


Comparison of the Gut Microbiota in Patients with Benign and Malignant Breast Tumors: A Pilot Study

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ABSTRACT: The microbiome plays diverse roles in many diseases and can potentially contribute to cancer development. Breast cancer is the most commonly diagnosed cancer in women worldwide. Thus, we investigated whether the gut microbiota differs between patients with breast carcinoma and those with benign tumors. The DNA of the fecal microbiota community was detected by Illumina sequencing and the taxonomy of 16S rRNA genes. The α -diversity and β -diversity analyses were used to determine richness and evenness of the gut microbiota. Gene function prediction of the microbiota in patients with benign and malignant carcinoma was performed using PICRUSt. There was no significant difference in the α -diversity between patients with benign and malignant tumors ($P=3.15e^{-1}$ for the Chao index and $P=3.1e^{-1}$ for the ACE index). The microbiota composition was different between the 2 groups, although no statistical difference was observed in β -diversity. Of the 31 different genera compared between the 2 groups, level of only *Citrobacter* was significantly higher in the malignant tumor group than that in benign tumor group. The metabolic pathways of the gut microbiome in the malignant tumor group were significantly different from those in benign tumor group. Furthermore, the study establishes the distinct richness of the gut microbiome in patients with breast cancer with different clinicopathological factors, including ER, PR, Ki-67 level, Her2 status, and tumor grade. These findings suggest that the gut microbiome may be useful for the diagnosis and treatment of malignant breast carcinoma.

KEYWORDS: Gut microbiome, breast carcinoma, benign breast tumor, *Citrobacter*, clinicopathological factors.

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Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide.¹ Genetic changes and environmental exposures that accumulate in the breast promote development of cancer in only a fraction of all cases.² Breast cancer may also be induced or promoted by some other poorly characterized or undescribed factors. Adverse outcomes in breast cancer have also been linked to genetic polymorphisms, obesity, diet, race, and chronic use of antibiotics.³ Additionally, all these factors are associated with disruption of commensal microbial homeostasis.

The microbiome plays a major role in preserving the human intestinal mucosal barrier, resisting colonization of pathogenic microorganisms, and promoting metabolic balance and immune homeostasis.⁴ There is an increasing interest in understanding the role of the altered gut microbiota that may substantially affect human health, such as being a general risk factor for cancer⁵ and diabetes,⁶ colorectal cancer,^{7,8} adiposity,⁹ inflammatory bowel disease,^{10,11} lung cancer,¹² cardiovascular diseases,¹³ mental diseases,^{14,15} etc. The microbiota impacts human metabolism and immunity.^{16,17} The composition of the microbial community can exert negative effects that promote disease occurrence or help in maintaining a healthy status. Studies suggest that pathogenic changes in breast carcinoma

may be influenced by microbe-host interactions.^{18,19} The most influential bacteria in patients with breast cancer were found at different sites, such as the breast tissue, skin, oropharynx, and gastrointestinal tract.³ Of these, the microbiome of the gastrointestinal tract plays a potential role in steroid hormone metabolism and synthesis of biologically active estrogen mimics during the development of breast cancer.²⁰ The microbial communities in breast cancer can also act as potential biomarkers for diagnosis, treatment, and prognosis, thereby promoting development of a new era of individualized medicines.²¹

Studies have demonstrated a correlation between the gut microbiota and estrogen levels, tumor grade, stage, and tumor type in patients with breast cancer.^{21–23} The microbiome in malignant and benign breast tumor tissues was reported to be dramatically different^{24,25}; but, there is limited research on the diversity of the microbiome in the gastrointestinal tract of patients with malignant and benign breast tumors.

In this study, we collected fecal samples from patients with malignant and benign breast tumors, performed 16S rRNA gene sequencing, and performed functional analysis of the gut microbiota. The results show the distinct richness of the gut microbial communities in patients with malignant or benign breast tumors, and provides an opportunity to explore potential role of the gut microbiome in breast carcinogenesis. Further, we evaluated the association of the gut microbiome with clinicopathological characteristics in patients with breast

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tumors, which may provide insights in breast cancer treatment and prognosis.

Materials and Methods

Patient enrollment

The study was performed in accordance with the principles of the Declaration of Helsinki concerning ethical research related to human subjects and was approved by the Institutional Review Board of the Quanzhou First Hospital affiliated to the Fujian Medical University. Informed consent was obtained from all the participants. Eighty-three patients with invasive ductal breast carcinoma who underwent surgery between January 2019 and December 2020 were enrolled in the study. Additionally, 19 patients with benign breast tumors who underwent segmental resection were recruited in the benign group. Segmental mastectomy is the mainstay of therapy for benign breast tumors. Patient data, including age, menopausal status, and tumor characteristics (tumor grade, diameter, lymph node involvement, Ki-67 index, estrogen receptor [ER]/progesterone receptor [PR] expression, and human epidermal growth factor receptor-2 [Her2] status), were obtained from pathology reports. The inclusion criteria for both the groups were as follows: female sex, good physical condition, no history of other cancers, active intestinal dysfunction or gastrointestinal inflammation, no bariatric surgery, celiac disease, pregnancy or nursing within the past 12 months, no special eating history (eg, vegetarian), and no antibiotic treatment prior to fecal specimen collection. Patients with breast cancer were diagnosed with nonspecific invasive carcinoma and did not receive surgery, chemotherapy, endocrine therapy, or radiation prior to sample collection. Subjects were excluded if they received antibiotic treatment within 6 months or other treatment before the onset of the disease, had undefined histological grade in pathological tests, had other pathological types of breast cancer or breast cancer during lactation, or were non-compliant.

Sample collection

We collected fresh stool samples, without undigested food residues or other solid substances, from patients who met the inclusion criteria, before treatment. The DNA preservation solution was used for sample storage at 18°C to 26°C, the main components of the solution were trihydroxy-methyl aminomethane buffer, disodium ethylenediamine tetra-acetic acid, sodium chloride, and sodium dodecyl sulfate.

