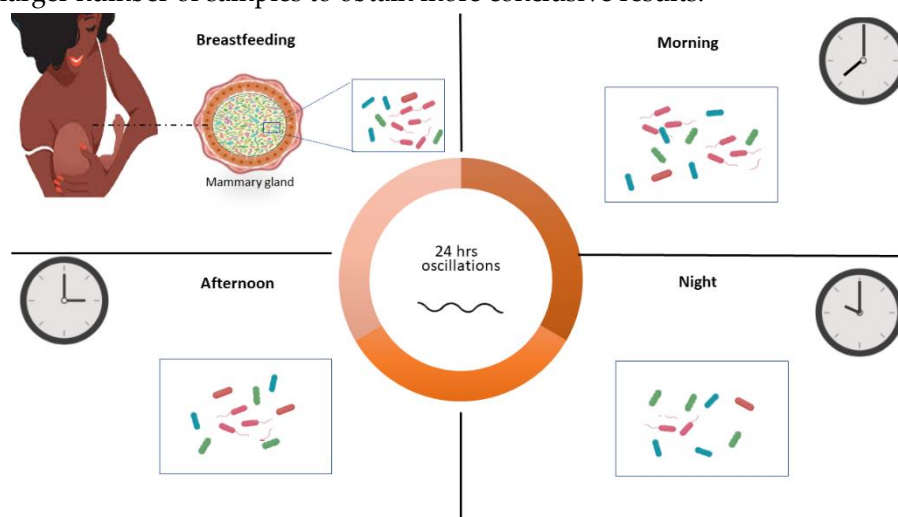


Figure 3. Model for the bacterial diversity change during the day. Bacterial microbiota shows variations in its diversity and composition between the morning and night. This could have an impact on the infant microbiota.

Author Contributions: Conceptualization, KC-C, and JG-M; methodology, KC-C, NGZ-T, FH-Q, AP-E and RG-G; software, KC-C and FH-Q; validation, KCC, NGZT, and JG-M; formal analysis, KC-C, and FH-Q; investigation, NGZ-T, and KC-C; resources, RG-G, and JGM; data curation, KC-C, and FH-Q; writing—original draft preparation, KCC, and NGZT; writing—review and editing KC-C, NGZ-T, and JG-M; visualization KC-C, FH-Q, and NGZ-T; supervision, JG-M; project administration, JG-M.; funding acquisition, JG-M.

Funding: This research received no external funding.

Acknowledgments: We are grateful to the participant mother who provide the samples. We thank CONACyT Doctoral Fellowships for KC-C (777953), and FH-Q (291236), and Master Fellowship for NGZ-T (997494). We are grateful to Ms. Viridiana Rosas Ocegueda for administrative assistance. JGM (19815) is a Fellow from the Sistema Nacional de Investigadores, Mexico.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Liang, X., Bushman, F. D., & FitzGerald, G. A. (2015). Rhythmicity of the intestinal microbiota is regulated by gender and the host circadian clock. *Proceedings of the National Academy of Sciences*, *112*(33), 10479-10484.
2. Costantini, C., Renga, G., Sellitto, F., Borghi, M., Stincardini, C., Pariano, M., ... & Romani, L. (2020). Microbes in the era of circadian medicine. *Frontiers in Cellular and Infection Microbiology*, *10*, 30.
3. Voigt, R. M., Forsyth, C. B., Green, S. J., Mutlu, E., Engen, P., Vitaterna, M. H., ... & Keshavarzian, A. (2014). Circadian disorganization alters intestinal microbiota. *PLoS one*, *9*(5), e97500.
4. Thaiss, C. A., Zeevi, D., Levy, M., Zilberman-Schapira, G., Suez, J., Tengeler, A. C., ... & Kuperman, Y. (2014). Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. *Cell*, *159*(3), 514-529.
5. Trinder, M., Bisanz, J. E., Burton, J. P., & Reid, G. (2015). Bacteria Need Sleep Too?: Microbiome Circadian Rhythmicity, Metabolic Disease, and Beyond. *University of Toronto Medical Journal*, *92*(3), 52-55.
6. Corona-Cervantes, K., García-González, I., Villalobos-Flores, L. E., Hernández-Quiroz, F., Piña-Escobedo, A., Hoyo-Vadillo, C., & García-Mena, J. (2020). Human milk microbiota associated with early colonization of the neonatal gut in Mexican newborns.
7. Italianer, M. F., Naninck, E. F., Roelants, J. A., van der Horst, G. T., Reiss, I. K., Goudoever, J. B. V., ... & Vermeulen, M. J. (2020). Circadian Variation in Human Milk Composition, a Systematic Review. *Nutrients*, *12*(8), 2328.
8. Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., ... Knight, R. QIIME allows analysis of high-throughput community sequencing data. *Nature methods*, (2010). *7*(5), 335-336. <https://doi.org/10.1038/nmeth.f.303>
9. Johnson, C. H., Zhao, C., Xu, Y., & Mori, T. (2017). Timing the day: what makes bacterial clocks tick?. *Nature Reviews Microbiology*, *15*(4), 232-242.

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



© 2020 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC-BY) license (<http://creativecommons.org/licenses/by/4.0/>).