ORIGINAL RESEARCH ARTICLE

Comparative study of breast and intestinal microecology in lactating women with and without mastitis

DOI: 10.29063/ajrh2024/v28i12.11

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Abstract

This study sought to compare bacterial abundance and diversity in milk and feces of healthy lactating women with patients suffering from lactation mastitis, explore the pathogenesis of lactation mastitis, and develop new ideas for its treatment and prevention from a microbiological perspective. A total of 19 lactating mastitis patients and 19 healthy lactating women were recruited. Milk and fecal Specimens were obtained from both groups, and microbial community structure was analyzed using 16S rRNA gene sequencing. Our results reveal significant alternations in microbial composition and distribution of milk and feces between healthy lactating women and mastitis patients. The findings suggest that lactating mastitis patients have dysregulated gut and milk microbiota, and modulating the gut-mammary axis may be a potential strategy for preventing or treating mastitis. *(Afr J Reprod Health 2024; 28 [12]: 97-107).*

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

Résumé

Cette étude visait à comparer l'abondance et la diversité bactériennes dans le lait et les selles de femmes allaitantes en bonne santé avec des patients souffrant de mammite de lactation, à explorer la pathogenèse de la mammite de lactation et à développer de nouvelles idées pour son traitement et sa prévention d'un point de vue microbiologique. Au total, 19 patientes atteintes de mammite allaitante et 19 femmes allaitantes en bonne santé ont été recrutées. Des échantillons de lait et de matières fécales ont été obtenus des deux groupes et la structure de la communauté microbienne a été analysée à l'aide du séquençage du gène de l'ARNr 16S. Nos résultats révèlent des alternances significatives dans la composition microbienne et la distribution du lait et des selles entre les femmes allaitantes en bonne santé et les patientes atteintes de mammite. Les résultats suggèrent que les patientes allaitantes atteintes d'une mammite ont un microbiote intestinal et laitier dérégulé, et que la modulation de l'axe intestin-mammaire pourrait constituer une stratégie potentielle pour prévenir ou traiter la mammite. *(Afr J Reprod Health 2024; 28 [12]: 97-107).*

Mots-clés: Lait maternel; microbiote microécologie ; voie intestinale-mammaire ; mammite de lactation

Introduction

The WHO recognizes breast milk as a crucial role in infants feeding[1]. It offers numerous health benefits for the baby and mother. Beyond essential nutrients, human milk boasts a wealth of bioactive materials, including growth factors, human milk oligosaccharides (HMOs), immunoglobulins, as well as microRNAs[2]. Human breast milk contains a rich microbial ecosystem (approximately 10^6 bacteria/mL), and is an ongoing source of symbiosis, mutualism, and/or potential probiotics in the developing infant's intestine [3], including Staphylococcus, Streptococcus, and some species of lactic bacteria[4]. Compared to formula-fed infants, breastfeeding is linked to a lower risk of various disorders including respiratory infections, irritable bowel syndrome, necrotizing enterocolitis, asthma, diabetes, obesity, Crohn's disease, and sudden infant death syndrome[5-7]. Breastfeeding improves the health and immune functions of the infant, and reduces morbidity and mortality from

gastrointestinal diseases. The indisputable evidence for breastfeeding endorses breast milk as the best functional food. Breastfeeding also offers mothers significant health advantages such as prevention of diabetes, and reduced risk of breast cancer and ovarian cancer[8]. Breast tissue inflammation (i.e., mastitis) affects up to 33% of breastfeeding women and is a major reason for discontinuation of breastfeeding[9].

Reports indicate that acute mastitis during lactation is 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 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 that time, there has been a surge in studies examining the bacterial colonies in breast milk through methods such as culture and gene sequencing[14-17]. Therefore, the traditional theory of bacterial invasion being the cause of mastitis has not been confirmed[12,13,18,19]. Similar to the flora of other body parts, the breast microflora also contains a diverse array of microorganisms, ranging from probiotics to potentially pathogenic bacteria, and forms a complex "microbiota-host" ecosystem with the human host. The ecosystem is the outcome of the co-evolution and mutual adaptation of hosts and microorganisms. Once the balance is disturbed, mastitis may occur. A growing body of research indicate that lactation mastitis is related to dysbiosis in mammary gland microbiome[19].