DNA extraction

DNA extraction was performed using the SDS lysis buffer freeze-thaw method. Genomic DNA was isolated using PowerMax extraction kit (MoBio Laboratories, Carlsbad, CA, USA) and stored at -20°C until further analysis. The quantity of DNA was determined using NanoDrop ND-1000

spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), followed by agarose gel electrophoresis.

16S rRNA gene sequencing and analysis

The 16S rRNA gene amplicons were prepared in a 50 µL reaction. The V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) with following primers: 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-G GACTACHVGGGTWTCTAAT-3').²⁶ The forward primer 515F was linked to A-adaptor, a specific 7-bp barcode, while the reverse primer 806R carried B-adaptor. The PCR were performed in a 50 µL mixture containing 25 µL of high-fidelity enzyme (Phusion HighFidelity PCR Master Mix with HF Buffer), 2 × 3 µL of each primer (5 µm), 9 µL of double distilled water, and 10 µL of template DNA. The PCR conditions were as follows: 8°C for 30 seconds (one cycle); 98°C for 15 seconds, 58°C for 15 seconds, and 72°C for 15 seconds (25 cycles); and 72°C for 1 minute (1 cycle). Further, the PCR products were purified using AMPure XP Beads (Beckman Coulter, Indianapolis, IN, USA), quantified using PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA), and then pooled amplicons were concentrated using HiSeq platform (Illumina HiSeq 4000).

The 16S rRNA gene sequencing analysis was performed using Quantitative Insights into Microbial Ecology (QIIME, version 1.9.1) software suite²⁷ and QIIME tutorial (<http://qiime.org/>) with some modifications. After sequencing, Vsearch (v2.4.4) was used to splice the reads in each sample to obtain the original tag data. Low-quality material or fragments, such as barcode and primer sequence mismatches, sequences sized <150bp, average mass value <20, sequences with ambiguous characters, single nucleotide repeats of >8bp, or sequencing errors and chimeras were removed during the pre-processing procedure. The extracted data were saved in the FastQ format. Each sample of the 2-terminal sequencing data contained FastQ1 and FastQ2 files, which were read at both ends of the sequence. The quality of the sequences was controlled and filtered, and splicing was concomitantly performed.

Cluster analysis of operational taxonomic units (OTUs)

Operational taxonomic units (OTUs) are hypothetical taxa in phylogenetic analyses or population genetic research. The OTU in this study represents sequence from the same source. OTU clustering analysis is usually performed at a threshold of 97% identity.

After clustering, the sequences were assigned taxonomy and referenced by GreenGene database.²⁸

Alpha diversity analysis

Alpha diversity (α -diversity) was determined using several indices to calculate the complexity of species diversity in

samples. Differences in α -diversity were computed using the Chao, ACE, and Shannon diversity indices. The Chao and ACE indices reflected species richness of the community. As a reflection of the species diversity, the Shannon value indicates both species richness and evenness—the value considers the abundance of each species.

Rarefaction curve

Rarefaction curves were computed from OTU tables using α -diversity, and random sampling of the sequence from each sample indicated community richness.

Beta diversity analysis

Beta diversity (β -diversity), computed using hierarchical cluster dendrograms (Bray-Curtis distance dissimilarities),²⁹ was used to compare the similarity between samples, which in this study is visibly displayed in the form of principal component analysis (PCA) and principal coordinate analysis (PCoA). The process of PCoA is using OTU sequence and based on UniFrac-weighted distances. The *P*-values were obtained by the similarities of the analysis of similarities (Anosim).³⁰

Kyoto encyclopedia of genes and genomes (KEGG) analysis

We performed linear discriminant analysis (LDA) effect size (LEfSe) in the relative abundance of genera to identify influences of biomarkers that were statistically significant.³¹ LDA values >2.0 were considered significantly enriched only when *P*-value $<.05$. The KEGG³² was used for abundance and functional annotation. Based on the sequencing data and comparison with the GreenGene database,²⁸ we used the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, version 1.1.4) to compare the OTU information and predict the functional composition of the metagenome.

Statistical analysis

Data analysis was performed using the Wilcoxon rank-sum test for binary variables and to explore differences in α -diversity between any 2 groups. The figures are prepared using R (v3.2.0). We used STAMP software (version 2.1.3)³³ to display the graphical representation and predict the KEGG function information. A *P*-value $<.05$ was considered statistically significant.

Results

Patient characteristics

To investigate the composition of the gut microbiome in patients with malignant and benign breast tumors, we collected fecal samples from 83 patients with invasive breast cancer and 19 patients with benign breast tumors (clinicopathological

Table 1. Demographic information of all subjects used in this study.

VARIABLE	BENIGN (N = 19)	MALIGNANT (N = 83)	<i>P</i> *
Age, years			
Average(range)	45.14 (31-56)	48.07 (29-68)	
Grade			
I	/	3	/
II	/	29	/
III	/	48	/
Unknown	/	3	/
Menopausal status			
Pre-menopause	13	53	
Post-menopause	6	30	
ER status			
Positive	/	51	/
Negative	/	32	/
PR status			
Positive	/	47	/
Negative	/	36	/
Ki67 status			
<30%	/	21	/
\geq 30%	/	62	/
HER-2			
Positive	/	37	/
Negative	/		/
Unknown	/	1	/

Abbreviations: ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; PR, progesterone receptor.
*Wilcoxon rank test between benign vs malignant group for menopausal status.

information is summarized in Table 1). The median age at diagnosis was 48.07 (range, 29–68) years and 45.14 (range, 31–56) years in the malignant and benign groups, respectively. Of these, there were 30 and 6 postmenopausal patients in the malignant and benign tumor groups, respectively. In the malignant tumor group, 51 (61.44%), and 47 (56.6%) tumors were ER-positive and PR-positive, respectively. Further, 29 (34.9%) and 48 (57.8%) patients had grade II and grade III diseases, respectively, and 37 (54.2%) were Her2 positive.

