ORIGINAL RESEARCH

Correlation between Anxiety, Depression, and Intestinal Flora in Breast Cancer Patients: Based on 16S rRNA Sequence Analysis

Chunfang Liu, MB; Tingting Yang, MB; Hongming Ji, MB; Shan Wang, MB; Xiuchuan Li, PhD

ABSTRACT

Objective • To explore intestinal flora differences in species diversity, community structure, and abundance of breast cancer and non-breast cancer populations with anxiety and depression and the corresponding group without anxiety and depression by 16S rRNA high-throughput sequencing technology.

Method • Breast cancer and non-breast cancer participants were recruited based on the inclusion and exclusion criteria as the research subjects. The study employed the anxiety self-assessment scale and the depression self-rating scale in the questionnaire survey to collect data.

Results • The scores of anxiety and depression of the four groups are as follows: In the breast cancer with anxiety and/or depression (BCAD) group, the anxiety score is 58.80 ± 5.27 and the depression score is 59.60 ± 4.94. In the breast cancer without anxiety and/or depression (BCWAD) group, the anxiety score is 36.53 ± 4.52 and the depression score is 38.20 ± 3.78. In the non-breast cancer group with anxiety and/or depression (HAD) group, the anxiety score is 57.87 ± 4.53 and the depression score is 59.13 ± 5.24. In the non-breast cancer group without anxiety and depression (HWAD) group, the anxiety score is 35.13 ± 5.28 and the depression score is 32.33 ± 4.37.

Conclusion • The intestinal flora of the breast cancer patients is significantly different from those of non-breast cancer patients, suggesting that there is an internal relationship between the changes in the intestinal flora and the occurrence and development of breast cancer. People with anxiety and depression without breast cancer show changes in their intestinal flora, suggesting that the changes of the intestinal flora can indeed trigger anxiety and depression. For the breast cancer patients with anxiety and depression, the intestinal flora shows a decrease in diversity and abundance, suggesting that the intestinal flora of the breast cancer patients with anxiety and depression undergo further changes. Thus the intestinal flora can become a new tool for monitoring, preventing, and treating the breast cancer and negative emotions. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

Breast Cancer (BC) is the most common cancer in the world and the leading cause of cancer death in women. Although the incidence is low in most parts of Asia compared to other countries, it is still the first in terms of incidence and the sixth leading malignancy associated with female mortality in China.[1-3] While the occurrence mechanism of BC is not entirely clear, DNA, genetic material, and environmental factors have been found to be closely related to its development.[4,5] In recent years, there has been a growing understanding of the role of intestinal flora in human health.[6] While the intestinal flora is recognized as a key factor in various diseases, this study specifically focuses on its relevance to breast cancer. More studies have confirmed the association between intestinal flora and diseases such as obesity,,[7] anxiety, depression.[8-10] Some research suggests that an unbalanced intestinal flora may contribute to the occurrence of non-alcoholic fatty liver disease[11] and Alzheimer's disease,[12] although the molecular mechanisms involved are not yet fully understood.

The intestinal flora has diverse functional roles, including providing nutrients and vitamins, protecting against pathogens, promoting immune system development, and maintaining

epithelial mucosal homeostasis.[13] In recent years, evidence from numerous studies has shown that microorganisms play a decisive role in diverse pathological conditions (including cancer).[14] It has been estimated that microbes may be involved in 15% to 20% of cancers.[15] In fact, the infiltration level of tumor-infiltrating lymphocytes in BC patients is found to be closely related to the diversity of intestinal flora.[16] Wang et al.[17] found that colon cancer patients show higher abundance of *Bacteroides fella*, Enterococcus, *Escherichia coli*, *Blebsella shigella*, *Streptococcus*, and *Peptococcus* than non-BC patients; and other bacteria such as *Streptococcus bovis* (a group of Gram-positive cocci) and *Enterotoxigenia fella* are also confirmed as risk markers for colon cancer.[18,19] These suggest that the intestinal flora may be used as an indicator to monitor the host for the non-BC status and predict and evaluate the host tumor occurrence.[20]

The intestinal tract contains nearly 100 trillion species of microbiome that share symbiotic properties with humans. The intestinal flora helps regulate mood and cognition by regulating the nervous, endocrine, and immune systems in two-way communication with brain.[21,22] Some studies have shown that intestinal flora plays an important role in regulating intestinal-brain function.[23] Brain-gut communication is driven by the vagus nerve. The vagus nerve connects to nearly 100 million neurons in the enteric nervous system, connecting afferent (vagus and spinal cord) and efferent adrenergic neurons (sympathetic and parasympathetic).[24] In addition, certain intestinal bacteria synthesize neurotransmitters[25] and nearly 20 neuropeptides are produced by the intestinal endocrine cells (central and peripheral neurons), and they act as second messengers to the brain, thereby regulating mood and cognition.[26] Negative emotions, along with other lifestyle factors of the non-BC can create an ecological imbalance, i.e., an imbalance between good and bad bacteria.[27] This imbalance will further aggravate negative emotions, which, in turn, will affect the intestinal flora, thus leading to further imbalance in the flora.

Many animal and human experiments have proved that the existence of negative emotions can change the intestinal flora. Yu et al.[28] has found that depression in rats leads to an increase in the abundance of *Bacteroidetes* while the abundance of *Firmicutes* decreases. Similarly, Lin et al.[29] found that the ratio of *Firmicutes* and *Bacteroidetes* is out of balance in patients with depression, with higher abundance of *Klebseria*, *Streptococcus*, *Prevotella*, and *Clostridium XI*. The intestinal biota is an important two-way interaction between the intestinal tract and the nervous system. Some changes, such as psychological distress or gastrointestinal infections, can affect these interactions and promote development and/or influence the development of anxiety and depression.[30]

Therefore, this paper intends to analyze the differences between the intestinal flora of the BC and the non-BC populations as well as those with anxiety and depression through high-throughput sequencing, so as to explore the changes in the intestinal flora of the BC patients and the changes in the intestinal flora of people with negative emotions on the basis of the changes in BC patients.