The gut lies at the intersection of health and illness, and its role is interwoven with that of other organs. In recent decades, we have witnessed a significant increase in research of axes that emphasize the multifaceted relationship between the gut and other human organ functions, e.g., 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. The intestinal and mammary gland adaptations in lactating women are synchronized due to the intricate interplay between the intestinal and mammary glands, which explains how alterations in the gut microbiota affect the mammary gland microecology and cause mastitis[24-25] detected healthy mature anaerobic intestinal flora in the breast and breast milk. A study of probiotics for acute mastitis during lactation revealed that upon oral ingestion of Lactobacillus fermentosa and Lactobacillus salivaris by mothers resulted in the probiotics reaching the mammary glands and being detected in breast milk[26-27]. These findings suggest that maternal gut microbes may migrate from gut to mammary glands via the gut-mammary axis.

Currently, lactation mastitis is treated using antibiotics, with poor therapeutic effects. Empiric antibiotics are the most commonly prescribed treatment for mastitis, which have been in use for many years. Unfortunately, many mastitis patients do not respond to treatment due to antibiotic resistance, which can arise from various mechanisms such as intrinsic resistance, genetic transmission, and biofilm formation[28]. Consequently, innovative approaches to managing mastitis are needed. It is pertinent to remember that among strategies for regulating mammary bacterial communities, using probiotic strains that are derived from breast milk is a promising approach in this context[18]. In that study, a probiotic bacterial strain derived from breast milk was used in treating mastitis.

In the present study, specimens of milk and stool were obtained from healthy lactating women and patients with lactation mastitis. A comparison of microbial communities in milk and stool specimens from the two groups was conducted through 16sRNA gene sequencing. The results enable us to explore the pathogenesis of lactation mastitis along the gut-mammary pathway and to explore novel approaches for its treatment.

Methods

Clinical data

Nineteen lactation mastitis patients were prospectively enrolled from June 2022 to May 2021 at the First Affiliated Hospital of Zhejiang Chinese Medicine University. In addition, 19 healthy lactating women served as controls. The study included lactating women aged 20–40 years, with breast lumps, pain, skin redness, fever (temperature of 37.5°C), and ultrasound showing lactation mastitis. No antibiotics or probiotics were administered to any of the patients within the preceding two weeks. The study was limited to

patients without other infections (e.g., puerperium infection), obesity, inflammatory bowel disease, asthma, and other inflammatory diseases. Additionally, all subjects gave their informed consent to participate in the study. 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. Milk and fecal specimens were obtained from the two groups of women, and the samples were categorized into the following groups: 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).

Breast milk sample collection:(1) Milk sample collection: Before collecting the milk, the breast, nipple, and areola were washed and disinfected with soapy followed with sterile water. While wearing sterile gloves, the first 5 mL of milk were discarded, and approximately 50 mL were collected 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 refrigerator at −80°C.

(2) *Fecal sample collection*: Before collecting stool samples, subjects were instructed 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. A soybean-sized sample (approximately 50 mg) was collected in sterile cryopreservation tubes, which was contained in a Ziploc bag in refrigerator at −80°C.