Microbiota analysis

We first evaluated the richness and evenness of the gut microbiome in the 2 groups (Figure 1). The α -diversity analysis showed that the community richness was not significantly different

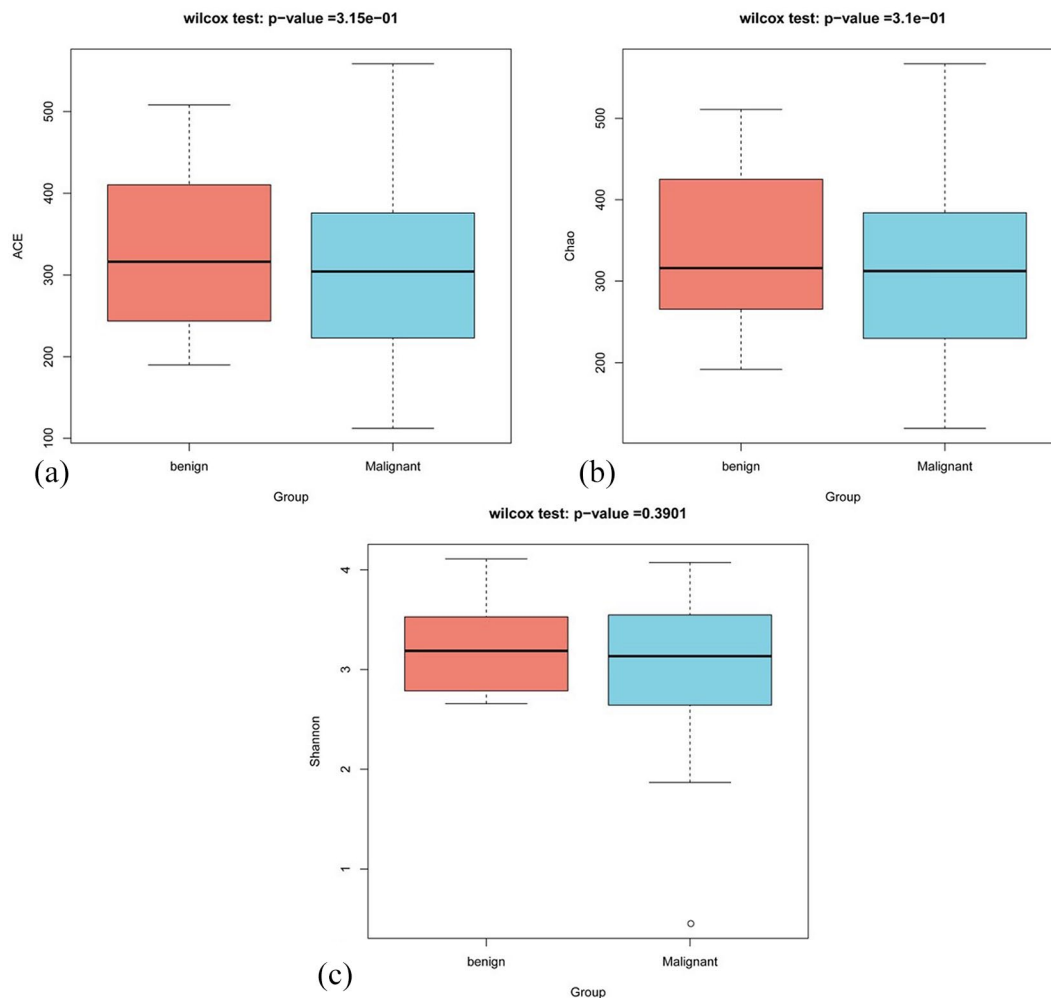


Figure 1. Alpha diversity indices boxplot between benign ($n=19$) and malignant ($n=83$) groups. comparison based on the (a) Chao, (b) ACE, and (c) Shannon. P -values from Wilcoxon test are shown.

between benign and malignant groups ($P=3.15e^{-1}$ for the Chao index and $P=3.1e^{-1}$ for the ACE index; Wilcoxon test; Figure 1a and b). No significant difference in community evenness was observed between the 2 groups using the Shannon index ($P=.3901$; Wilcoxon test; Figure 1c). The rarefaction curve tended to be flat, suggesting that community richness approached saturation in both groups (Supplemental Figure S1).

We investigated the taxonomic profiles of the gut microbiota of patients with malignant and benign tumors at the phylum and genus levels and found that the abundances of major taxa in both the groups were similar (Figure 2a). The gut microbiota of patients with malignant and benign breast tumors is illustrated by a heat map, where an increase in the number of species in the gut microbiota was observed in the malignant group than that in benign group (Figure 2b). The β -diversity analysis was used to compare the similarities between samples, including PCA and PCoA. After visualization, some differences in community membership between the 2 groups were observed in the PCA (Figure 2c), but they were not significant in the PCoA (Anosim $P=.953$; Figure 2d).

To further assess the differences in the microbiome between the benign and malignant groups, an LEfSe analysis was performed to identify differences in the microbiota and detect significant biomarkers. The relative distribution of 31 genera was found to be significantly different between the benign and malignant groups ($P<.05$), including *Faecalibacterium*, *Lachnospira*, *Clostridium*, *Brachybacterium*, *Butyrivibrio*, and *Mobiluncus*. Furthermore, LEfSe showed that *Clostridium*, *Faecalibacterium*, *Lachnospira*, *Erysipelotrichaceae*, *Romboutsia*, *Fusicatenibacter*, *Xylophilus*, and *Arcanobacterium* were significantly more abundant in the benign tumor group than that in malignant tumor group. The amount of *Citrobacter* was significantly higher only in the malignant tumor group (LDA score >3 ; Figure 3a). Differential analysis of taxa in the gut microbiota of the malignant and benign groups based on a permutation test is shown in Figure 3b.

