Original Article

Assessment of Breastmilk Microbiome Focusing on Probiotics

Abstract

Background: Breastmilk is an important factor affecting the infant's gut microbiota and health. Both milk microbiome and the content of oligosaccharides play a role in this effect. The mother's lifestyle can affect the breast milk microbiome. In this study, we assessed the breast milk microbiome in healthy mothers in Iran and its relationship to the mother's lifestyle during lactation. **Materials and Methods:** In a cross-sectional study, from May 2022 to January 2023, 20 mother-neonates participated by donating their breastmilk samples and completing two questionnaires (food recall for nutritional status and DASS-21 for psychological status). Milk samples in aerobic and anaerobic conditions were cultured to isolate bacterial strains, and the probiotic strains were determined. Data entrees and analysis were done using SPSS-15, and the independent T-student or Mann–Whitney U test was used for statistical analysis. **Results:** Seven bacterial species were isolated, including *Pedicoccus acidilactici, Lactobacillus fermentum* (potential probiotics), *Corynebacterium kroppenstedtii*, *Staphylococcus epidermidis, Rothia kristinae, Streptococcus rubneri*, and *Streptococcus parasanguinis.* It was shown that the consumption of dairy products in the diet was related to probiotic strains in breast milk (p value = 0.03). **Conclusions:** Among the microbiome isolated from breast milk, two probiotics, *Pedicoccus acidilactici* and *Lactobacillus fermentum,* were detected. In the mother's diet, dairy product intake during lactation can increase the probiotics in breast milk and give the infant more valuable content.

Keywords: *Breastmilk, lifestyle, microbiome, probiotics*

Nazila Kassaian[1], Shahrzad Ahangarzadeh[2], Bahareh Vakili[2], Parisa Shoaei[2], Abbas Alibakhshi[3], Soodabeh Rostami[1], Shahin Gavanji[4], Shayan Arbabnia[5], Elmira Mohammadi[6]

[1]Nosocomial Infection Research Center, Isfahan University of Medical Sciences, Isfahan, Iran, [2]Infectious Diseases and Tropical Medicine Research Center, Isfahan University of Medical Sciences, Isfahan, Iran, [3]Molecular Medicine Research Center, Hamadan University of Medical Sciences, Hamadan, Iran, [4]Department of Plant Biotechnology, Medicinal Plants Research Centre, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran, [5]Department of Food Science and Technology, Islamic Azad University, Najafabad Branch, Isfahan, Iran, [6]Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

Address for correspondence:
Dr. Elmira Mohammadi,
Pharmaceutical Research Center, Isfahan University of Medical Sciences, Isfahan - 8174673461, Iran.
E-mail: el.mohammadi@pharm.mui.ac.ir

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Introduction

The transmission of useful bacteria to the infant's gastrointestinal tract via breastfeeding is critical for immunity development. Breast milk plays a crucial role in the infant's gut microbiota. In addition to breast milk microbiota, prebiotics, including human milk oligosaccharides (HMOs) and antimicrobial factors, are important in this process.[1] Breast milk has some protective effects for infants against some infections, such as respiratory[2] and gastrointestinal infections,[3] reducing the occurrence of necrotizing enterocolitis[4] and allergies.[5] Although breast milk is primarily considered a sterile fluid to date, many studies have reported that various bacterial species can be detected in breast milk.[6-8] In breast milk, the origin of bacteria can be related to internal bacteria, which are translocated to the breast with the "enter-mammary pathway" or external bacteria, which flow back into the breast during sucking.[9,10] In previous studies, with the aid of culture base methods, bacterial species from Micrococcaceae, Streptococcaceae, Neisseriaceae, Lactobacillaceae, Bifidobacteriaceae, Corynebacteriaceae, and Staphylococcaceae have been isolated from breastmilk samples.[11,12] Several studies have reported a diverse abundance of live bacteria in breast milk (up to 1 × 10^7 CFU/mL).[5] Meanwhile, the presence of probiotics can be very important. Probiotics, which are live and nonpathogenic microorganisms, whether bacteria or yeasts, can benefit the host if administrated sufficiently.[13] Lactobacillus and Bifidobacterium are the most known probiotics with a long history of safe use in humans. The probiotics can improve pediatric health and may also expand in nutraceutical applications.[14]