(3) *DNA extraction and PCR amplification*: The reagent utilized in the study can salvage DNA from minimal sample amounts and is effective enough in preparing DNA from samples in this study. Nuclear-free water was used in this study. The extracted DNA was resuspended in 50 µL of elution buffer and then preserved at -80°C prior to PCR (LC-Bio Tech, China). We extracted the DNA from stool and water samples (milk) using the Stool and Water DNA Kit (Omega Bio, GA, USA). Extracted DNA was analyzed using electrophoresis on agarose gels. The amount of DNA was determined using UV spectrophotometer. The universal primer 341F (5'-CCTACGGGNGGCWGCAg-3') was used. Then, 805R (5'-gactachVggGTATctaatcc-3') was employed to amplified the V3-V4 region of the 16S RNA gene of bacteria. Using primers F (5'-gtGartCATCGAATCTTTG-3') and R (5'-TCCTCCGCTTATTGATATGC-3'), we amplified the ITS2 region of bacteria. In 12.5 U of Phusion Hot Start Flex 2X Master Mix L, we added 2.5 µl of forward primer, 2.5 µl of reverse primer, 25 µl of DDH2O, and 50 ng of template DNA. During the thermal cycle, following conditions were observed: initial denaturation at 98°C for 30 s, subsequent denaturation at 98°C for 10 s, subsequent denaturation at 54°C for 30 s, cycle repeated for 30 times, heating at 72°C for 30 s, extension at 72°C for 10 min, endless extension at 4°C. In addition, the 5' ends of the primers were individually encoded, and universal primers were sequenced. A 2% agarose gel electrophoresis was employed to confirm the PCR results. In the negative control, ultrapure water was substituted for the sample solution in the DNA extraction. AMPure XT beads (Beckman coulter, MA, USA) was used to purified PCR products, and quantified them using a Qubit fluorometer (Invitrogen, MA, USA). The amplicon libraries were generated using an Agilent 2100 Bioanalyzer (Agilent, DE, USA) and then analyzed for size and quantity using the Illumina Library Quantification Kit (Kapa Bio, MA, USA). The sequencing of libraries was carried out using NovaSeq PE250 platform.

Data analysis

Based on unique barcodes assigned to each sample, paired-end reads were truncated by removing 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.

Simpson index is used to measure the diversity of species in a community. Denoted as D, this index is calculated as: $D = 1 - \Sigma n_i(n_i-1) / N(N-1)$, where: n_i: The number of organisms that belong to species i; N: The total number of organisms. The higher the value for this index, the higher the diversity of species. The observed OTUs index is employed to measure the richness of species in a community. It is the number of OTUs observed. As amplicon sequence variants (ASVs) were obtained

with DADA2 for processing amplicon sequencing data instead of OTUs in this study, this index is the number of ASVs observed. The higher the value for this index, the higher the richness of species.

Ethical consideration

Prior to participating in any trial activities, informed consent was obtained in writing from each individual from both groups. The original trial protocol received approval from the Institutional Review Board (IRB) of Zhejiang Hospital of Traditional Chinese Medicine on July 16, 2020 (approval number 2021-K-214-01)

Results

Sequencing summary

76 samples were analyzed, including 19 milk and 19 fecal specimens from both healthy lactating women and lactation 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. Figure 1A illustrated a significant difference in the observed OTUs index between milk samples from groups C and BM ($p < 0.05$; $p = 0.0079$). According to Figure 1B, a difference in the OTUs was noted between stool samples from FM and FH groups but without statistical significance ($p = 0.63$). The relative abundance of the top 20 phyla from the two groups of milk and fecal samples were shown in Figure 1C and 1D.

Variations in the evenness of microflora among samples

A comparison of the Simpson index between the two groups suggests a significant difference in evenness of microflora among the milk samples from lactation mastitis patients and lactating healthy women ($p = 0.00037$; Figure 2A). Nevertheless, among stool samples, there is no significant difference in microbial diversity from the lactation mastitis patients and healthy lactating women ($p > 0.05$; $p = 0.91$; Figure 2B).

Variations in the beta-diversity of microflora among samples

The PCoA of weighted unifrac of milk and fecal samples from the two groups indicated that the milk samples from the lactation mastitis patients and lactating healthy women differed significantly in terms of microflora composition and distribution ($p < 0.05$ and $p = 0.001$, respectively; Figure 2C). It's noteworthy that the composition and distribution of bacteria in the stool samples differed significantly between lactation mastitis patients and healthy lactating women ($p < 0.05$ and $p = 0.021$, respectively; Figure 2D).

Analysis of microflora composition

Relative abundance analysis revealed that the breast milk microbiome is primarily composed of three phyla: Proteobacteria, Firmicutes, and Actinobacteria. The relative abundances of these bacteria varied between lactating healthy women and patients with lactating mastitis. As shown in Figure 3A, there is significant difference in the abundances of Firmicutes and Actinobacteria differed significantly between the two groups ($p < 0.05$).