We used PICRUST with STAMP software to calculate the KEGG pathways in the gut microbiome of patients with benign and malignant tumors. The gut microbiota in the malignant tumor group showed an increase in poorly characterized glycan biosynthesis and metabolism, whereas there was

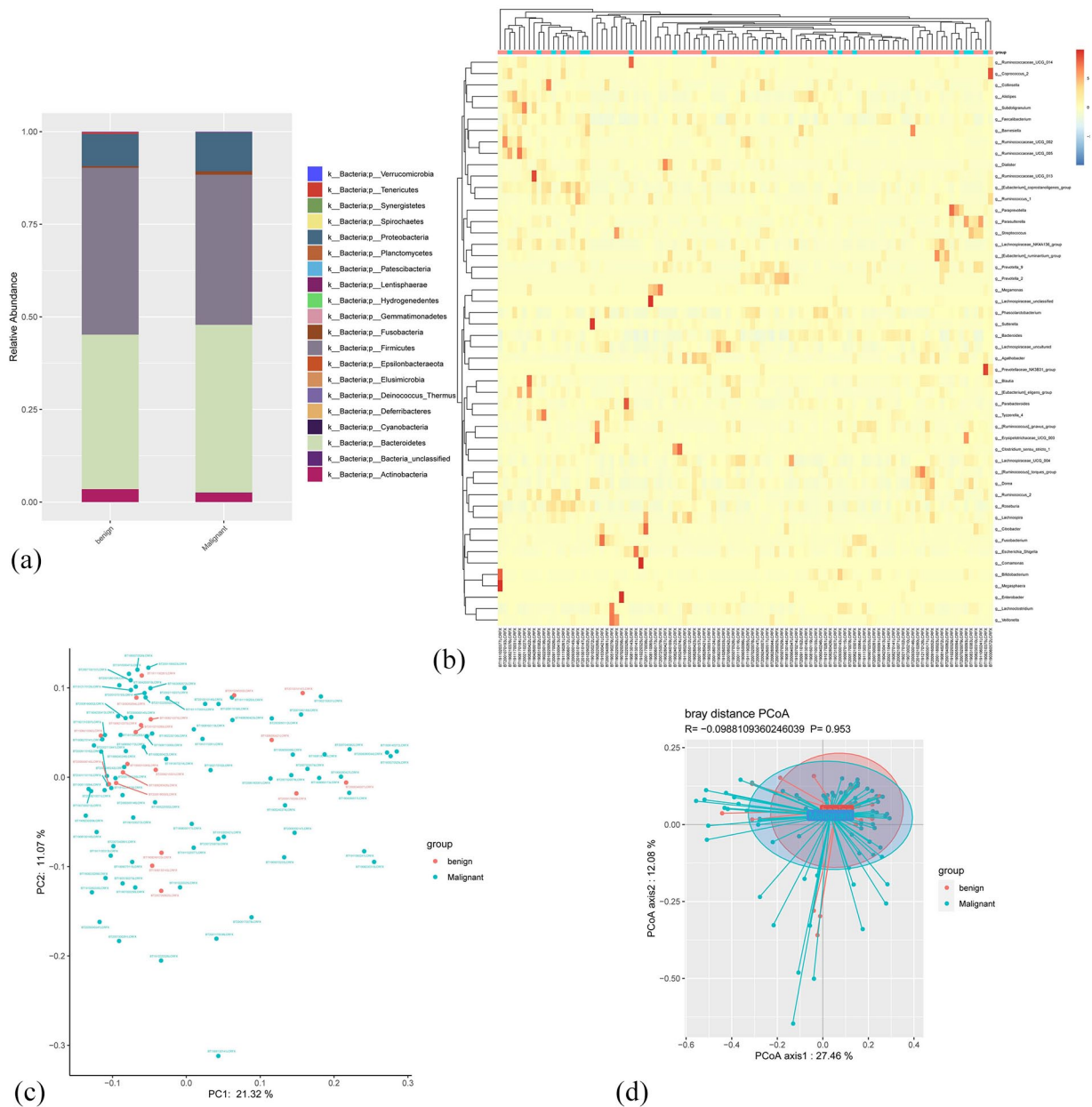


Figure 2. Comparison of the gut microbiota taxonomic profiles from patients with benign (n=19) and malignant (n=83) tumors. (a) Barplots of the gut microbiota taxonomic profiles of the benign and malignant cases at phylum level. (b) Heatmap shows the OTU presence and absence of the top 50 fecal samples at genus level. (c) The analysis of principal component analysis (PCA) based on OTU between groups. (d) Principal co-ordinates analysis (PCoA) based on OTU between groups (*P*-value from Anosim analysis are shown).

a reduction in transcription than that in benign group ($P < .05$, Figure 3c). A more detailed analysis of KEGG pathways indicated significant differences in 26 pathways ($P < .05$), including lipopolysaccharide biosynthesis, glycolysis/gluconeogenesis, folate biosynthesis, glycerophospholipid metabolism, and sporulation between the 2 groups (Figure 3d).

Microbial profiles associated with clinicopathological factors in breast cancer

Further, we evaluated the association between the gut microbial communities and clinicopathological factors in breast cancer. Patients with PR-positive breast cancer displayed an

enrichment of *Prevotellaceae* and *Tyzzerella*, whereas those with PR-negative breast cancer had an abundance of *Barnesiellaceae*, *Lactobacillaceae*, *Lactobacillus*, *Prevotellaceae*, *Cloacibacillus*, *Acinetobacter*, *Hydrogenophilus*, *Rhodobacteriae*, and *Hydrogenophilaceae* (Figure 4a). Microbial species of *Megasphaera*, *Roseburia*, and *Prevotellaceae* were found to be of distinct richness in patients with ER-positive breast cancer, while *Bacteroides*, *Bacteroidaceae*, *Puniceicoccaeae*, *Opitutales*, *Hydrogenophilus*, and *Hydrogenophilaceae* were significantly enriched in those with ER-negative breast cancer (LDA score > 3) (Figure 4b). The gut microbes, such as *Megasphaera*, *Barnesiellaceae*, *Alloprevotella*, *Lachnospiraceae*, *Moraxellaceae*, *Acinetobacter*, *Enorma*, *Flavonifractor*, *Burkholderiaceae*, and

