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Analysis of Gut Microbiota in Patients with Breast Cancer and Benign Breast Lesions

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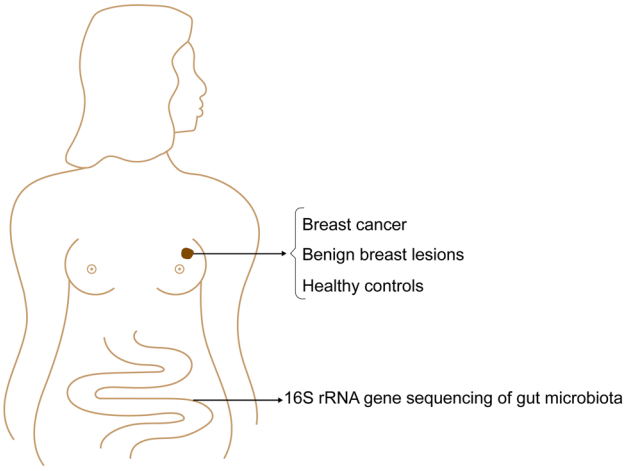
Abstract

Breast cancer (BC) and benign breast lesions (BBLs) are common diseases in women worldwide. The gut microbiota plays a vital role in regulating breast diseases' formation, progression, and therapy response. Hence, we explored the structure and function of gut microflora in patients with BC and BBLs. A cohort of 66 subjects was enrolled in the study. Twenty-six subjects had BC, 20 subjects had BBLs, and 20 matched healthy controls. High throughput 16S ribosomal RNA (16S rRNA) gene sequencing technology was used to determine the microbial community structure. Compared with healthy individuals, BC patients had significantly lower alpha diversity indices (Sobs index, $p = 0.019$; Chao1 index, $p = 0.033$). Sobs and Chao1 indices were also lower in patients with BBLs than healthy individuals, without statistical significance ($p = 0.279$, $p = 0.314$, respectively). Both unweighted and weighted UniFrac analysis showed that beta diversity differed significantly among the three groups ($p = 3.376e{-}14$, $p < 0.001$, respectively). Compared with healthy individuals, the levels of *Porphyromonas* and *Peptoniphilus* were higher in BC patients ($p = 0.004$, $p = 0.007$, respectively), whereas *Escherichia* and *Lactobacillus* were more enriched in the benign breast lesion group ($p < 0.001$, $p = 0.011$, respectively). Our study indicates that patients with BC and BBLs may undergo significant changes in intestinal microbiota. These findings can help elucidate the role of intestinal flora in BC and BBLs patients.



K e y w o r d s: gut microbiota, 16S rRNA gene sequencing, breast cancer, benign breast lesions

Introduction

Breast cancer (BC) is the most common cancer among women worldwide. BC remains a significant cause of mortality in women, despite the use of adjuvant chemotherapeutic and hormonal agents (Braden et al. 2014). Genetic and other established risk factors such as early menarche age, high body mass index (BMI), and sedentary lifestyle have been associated with the onset and progression of BC. Benign breast lesions (BBLs), including fibroadenoma, are commonly found in young women. Estrogens and their receptors are implicated in the onset and progression of BBLs. Accumulating data have indicated that alterations in the host microbiome, primarily intestinal microbiota, may contribute to the pathogenesis of both gastrointestinal and extra-intestinal tumors (Belkaid and Hand 2014; Dzutsev et al. 2017).

The number of genes in the human intestinal microbiota, regarded as an alternative genome in humans, is nearly 150 times higher than that of the human genome (Zhu et al. 2010). This intestinal ecosystem is involved

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in a dynamic interaction with host cells, microbes, and food. Besides, it acts as a multi-dimensional "microbial organ" by enhancing the synthesis of essential amino acids and vitamins, producing small molecules, nutritional absorption, metabolism of bile acids, activation of immune cells, and inactivation of toxins and carcinogens (Eslami-S et al. 2020). The remarkable contribution of the gut microflora to human health and disease has been extensively recognized. It has been speculated that changes in the constitution and functions of the gut microbiome might contribute to the onset and progression of BC and BBLs.

Although some studies reported higher microbial diversity in BC patients than healthy controls (Gopalakrishnan et al. 2018; Zhu et al. 2018), other investigations found less microbial diversity in postmenopausal BC subjects (Goedert et al. 2015; 2018). In addition, gut dysbiosis in individuals with BBLs is not fully understood. Therefore, 16S ribosomal RNA (16S rRNA) gene sequencing technology was utilized to explore intestinal microbiota dysbiosis in BC and BBL patients.

Experimental

Materials and Methods

Patient selection. Seventy subjects, including 27 BC patients, 22 BBL patients, and 21 healthy controls, were recruited from The Affiliated Hospital of Qinghai University between November 2020 to February 2021. Pathology reports confirmed the diagnosis of all cases. Healthy controls with color Doppler ultrasound showing no breast lesions were enrolled from the physical examination center and matched with cases by age, gender, BMI, and geographic region. Exclusion criteria included diabetes, inflammatory bowel disease, autoimmune diseases, and past treatment with chemotherapy, surgery, or radiation prior to obtaining fecal samples. None of the subjects had received antibiotics or probiotics within one month of stool collection. All subjects provided written consent.