MATERIALS AND METHODS

Study subjects

Selection of the BC patients. Female BC patients treated in a Three-A hospital in Anhui Province from October, 2019 to October, 2020 are selected as the subjects of the BC group.

Inclusion criteria: (1) Patients with the BC confirmed through pathology or puncture results without surgery or chemoradiotherapy. (2) Patients between the ages of 18 and 55 years, being able to understand questionnaire questions, and complete them independently. (3) Distant metastasis of the tumor has not yet occurred. (4) Patients are cognitively normal and can communicate in Standard Chinese. (5) Patients and their families who have volunteered to participate in the study.

Exclusion criteria: (1) Patients with a variety of primary and other diseases. (2) In the three months prior to the fecal sample being taken, patients have taken antibiotics, steroid isohormone, or other hormone herbal preparations (including oral, intramuscular, or intravenous), and have taken probiotics such as probiotics or yoghurt. (3) Patients having hereditary BC. (4) Patients having systemic infection, or important organ failure. (5) Patients with mental illness, such as anxiety or depression.

Selection of women without BC. Patients in the psychological clinic of the hospital and medical staff of the hospital in the same period are selected as the non-BC control group.

Inclusion criteria: (1) Patients showing no obvious breast lesions after physical examination, B-ultrasonography, or ductoscopy; the report suggests normal. (2) Patients between the ages of 18 and 55 years, being able to understand questionnaire questions and complete them independently. (3) Patients are cognitively normal and can communicate in Standard Chinese. (4) The patients and their families have volunteered to participate in the study.

Exclusion criteria: (1) Patients with various primary diseases and other diseases. (2) In the three months prior to the fecal sample being taken, patients have taken antibiotics, steroid isohormone, or other hormone herbal preparations (including oral, intramuscular, or intravenous), and have taken probiotics such as probiotics or yoghurt. (3) Patients having mental illness, such as anxiety or depression.

Research methods

General data questionnaire is used to investigate the basic information of the BC patients and the non-BC population who meet the enrollment conditions. The self-rating depression scale and the self-rating anxiety scale are used to investigate their anxiety and depression emotions and then divide them into groups.

In the BC group, those who have reached the standard score of anxiety (elaborated later) and depression are classified as the BC with anxiety and/or depression group (BCAD group), while those who have not reached the standard score are classified as the BC without anxiety and

depression group (BCWAD group). Similarly, in the non-BC group, those who have reached the standard score of the anxiety and depression are classified as the non-BC group with anxiety and/or depression (HAD group), while those who have not reached the standard score are classified as the non-BC group without anxiety and depression (HWAD group). Feces samples are collected from all enrolled subjects for their intestinal flora sequencing analysis.

Questionnaire and scale. General information questionnaire. Self-made general information questionnaire, including name, age, occupation, educational level, family income, and other general demographic data was used.

Self-rating depression scale (SDS): It is used to measure the degree of depression and its change with treatment. There are 20 items in the scale, and each item is divided into 4 grades, 1 = no or very little time, 2 = a small amount of time, 3 = a considerable amount of time, 4 = most time or all time. The standard score (Y) is equal to the sum of the scores of each item multiplied by 1.25. The demarcating value of the standard score is 53 points, mild depression is between 53-62, moderate depression is 63-72, and major depression is ≥ 72. The reliability and validity of the scale are 0.806 and 0.921 respectively.[31]

Self-rating anxiety scale (SAS): It is a psychological scale used to measure the severity of anxiety state and its changes with treatment. SAS scale has 20 items and the scoring method is the same as SDS. According to the results of Chinese norm, the cut-off value of the standard score of the self-rating anxiety scale is 50 points, with a range of 50-59 points for mild anxiety, 60-69 points for moderate anxiety, and over 70 points for severe anxiety. The Cronbach's α coefficient of this scale is 0.791.[32]

Sequencing analysis method of the intestinal flora. Feces specimens are kept. All the BC patients in the group are instructed to take feces specimens in the morning of the day before their surgery, and those in the non-BC group are instructed to take feces specimens in the morning of the day after the questionnaire. The samples are taken in strict accordance with the instructions of the specimen box, sealed and stored in a -80°C refrigerator.

Subsequently, the feces samples are extracted for DNA, PCR amplification.

Main laboratory reagents: E.Z.N.ATM Mag-Bind Soil DNA Kit, Qubit 3.0 DNA, Detection kits, 2× Taq Master Mix, and MagicPure Size Selection DNA Beads.

Main experimental instruments: PICO-21 table top centrifuge, GL-88B vortex mixer, TND03-H-H mixed dry thermostats, electrophoresis apparatus power supply, electrophoresis tank, electrophoresis tank, gel imaging system, Qubit 3.0 fluorometer, PCR instrument, and pipette.

Sample pretreatment: To weigh 200 mg sample and put it into a 2 mL sterilized centrifuge tube. Add 1 ml of 70% ethanol, stir and mix, centrifuge at 10000 rpm room temperature for 3 minutes, and discard the upper liquid. To add 1x PBS solution, stir and mix, centrifuge at 10000 rpm room temperature for 3 minutes, and discard the upper liquid. To invert the 2 mL tube on the absorbent paper for 1 minutes, until no liquid flows out. To put the sample tube into the 55-metal bath for 10 minutes to completely volatilize the residual alcohol and ensure subsequent experimental operations.

Extract DNA: For specific extraction steps, to refer to the instruction manual of OMEGA Kit E.Z.n.tm Mag-Bind Soil DNA Kit.

Specimens quality test: A total of 60 cases of bacterial DNA are extracted from stool samples of the BC and the non-BC population. To test the ratio of A260/280 and determine the total DNA concentration, and make the electrophoretic map.