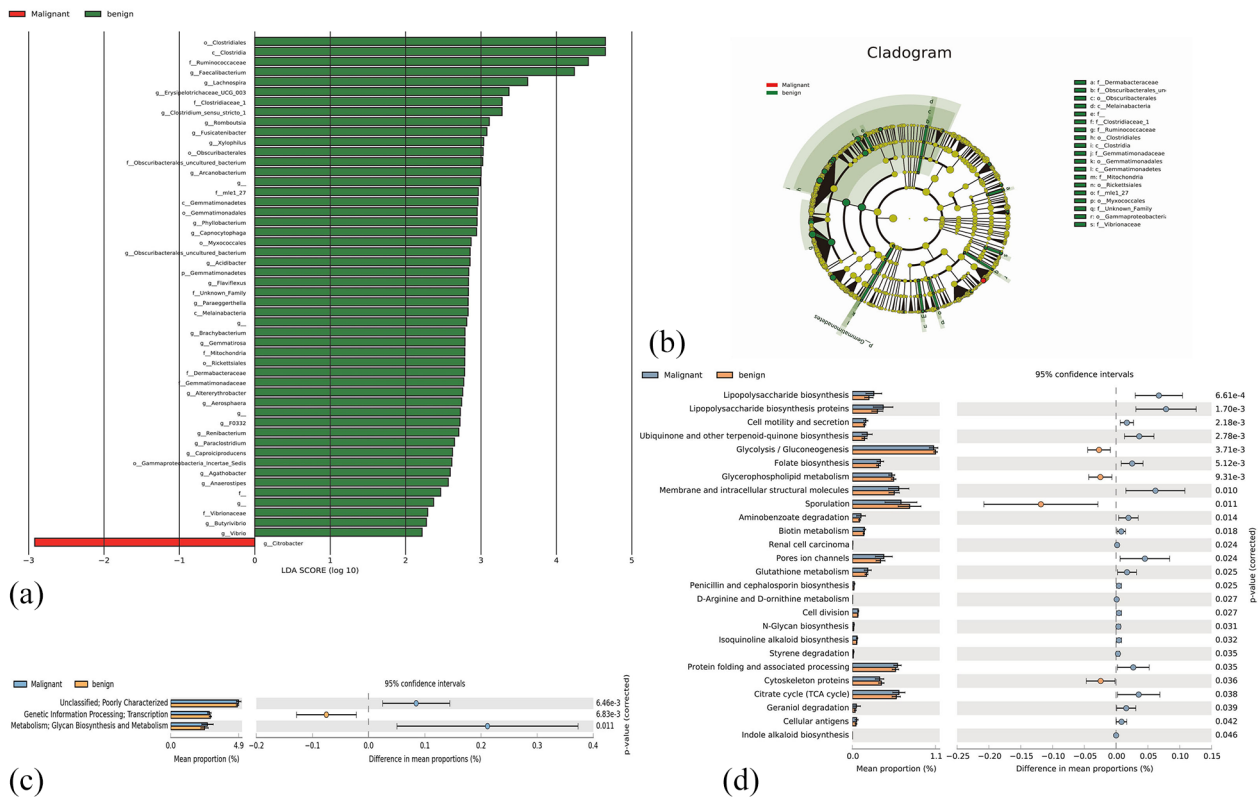


Figure 3. Comparison of gut microbiota using LDA and KEGG. (a) Differential taxa between benign and malignant gut microbiota based on a Linear Discriminant Analysis (LDA). Taxa with LDA score >2 at the family and genus level are defined as statistical differences. (b) Differential taxa between the gut microbiota of the 2 groups based on a permutation test. (c and d) Histogram of Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway of gut microbiota.

Eubacterium were more abundant in patients with Her2-positive cancer than in those with Her2-negative cancer (Figure 4c). In patients with low Ki-67 expression (Ki-67 $< 30\%$), the gut microbiomes were enriched for *Lactobacillus*, *Clostridium*, *Clostridiaceae*, *Megasphaera*, *Proteus*, and *Burkholderiaceae*. However, *Ruminiclostridium*, *Tenericetes*, *Mollicutes*, *Ruminococcaceae_UCG*, *Izimaplasmatales*, *Sporobacter*, *Syntrophomonadaceae*, and *Clostridiales_vadinBB60* were enriched in the Ki-67 high expression group (Ki67 $\geq 30\%$) than in Ki-67 low expression group (Figure 4e). Enrichment of *Coriobacteriaceae*, *Collinella*, and *Faecalitalea* was found in patients with histologic grade III disease (Figure 4f). Additionally, an increase in numbers of microbial species, such as *Enterobacter*, *Erysipelotrichaceae*, *Romboutsia*, *Anaerostipes*, *Granulicatella*, and *Carnobacteriaceae* was particularly observed in premenopausal patients; whereas, numbers of *Herbinix*, *Ruminococcaceae*, and *Epulopiscium* were increased in postmenopausal women (Figure 4d).

Discussion

We investigated the gut microbiome of patients with benign and malignant breast tumors using 16S rRNA gene sequencing and compared the microbial communities based on clinicopathological factors in patients with breast cancer. Microbiome community richness was higher in benign group than in malignant group. The metabolic pathways in patients with malignant

tumors were significantly different from those in patients with benign tumors. The study also established the distinct abundance of the gut microbiome in patients with breast cancer with different clinicopathological factors, including ER, PR, and Ki-67 levels, Her2 status, and tumor grade.

Zhu et al¹⁸ found that the gut microbial communities differed between patients with breast cancer and healthy controls both in pre- and post-menopausal women using the Chao and Shannon indices. Goedert et al¹⁹ suggested that postmenopausal patients with breast cancer had a less diverse and compositionally different fecal microbiota than postmenopausal healthy women. Moreover, the present study also showed that the fecal microbiota of patients with malignant tumor than those with benign tumor had a lower α -diversity with the Chao and ACE indices, but not with Shannon index. Therefore, patients with malignant tumors have less species richness, but indistinctive evenness of the gut microbial community than those with benign tumors.

Antibiotic use can cause disorder of host immune system, change the composition of gut microbiota^{34,35} and possibly aggravate chronic inflammation and carcinoma.^{36,37} However, the debate on the role of antibiotics remains inconclusive. Several observational studies indicate that antibiotic use won't increase the risk of breast cancer.³⁸⁻⁴² Elkrief et al concluded that the effect of broad-spectrum antibiotics on the intestinal flora of cancer patients receiving immunotherapy is also controversial.

Other studies show that antibiotic-induced turbulence of gut microbiota can aggravate breast tumor and metabolic disorder.^{43,44} A systematic review demonstrates that the risk of breast cancer modestly increases for individuals who have ever used antibiotics.⁴⁵ The causality between antibiotics and breast cancer remains unclear, so further explanation and mechanistic studies still need to be done.