Specimen collection. Fresh stool specimens were obtained from eligible subjects and then frozen at −80°C 2 h before use.

Fecal specimen processing and analysis. Microbiota evaluations were conducted at the Wuhan Huada Medical Laboratory Co., Ltd. Four samples had remarkably low numbers of reads and were ultimately excluded from the analysis. Hence, the final analysis was based on data collected from 26 BC patients, 20 BBL patients, and 20 healthy subjects. Polymerase chain reaction (PCR) amplification was performed in a 50-μl reaction mixture containing 30 ng of genomic DNA, and specific primers were designed. Agencourt AMPure XP beads were used to purify the amplicons. RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent, USA). High-quality libraries were sequenced on the Illumina HiSeq 2500 sequencing platform (BGI, China). The 16S rRNA V3-V4 hypervariable region was amplified with degenerate PCR primers: 341F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Raw reads were filtered to remove adaptors and low-quality and ambiguous reads. Next, fast length adjustment of short reads (FLASH, v1.2.11) software was used to merge paired-end reads. UPARSE implemented within USEARCH (v7.0.1090) was used to cluster effective tags to obtain operational taxonomic units (OTUs) at 97% sequence similarity, and chimeras were identified and removed with UCHIME (v4.2.40). Taxonomy was assigned to each OTU with the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu) database using usearch_global of USEARCH.

Statistical analysis. Statistical analyses were conducted using SPSS Statistics 25, R software (v3.2.1), and other online analysis tools. A p-value of < 0.05 was considered statistically significant. A Petaline graph was generated using R (v3.1.1). Microbial diversity was evaluated using alpha and beta diversity indices. Alpha diversity was measured using Sobs and Chao1 diversity indices to estimate community richness and was compared using the Wilcox statistical test. The alpha diversity boxplot, and the statistical tests, were performed using R (v3.2.1). Beta diversity was calculated using unweighted and weighted UniFrac distance metrics, principal coordinate analysis (PCoA), and partial least squares-based discriminant analysis (PLS-DA) models. The beta diversity boxplot was generated using the R (v3.4.1) package ggplot. PCoA plots were generated to visually display patterns of beta diversity after 100 iterations using QIIME (v1.80). PLS-DA was implemented in the R mixOmics package. The linear discriminant analysis (LDA) coupled with effect size (LEfSe) was performed using the LEfSe program to determine differentially abundant taxa in each group, and a logarithmic LDA score > 2 was considered significant.

Results

Patient characteristics. All participants were Chinese women (healthy controls, n = 20; BC: n = 26; BBL: n = 20). There were no significant differences in age and BMI among the three groups (Table I).

Different groups showed different OTUs. Sequencing showed 3,567,593 effective sequences (average of 54,054 sequences per sample). In the three groups, 723 OTUs were detected. Among them, 517 OTUs were

Table I
Baseline characteristics of the patients enrolled.

	Healthy controls	Breast cancer	Benign breast lesions
No. of individuals	20	26	20
Gender (male/female)	0/20	0/26	0/20
Mean age (± SD, years)	46.90 (10.87)	49.62 (7.33)	48.95 (8.73)
Mean BMI (± SD, kg/m^2)	22.80 (2.02)	22.88 (1.98)	21.71 (2.20)

common to the three groups, but 64 were exclusive to BC patients, 77 were exclusive to individuals with BBLs, and 65 were exclusive to healthy controls (Fig. 1).

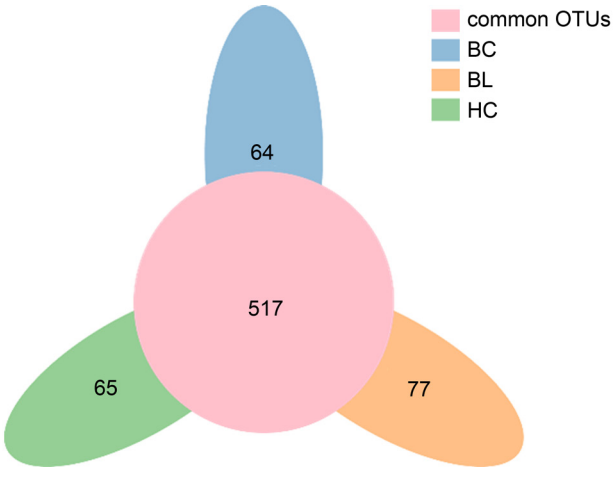

Fig. 1. The Petaline graph for calculated OTUs. Different colors designate different groups. The central circular area designates the set of OTUs often present in the counterpart groups, and the single-layer zone designates the number of OTUs uniquely found in each group; BC – breast cancer, BL – benign breast lesions, HC – healthy controls.