PCR amplification and DNA purification recovery: First round amplification: Quantify the genomic DNA accurately by using the Qubit 3.0 DNA test kit to determine how much DNA should be added to the PCR reaction. The primers used for PCR are fused with 16SV3-V4 universal primers from Miseq sequencing platform.

341F primers: CCCTACACGACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCWGCAG

805R primers: GACTGGAGTTCCTTGGCACCCGA GAATTCCA GACTACHVGGGTATCTAATCC

To use a pipette to gently blow or shake the mixture, and briefly centrifuge the reaction liquid to the bottom of the tube.

PCR amplification is performed in the configured PCR system according to the following reaction conditions: 95°C, 3 min →→ (94°C, 20 sec → 55°C, 20 sec → 72°C, 30 sec) 5 cycles →→ 72°C, 5 min →→ 10°C, ∞.

Second round amplification: To introduce Illumina bridge PCR compatible primers by using the products of the first round of amplification.

In sterile PCR tubes, the PCR tube is placed in a PCR instrument for amplification.

PCR reaction conditions: 95°C, 3 min →→ (94°C, 20 sec → 55°C, 20 sec → 72°C, 30 sec) 5 cycles →→ 72°C, 5 min →→ 10°C, ∞.

After the amplification, product purification is performed. For the PCR products amplified by bacteria and archaea and the PCR products with normal amplified fragment more than 400 bp, Agencourt AMPure XP is treated with 0.6 times of magnetic beads. For the fungal PCR products and other PCR products with amplification fragment less than 400 bp, Agencourt AMPure XP is treated with 0.8 times of magnetic beads.

Quantitative hybrid: To quantify the recovered DNA accurately, the Qubit 3.0 DNA test kit is used to facilitate the sequencing of 1:1 equal amount of mixing. 10 ng DNA is measured from each sample mixture, and the final sequencing concentration is 20 pmol.

Library quality test: The library size is determined by 2% agarose gel electrophoresis. The library concentration is determined by Qubit 3.0 fluorescence quantifier.

Sequencing project analysis process: Paired-end (PE) reads obtained from the second-generation sequencing are

first spliced according to the overlap relationship, and the sequence quality is controlled and filtered after sample distinction, and then operational taxonomic units (OTU) clustering (amplicon sequence variant (ASV) denoising) analysis and taxonomic analysis are carried out. Based on the results of OTU cluster analysis, diversity index analysis and sequencing depth detection can be performed. Based on taxonomic information, statistical analysis of community structure can be performed at each classification level. On the basis of the above analysis, a series of in-depth statistical and visual analyses, such as Beta diversity analysis, grouping test, difference significance test, correlation analysis of environmental factors, correlation and model prediction analysis, and functional prediction, can be carried out on the community composition and phylogenetic information of diverse species.

Sequence software and statistical methods

USEARCH, Cutadapt, Mother, STAMP, and R software package are used for statistical analysis during sequencing. Other data are analyzed by SPSS 25.0 statistical software.

Enumerative data are represented by (n, %), χ^2 test is used, the measurement data of normal distribution are expressed by mean standard deviation, *t* test and variance analysis are performed, and $P < .05$ indicates that the difference is statistically significant.

RESULTS

Results of questionnaire and scale

Results of general information. A total of 60 people is included in this experiment, all are of Han nationality, and do not smoke or drink. The age of the BC group members ranged from 29 to 50 years, while the age of the non-BC group members ranged from 25 to 49. There is no statistically significant difference ($P > .05$) in general data among the four groups, which is comparable. See table 1 for comparison.

Scores of anxiety and depression of the four groups. The score of anxiety in the BC group is 47.67 ± 12.31, which corresponds to 58.80 ± 5.27 in the BCAD group (including 9 patients with mild symptoms, 5 patients with moderate symptoms, and 1 patient with severe symptoms) and 36.53 ± 4.52 in the BCWAD group. The comparison between the two groups is statistically significant ($P < .05$). The score of anxiety in the non-BC group is 46.50 ± 12.53, which corresponds to 57.87 ± 4.53 in the HAD group (including 10 patients with mild symptoms, and 5 patients with moderate symptoms) and 35.13 ± 5.28 in the HWAD group. The comparison between the two groups is statistically significant ($P < .05$). The comparison of the four groups reveals statistically significant differences ($P < .05$) in terms of anxiety but there is no overall difference between the BC group and the non-BC group ($P > .05$). See table 2.

The score of depression in the BC group is 48.90 ± 11.71, which corresponds to 59.60 ± 4.94 in the BCAD group (including 12 patients with mild symptoms and 3 patients with moderate symptoms) and 38.20 ± 3.78 in the BCWAD group. The comparison between the two groups is statistically significant ($P < .05$). The score of depression in the non-BC group is 45.73 ± 14.43, which corresponds to 59.13 ± 5.24 in the HAD group (including 11 patients with mild symptoms and 4 patients with moderate symptoms and 32.33 ± 4.37 in the HWAD group. The comparison between the two groups is statistically significant ($P < .05$). The comparison of the four groups reveals statistically significant difference ($P < .05$) in terms of depression but there is no overall difference between the BC groups and the non-BC groups ($P > .05$), See table 3.

