**Comparative study of breast and intestinal microecology between healthy women during lactation and patients with mastitis**

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**Abstract：Objective:** Our objectives were to compare the abundance and diversity of bacteria in milk and feces of healthy lactating women and patients with lactation mastitis; explore the pathogenesis of lactation mastitis; and formulate new ideas for the treatment, and prevention of lactation mastitis from a microbiological perspective. **Methods:** Prospectively recruited subjects included 19 breastfeeding mastitis cases and 19 lactating healthy subjects. Both healthy lactating women and patients with lactation mastitis provided feces and milk samples for collection. Applying 16sRNA sequencing techniques, the microbial diversity and abundance of populations of bacteria detected in milk and feces were compared. **Results:** The examination of bacterial composition and distribution in the milk and feces of lactating women who were healthy and those who had mastitis differed significantly, according to the analysis of α and β diversity. **Conclusions:** Lactating mastitis patients exhibit gut and milk microbiome dysregulation. Modulation of the gut-mammary axis might be a viable method for the prevention or treatment of mastitis.

**Keywords:** Breast milk; microecology microbiota; gut-mammary pathway; lactation mastitis

The World Health Organization considers breastfeeding to be the gold standard for infants feeding [1]. Breastfeeding has many health benefits for the baby and mother[2]. In the developing infant's stomach, human breast milk provides Staphylococcus, Streptococcus, and lactic acid bacteria through symbiosis, mutualism, and/or possible probiotics at a concentration of about 106 bacteria per milliliter[3,4]. Breast-fed babies are more resistant to a number of illnesses than formula-fed babies are, including irritable bowel syndrome, necrotizing enterocolitis, respiratory infections, asthma, diabetes, obesity, Crohn's disease, and syndrome of sudden infant death[5].In summary, breastfeeding improves the infant's health and immune function, and reduces morbidity and mortality from gastrointestinal diseases. The indisputable evidence for breastfeeding endorses breast milk as the best functional food[5-7]. At the same time, breastfeeding is also with maternal protection against breast cancer, ovarian cancer, and diabetes [8]. Approximately to 33percent of the surveyed of breastfeeding women experience breast tissue inflammation, which is a crucial risk factor in the decision to stop nursing[9].

Previous studies reported that acute mastitis during lactation was caused by milk stasis and exogenous bacterial infection. The wide use of second-generation gene sequencing technology has promoted human microbial research. The human microbiome project (HMP), which started in 2000, found that thousands of bacterial species live in the human gut, reproductive system, skin, mouth, and breast [10,11]. In 2003, the diversity of bacteria in healthy breast milk was first reported through bacterial culture [12,13]. Since then, a growing volume of investigations have used gene sequencing and culture to analyze the bacterial colonies in breast milk[14-17]. Therefore, the traditional theory of bacterial invasion being the cause of mastitis cannot be confirmed [12,13,18,19]. Similar to the flora of other body parts, the breast microflora also contains a variety of microorganisms, ranging from probiotics to potentially pathogenic bacteria, and forms a complex "microbiota-host" ecosystem with the human host. The ecosystem is the result of the co-evolution and mutual adaptation of hosts and microorganisms. once the balance is disturbed, mastitis may occur. The dysbiosis of the mammary gland microbiome is linked to lactation mastitis, according to a growing number of research [19].

The gut lies at the intersection of health and illness, and its role is interwoven with that of other organs. Researchers have investigated an increasing number of axes that emphasize the connection between the gut and certain other human organ functions over the last few decades, such as the gut-brain, gut-liver, gut-kidney, and gut-vaginal axes[20-23]. The gut-mammary axis, which is fundamental for animal evolution, is frequently overlooked. Because of the complex interactions between the intestinal and mammary glands, the intestinal and mammary gland adaptations in lactating women are synchronized, which explains how changes in the intestinal microecology affect the mammary gland microecology and cause mastitis[24]. Healthy mature anaerobic intestinal flora were detected in the breast and human milk by Loy et al[25]. After oral delivery of Lactobacillus fermentosa and Lactobacillus salivaris to the mothers, the probiotics reached the mammary glands and were found in breast milk, according to a research of probiotics for acute mastitis during lactation [26,27]. Based on these results, the gut-mammary axis may be a way for bacteria from the mother's gut to move from the gut to the mammary glands and breast milk while the mother is breastfeeding.