Microbiota composition analysis. The same four bacteria were identified at the phylum level to be dominant in all three groups, including Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, of which Firmicutes and Bacteroidetes were predominant in each group (Fig. 2A). In addition, 23 bacteria genera with relative abundance higher than 0.5% were identified among the three groups, including *Veillonella*, *Dialister*, *Oscillibacter*, *Lachnospiracea_incertae_sedis*, *Parasutterella*, *Megasphaera*, *Prevotella*, *Roseburia*, *Bifidobacterium*, *Clostridium_XlVa*, *Barnesiella*, *Eubacterium*, *Escherichia*, *Faecalibacterium*, *Phascolarctobacterium*, *Bacteroides*, *Blautia*, *Megamonas*, *Gemmiger*, *Parabacteroides*, *Ruminococcus*, *Alistipes*, and *Succinivibrio* (Fig. 2B). The patterns of microbial composition were highly variable among these three groups.

As shown in Table II, compared with the healthy control group, the relative richness of five bacterial genera was increased in the BC group (*Escherichia*, *Peptoniphilus*, *Bilophila*, *Lactobacillus*, and *Porphyromonas*) while the relative richness of fifteen bacterial genera was decreased (*Faecalibacterium*, *Lachnospiracea_incertae_ sedis*, *Collinsella*, *Alistipes*, *Anaerofilum*, *Christensenella*, *Butyricimonas*, *Erysipelothrix*, *Acidaminococcus*,

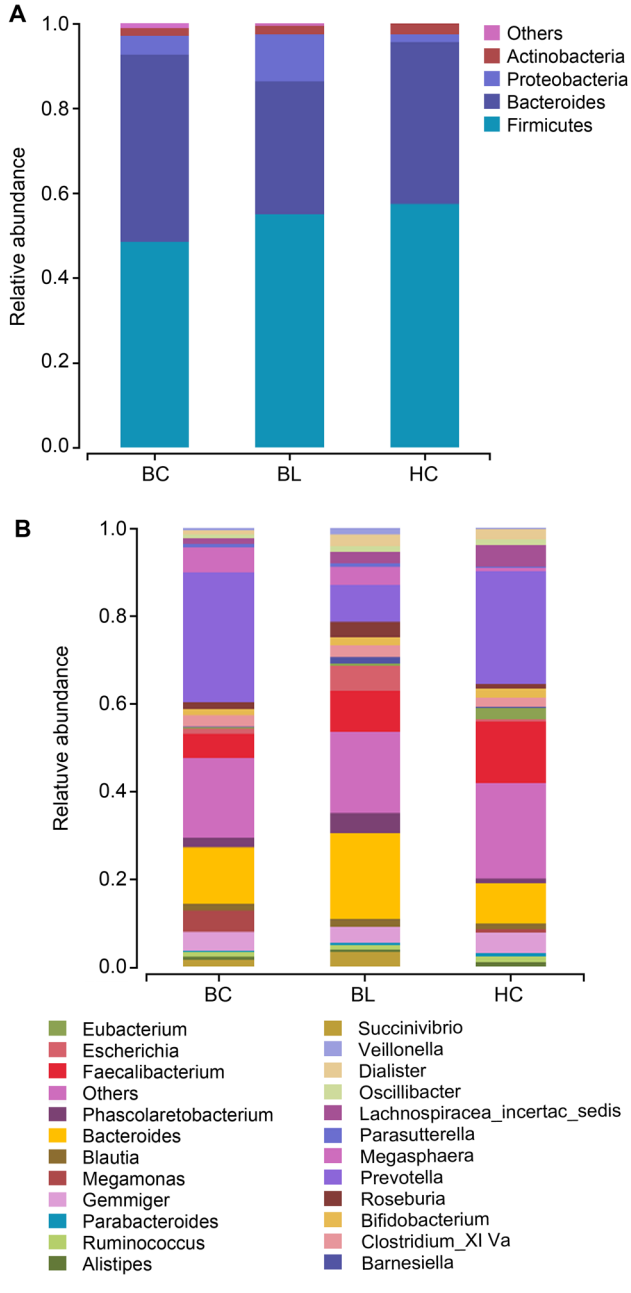

Fig. 2. Comparison of relative taxa richness among breast cancer patients, benign breast lesion patients, and healthy controls.
A) Comparison at the phylum level; B) comparison at the genus level; BC – breast cancer, BL – benign breast lesions, HC – healthy controls.

Table II
Changes in bacterial abundance at the genus level in patients with breast cancer and benign breast lesions.