Table 1. General Information Comparison of the Four Groups

Item	BCAD Group	BCWAD Group	HAD Group	HWAD Group	F/χ^2	*P* value
Age (age, $\bar{x} \pm s$)	42.67 ± 7.29	44.67 ± 5.64	39.13 ± 6.08	39.40 ± 6.71	2.57	.064
Career (n, %)						
Peasant	7 (46.67)	5 (33.33)	5 (33.33)	4 (26.67)		
On job	5 (33.33)	6 (40.00)	8 (53.33)	10 (66.67)	4.94	.552
Unemployed	3 (20.00)	4 (26.67)	2 (13.33)	1 (6.67)		
Education (n, %)						
Junior high and below	5 (33.33)	6 (40.00)	4 (26.67)	3 (20.00)		
Senior high/Secondary technical	4 (26.67)	4 (26.67)	5 (33.33)	4 (26.67)	3.52	.940
Junior college	4 (26.67)	3 (20.00)	3 (20.00)	3 (20.00)		
College and above	2 (13.33)	2 (13.33)	3 (20.00)	5 (33.33)		
Menstrual status (n, %)						
Regular	9 (60.00)	10 (66.67)	11 (73.33)	11 (73.33)		
Menolipsis	2 (13.33)	3 (20.00)	2 (13.33)	1 (6.67)	2.27	.893
Irregular (excessive menstruation/ hypomenorrhea /Irregular menstrual cycle)	4 (26.67)	2 (13.33)	2 (13.33)	3 (20.00)		
Monthly family income (n, %)						
<2000	5 (33.33)	5 (33.33)	3 (20.00)	2 (13.33)		
2000-3000	3 (20.00)	2 (13.33)	2 (13.33)	3 (20.00)		
3000-4000	4 (26.67)	3 (20.00)	3 (20.00)	3 (20.00)	5.10	.955
4000-5000	2 (13.33)	3 (20.00)	4 (26.67)	3 (20.00)		
>5000	1 (6.67)	2 (13.33)	3 (20.00)	4 (26.67)		
Neoplasms staging (n, %)						
I-II Period	6 (40.00)	8 (53.33)	-	-	0.54	.464
III-IV Period	9 (60.00)	7 (46.67)				

Note: Measurement data are described by mean ± standard deviation and variance analysis. Count data is described by case and percentage, and test by 2 or Fisher exact probability method.

Table 2. Anxiety Score Comparison of the Four Groups ($\bar{x} \pm s$)

Grouping	Score	Grouping	Score	*t*	*P* value
BC group	47.67 ± 12.31	BCAD	58.80 ± 5.27	12.43	.000
		BCWAD	36.53 ± 4.52		
Non-BC group	46.50 ± 12.53	HAD	7.87 ± 4.53	12.66	.000
		HWAD	35.13 ± 5.28		
F/t	0.36		105.17	-	-
P value	.717		.000	-	-

Table 3. Depression Score Comparison of the Four Groups ($\bar{x} \pm s$)

Grouping	Score	Grouping	Score	*t*	*P* value
BC group	48.90 ± 11.71	BCAD	59.60 ± 4.94	13.32	.000
		BCWAD	38.20 ± 3.78		
Non-BC group	45.73 ± 14.43	HAD	59.13 ± 5.24	15.22	.000
		HWAD	32.33 ± 4.37		
F/t	0.93	-	140.37	-	-
P value	0.355	-	.000	-	-

Results of sequencing analysis of the intestinal flora

Analysis results of sequence number of each group of samples. Using high-throughput sequencing of V3-V4 region of fecal DNA, and taxonomic analysis of OTU sequence number at 97% similar level, 4585137 effective sequences are obtained from all sample data of the four groups after quality control filtering, with a total base number of 1889546487 bp and an effective average length of 412.01

bp, of which the effective length of 95% samples is between 405-420 bp, and the sample distribution is shown in Figure 1.

A total of 2695227 effective sequences are obtained in the BC group, with a total base number of 1114087449 bp, the original mean length of 12411.71 bp, and the mean effective sequences of 89840 ± 21945.64, among which the mean effective sequences of BCAD group and BCWAD group are 89924.80 ± 2275.126 and 89757.00 ± 21908.62 respectively. There is no statistically significant difference between the two groups ($P > .05$).

A total of 1889911 effective sequences are obtained in the non-BC group, with 775459038 bp base number and original mean length of 12308.80 bp. The average effective sequences are 62997 ± 4065.43, among which the average effective sequences in the HAD group and HWAD group are 63726.93 ± 3595.93 and 62267.07 ± 4490.39 respectively. There is no statistically significant difference between the two groups ($P > .05$).

The difference in the mean effective sequence number between the BC group and the non-BC group is statistically significant ($P < .05$). Similarly, the difference in the mean effective sequence number among the four groups is also statistically significant ($P < .05$). See table 4 for details.

Rank-abundance curve analysis results of each group of samples. According to rank-abundance curve (Figure 2), this experiment involves a sample size of abundance, that is, the length of the horizontal axis is wider, without any obvious difference. OTU grade is greater than 200, which shows that the intestinal flora richness in various samples is consistent, eliminates the error in experimental results caused due to the large gap between individual samples, and increases the credibility of the experimental results. However, for the evenness of species composition, it can be seen from the shape of the curve that the abundance of all samples decreases with the increase of the OTU level, and all samples tend to be flat when OTU grade is greater than 200 and abundance is less than 0.01. The flat curve indicates that the species composition of the sample is highly uniform. In this study, the species richness of all samples is consistent (OTU grade is greater than 200), and the evenness is high (OTU abundance is below 0.01 and gradually reaches a plateau), implying that it has good representativeness, the data obtained by sequencing is reliable, and the results are accurate.

Diversity index results analysis. In order to verify that the amount of sequencing data of each group of samples at the 97% similarity level meets the requirements of this experiment, the dilution curve of all samples is first drawn (Figure 3). The dilution curve of each sample increases slowly when the number of sequences is about 10 000, and the curves tend to flatten gradually with the increasing number of detection sequences. When the number of sequences reaches 30 000, all the four groups are in a plateau stage. The sample dilution curves of each group confirm that the amount of sequencing data in this experiment is sufficient to cover all bacterial species in the intestinal tract. In addition, it also indicates that the species abundance in the samples meets the requirements of subsequent sequencing. It further verifies that the high species abundance of the above rank-abundance curve, and the results are credible. To further illustrate that the samples in this experiment reach the required amount for sequencing, the dilution curve of Chao is plotted (see Figure 4). Chao curve shows that the sequence number of dilution curve of each sample also increases slowly when it is about 10 000, and tends to be flat when it reaches 30 000, which further verifies that the required sequencing quantity is sufficient and the sample richness is good in this study.