Breast milk microbiome may be a candidate for shifting probiotics from the mother to the newborn's gut through the gut-breast axis. In recent years, research on breast milk probiotics has gained significant attention, highlighting the critical role that human milk oligosaccharides (HMOs) and beneficial

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bacteria play in infant gut health and development.[15] However, geographical, demographic, lifestyle, and genetic factors can affect milk component variation[16-18] The mode of delivery (vaginal birth vs Cesarean) can also affect the breast milk microbiota.[19] Considering the importance of breast milk in infant health and the potential effect of the mother's lifestyle on the content of breastmilk, investigating this effect can guide mothers to adopt a lifestyle that will create favorable effects on breastmilk. Advances in microbiome analysis techniques have allowed researchers to understand better the specific strains present in breast milk and their functional benefits. Additionally, there has been growing interest in developing probiotic supplements derived from breast milk to support infants who are not exclusively breastfed, further emphasizing the importance of maternal health and nutrition in shaping the microbial landscape of newborns.[20] Despite the growing body of research on breast milk probiotics, several informational and research gaps remain. There is limited research on the variability of breast milk probiotics across different populations, maternal diets, and health conditions, which could influence the composition and efficacy of these microbial communities. Variability in the composition of breast milk probiotics due to these factors has not been sufficiently studied, limiting the generalizability of findings across diverse populations.[21]

Addressing these gaps could significantly advance our understanding of the role of breast milk probiotics in infant health and development. Nowadays, a major challenge in the breast microbiome and interactions between infants and mothers lies in lack of databases. The main aim of this study, which was conducted with the participation of healthy mothers who donated milk samples and completed the questionnaires, was to identify the microbiome and factors that could optimize the composition of breast milk microbiota. So, we assessed the breast milk microbiome in healthy mothers in Iran and its relationship to the mother's lifestyle during lactation.

Materials and Methods

This cross-sectional study was performed from May 2022 to January 2023. Of the 28 nominated mothers to participate in this study, 20 healthy mother-neonates who experienced term birth (all infants were born healthy from the 37th to 40th week of pregnancy onward) and had not used any antibiotics during the past 3 months were enrolled. Their breast milk was sampled, and questionnaires were filled out at the door of houses in a standard protocol. One clean sample, 12–16 weeks after delivery, was collected using an aseptic technique from their breast in a sterile condition and has been learned by the mothers. The samples were collected in the afternoon and transferred fast to the laboratory to be collected in a − 80˚C freezer. Also, two questionnaires related to the lifestyle of mothers during breastfeeding (nutritional and psychological conditions) were completed by mothers. For bacterial isolation, 20 μL of each breast milk sample was cultured in a Man Rogosa Sharp (MRS) agar plate in aerobic and anaerobic conditions at 37°C for 48–72 hours. Also, a sample culture in MRS agar medium consisting of cysteine hydrochloride and mupirocin was performed to isolate bifidobacteria at 37°C for 48–72 h in anaerobic conditions.[22] After that, all the colonies were subcultured, and a gram stain was used for initial identification. Chromosomal DNA was extracted from each isolated bacterium via the AddPrep Genomic DNA Extraction Kit (http://addbio.net). The polymerase chain reaction (PCR) with universal primers for 16S rRNA gene The universal 16S rRNA bacterial primers 27F (5′AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′) were used to amplify the 16S rDNA sequence under the following conditions: 94°C for 3 min, 94°C for 1 min, 58°C for 45 s, 72°C 1 min, 34 cycles, and a final extension step of 72°C for 10 min. After that, the PCR product was assessed via electrophoresis in a 1% (w/v) agarose gel. For final identification, PCR products were sequenced with an Applied Biosystems SeqStudio Genetic Analyzer (Thermo Fisher Scientific) according to the standard protocol of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific Inc.). Finally, we used the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare the sequencing results with identified sequences in the database of the Gene Bank.