Breast cancer		Benign breast lesions	
More abundant genera	Less abundant genera	More abundant genera	Less abundant genera
Escherichia	*Faecalibacterium*	*Escherichia*	*Collinsella*
Peptoniphilus	*Lachnospiracea_incertae_sedis*	*Peptoniphilus*	*Alistipes*
Bilophila	*Collinsella*	*Coprobacillus*	*Megamonas*
Lactobacillus	*Alistipes*	*Lactobacillus*	*Butyricimonas*
Porphyromonas	*Anaerofilum*	*Porphyromonas*	*Acidaminococcus*
	Christensenella		*Asaccharobacter*
	Butyricimonas		*Tissierella*
	Erysipelothrix		*Cloacibacillus*
	Acidaminococcus		
	Victivallis		
	Eubacterium		
	Tissierella		
	Hydrogenoanaerobacterium		
	Cloacibacillus		
	Oxalobacter		

Victivallis, *Eubacterium*, *Tissierella*, *Hydrogenoanaerobacterium*, *Cloacibacillus*, and *Oxalobacter*). Also, compared with the healthy controls, the relative richness of five bacterial genera was increased in patients with BBLs (*Escherichia*, *Peptoniphilus*, *Coprobacillus*, *Lactobacillus*, and *Porphyromonas*), whereas the relative richness of eight bacterial genera was decreased (*Collinsella*, *Alistipes*, *Megamonas*, *Butyricimonas*, *Acidaminococcus*, *Asaccharobacter*, *Tissierella*, and *Cloacibacillus*).

Biodiversity analysis. Alpha diversity indices (Sobs and Chao1) are shown in Fig. 3. Compared with the healthy controls, BC patients had significantly lower alpha diversity indices (Sobs index, $p = 0.019$; Chao1 index, $p = 0.033$). There were no differences in Sobs and Chao1 indices between patients with BBLs and healthy individuals ($p = 0.279$, $p = 0.314$, respectively).

In addition, beta diversity assessments based on weighted UniFrac were markedly different among the three groups (both $p < 0.001$, Fig. 4A, B). These results suggested an altered gut microbiota composition in BC and BBL patients.

The weighted UniFrac PCoA plot showed no visible separation among the three groups (Fig. 5A), but the PLS-DA analysis separated the three groups (Fig. 5B). Collectively, this observation revealed that the structure of the gut microbiota community was different among the three groups.

Bacterial taxonomic differences. *Prevotella*, *Porphyromonas*, *Peptoniphilus*, and *Megamonas* were the major taxonomic groups in the BC group, whereas *Lactobacillus*, *Escherichia*, and *Coprobacillus* were the major taxonomic groups in the BBL group. *Cloacibacillus*, *Asaccharobacter*, *Christensenella*, *Alistipes*, *Tissierella*, *Hydrogenoanaerobacterium*, *Butyricimonas*, *Acidaminococcus*, *Oxalobacter*, *Collinsella*, and *Eubacterium* were the major taxonomic groups in the healthy controls (Fig. 6).

Discussion

This cross-sectional study reveals a decreasing trend in gut diversity of BC and BBL subjects. It was similar to a previous case-control study, which showed a lower diversity and altered composition of microbiota in the fecal samples of postmenopausal BC patients (Goedert et al. 2015). In another study, milk from mastitis patients demonstrated microbiota dysbiosis, including lower microbial diversity with increased opportunistic pathogens and reduced commensal organisms (Patel et al. 2017). The diversity of gut microbiota is essential for maintaining health (Katagiri et al. 2019). A low diversity of gut microbiota is often a hallmark of intestinal dysbiosis and has been linked to inflammatory bowel disease, obesity, allergic rhinitis, and gastric carcinoma (Ferreira et al. 2018; Watts et al. 2021). Mounting evidence has shown that growing up in microbe-rich environments, for instance, traditional farms, improves children's health, and a high diversity has been associated with increased health in the elderly (Claesson et al. 2012; Le Chatelier et al. 2013). In addition, prebiotics, probiotics, and diverse nutrition have been shown to decrease the risk of BC (Newman et al. 2019; Goubet et al. 2021; Jiang and Fan 2021; Méndez Utz et al. 2021;