Table 4. Comparison of the Number of Valid Sequences Among the Four Groups ($\bar{x} \pm s$)

Grouping	Effective sequence number	Grouping	Effective sequence number	*t*	*P* value
BC group	89840 ± 21945.64	BCAD	89924.80 ± 2275.126	0.02	.984
		BCWAD	89757.00 ± 21908.62		
Non-BC group	62997 ± 4065.43	HAD	63726.93 ± 3595.93	0.98	.334
		HWAD	62267.07 ± 4490.39		
F/*t*	6.59	-	14.00	-	-
P value	.000	-	0.000	-	-

Figure 1. Length Distribution of Valid Sequence Data

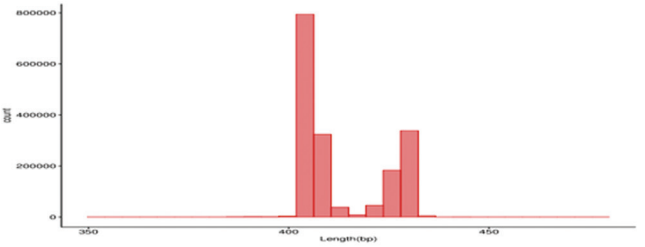
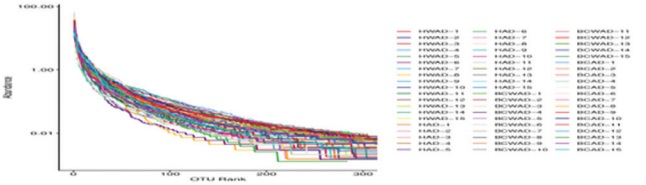
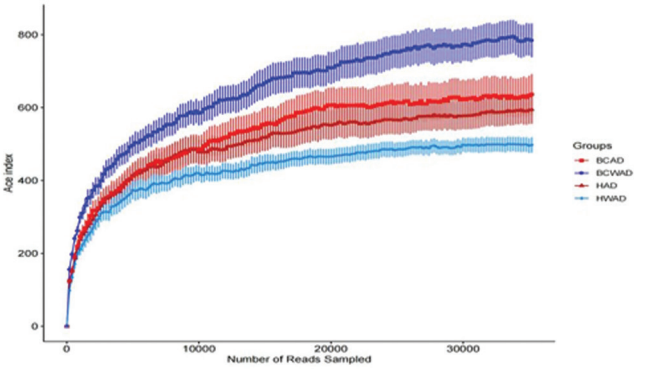


Figure 2. Rank-Abundance Graph



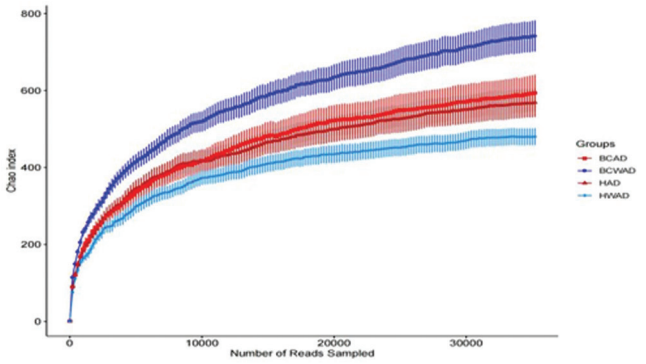
Note: The Horizontal Axis is the OTU Rank Value Arranged in Descending Order According to the Relative Abundance Content Level of OTU, and the Vertical Axis is the Abundance Value of Samples Under This OTU. Each Curve is a Sample.

Figure 3. Alpha Index Dilution Curves



Note: Horizontal Axis is the Number of Sequences Randomly Selected from the Sample, While Vertical axis is the Corresponding Ace Index Obtained. Each Curve is a Group.

Figure 4. Chao Dilution Curves



Note: Horizontal Axis is the Number of Sequences Randomly Selected from the Sample, While Vertical axis is the Corresponding Chao Index Obtained. Each Curve is a Group.

Table 5. Diversity Index Analysis Between the Breast Cancer Group and the Non-Breast Cancer Group ($\bar{x} \pm s$)

Index classification	BC group	Non-BC group	*t*	*P* value
Shannon index	3.50 ± 0.50	3.38 ± 0.56	0.85	.400
Chao index	740.68 ± 204.42	565.25 ± 138.62	3.89	.000
Ace index	755.15 ± 210.8	572.94 ± 135.75	3.98	.000
Simpson index	0.09 ± 0.08	0.10 ± 0.09	-0.20	.841
Shannon-even index	0.55 ± 0.0	0.55 ± 0.07	0.19	.852
Coverage index	1.00 ± 0.00	1.00 ± 0.00	-5.09	.000

Table 6. Diversity Index Analysis Between the BCAD Group and the BCWAD Group ($\bar{x} \pm s$)

Index classification	BCAD group	BCWAD group	*t*	*P* value
Shannon index	3.28 ± 0.57	3.72 ± 0.31	-2.65	.013
Chao index	666.79 ± 207.84	814.58 ±177.93	-2.09	.046
Ace index	688.16 ± 220.21	822.13 ± 184.26	-1.81	.082
Simpson index	0.12 ± 0.11	0.07 ± 0.04	1.66	.108
Shannon-even index	0.53 ± 0.08	0.58 ± 0.05	-2.27	.031
Coverage index	1.00 ± 0.00	1.00 ± 0.00	-2.89	.007

Table 7. Diversity Index Analysis of the HAD Group and the HWAD Group ($\bar{x} \pm s$)

Index classification	HAD Group	HWAD Group	*t*	*P* value
Shannon index	3.61 ± 0.44	3.15 ± 0.59	2.40	.023
Chao index	615.48 ± 157.98	515.01 ± 97.14	2.10	.045
Ace index	621.11 ± 155.63	524.76 ± 94.76	2.05	.050
Simpson index	0.07 ± 0.03	0.13 ± 0.11	-2.28	.030
Shannon-even index	0.58 ± 0.06	0.52 ± 0.88	2.30	.029
Coverage index	1.00 ± 0.00	1.00 ± 0.00	-1.61	.119