Currently, lactation mastitis is treated using antibiotics, with poor therapeutic effects. The most common treatment for mastitis is empiric antibiotics, which have been available for use for a long time. Unfortunately, a large number of mastitis patients do not improve with treatment because of antibiotic resistance brought on by a variety of causes, comprising intrinsic resistance, antibiotic resistance gene transfer and/or biofilm building [28]. Therefore, new management strategies for mastitis are needed. It is important to keep in mind that the use of probiotic strains generated from breast milk is appropriate in this context among techniques for managing the mammary bacterial communities[18]. In that study, a strain of probiotic bacteria isolated from breast milk was used to prevent and treat mastitis.

Milk and stool samples from lactation women in good health and patients with lactation mastitis were collected for this investigation. Using 16sRNA gene sequencing, the bacterial populations in milk and stool samples from the two groups were compared. This study investigates the pathophysiology of lactation mastitis along the gut-mammary axis and provides novel thoughts for its therapy.

**Materials and methods**

**Clinical data**

From June 2022 to May 2021, 19 individuals with lactation mastitis were proactively chosen at the hospital. 19 healthy lactating women served as the study's controls. The study included lactation women aged 20–40 years, with breast lumps, pain, skin redness, fever (temperature of 37.5°C), and ultrasound showing lactation mastitis. The patients gave consent to take part in the study and had not recently received probiotics or antibiotics. Patients with additional infections (e.g., puerperium infection), obesity, asthma, inflammatory bowel disease, and other inflammatory illnesses were excluded from the study. In addition, the participants provided consent for study participation. We excluded healthy lactating women with a history of mastitis indicated by medical history, physical examination, or imaging (B-ultrasound), acute or chronic diarrhea, constipation, or antibiotic use within the last two weeks. The following categories were created from the milk and feces samples that were gathered from the two groups of women: milk of healthy women during lactation (C), milk of patients with lactation mastitis (BM), stool of healthy women during lactation (FH), and stool of patients with lactation mastitis (FM).