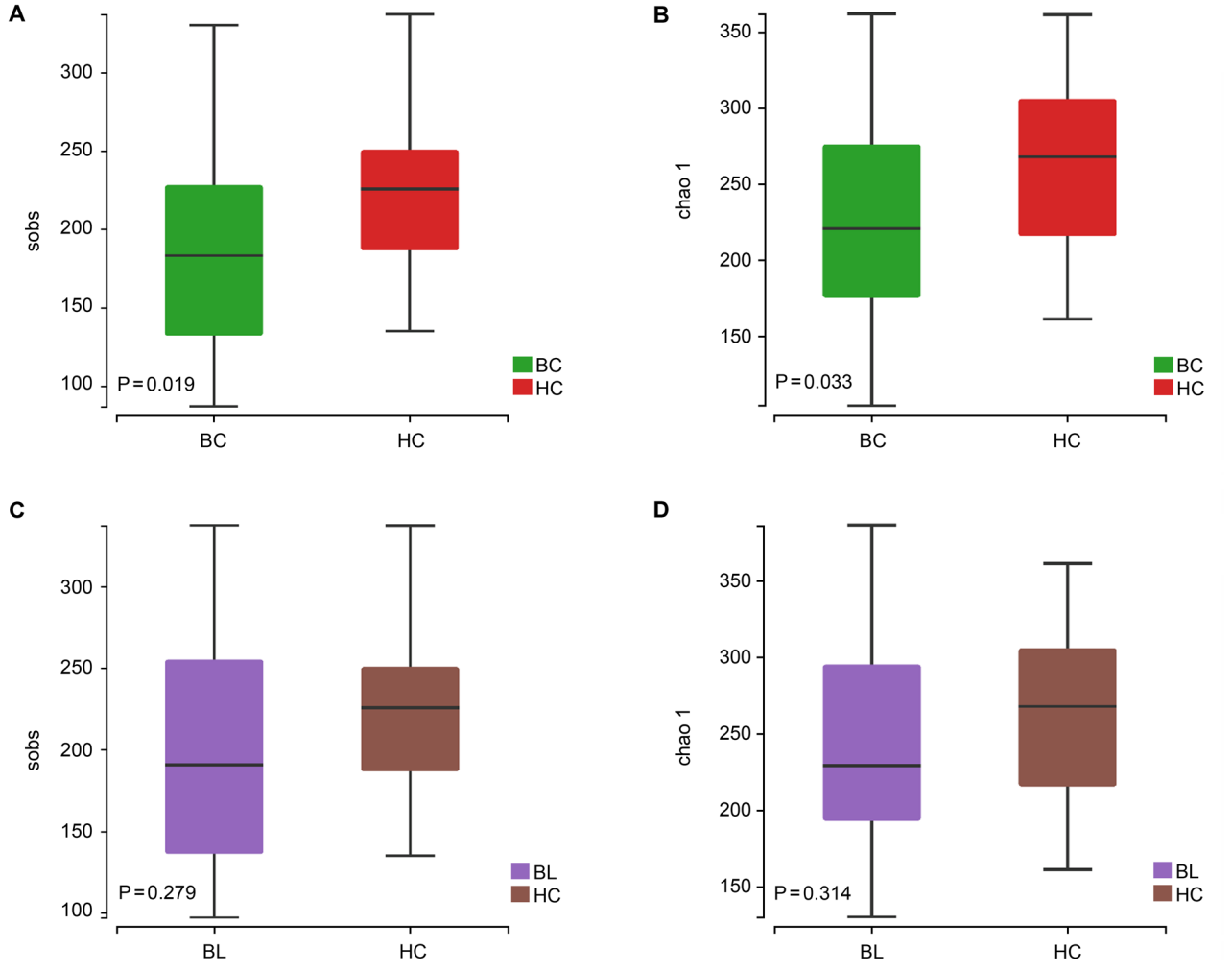

Fig. 3. Alpha diversity metrics (Sobs and Chao1 index).

A, B) Boxplots for species richness between breast cancer patients and healthy controls; C, D) boxplots for species richness between benign breast lesion patients and healthy controls; BC – breast cancer, BL – benign breast lesions, HC – healthy controls.

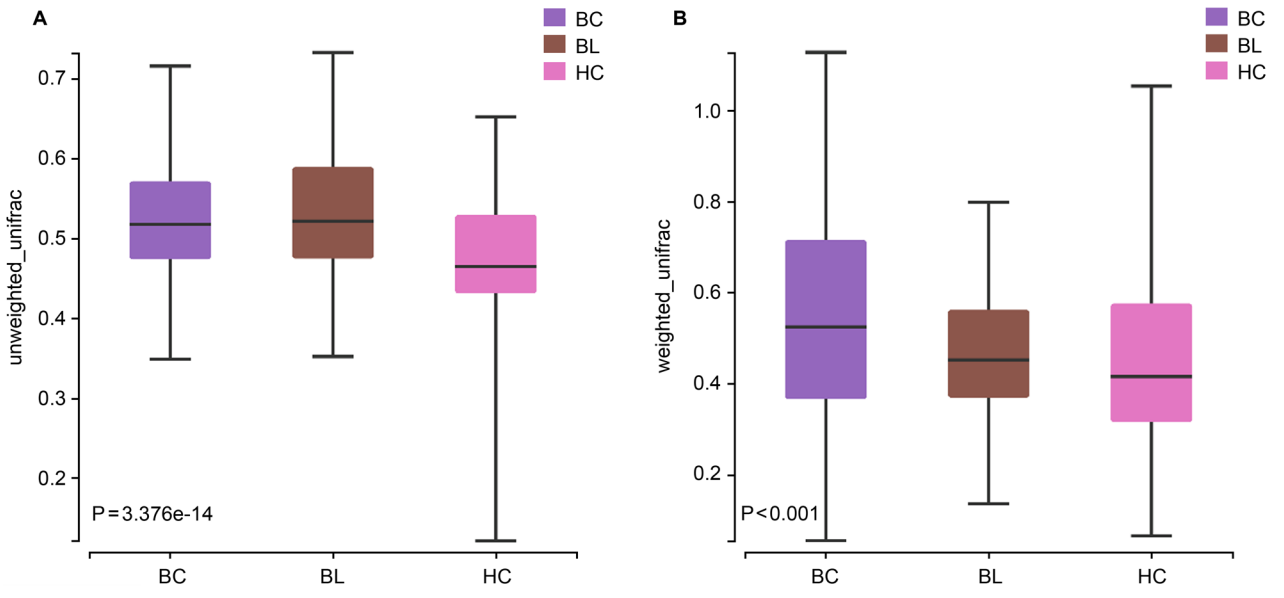

Fig. 4. Beta diversity assessment based on unweighted and weighted UniFrac.