Further, the study revealed that patients with malignant tumors possessed elevated levels of *Citrobacter*, whereas a great majority of the microbiota elevated in those with benign tumors included *Clostridium*, *Faecalibacterium*, *Lachnospira*, *Erysipelotrichaceae*, *Romboutsia*, *Fusicatenibacter*, *Xylophilus*, and *Arcanobacterium*. Zhu et al¹⁸ found that 38 species were enriched in the postmenopausal group, including *Citrobacter*, *Escherichia*, and *Klebsiella*. In contrast, a previous analysis demonstrated that the levels of *Clostridaceae*, *Faecalibacterium*, and *Ruminococcaceae* were higher, while those of *Dorea* and *Lachnospiraceae* were lower in postmenopausal patients with breast cancer than levels in healthy controls.¹⁹ Preclinical studies in a mouse model showed that infection with *Citrobacter* increased epithelial cell proliferation and promoted growth of chemically-induced colon tumors.⁴⁶ In contrast to previous studies,^{18,47,48} *Clostridium* richness was found in patients with benign breast tumor than in those with malignant tumor. Diversity in the gut microbiota composition of patients with breast carcinoma suggests that microbial metabolism or dysbiosis may play an important role in breast cancer development.

Dysbiosis in the microbial community can regulate systemic immune function and increase production of inflammatory mediators that correlate with poor outcome in many diseases, including malignant tumors.^{3,49,50} It is established that estrogen metabolism is associated with some species of the gastrointestinal microbes.^{51,52} However, the metabolic output of the microbiota often reflects the condition of the commensal ecosystem.³ Microorganisms and their metabolites have variable effects on breast cancer through different metabolic pathways. For instance, bacterial metabolites, such as lithocholic acid and cadaverine, have been shown to exert an inhibitory effect on breast cancer.⁵³⁻⁵⁵ Kovács et al showed that lithocholic acid increases oxidative stress in breast cancer. Based on the present analysis, the gut microbial metabolism in patients with malignant and benign tumors was significantly different. We observed an increase in a majority of metabolic pathways in patients with malignant tumors, especially the lipopolysaccharide biosynthesis pathways, than that in patients with benign tumors. In contrast, a significant increase in sporulation in patients with benign tumors was found by KEGG analysis. A previous study has shown that bacterial lipopolysaccharides are frequently present in many human solid tumors.⁵⁶ Moreover, high-fat diets may increase the serum levels of bacterial lipopolysaccharide, thereby impacting breast cancer risk in mouse models.⁵⁷

Patients with breast cancer with different clinical characteristics show diverse compositions of the gut microbiota.^{21,58} A

study conducted by Bard et al⁵⁹ showed that the number of *Blautia* sp. was higher in patients with grade III disease than in those with grade I disease, and the enrichment or proportions of some bacteria was significantly different according to the clinical stage and body mass index (BMI). Moreover, Luu et al²² suggested that the microbiome in patients with breast cancer significantly varied with different clinical stages, histoprognostic grades, and BMI. Similar to a previous study, the present study found that the number and abundance of the gut microbial communities were extraordinarily higher in patients with grade III than in those with grade I/II breast cancer. Furthermore, an abundance of *Herbinix*, *Ruminococcaceae*, and *Epulopiscium* was detected in postmenopausal patients.

Additionally, patients with breast cancer with different expression of Ki-67 (<30%/≥30%), ER (+/-), PR (+/-), and Her2 (+/-) showed microbiome diversity in the present study. A study by Banerjee et al²³ demonstrated similar microbial signatures in ER+ and Her2+ samples, while the triple negative and triple positive samples presented with distinct patterns of microbiomes. Similarly, Nejman et al⁵⁶ revealed that the prevalence of multiple bacterial taxa was various in different breast cancer subtypes based on their ER, PR, and Her2 status. It is suggested that each breast cancer type might have type-specific communities. The connection of breast cancer type and gut microbiome might via enterohepatic circulation, which were thought to affect circulating and excretory estrogen levels in breast cancer.

According to analysis of different clinicopathological grouping, Wu et al⁶⁰ found that Her2 status and age at menarche had significant association with α -diversity measures of the gut microbiome and specific microbial composition. He et al⁴⁸ found that the composition and symbiosis of the gut microbiota in patients with premenopausal breast cancer changed significantly in comparison with that in healthy premenopausal women. However, further studies are needed to confirm the characteristics of the human microbiome and intrinsic connection between the pathological and clinical features of breast cancer and established risk factors.

Enrichment of *Megasphaera* was identified by 16S rRNA sequencing analysis in patients with ER+ and Her2+ tumors in the present study. It is known that estrogen levels are influenced by the diversity of the gut microbiome, and that increased levels of endogenous and circulating estrogen are related to a high risk of breast cancer in postmenopausal women.⁶¹ Interestingly, in the present study, members of only the *Prevotellaceae* family were abundant in both patients with PR+ and ER+ tumors, while microbes such as *Hydrogenophilus*, *Lactobacillus*, and *Acinetobacter* were highly abundant in patients with PR- and ER- tumors. Therefore, the function and connection of the gut microbiome in estrogen production warrants further investigation. Moreover, it will be interesting to investigate the role of the gut microbiome in the treatment response, including chemotherapy, endocrine therapy, and targeted therapy.

Previous studies have attempted exploration of similar topics. Terrisse et al⁶² found that chemotherapy drastically shifted the microbiome composition, by reducing abundance of microflora associated with side effects and increasing abundance of favorable commensals after adjuvant or neoadjuvant treatment with anthracyclines and taxanes. A study by Di Modica et al⁶³ revealed the existence of specific microbiota in patients with Her2+ breast cancer that can influence their response to targeted therapy. This suggests that manipulation of the fecal microbiota is an optimal future regimen to achieve effective treatment or to explore its potential as a biomarker for therapeutic response. Moreover, anticancer properties of the intestinal flora should be closely studied. It is possible that the gut microbiome community in patients with breast cancer may contribute to the prognosis and survival outcome. However, a lack of a healthy control group and a low number of samples were limitations in this study. Thus, further analysis of a healthy cohort and a larger sample size is imperative for whole-genome sequencing.