Participants were asked to write down the type and amount of food and beverages and may use supplement intake during a regular day via 1-day food recall questionnaire,[23] and then the portion sizes were extracted by a trained dietitian. Food diaries and dietary frequency questionnaires (FFQs) are standard dietary assessment methods used to evaluate individual food intake. Food diaries involve detailed logging of all foods and beverages consumed over a specific period, allowing for real-time tracking of dietary habits. In contrast, FFQs consist of questions about the frequency and portion size of various foods consumed over a timeframe, which can provide a broader overview of nutritional intake but is memory-based. Both methods aim to gather information about dietary patterns but differ in their approach and granularity. Dietary recall is an accurate and common method used to assess individual dietary intakes and includes details about what types of food and beverages have been consumed. This recall also included the use of any probiotic supplements. Depression, anxiety, and stress were assessed via the DASS-21 questionnaire.[24] The DASS-21 is a self-report scale designed to measure emotional states. Subjects were asked to use 4-point scales to rate the extent of their experiences over the past week, and depression, anxiety, or stress was calculated by summing the scores. The determination of depression, anxiety, and stress severity has been evaluated according to Table 1.

We analyzed all data in SPSS 15 (SPSS Inc., Chicago, IL, USA). Categorical and continuous data were presented as frequency (percentage) and mean ± standard deviation (SD). After the assessment of the normal distribution by the Kolmogorov–Smirnov test, the differences between the two independent experiments were analyzed using the independent T-student or Mann–Whitney U test. A level of difference at $p \leq 0.05$ was considered as significant.

Ethical considerations

The research protocol has been approved by the Isfahan University of Medical Sciences Ethical Research Committee, Iran (approval number: IR.MUI.MED.REC.1400.083). Written informed consent was obtained from all the women before enrollment by considering all the rules of research ethics, including providing voluntary entry, complete information, ease of access to facilitators, and the right to withdraw from cooperation.

Results

Three subjects were excluded from the study due to not completing the questionnaires, the low sample size of breast milk, and low cooperation. Breast milk was collected from 17 women aged from 21 to 40 years. The majority of participants, 11 (65%), had undergone Cesarean section, while 6 (35%) had normal vaginal births. The mean maternal age was 32 ± 5 years. Of all participants, five subjects (29.41%) were complicated with hypothyroidism, which was administered levothyroxine, and one woman (5.91%) had minor thalassemia. The probiotic bacteria were screened from 3 isolates (17.61%) on MRS agar plates. Based on the results of biochemical characteristics and 16S rRNA sequence analysis, the strains of *Pedicoccus acidilactici* (in 1 sample) and *Lactobacillus fermentum* (in 2 samples) were detected.

The nonprobiotic strains were isolated from 7 samples. They were *Corynebacterium kroppenstedtii*, *Staphylococcus epidermidis, Rothia kristinae, Streptococcus rubneri*, and *Streptococcus parasanguinis.* Among them, *S. epidermidis* was abundant [Figure 1]. None of the women were exposed to probiotic supplements. However, five women (29%) were probiotic dairy consumers. We did not observe any significant correlation between probiotic content in human milk and stress, depression, or anxiety [Table 2]. Also, no correlation was observed between the mother's age, underlying disease, or delivery mode and breast milk probiotic existence (p value > 0.05).

There was no significant difference between the abundant intake of fruits, vegetables, meat, oil/nuts, bread, and cereals in women with and without probiotic bacteria in their breast milk (p value > 0.05). However, the intake of dairy products was significantly higher in mothers with probiotics in their breast milk (p value = 0.03) [Table 3]. However, consuming dairy products enriched with probiotics did not significantly affect the probiotic content in breast milk (p value = 0.51).