**Methods**

Sample collection: (1) Milk sample collection: Before the milk was collected, sterile water was used to clean and sterilize the breast, nipple, and areola. Choosing to discard the first 5 milliliters of milk, the researcher put on sterile gloves and then collected 50 milliliters of milk in sterile test tubes.To remove the upper fatty layer, we centrifuged 50 mL of milk at a low speed for 5–10 min and stored the liquid that had settled down in a refrigerator at −80°C until DNA extraction.(2) Fecal sample collection: Before fecal sample collection, subjects were asked to pass urine to avoid fecal contamination. A sterile stool collection box was placed in the back of a squat toilet to ensure that the stool was emptied into the box. The cotton tip of a sterilized cotton swab was inserted into the feces and lightly stirred. Until DNA extraction, a sample the size of a soybean (approximately 50 mg) was collected in sterile cryopreservation tubes and kept in a Ziploc bag in the refrigerator at -80°C. PCR amplification and DNA extraction: The study's reagent is efficient in preparing DNA from the majority of bacteria and can recover DNA from minuscule amounts of samples. Blank water was produced by using water that was free of nuclear contamination. The complete DNA sample was eluted into 50 microliters of elution buffer, which was then frozen at -80 degrees Celsius until the PCR analysis could be performed (LC-Bio Technology Co., Ltd., Hang Zhou, Zhejiang Province, China). Stool and milk DNA was extracted using the E.Z.N.A.® Feces DNA Kit and E.Z.N.A.® Water DNA Kit, respectively, according to the official guidelines. We assessed the purity of the isolated DNA by electrophoresis on agarose gels. A UV spectrophotometer was used to quantify the amount of DNA present. Utilized is the general primer primer 341F (5'-CCTACGGGNGGCWGCAg-3'). The bacterial 16S rRNA gene's V3-V4 region was subsequently amplified by the 805R (5'-gactachVggGTATctaatcc-3') . The bacterial ITS2 region was amplified with the primers F (5'-gtGartCATCGAATCTTTG-3') and R (5'-TCCTCCGCTTATTGATATGC-3'). In 12.5 U of Phusion Hot Start Flex 2X Master Mix L, we added 2.5 microliter of forward primer, 2.5 microliter of reverse primer, 25 microliter of DDH2O, and 50 anogram of template DNA. Initial denaturation was conducted at 98 degrees Celsius for 30 seconds, followed by denaturation at 98 degrees Celsius for 10 seconds, followed by denaturation at 54 degrees Celsius for 30 seconds; this cycle was repeated 30 times, followed by heating at 72 degrees Celsius for 30 seconds, extension at 72 degrees Celsius for 10 minutes, and indefinite extension at 4 degrees Celsius. Universal primers were sequenced, and each sample's 5' ends were barcoded. A 2% agarose gel electrophoresis confirmed PCR results. In order to prevent false-positive PCR results, the DNA extraction negative control used ultrapure water. We utilized AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and Qubit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) instruments in order to accomplish purification and quantification of PCR output. An Agilent 2100 Bioanalyzer (Agilent, Wilmington, DE, USA) and the Illumina Library Quantification Kit were used to measure the size and quantity of the amplicon libraries before sequencing (Kapa Biosciences, Woburn, MA, USA). NovaSeq PE250 sequenced libraries.

**Data analysis**

Based on the unique barcodes assigned to each sample, paired-end reads were truncated by removing the primer sequences from each sample:Software: cutadapt;Version: cutadapt-1.9;Paired-end reads into long amplicon fragments 16S (V3+V4):Software: FLASH;Version:FLASH-1.2.8;ITS2:Software:PEAR;Version:PEAR-0.9.6; Denoise; Software:qiime2DADA2; Version:2019.7; Sequence alignment;Software:qiime2 feature-classifier

**Results**

**Sequencing summary**

76 samples were evaluated in total (19 milk samples from healthy lactating women, 19 milk samples from lactating mastitis patients, 19 stool samples from healthy lactating women, and 19 stool samples from lactating mastitis patients).

**Variations in the abundance of microflora among samples**

We compared the observed OTUs index between the two groups of same kind of sample. Milk samples from groups C and BM showed a significantly different index of observed otus in Figure 1A (p < 0.05; p = 0.0079). The OTUs in stool samples from the FM and FH groups differed, although the difference was not statistically significant (p = 0.63), as shown in Figure 1B. The composition and distribution of the top 20 species with the highest relative abundances differed between the milk and fecal samples collected from the two groups (Figure 1C and 1D).

**Variations in the evenness of microflora among samples**

The Simpson index shows a significant difference in the evenness of microflora between the milk samples from lactating healthy women and lactation mastitis patients (p = 0.00037; Figure 2A) when compared between the milk and stool samples from the two groups. The microbial diversity of the stool samples from lactation mastitis patients and healthy lactating mothers did not differ significantly, however (p > 0.05; p = 0.91; Figure 2B).

**Variations in the beta-diversity of microflora among samples**

The main coordinates analysis of weighted unifrac of milk and fecal samples from the two groups revealed that milk samples from lactation mastitis patients and lactating healthy women differed considerably in terms of microbiota composition and distribution (p < 0.05; p = 0.001; Figure 2C). It should be highlighted that lactation mastitis patients and healthy breastfeeding mothers had significantly different bacterial compositions and distributions in their stool samples (p < 0.05; p = 0.021; Figure 2D).