A) Boxplots showing the comparison of beta diversity based on unweighted UniFrac among groups; B) boxplots showing the comparison of beta diversity based on weighted UniFrac among groups; BC – breast cancer, BL – benign breast lesions, HC – healthy controls.

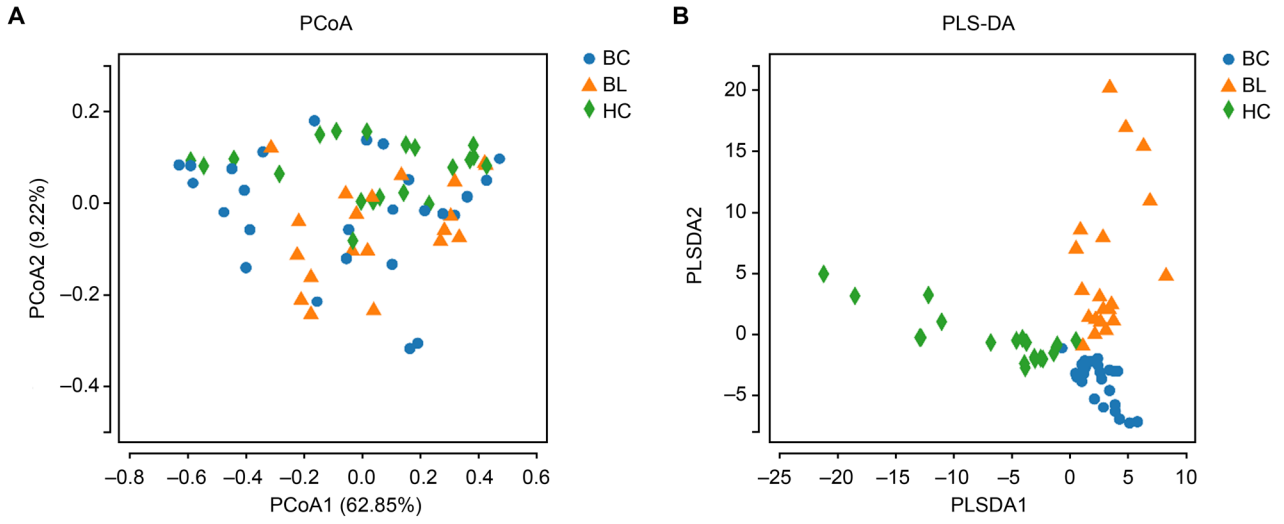

Fig. 5. PCoA and PLS-DA analysis of microbiota among breast cancer patients, benign breast lesion patients, and healthy controls. Blue circles, orange triangles, and green diamonds represent samples in different groups. The closer the spatial distance of the sample, the more similar the species composition of the sample.

A) PCoA plot based on weighted Unifrac; B) PLS-DA plot; BC – breast cancer, BL – benign breast lesions, HC – healthy controls.

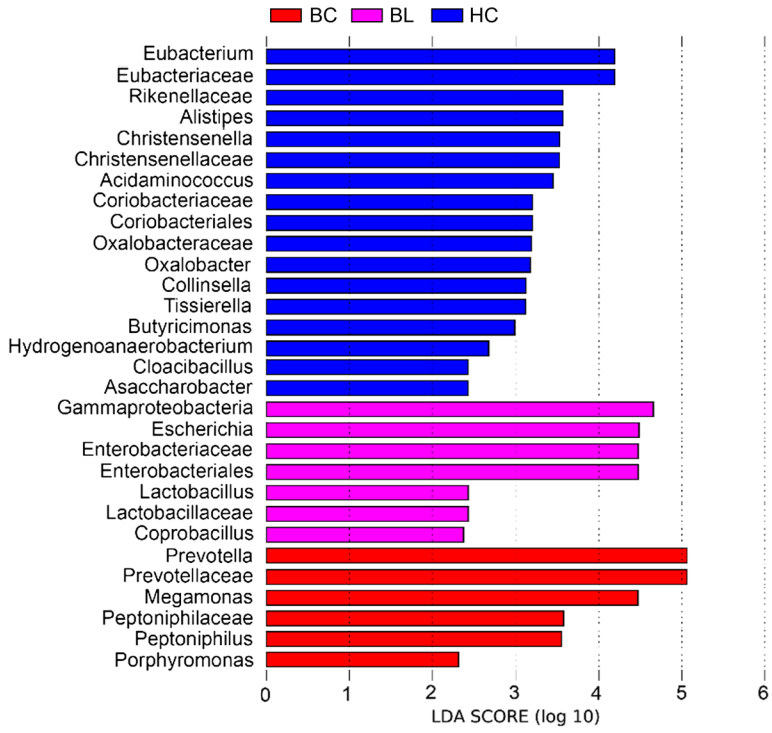

Fig. 6. Characteristics of bacterial community composition in breast cancer patients, benign breast lesion patients, and healthy control groups. The linear discriminant analysis (LDA) coupled with effect size (LEfSe) was performed using the LEfSe program. An LDA (log10) score of > 2.0 was considered significant; BC – breast cancer, BL – benign breast lesions, HC – healthy controls.