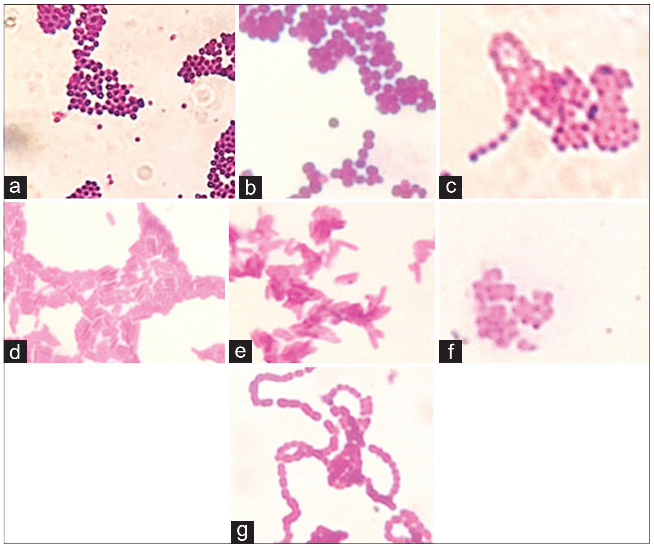

Figure 1: Bacterial species isolated from breast milk samples. (a) *Staphylococcus epidermidis*, (b) *Pediococcus acidilactici*, (c) *Streptococcus parasanguinis*, (d) *Lactobacillus fermentum*, (e) *Corynebacterium kroppenstedtii*, (f) *Rothia kristinae*, (g) *Streptococcus rubner*

Table 1: Recommended cutoff scores for conventional severity labels according to DASS-21 questionnaire[21]

	Depression	Anxiety	Stress
Normal	0–9	0–7	0–14
Mild	10–13	8–9	15–18
Moderate	14–20	10–14	19–25
Severe	21–27	15–19	26–33
Extremely severe	28+	20+	34+

Table 2: Relationship between stress, anxiety, and depression in women with and without probiotics in breast milk

Mental disorders Score	Groups of breast milk	Mean score (SD)	*p*
Stress	Non- probiotic	1 (1.10)	0.67
	Probiotic	1.31 (1.10)	
Anxiety	Non- probiotic	1.31 (1.80)	0.86
	Probiotic	1.61 (1.50)	
Depression	Non- probiotic	0.71 (1.40)	0.86
	Probiotic	1 (1.70)	

Discussion

In this study, probiotic and nonprobiotic species were isolated from breast milk. Probiotic isolated species were *Lactobacillus fermentum* and *Pediococcus acidilactici.* Other studies have also reported these species' isolations from breast milk.[25,26] *Pediococcus acidilactici* is a homofermentative Gram-positive coccus that usually appears

Table 3: Relationship between mothers' daily dietary consumption and the probiotic existence in their breast milk

Food groups in mothers' diet	Servings/day in group 1* Mean (SD)	Servings/day in group 2* Mean (SD)	*p*
Fruits	3.31 (1.50)	2.31 (1.90)	0.42
Vegetables	1 (0.10)	1.90 (1)	0.16
Dairy products	1.31 (0.60)	0.60 (0.50)	0.03**
Meat/fish/poultry	4 (1.70)	3.61 (1.30)	0.71
Oil/nuts	2 (2.60)	1.11 (1.20)	0.38
Bread and cereals	14.30 (1.50)	13.80 (4.40)	0.86

*group 1: probiotic existence in breast milk. *Group 2: breast milk without any probiotic. **: Statistically significant

in pairs or tetrads. It grows under various temperatures, pH levels, and osmotic pressure, allowing it to colonize the digestive tract.[27] This potential probiotic has benefits such as being an immune modulators and antiparasitic effects.[28,29] It has been demonstrated that *Pediococcus acidilactici* is usually found in fermented dairy products, fermented vegetables, and meat.[27] *Lactobacillus fermentum* is also a Gram-positive bacillus that has probiotic characteristics. It can be isolated from different environments, such as bread, dairy products, sausages with natural fermentation, and breast milk.[30] Varied benefits related to this species were reported, including enhancement in immunologic response, averting gastrointestinal and upper respiratory infections, acting as cholesterol-lowering agents, and the ability to be used as a food preservative or antibiotic.[31] According to our results, the presence of this species in breast milk can be related to dairy product consumption. In our study, some other bacterial species were also isolated from breast milk samples, including *Corynebacterium kroppenstedtii*, *Staphylococcus epidermidis, Rothia kristinae, Streptococcus rubneri*, and *Streptococcus parasanguinis. Staphylococcus epidermidis* is a Gram-positive member of the normal human skin flora. *Streptococcus parasanguinis* is a Gram-positive coccus that belongs to the human oral microbiome, especially the infant oral cavity. Studies indicate that this presence can result from bidirectional transmission between the mammary gland and the infant's mouth.[32] *Streptococcus rubneri* is an ovoid-shaped Gram-positive lactic acid bacteria isolated first from healthy human throat samples.[33] Little information about this species has been reported so far. The origin of this strain in the milk sample needs to be clarified. *Corynebacterium kroppenstedtii* was first isolated from a human sputum sample in 1998.[34] *Corynebacterium kroppenstedtii* is a Gram-positive bacterium that can lead to opportunistic human infections, such as granulomatous mastitis and breast abscesses.[35] Although *Corynebacterium kroppenstedtii* was found in some samples of this study, they did not complain about specific clinical symptoms. The role of *Corynebacterium kroppenstedtii* in breast pathologies remains unclear.[36]