**Analysis of microflora composition**

According to the relative abundance analysis, we concluded that the predominant phyla in breast milk are Proteobacteria, Firmicutes, and Actinobacteria. The relative abundances of these bacteria varied between lactating healthy women and patients with lactating mastitis. The abundances of Firmicutes and Actinobacteria between the two groups are significantly different, as shown in Figure 3A(p < 0.05). the microbial composition of Stool samples from lactating women were predominanted byfour phyla: Firmicutes(FH group: 35.28%; FM group: 51.81%), Bacteroides(FH group: 25.87%; FM group: 24.63%), Actinobacteria(FH group: 24.08%; FM group: 19.02%), and Proteobacteria(FH group: 12.97%; FM group: 3.72%); their relative abundance differed between lactating mastitis patients and healthy lactating women. Firmicutes and Proteobacteria abundances between the two groups were significantly different (p < 0.05; Figure 3C). And furthermore, as shown in Figure 3B and 3D, both groups differed significantly in terms of the abundances of several bacterial communities at the genus level (p < 0.05).

For assessing differences between species at many levels, a linear discriminant analysis effect size (LEfSe) was carried out (with a threshold of LDA value > 3 and p=0.05). Individual samples from the two groups were compared to identify taxa with significant differences from the phylum to genus levels. Figure 4A and 4B shows significantly different species with LDA values > 3. The biomarkers with significant differences are indicated by different colors in the bar chart, whereas the length reflects the LDA value (i.e., the degree of influence of the differences in species between the groups). Generally, longer lengths correlate with greater influence.

Using LEfSe analysis, we identified two differentially abundant phyla in milk samples from the two groups (Figure 3A). The milk of healthy breastfeeding women contained a total of 13 differently enriched genera, including *Acinetobacter, Bradyrhizobium, Bradyrhizobiaceae\_unclassified, Ralstonia, Comamonas, Gemella, Enhydrobacter,* and *Neisseria*. In contrast, *Burkholderia, Staphylococcus, Brevundimonas, Sphingopyxis,* and *Actinobacteria\_unclassified* were more abundant in the milk of lactating mastitis patients. In addition, 16 differentially enriched families were identified.

According to the LEfSe analysis, the feces of the two groups contained two distinct phyla: Proteobacteria and Firmicutes. Ten highly differential genera were identified in the feces of healthy lactating women, including *Escherichia\_Shigella, Pantoea, Enterobacter, prevotellace\_unclassified, Coprococcus\_3*, and *Anaerococcus*. in contrast, *Faecalibacterium, Ruminococcus\_1, Gammaproteobacteria\_unclassified* and other bacteria had greater abundance in the feces of lactation mastitis patients.

**Discussion**

Previous research on the microecology of breast milk has revealed that it is a source of beneficial flora for the infant gastrointestinal tract and that mastitis has a substantial impact on weaning in nursing moms. However, few studies have examined the etiology of mastitis from the microbiological perspective. This study aimed to examine the differences between healthy lactating women and patients with mastitis from the standpoint of mammary gland and intestinal microbes, as well as to investigate the pathogenesis of mastitis.

The microecological studies that have examined lactation mastitis are limited. Bacterial species diversity is shown to be lower in patients with autoimmune illnesses, diabetes, obesity, cardiovascular and inflammatory enteritis, cerebrovascular diseases, and gastrointestinal cancer in earlier studies **[**29,30]. We present a thorough picture of the microbial dysbiosis related to mastitis using α and β diversity measures. In agreement with prior studies [30-32], we found that mastitis-affected milk samples contained less microbial diversity and species richness than controls. By analyzing the β diversity using weighted UniFrac matrices, significant microbial differences were identified between healthy controls and mastitis patients. The microbial composition of breast milk mainly consists of Proteobacteria, Firmicutes, and Actinobacteria. Their relative quantity varies based on the mother's state of health(i.e., healthy or suffering from mastitis). These findings are consistent with previous studies. Firmicutes and Actinobacteria had substantially different abundances between the groups (p < 0.05; Figure 3A), consist with earlier report[30].