The stool microbiome of lactating women was primarily composed of four 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. the abundances of Firmicutes and Proteobacteria also differed significantly between the two groups ($p < 0.05$; Figure 3C). In addition, as shown in Figure 3B and 3D, both groups differed significantly in terms of the abundances of several microbial communities at genus level ($p < 0.05$).

Linear discriminant analysis effect size (LEfSe) was conducted for multilevel species differences (the threshold was LDA value > 3 and $p < 0.05$). A comparison of individual samples from the two groups was conducted to identify taxa exhibiting significant differences from the phylum to genus levels. Figure 4A and 4B shows significantly differented species with LDA values > 3.

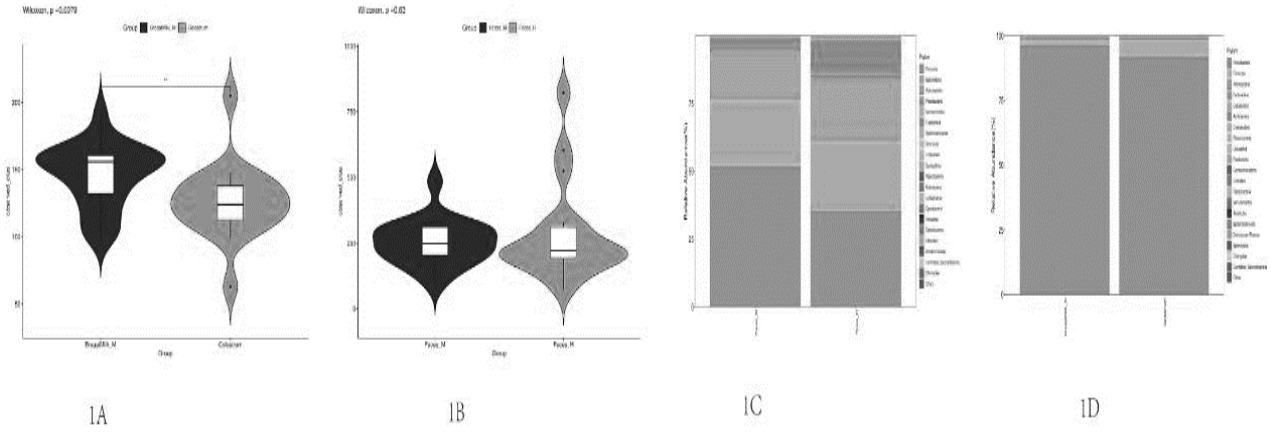

Figure 1: Differences in microbes' abundance between samples. (1A) The OTUs index of milk samples from the two group. (1B) the OTUs index of stool samples from the two groups. (1C) The relative abundance of the top 20 phyla of milk samples from the two groups. (1D) The relative abundance of the top 20 phyla of stool samples from the two groups.

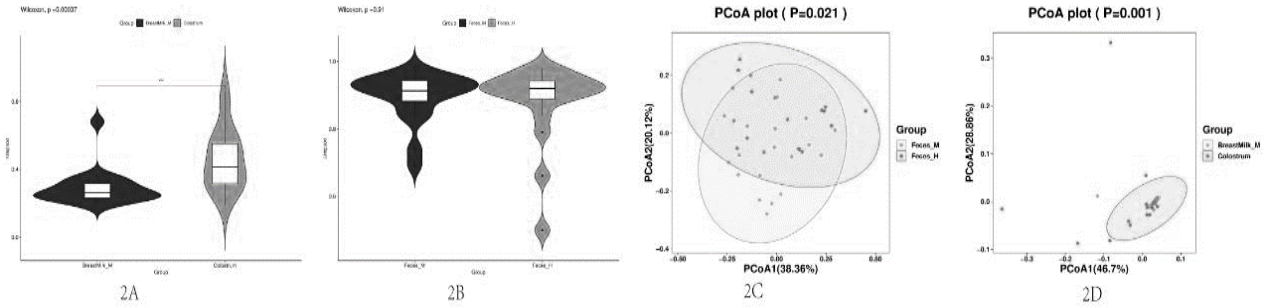

Figure 2: Differences in the microflora's uniformity and diversity between samples. (2A). The Simpson index of milk samples from the two group. (2B) the Simpson index of stool samples from the two groups. (2C) beta diversity of milk samples from the two group. (2D) beta diversity of stool samples from the two group