Table 8. Diversity Index Analysis of the Four Groups of Research Objects ($\bar{x} \pm s$)

Index classification	BCAD Group	BCWAD Group	HAD Group	HWAD Group	*F*	*P* value
Shannon index	3.28 ± 0.57	3.72 ± 0.31	3.61 ± 0.44	3.15 ± 0.59	4.49	.007
Chao index	666.79 ± 207.84	814.58 ± 177.93	615.48 ± 157.98	515.01 ± 97.14	8.56	.000
Ace index	688.16 ± 220.21	822.13 ± 184.26	621.11 ± 155.63	524.76 ± 94.76	8.10	.000
Simpson index	0.12 ± 0.11	0.07 ± 0.04	0.07 ± 0.03	0.13 ± 0.11	2.68	.056
Shannon-even index	0.53 ± 0.08	0.58 ± 0.05	0.58 ± 0.06	0.52 ± 0.88	3.49	.021
Coverage index	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	15.00	.000

The species-based dilution curves show that the sequencing quantity is sufficient. Statistical analysis of the diversity indices of the four groups shows that:

1) The analysis of all the specimens from the BC and the non-BC groups shows that the Chao index and the ACE index reflecting species abundance in the BC group are higher than those in the non-BC group, while the Coverage index reflecting species diversity in the BC group is lower than that in the non-BC group. The two groups had statistical significance ($P < .05$). However, there is no statistical significance between the Shannon index and the Simpson index reflecting species diversity, as well as the Shannon-even index reflecting species uniformity ($P > .05$). See Table 5.

2) The analysis of the BCAD group and the BCWAD group in the BC group shows that in the Chao index reflecting species abundance, the Shannon index and the Coverage index reflecting species diversity, and the Shannon-even index reflecting species evenness, the BCAD group is lower than the BCWAD group. There are differences between the two groups ($P < .05$). However, there is no statistically significant difference between the Ace index reflecting species abundance and the Simpson index reflecting species diversity in the two groups ($P > .05$). See Table 6.

3) The analysis of the HAD group and the HWAD group in the non-BC group shows that in the Chao index reflecting species abundance, the Shannon index reflecting species diversity, and the Shannon-even index reflecting species evenness, are all higher in the HAD group than the HWAD group. In case of the Simpson index reflecting species diversity, the HAD group is lower than the HWAD group (the higher the Simpson index value, the lower the community diversity), and there are statistically significant differences between the two groups ($P < .05$). However, here is no differences between the Chao index reflecting species abundance and the Coverage index reflecting species diversity ($P > .05$). See Table 7.

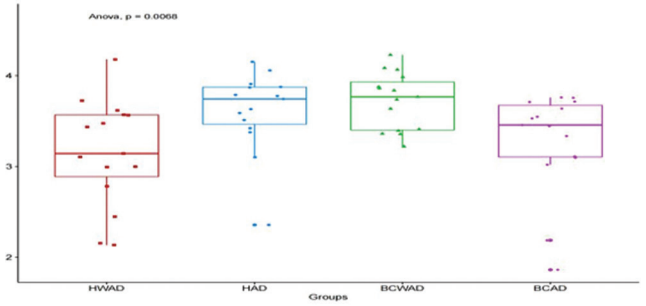
4) Further analysis of all specimens of the four groups shows that there is statistically significant difference between the Chao index and the Ace index reflecting species abundance, the Shannon index and the Coverage index reflecting species diversity, and the Shannon-even index reflecting species evenness in the four groups ($P < .05$). However, the Simpson index reflecting species diversity has no statistically significant difference ($P > .05$). See Table 8.

The above research results show that there are significant differences in the diversity of the intestinal flora among the four groups.

In order to verify and further confirm the authenticity and reliability of the above results, the Alpha index difference test box plot between groups is made, and the results show that the index line graphs are consistent with the above results, see Figures 5A, 5B, 5C, 5, 5, 5F.

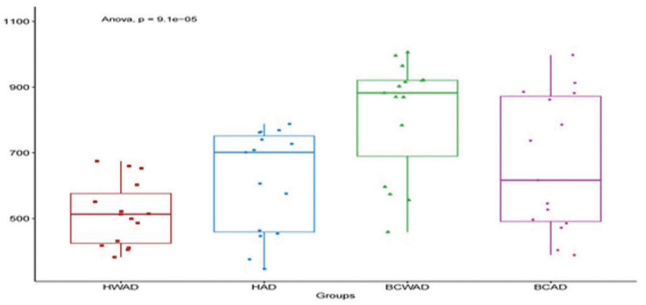
Analysis results of differences in sample communities. Phylum level analysis: 1)Analysis of all specimens from the BC and non-BC groups shows that there are statistical differences between the two groups in 19 species of bacteria including *Armatimonadetesm*, *Acidobacteria*, *Firmicutes*, and *Bacteroidetes* etc. In the non-BC group, the abundance of 6 species of bacteria (*Nitrospirae*, Unclassified, *Acidobacteria*, *Gemmatimonadetes*, *Actinobacteria*, and *Firmicutes*) is higher than that of the BC group. Whereas, the abundance of other bacteria is lower in the non-BC group than that of the BC group ($P_{average} < .05$). See Figure 6 and Table 9.