Two significant recent global studies about mothers' and infants' microbiome exist. The Microbiome Atlas of Mothers and Infants (MAMI) is a comprehensive database that has collected over 39,000 maternal and neonatal microbial samples from 29 countries across six continents. This initiative aims to archive data on edible probiotic strains found in various body locations, including the nasal and oral cavities, gastrointestinal tract, vagina, skin, and breast milk. In this study, the breast milk microbiota from Africa differs from Asia and North America. However, this project has no data from Iran and Middle East that are qualified. This study demonstrated that *Streptococcus* was the most abundant and relatively stable genus overall.[37] In another large investigation named "Human Milk-Gest Study", aimed to investigate potential association of mode of delivery, preterm birth, birth weight, and gestational age with breast milk microbiota, the milk microbiota at different sampling times was significantly different. In mature human milk samples, *Ralstonia*, *Burkholderiaceae_uc*, and *Pelomonas* were dominant.[38]

Breast milk microbiome can be influenced by variable factors such as maternal health, genetics, diet, mode of delivery, and environmental situations.[17,39,40] In the study conducted by Khodayar-Pardo *et al.*,[41] it was found that delivery mode, gestational age, and the stage of lactation can affect the milk microbiota. According to their study, breast milk from mothers who underwent Cesarean sections contained a higher bacterial concentration during early lactation (days 1–16) than those with natural deliveries. It also showed significantly higher levels of *Streptococcus* spp. and lower levels of *Bifidobacterium* spp. In our study, no significant relationship was observed between the mode and age of delivery and milk microbiome, which may be due to the small sample size. The effect of the mother's diet on the composition of breast milk is not clearly demonstrated. Moreover, the evidence of the relationship between the mother's dietary intake and their breast milk probiotic content is limited. In a survey conducted by Drago *et al.*,[25] with 50 mother precipitants, no association between diet and breast milk microbiota was observed. However, in the study of Nikolopoulou *et al.*,[42] on 100 healthy women, regular yogurt consumption was reported to increase bacteria in breast milk. Our study showed an increasing relationship between dairy consumption and the presence of specific probiotics in breast milk. Interestingly, consistent with the reported result by Nikolopoulou *et al.*,[42] our study showed no significant relation between probiotic-enriched foods and an increase in breast milk probiotics. It is recommended that mothers increase their intake of dairy products to enhance the probiotics in breast milk and benefit infants.

In terms of the relationship between the mother's psychological characteristics and milk microbiota, in our study, we did not obtain a significant association between stress, anxiety, or depression in mothers and their breast milk microbiota. However, Browne *et al*.[40] reported a potential relationship between milk microbiota and psychosocial distress in lactating mothers ($N = 77$). This may be due to low psychological problems in our subjects.

The biggest limitation of this study was the small sample size which can reduce internal and external validity, making it difficult to generalize results. However, this study can be considered as a pilot for the future longitudinal studies. Longitudinal studies of the breast milk microbiome alongside maternal diet over time can help establish causal relationships by analyzing how maternal nutrition variations influence breast milk composition and diversity.

Conclusion

In our study, several strains were isolated from breast milk, and their presence was not related to stress, depression, anxiety, mother's age, underlying disease, or delivery mode. This study demonstrated that women who consumed dairy products had the most value for probiotic contents in breast milk among the food groups. The long-term effects of breast milk-derived probiotics on health outcomes beyond infancy, such as their role in preventing chronic diseases, require further investigation. It is suggested that causal relationships be established by analyzing how maternal nutrition variations influence breast milk composition and diversity during a longitudinal study. Also, there is a need for standardized methodologies to isolate and characterize breast milk probiotics, which would facilitate comparisons across studies and enhance the reproducibility of findings.

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Conflicts of interest

Nothing to declare.

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