Pourbaferani et al. 2021). On the other hand, decreased microbial diversity caused by long-term use of antibiotics has been shown to increase the BC risk (Sergentanis et al. 2010; Wirtz et al. 2013; Simin et al. 2020), although conflicting results have been observed (García Rodríguez and González-Pérez 2005; Sørensen et al. 2005).

Previous studies showed a direct and strong association between fecal microbiota diversity and estrogen levels in women (Flores et al. 2012). Generally, estrogens and their metabolites undergo sulfation and glucuronidation in the liver. The conjugated estrogens can then be excreted via stool and urine. Intestinal bacteria can directly affect estrogen production by secreting β-glucuronidase (GUS), an enzyme that depolymerizes estrogens into their active forms, to control the concentration of estrogens reabsorbed into the

enterohepatic circulation. In addition, gut microbes synthesize estrogen-like compounds or estrogen mimics from the daily diet.

Furthermore, multiple bacterial metabolites (e.g., short-chain fatty acids, acetate, butyrate, pyruvate, formate, active amines, bile acids and derivatives, indole derivatives, etc.) can be involved in cancer cell growth, apoptosis, and invasion, epithelial-to-mesenchymal transition, and antitumor immune activity (Kovács et al. 2021). Changes in microbiome composition will lead to changes in the profiles of metabolites (Kovács et al. 2021). We, therefore, speculate that the proportion of microbiota-encoded GUS enzymes changed, thus affecting the metabolism of steroid hormones, metabolite profiles, and alpha diversity of intestinal microorganisms in BC and BBL patients.

Furthermore, microbial diversity can affect the efficacy of anticancer therapy. Fecal samples from melanoma patients receiving anti-PD-1 treatment exhibited a more diverse microbiome, and patients had significantly longer progression-free survival. The microbiota of immune therapy responders may upregulate the immune response by enhancing antigen presentation or increasing T cell recruitment in the local tumor environment (Gopalakrishnan et al. 2018).

Similarly, gut microbiota conditions the metastasis and therapeutic efficacy of trastuzumab in HER2-positive BC (Ingman 2019; Di Modica et al. 2021). Probiotic administration can significantly increase the number of bacterial species and the bacterial diversity assessed with the Chao1 index in overweight BC survivors (Pellegrini et al. 2020). Therefore, we hypothesize that the reduced microbial diversity may affect the treatment efficacy of BC patients.

According to the LEfSe analysis, *Prevotella*, *Porphyromonas*, *Peptoniphilus*, and *Megamonas* were indicator bacterial species in BC patients. *Prevotella* and *Porphyromonas* were also identified as potential microbial markers for postmenopausal BC patients (Amanatullah et al. 2017; Zhu et al. 2018). The two genera are also associated with colorectal cancer and precancerous adenomas (Warren et al. 2013; Lasry et al. 2016). Notably, *Prevotella* has been found on breast skin and mammary tissue (Urbaniak et al. 2014; Hieken et al. 2016; Urbaniak et al. 2016). Transferring microorganisms from the intestine to the breast tissue leads to increased systemic inflammation in BC and is therefore considered a cause of BC (Rao et al. 2007). Previous studies have shown that inflammatory indicators, such as platelet/lymphocyte ratio and lymphocyte/monocyte ratio, significantly influence the prognosis of various cancers, and neutrophilia is associated with a poor prognosis of BC (Lakritz et al. 2015). Systemic inverse interactions among microbes, interleukin-6 (IL-6), and neutrophils have been noted in BC (Rutkowski et al. 2015). A high lymphocyte/neutrophil ratio increases the risk of relapse in BC patients (Margolis et al. 2007). Therefore, we hypothesize that *Prevotella* was involved in the inflammatory response in BC patients. In addition, *Prevotella* can activate Toll-like receptor 2, leading to the production of Th17-polarizing cytokines by antigen-presenting cells, including IL-23 and IL-1. *Prevotella* can also stimulate epithelial cells to produce IL-8, IL-6, and CCL20, which promote mucosal Th17 immune responses (Larsen 2017). *Porphyromonas uenonis* showed a weak positive correlation with CD19 in BC patients (Zhu et al. 2018). *Peptoniphilus* were abundant in endocrine receptor-positive, human epidermal growth factor receptor 2-positive, and triple-negative BC types (Banerjee et al. 2018). *Megamonas* decreased significantly in patients with Bechet's disease, and this alteration may be associated with immune aberration (Shimizu et al. 2019). Thus, we infer that the bacterial abundance changes were involved in the disruption of immune homeostasis in BC patients.