Figure 5A. Exponential Difference Test Boxplot of the Shannon Index Between Groups



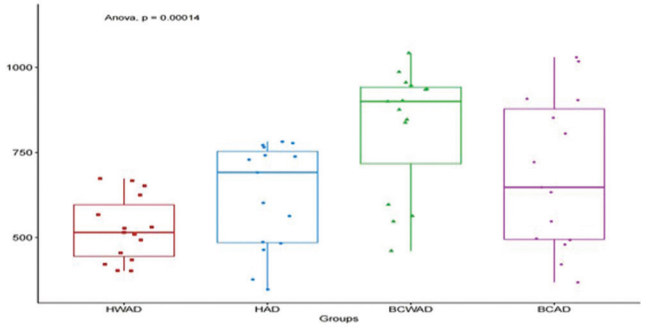
Note: The Horizontal Axis is the Group Name, and the Vertical Axis is the Alpha Index of Each Group. The *t* test is Used for the Two Groups, and the Anova Test is Used for Inter-Group Comparison.

Figure 5B. Exponential Difference Test Boxplot of the Chao Index Between Groups



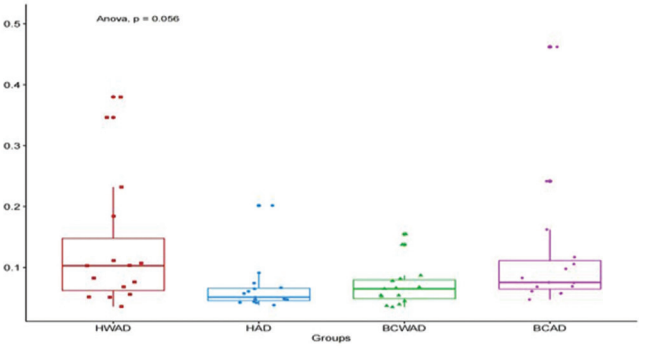
Note: The Horizontal Axis is the Group Name, and the Vertical Axis is the Alpha Index of Each Group. The *t* test is Used for the Two Groups, and the Anova Test is Used for Inter-Group Comparison.

Figure 5C. Exponential Difference Test Boxplot of the Ace Index Between Groups



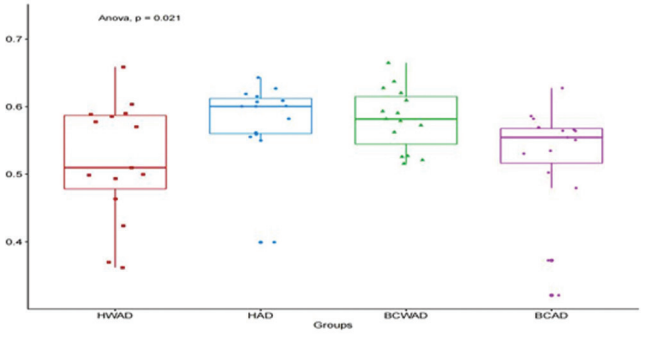
Note: The Horizontal Axis is the Group Name, and the Vertical Axis is the Alpha Index of Each Group. The *t* test is Used for the Two Groups, and the Anova Test is Used for Inter-Group Comparison.

Figure 5D. Exponential Difference Test Boxplot of the Simpson Index Between Groups



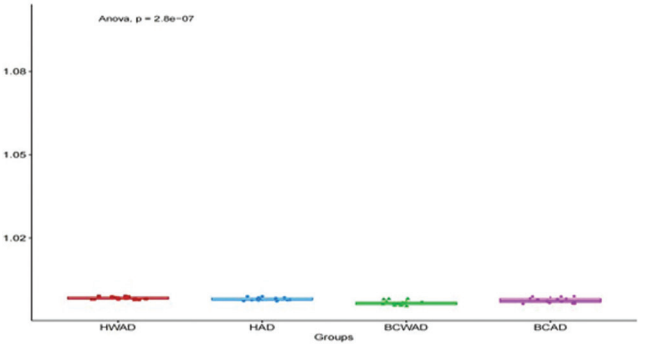
Note: The Horizontal Axis is the Group Name, and the Vertical Axis is the Alpha Index of Each Group. The *t* test is Used for the Two Groups, and the Anova Test is Used for Inter-Group Comparison.

Figure 5E. Exponential Difference Test Boxplot of the Shannon-even Index Between Groups



Note: The Horizontal Axis is the Group Name, and the Vertical Axis is the Alpha Index of Each Group. The *t* test is Used for the Two Groups, and the Anova Test is Used for Inter-Group Comparison.

Figure 5F. Exponential Difference Test Boxplot of the Coverage Index Between Groups



Note: The Horizontal Axis is the Group Name, and the Vertical Axis is the Alpha Index of Each Group. The *t* test is Used for the Two Groups, and the Anova Test is Used for Inter-Group Comparison.

2) Upon comparison of the BCAD group with the BCWAD group, the abundance of unclassified bacteria and *synergistetes* in the BCAD group are lower than that found in the BCWAD group. The difference is statistically significant ($P < .05$). See Table 10 and Figure 7.

3) Upon comparison of the HAD group with the HWAD group, the abundance of Planctomycetes in the HAD group is found to be higher than that in the HWAD group. The difference is statistically significant ($P < .05$). See Table 10 and Figure 7.

Class level analysis: 1) Analysis of all specimens from the BC and the non-BC groups show that there are statistical differences between the two groups in 33 species of bacteria including *Nitrospira*, *Bacteroidia*, *Epsilonproteobacteria*, and *Chlorobia*, etc. In the non-BC group, in addition to *Acidobacteria* (including Gp6, Gp17, unclassified, Gp16,

Figure 6. Relative Abundance of the Intestinal Flora in the Four Groups at the Phylum Level

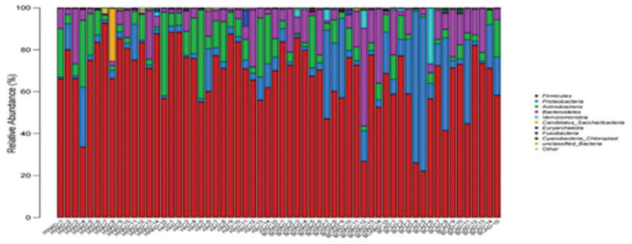


Table 9. Difference Analysis of Phylum Flora Between the Breast Cancer Group and the Non-Breast Cancer Group ($\bar{x} \pm s$)