There were notable differences in the composition and distribution of fecal flora between the two groups, despite the fact that there was no discernible difference between the healthy control and mastitis groups in terms of the richness and variety of fecal flora overall.

Intestinal flora abnormalities are tightly associated to metabolic diseases including diabetes and nonalcoholic fatty liver disease in addition to causing intestinal diseases[33], inflammatory bowel disease [34], and adiposity. Additionally, previous studies suggest that intestinal flora may be related to infectious diseases, such as mastitis, and that regulating the intestinal flora can reduce the risk of pneumonia due to Streptococcus pneumoniae[35]. Numerous follow-up studies have confirmed the link between intestinal flora and mastitis via the bacterial metabolites, such as lipopolysaccharides (LPSs) [36] and short-chain fatty acids [37,38]. LPSs produced in the rumen are passed through the epithelium of the rumen to the bloodstream and then throughout the body to reach the organs and tissues. Large amounts of LPS from the blood reach the mammary gland during breastfeeding, eventually causing inflammation of the mammary gland [36]. When dietary fiber is fermented in the intestines, it produces short-chain fatty acids that have anti-inflammatory properties. According to Several studies, they decreased the degenerative modification of breast tissue brought on by LPS and suppressed the generation of pro-inflammatory cytokines[38,39]. The manipulation of gut flora is therefore promising for reducing mammary gland inflammation.

The specific mechanism of such gut-mammary axis is still unclear. Several investigations have shown that dendritic cells (DCs) and CD18+ cells can penetrate the tight junctions of intestinal epithelial cells, ingest non-invasive bacteria, and transport them to other MALT locations, including the mammary gland during lactation. Due to DC-expressed tight junction proteins, the integrity of the intestinal epithelial barrier is maintained[40-42]. Additionally, it has been demonstrated that specific strains of lactic acid bacteria isolated from breast milk can enter monolayer Caco-2 cells via CD-mediated pathways[1]. Multiple in vitro investigations, animal studies, and human research have supported the notion that physiological bacterial transmission occurs throughout late pregnancy and breastfeeding[4346].Recent studies have shown that in spite of the differences in bacterial composition between maternal feces and milk, a strong canonical correlation has been demonstrated between them[47].

Immunological and microbiological environments are present in the mammary gland, which, like the gastrointestinal system, contains immunoglobulin A (IgA) as well as microflora. Interleukin-1, a pro-inflammatory cytokine, bacterial flagellin, and the microbial metabolite butyrate all have the ability to increase CCL28 production in the colon[48,49]. Butyrate also helps colonic epithelial cells express pIgR in a constitutive manner [50]. IgA synthesis in milk is dependent on the mammary gland's CCL28 and pIgR expression levels, which are both critically important [48]. Therefore, we suggest that in healthy mammary glands, immune cells and bacteria have a complex, reciprocal connection. Milk somatic cell counts (SCCs) and relative Staphylococcus abundance were shown to be positively correlated by Boix-Amorós et al. in their study of the association between total somatic cell counts and breast milk microorganisms[51]. Sphingomonas, Novosphingobium, Serratia, Ralstonia, Pseudomonas, and Bradyrhizobium were found to have inverse correlations with neutrophil and SCCs numbers in a separate investigation.The proportional numbers of macrophage/secretory epithelial (MSE) cells, however, were favorably correlated with Bradyrhizobium, Sphingomonas, Gemella, Granulicatella, and Actinomyces[52]. Further study is required to determine the exact mechanism underlying the relationship between milk cytokines/chemokines and the bacterial population makeup.