Among the genera with a decreased abundance in patients with BC, *Collinsella* has been associated with a cancer-free status and a better prognosis in BC patients (Terrisse et al. 2021). In the present study, *Alistipes* was decreased in the gut of patients with BC but was increased in the nipple aspiration fluid of patients with BC in a previous study (Laborda-Illanes et al. 2020). In BC, the number of *Anaerofilum* in the gut appears to be associated with the number of tumor-infiltrating lymphocytes (Shi et al. 2019). A lower number of gut *Butyricimonas* have been reported before in BC (Bobin-Dubigeon et al. 2021), supporting the present study. *Acidaminococcus* and *Cloacibacillus* have been observed with different gut abundance among BC subtypes associated with prognoses (Wu et al. 2020; Yang et al. 2021).

Even if the association between gut dysbiosis and BC has been extensively studied (Kovács et al. 2021), the association between gut dysbiosis and BBLs has not been extensively explored before. The present study suggests that women with BBLs display changes in the gut microbiome compared with healthy women. Many BBLs are precursor lesions in a spectrum of lesions leading to BC or to be markers of increased risk of breast cancer (Hartmann et al. 2005; Worsham et al. 2009; Johansson et al. 2021). Some of the bacteria found to be increased or decreased in patients with BBLs were also observed in patients with BC (increased *Escherichia*, *Peptoniphilus*, *Lactobacillus*, and *Porphyromonas*; decreased: *Collinsella*, *Alistipes*, *Butyricimonas*, *Acidaminococcus*, *Tissierella*, and *Cloacibacillus*). It is supported by Yang et al. (2021), who showed that among 31 genera of gut microbiota, only one (*Citrobacter*) was different between patients with BC and BBL. Still, Meng et al. (2018) reported differences in gut microbiota between BBL and BC. The roles of the various bacteria in immunity

and cancer development discussed above might also apply to BBLs. Therefore, it could be hypothesized that a gut dysbiosis is an early event in the development of BBLs and BC and that the changes in gut microbiota are an early event in the spectrum of events from normal breast tissue to BBLs to BC. Ideally, longitudinal studies should be performed to examine this point.

In this study, the populations of *Escherichia* and *Lactobacillus* were significantly upregulated in BBL patients. The impaired barrier function allows bacterial access to the intestinal epithelium, enabling the delivery of toxins. *Escherichia coli* can putatively induce tumorigenesis by generating DNA mutagens such as genotoxin colibactin (Arthur et al. 2012). *Staphylococcus aureus* is an important factor inducing mutation of the MED12 gene, which may contribute to uterine leiomyomas and breast fibroadenomas (Bullerdiek and Rommel 2018). However, no significant changes in *S. aureus* were detected. Surprisingly, *Lactobacillus* was upregulated in BBL patients. *Lactobacillus* is usually considered to be a beneficial bacterium. Oral consumption of *Lactobacillus acidophilus* can decrease in fecal enzyme activity of GUS (Kwa et al. 2016), thereby reducing the estrogen burden in the body. *Lactobacillus reuteri* was found to be helpful in suppressing mammary tumorigenesis in genetically susceptible Her2 mutant mice (Lakritz et al. 2014). In addition, *Lactobacillus* exhibited anti-inflammatory properties in *E. coli*-stimulated bovine mammary epithelial cells (Bouchard et al. 2015). Hence, the upregulation of *Lactobacillus* in BBL patients may be due to the presence of the tumor, which allows the intestine to attract more beneficial bacteria to fight it.

In conclusion, non-malignant breast diseases have been far less studied. However, the great potential of intestinal microbiota in the development and treatment of benign breast diseases cannot be overlooked. The use of probiotics to treat mastitis in breastfeeding women has been reported. Probiotics are potentially effective at eliminating chronic subclinical infections as antibiotic treatment (Arroyo et al. 2010). Therefore, more related studies are required in the future.

Herein, we performed 16S rRNA gene sequencing of fecal samples collected from BC and BBL patients and healthy controls matched by gender, age, and BMI. Compared with healthy controls, BC and BBL patients showed a decreasing trend in intestinal microbiota diversity, which may be associated with their pathogenesis. The up- or down-regulated strains may be an essential indicator of the initiation of BC and BBLs. These results may provide a valuable reference for future related studies. However, several limitations must be addressed in future studies. First, species-level differences were not captured due to the limitations of 16S rRNA sequencing. More studies with whole-genome sequencing are needed. Second, this study was a single-center study with relatively small sample size. Third, the dietary structures differed among individuals, which might have influenced the results. Additional studies should be conducted with larger samples to explore the functions of intestinal flora in BC and BBLs.

Ethical statement

The study protocol was approved by the Ethics Committee of The Affiliated Hospital of Qinghai University (approval number: P-SL-2020035)

Author contributions

ZJM and MLQ collected and analyzed data and wrote the manuscript. XWW designed and edited the manuscript.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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