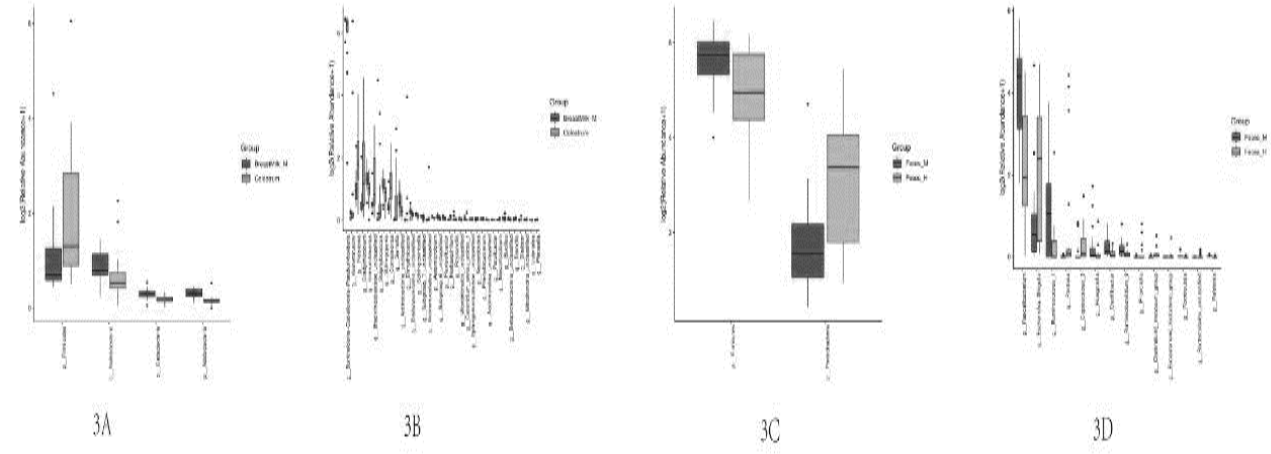

Figure 3: The variations in composition of each group. (3A) The relative abundance of phyla with significant difference between the two groups of milk samples (3B) The relative abundance of microbiota in milk samples from the two groups at genus level (3C) The relative abundance of phyla with significant difference between the two groups of stool samples (3D) The relative abundance of bacteria in stool samples from the two groups at genus level