In summary, the study demonstrates that the proportion and metabolic pathways of the gut microbiome in patients with malignant breast tumors are significantly different from those in patients with benign tumors. Furthermore, we observed a distinct enrichment of the gut microbiome in patients with breast cancer with different clinicopathological factors, including ER, PR, Ki-67 levels, Her2 status, and tumor grade. These findings suggest that the gut microbiome may be useful for the diagnosis and treatment of malignant breast tumors.

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Authors Contributions

D.B.C. contributed to the conception and design of the study; P.D.Y., Z.T.W., and W.B.L. contributed to the acquisition of data and analysis; P.D.Y. and Q.Q.P. contributed to drafting the article, and/or revised the manuscript. D.B.C. did study supervision.

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Supplemental Material

Supplemental material for this article is available online.

REFERENCES

- Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71:209-249.
- Mani S. Microbiota and breast cancer. *Prog Mol Biol Transl Sci*. 2017;151:217-229.
- Buchta Rosean C, Bostic RR, Ferey JCM, et al. Preexisting commensal dysbiosis is a host-intrinsic regulator of tissue inflammation and tumor cell dissemination in hormone receptor-positive breast cancer. *Cancer Res*. 2019;79:3662-3675.
- O'Hara AM, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep*. 2006;7:688-693.
- Vogtmann E, Goedert JJ. Epidemiologic studies of the human microbiome and cancer. *Br J Cancer*. 2016;114:237-242.
- Karlsson FH, Tremaroli V, Nookaew I, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature*. 2013;498:99-103.
- Scanlan PD, Shanahan F, Clune Y, et al. Culture-independent analysis of the gut microbiota in colorectal cancer and polyposis. *Environ Microbiol*. 2008;10:789-798.
- Gao R, Kong C, Huang L, et al. Mucosa-associated microbiota signature in colorectal cancer. *Eur J Clin Microbiol Infect Dis*. 2017;36:2073-2083.
- Turnbaugh PJ, Hamady M, Yatsunenko T, et al. A core gut microbiome in obese and lean twins. *Nature*. 2009;457:480-484.
- Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA*. 2007;104:13780-13785.
- Ott SJ, Musfeldt M, Wenderoth DF, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut*. 2004;53:685-693.
- Lee SH, Sung JY, Yong D, et al. Characterization of microbiome in bronchoalveolar lavage fluid of patients with lung cancer comparing with benign mass like lesions. *Lung Cancer*. 2016;102:89-95.
- Wang Z, Klipfell E, Bennett BJ, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 2011;472:57-63.
- Song Y, Liu C, Finegold SM. Real-time PCR quantitation of clostridia in feces of autistic children. *Appl Environ Microbiol*. 2004;70:6459-6465.
- Parracho HM, Bingham MO, Gibson GR, McCartney AL. Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. *J Med Microbiol*. 2005;54:987-991.
- Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. *Nature*. 2012;489:231-241.
- Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell*. 2014;157:121-141.
- Zhu J, Liao M, Yao Z, et al. Breast cancer in postmenopausal women is associated with an altered gut metagenome. *Microbiome*. 2018;6:136.
- Goedert JJ, Jones G, Hua X, et al. Investigation of the association between the fecal microbiota and breast cancer in postmenopausal women: a population-based case-control pilot study. *J Natl Cancer Inst*. 2015;107:djv147. doi:10.1093/jnci/djv147
- Parida S, Sharma D. The microbiome-estrogen connection and breast cancer risk. *Cells*. 2019;8:E1642.
- Chadha J, Nandi D, Atri Y, Nag A. Significance of human microbiome in breast cancer: tale of an invisible and an invincible. *Semin Cancer Biol*. 2021;70:112-127.
- Luu TH, Michel C, Bard JM, Dravet F, Nazih H, Bobin-Dubigeon C. Intestinal proportion of *blautia* sp. Is associated with clinical stage and histoprognostic grade in patients with early-stage breast cancer. *Nutr Cancer*. 2017;69:267-275.
- Banerjee S, Tian T, Wei Z, et al. Distinct microbial signatures associated with different breast cancer types. *Front Microbiol*. 2018;9:951.
- Urbanik C, Gloor GB, Brackstone M, Scott L, Tangney M, Reid G. The microbiota of breast tissue and its association with breast cancer. *Appl Environ Microbiol*. 2016;82:5039-5048.
- Hieken TJ, Chen J, Hoskin TL, et al. The microbiome of aseptically collected human breast tissue in benign and malignant disease. *Sci Rep*. 2016;6:30751.
- Michelsen CF, Pedas P, Glaring MA, Schjoerring JK, Stougaard P. Bacterial diversity in greenlandic soils as affected by potato cropping and inorganic versus organic fertilization. *Polar Biol*. 2014;37:61-71.
- Bokulich NA, Subramanian S, Faith JJ, et al. Quality-filtering vastly improves diversity estimates from illumina amplicon sequencing. *Nat Methods*. 2013;10:57-59.
- DeSantis TZ, Hugenholtz P, Larsen N, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with arb. *Appl Environ Microbiol*. 2006;72:5069-5072.
- Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*. 2007;73:5261-5267.
- Elahi R, Birkeland C, Sebens KP, Turner KR, Dwyer TR. Limited change in the diversity and structure of subtidal communities over four decades. *Mar Biol*. 2013;160:3209-3219.
- Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biol*. 2011;12:R60.
- Kanehisa M, Goto S, Kawashima S, Okuno Y, Hattori M. The KEGG resource for deciphering the genome. *Nucleic Acids Res*. 2004;32:D277-D280.
- Parks DH, Beiko RG. Identifying biologically relevant differences between metagenomic communities. *Bioinformatics*. 2010;26:715-721.
- Erdman SE, Pouthahidis T. Gut bacteria and cancer. *Biochim Biophys Acta*. 2015;1856:86-90.
- Francescone R, Hou V, Grivnenkov SI. Microbiome, inflammation, and cancer. *Cancer J*. 2014;20:181-189.