According to earlier research, the microbiota in the mother's milk may be influenced by things like her food and/or the bacteria in her digestive system [47]. In the research conducted at our laboratory, we found the abundance of Firmicutes in the two groups' milk and stool samples were drastically different. In contrast to a control group of healthy individuals, stool samples had elevated Firmicutes whereas milk samples had lower amounts of this bacteria. Furthermore, the DNA of some enteric-related strictly anaerobic bacteria (Faecalibacterium and Veillonella) was detected in our study, which indirectly supports the enteric-mammary pathway hypothesis. Previous studies have shown that Lactobacillus salivarius Ren significantly reduces the number of ruminococcus, while in our study, the concentration of g\_\_Ruminococcus\_1 was higher in the feces of patients with lactation mastitis. We speculate that regulating the gut-mammary axis by reducing the number of ruminococcus in the intestinal flora of lactation mastitis may be useful for treating mastitis. However, the study only provides preliminary conjectures, and further research is required to confirm our speculation.

Mastitis treatment using probiotics and other medications that affect the gut-breast axis represents an innovative and potentially fruitful direction to pursue. Considering that mastitis is a disorder, rebalancing the microbiome with probiotics seems to be a possible corrective measure. The term probiotics refers to living microbes that, when provided in sufficient quantities, may benefit the host's health. Probiotics can be isolated from milk because certain gut bacteria migrate to the breast, and there is a symbiotic relationship between milk bacteria and the human digestive system. There is some evidence that these microbes may prevent breast infections. Several bacterial strains, including Lactobacillus salivary, Lactobacillus paracalactium, Lactobacillus plantarum, and Lactobacillus fermentosa, have been identified from human milk and are classified probiotics and safe by the European Food Safety Authority [53].

**Conclusion**

Several previous studies have indicated that lactation mastitis is related to disturbed flora in both the milk and feces of patients. Furthermore, the DNA of some enteric-related strictly anaerobic bacteria (Faecalibacterium and Veillonella) was detected in milk samples, and pro-inflammation taxa ruminococcus was enriched in fecal sample of mastitis suggesting potential value of gut-mammary axis targeting mocrobiome treatment in lactation mastitis. However, further research is required to elucidate the specific mechanism underlying this disorder.

**Declarations**

**Acceptance of participation and ethical clearance**

Acceptance of participation and ethical clearance The initial trial protocol was authorized by a central institutional review board at the Zhejiang Hospital of Traditional Chinese Medicine in Hangzhou, Zhejiang, China, and it was carried out in accordance with the 2013 amendments to the Declaration of Helsinki and best practices for clinical research. Each subject's written informed consent was obtained before they took part in any trial-related activity.

**Access to materials and data**

All of the raw dataand/or analyzed results from this study may be found in the NCBI archive.<https://dataview.ncbi.nlm.nih.gov/object/PRJNA913391?reviewer=gpvq2eb6ajeq5eau2lcas22bct>.

**Permission to publish**

Not relevant.

**conflicts of interest**

The authors claim to have no conflicts of interest.

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**Contributions of authors**

The study questions were conceived by Wenjun Wang, who also collected the data, performed the statistical analysis, produced the initial and revised articles, and approved the submitted version of the paper.

Fenhua Wang, Chaonan Li, Xiaohong Xie, Qi Zhu, Shuyao Fan, and Xidong Gu assisted with the research design, data collection and entry, statistical analysis, initial article drafting, and final article submission.

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Figure1:1A,1B:Comparison of OTU quantity observed between the two specimen groups;1C,1D:Composition and distribution of the 20 species with the hightest relative abundance of species at the phylum level across the two specimen groups.



Figure2：2A,2B:Simpson Index comparison among different samples;2C,2D:PCoA analysis between different samples (Weighted\_UniFrac).



Figure 3:Analysis of significant differences among groups of bacteria(3A:phylum-level analysis of milk samples;3B:generic-level analysis of milk samples;3C:phylum-level analysis of fecal samples;3D:genus-level analysis of fecal samples)



Figure 4:4A;4B:Categories corresponding to diverse microbial communities.