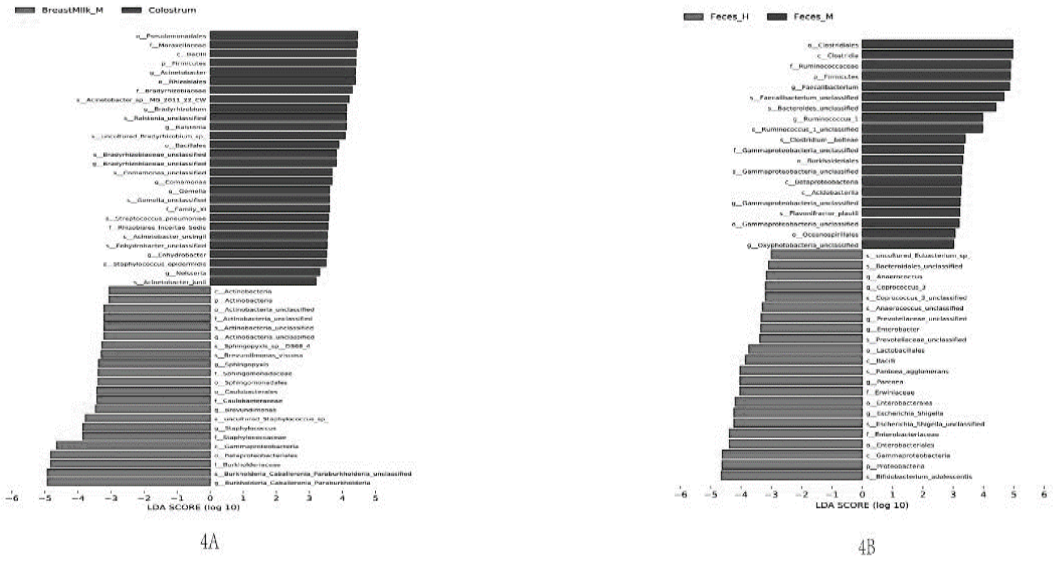

Figure 4: The variations in the multilevel species within each group. (4A) LEfSe of milk samples from the two groups. (4B) LEfSe of stool samples from the two groups.

The biomarkers with significant differences were indicated by different colors in the bar chart, whereas the length reflects the LDA value (i.e., the extent to which the differences in species between the groups influence the results). 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). In total, 13 differentially enriched genera were identified in the milk from healthy lactating women, 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 LEfSe analysis, two distinct phyla were detected in fecal samples of the two groups: 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 fecal samples of lactation mastitis patients

Discussion

Prior research has demonstrated that breast milk harbours a diverse community of beneficial bacteria for the neonatal intestinal tract and that mastitis has a significant influence on weaning in lactating women. However, there is a paucity of research on the etiology of mastitis from the microbiological perspective. The goal of this study is to analyze differences between healthy lactating women and mastitis patients from the standpoint of mammary gland and intestinal microbes, as well as to investigate the pathogenesis of mastitis.

Only a few microecological studies have evaluated lactation mastitis. Bacterial diversity is strongly linked to disease status, with prior studies reporting a reduction in species diversity among individuals with obesity, diabetes, cardiovascular diseases, inflammatory enteritis, gastrointestinal cancer, and autoimmune diseases[29-30]. Using α and β diversity measures, we present a overview of the microbial dysbiosis associated with lactating mastitis. 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 observed between healthy controls and mastitis patients. The microbial composition of breast milk mainly consists of Proteobacteria, Firmicutes, and Actinobacteria. Their relative abundance varied according to health status of mother (i.e., healthy or mastitis patients). Results of this study are consistent with prior studies. Firmicutes and Actinobacteria had substantially different abundances between groups ($p < 0.05$; Figure 3A), consist with earlier report[30].

Despite lack of significant difference between healthy control and mastitis groups in terms of overall richness and diversity of fecal flora, significant variations in microbial community structure of fecal flora between the two groups were observed.

Disorders of intestinal flora not only cause intestinal diseases but also are highly correlated with metabolic diseases, such as diabetes, nonalcoholic fatty liver disease[33], inflammatory bowel disease[34], as well as adiposity. Furthermore, prior research has shown 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 indicated the link between intestinal flora and mastitis via bacterial metabolites, including lipopolysaccharides (LPSs)[36] and short chain fatty acid (SCFA)s[37-38]. LPS produced in the intestine passes through intestinal epithelium into bloodstream and then throughout the system. During breastfeeding, large quantities of LPS enter the mammary gland, eventually causing inflammation of the mammary gland[36]. By fermenting the dietary fiber in the intestine, SCFAs can be produced that have anti-inflammatory properties. Prior studies indicated that they inhibited production of pro-inflammatory cytokines and reduced pathological alteration of breast tissue induced by LPS[38-39]. Thus, it is promising to reduce inflammation in mammary gland through manipulate intestinal flora.

The specific mechanism of such gut-mammary axis is still unclear. Several studies have revealed that dendritic cells (DCs) and CD18+ cells can penetrate gut epithelium and absorb and transport non-invasive bacteria to the other MALT sites, including the mammary gland during lactation. The integrity of intestinal epithelial barrier is positively corelated with DCs[40-42]. Additionally, certain strains of lactic bacteria derived from milk were shown to cross into monolayer Caco-2 cells by CD-mediated mechanisms[1]. A range of studies have demonstrated the physiological transfer of bacteria during both late pregnancy and lactation[43-46]. Recent studies indicated that in spite of differences in bacterial composition between maternal feces and milk, a strong canonical correlation has been demonstrated between them[47].