36. Lamb R, Ozsvári B, Lisanti CL, et al. Antibiotics that target mitochondria effectively eradicate cancer stem cells, across multiple tumor types: treating cancer like an infectious disease. *Oncotarget*. 2015;6:4569-4584.
37. Nanda N, Dhawan DK, Bhatia A, Mahmood A, Mahmood S. Doxycycline promotes carcinogenesis & metastasis via chronic inflammatory pathway: an in vivo approach. *PLoS One*. 2016;11:e0151539.
38. Velicer CM, Heckbert SR, Lampe JW, Potter JD, Robertson CA, Taplin SH. Antibiotic use in relation to the risk of breast cancer. *JAMA*. 2004;291:827-835.
39. Sørensen HT, Skriver MV, Friis S, McLaughlin JK, Blot WJ, Baron JA. Use of antibiotics and risk of breast cancer: a population-based case-control study. *Br J Cancer*. 2005;92:594-596.
40. García Rodríguez LA, González-Pérez A. Use of antibiotics and risk of breast cancer. *Am J Epidemiol*. 2005;161:616-619.
41. Velicer CM, Heckbert SR, Rutter C, Lampe JW, Malone K. Association between antibiotic use prior to breast cancer diagnosis and breast tumour characteristics (united states). *Cancer Causes Control*. 2006;17:307-313.
42. Friedman GD, Oestreicher N, Chan J, Quesenberry Cp Jr, Udaltsova N, Habel LA. Antibiotics and risk of breast cancer: up to 9 years of follow-up of 2.1 million women. *Cancer Epidemiol Biomarkers Prev*. 2006;15:2102-2106.
43. McKee AM, Kirkup BM, Madgwick M, et al. Antibiotic-induced disturbances of the gut microbiota result in accelerated breast tumor growth. *iScience*. 2021;24:103012.
44. Kirkup BM, McKee A, Makin KA, et al. Perturbation of the gut microbiota by antibiotics results in accelerated breast tumour growth and metabolic dysregulation. Preprint. Posted online January 1, 2019. BioRxiv 553602. doi:10.1101/553602
45. Elkrief A, Derosa L, Kroemer G, Zitvogel L, Routy B. The negative impact of antibiotics on outcomes in cancer patients treated with immunotherapy: a new independent prognostic factor? *Ann Oncol*. 2019;30:1572-1579.
46. Newman J, Kosaka T, Sheppard B, Fox J, Schauer D. Bacterial infection promotes colon tumorigenesis in *Apc(min/+)* mice. *J Infect Dis*. 2001;184:227-230.
47. Goedert JJ, Hua X, Bielecka A, et al. Postmenopausal breast cancer and oestrogen associations with the IgA-coated and IgA-noncoated faecal microbiota. *Br J Cancer*. 2018;118:471-479.
48. He C, Liu Y, Ye S, Yin S, Gu J. Changes of intestinal microflora of breast cancer in premenopausal women. *Eur J Clin Microbiol Infect Dis*. 2021;40:503-513.
49. Buchta Rosean CM, Rutkowski MR. The influence of the commensal microbiota on distal tumor-promoting inflammation. *Semin Immunol*. 2017;32:62-73.
50. Willing BP, Russell SL, Finlay BB. Shifting the balance: antibiotic effects on host-microbiota mutualism. *Nat Rev Microbiol*. 2011;9:233-243.
51. Yang J, Tan Q, Fu Q, et al. Gastrointestinal microbiome and breast cancer: correlations, mechanisms and potential clinical implications. *Breast Cancer*. 2017;24:220-228.
52. Kwa M, Plottel CS, Blaser MJ, Adams S. The intestinal microbiome and estrogen receptor-positive female breast cancer. *J Natl Cancer Inst*. 2016;108:djw029. doi:10.1093/jnci/djw029
53. Kovács T, Mikó E, Vida A, et al. Cadaverine, a metabolite of the microbiome, reduces breast cancer aggressiveness through trace amino acid receptors. *Sci Rep*. 2019;9:1300.
54. Kovács P, Csonka T, Kovács T, et al. Lithocholic acid, a metabolite of the microbiome, increases oxidative stress in breast cancer. *Cancers*. 2019;11:1255.
55. Mikó E, Vida A, Kovács T, et al. Lithocholic acid, a bacterial metabolite reduces breast cancer cell proliferation and aggressiveness. *Biochim Biophys Acta Bioenerg*. 2018;1859:958-974.
56. Nejman D, Liviyatan I, Fuks G, et al. The human tumor microbiome is composed of tumor type-specific intracellular bacteria. *Science*. 2020;368:973-980.
57. Soto-Pantoja DR, Gaber M, Arnone AA, et al. Diet alters entero-mammary signaling to regulate the breast microbiome and tumorigenesis. *Cancer Res*. 2021;81:3890-3904.
58. Fernández MF, Reina-Pérez I, Astorga JM, Rodríguez-Carrillo A, Plaza-Díaz J, Fontana L. Breast cancer and its relationship with the microbiota. *Int J Environ Res Public Health*. 2018;15:1747.
59. Bard J, Luu HT, Dravet F, et al. Relationship between intestinal microbiota and clinical characteristics of patients with early stage breast cancer. *FASEB J*. 2015;29:914-922.
60. Wu AH, Tseng C, Vigen C, et al. Gut microbiome associations with breast cancer risk factors and tumor characteristics: a pilot study. *Breast Cancer Res Treat*. 2020;182:451-463.
61. Jarman R, Ribeiro-Milograna S, Kalle W. Potential of the microbiome as a biomarker for early diagnosis and prognosis of breast cancer. *J Breast Cancer*. 2020;23:579-587.
62. Terrisse S, Derosa L, Iebba V, et al. Intestinal microbiota influences clinical outcome and side effects of early breast cancer treatment. *Cell Death Differ*. 2021;28:2778-2796.
63. Di Modica M, Gargari G, Regondi V, et al. Gut microbiota condition the therapeutic efficacy of trastuzumab in her2-positive breast cancer. *Cancer Res*. 2021;81:2195-2206.