The mammary gland, like the gastrointestinal system, contains microbial communities that interact with host immunological system. CCL28 expression can be stimulated by pro-inflammatory cytokine interleukin-1, flagellin, and a short-chain fatty acid produced by microbial metabolite like butyrate[48-49]. Butyrate also helps colonic epithelial cells express pIgR in a constitutive manner[50]. Meanwhile, the levels of CCL28 and pIgR in mammary gland are reported pivotal for the level of IgA in breast milk[48]. Herefore, we propose that immune cells and microbiome in healthy mammary glands have a complicated, bidirectional relationship. Boix-Amorós *et al*. used 16S rRNA sequencing to study microbiota in human milk, discovering a positive correlation between the Staphylococcus level and milk somatic cell counts (SCCs)[51]. A related study discovered a link between maternal cells and microbial communities: the abundances of Sphingomonas, Serratia, Novosphingobium, Pseudomonas, Ralstonia, and Bradyrhizobium were inversely linked with SCCs and neutrophil concentrations. However, Bradyrhizobium, Sphingomonas, Gemella, Granulicatella, and Actinomyces were positively linked with index of macrophage/secretory epithelial (MSE) cells[52]. The precise process is unknown, and more research is necessary to explore the association between milk microbial communities and milk cytokines/chemokines.

Previous studies suggested that factors such as microbiota of the mother's digestive tract and/or her diet may influence the milk microbiota[47]. In the research conducted at our laboratory, a significant difference between abundance of Firmicutes in milk and stool samples were found between groups. In comparison to the healthy control, abundance of Firmicutes in milk samples were downregulated, whereas an upregulation in stool samples. 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. Prior studies indicated that Lactobacillus salivarius Ren significantly reduces the abundance 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 abundance of ruminococcus in intestinal microbiota of lactation mastitis may be useful for treating mastitis. However, the study only provides preliminary conjectures, and more research is needed to validate our speculation.

Regulating the gut-breast pathway by probiotics and drugs could represent a novel and promising method for treating mastitis. 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 are evidences that these microbes may prevent breast infections. There are several bacterial strains sourced from human milk, including Lactobacillus salivary, Lactobacillus plantarum, Lactobacillus paracalactium, and Lactobacillus fermentosa, which are considered probiotics and have been recognized as safe by the European Food Safety Authority[53].

Strengths and limitations

We used Firmicutes abundance (FH group: 35.28%; FM group: 51.81%) of stool samples from the 2 groups to assess the statistical power of this study. R was used and a two-sample test was performed. Type I error rate(α) was set to 5% and MDE was set to 20%. The statistical power is 0.7482. We must admit that the statistical power of this study is insufficient because the number of subjects is small. The current report is only a preliminary conclusion. In the future, more samples and manpower need to be invested for further in-depth research.

While we have successfully distinguished bacteria associated with mastitis in both milk and stool samples, the specific interactions between immune cells and the microbiome in the mammary gland warrant additional study. Moreover, although preliminary evidence supports the gut-mammary axis hypothesis, the exact mechanisms by which the gut microbiome contributes to mastitis remain unclear.

Despite some limitations, this study lays the foundation for further research into the role of the gut-mammary axis in the pathogenesis of mastitis and the development of novel probiotic treatment strategies.

Conclusion

The results of this study demonstrated 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 microbiome treatment in lactation mastitis.

Availability of data and materials

The datasets employed in this study are accessible as reasonable request from the corresponding author.

Competing interests

The authors state that there are no competing interests.

Authors' contributions

Wenjun Wang conceived the research questions, collected and analyzed the data, wrote the initial and revised drafts of the article, and approved the final submission. Fenhua Wang, Chaonan Li, Xiaohong Xie, Qi Zhu, Shuyao Fan, Xidong Gu contributed to the research design, collected and entered the data, performed statistical analyses, assisted with the initial draft of the article, and approved the final submission.

Funding

This research was supported by the Zhejiang Provincial Natu-ral Science Foundation of China under grant NO.LY22H290004.

Acknowledgments

We acknowledge the linguistic support provided by LetPub (www.letpub.com) during the preparation of this manuscript.

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