Flora name	BC group	Non-BC group	*t*	*P* value
Armatimonadetes	0.05 ± 0.04	0.01 ± 0.0	4.90	.000
Planctomycetes	0.01 ± 0.01	0.00 ± 0.00	4.59	.000
Chloroflexi	0.12 ± 0.10	0.04 ± 0.06	3.94	.000
Aminicenantes	0.00 ± 0.00	0.00 ± 0.00	4.86	.000
Nitrospirae	0.01 ± 0.01	0.07 ± 0.08	-4.04	.000
Unclassified	0.00 ± 0.00	0.38 ± 0.53	-3.88	.000
Chlamydiae	0.00 ± 0.00	0.00 ± 0.00	4.50	.000
Ignavibacteriae	0.00 ± 0.00	0.00 ± 0.00	3.60	.001
Acidobacteria	0.04 ± 0.03	0.13 ± 0.14	-3.54	.001
Deferribacteres	0.01 ± 0.01	0.00±0.00	3.18	.002
Gemmatimonadetes	0.01 ± 0.01	0.02 ± 0.02	-3.04	.004
Proteobacteria	16.73 ± 20.27	5.39 ± 6.56	2.92	.005
Actinobacteria	6.00 ± 5.35	12.68 ± 11.26	-2.93	.005
Firmicutes	62.91 ± 17.18	73.13 ± 12.94	-2.60	.012
Deinococcus-Thermus	0.00 ± 0.00	0.00 ± 0.00	2.39	.020
Chlorobi	0.00 ± 0.00	0.00 ± 0.00	2.28	.026
Synergistetes	0.01 ± 0.01	0.00 ± 0.00	2.27	.027
Bacteroidetes	11.82 ± 10.93	6.71 ± 7.46	2.12	.039
Thermotogae	0.00 ± 0.00	0.00 ± 0.00	2.11	.039

Table 10. Difference Analysis of Phylum Flora Between the BCAD Group and the BCWAD Group, along with the Difference Analysis of Phylum Flora Between the HAD Group and the HWAD Group ($\bar{x} \pm s$)

Flora name	BCAD Group	BCWAD Group	*t*	*P* value
unclassified_Bacteria	0.09 ± 0.10	0.29 ± 0.27	-2.70	.015
Synergistetes	0.00 ± 0.00	0.01 ± 0.02	-2.45	.028
Flora name	**HAD Group**	**HWAD Group**	***t***	***P value***
Planctomycetes	0.00 ± 0.00	0.00 ± 0.00	2.49	.024

Gp10, and Gp4), the abundance of 12 species of bacteria, such as *Nitrospira*, *Gemmatimonadetes*, *Actinobacteria*, and so on, is higher than that of the BC group. Other species in the non-BC group are all lower than that of the BC group ($P_{average} < .05$). See Table 11 (only the 25 bacteria with the lowest *P* values are listed).

2) Upon comparing the BCAD group with the BCWAD group, the abundance of 6 species of bacteria (including *Cytophagia*, unclassified-Bacteria, *Epsilonproteobacteria*, *Synergistia*, *Flavobacterii*, and *Acidobacteria*_Gp6) in the BCAD group is found to be lower than the BCWAD group. The difference is statistically significant (*P average* < .05). See Table 12 and Figure 9.

3) Upon comparing the HAD group with the HWAD group, the abundance of *Planctomycetia* in the HAD group is found to be higher than the HWAD group. The difference is statistically significant ($P < .05$). See Table 13 and Figure 9.

Order level analysis: 1) Analysis of all specimens from the BC and the non-BC groups shows that there are statistical differences between the two groups in 55 species of bacteria

Figure 7. Results of the Phylum Level Difference Analysis Between the BCAD Group and the BCWAD Group, and Between the HAD group and the HWAD group

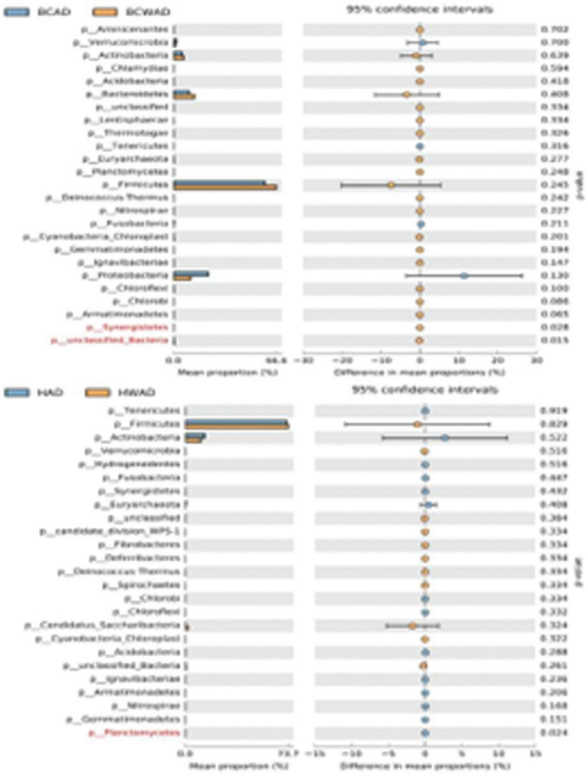


Figure 8. Relative Abundance of the Intestinal Flora in the Four Groups at the Class Level

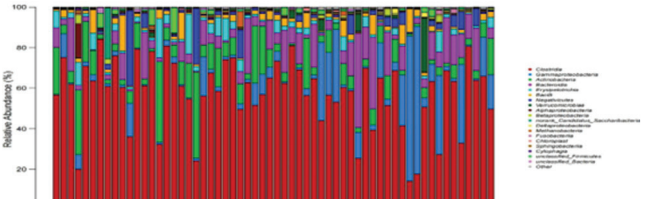


Table 11. Difference Analysis of the Class Level Flora Between the Breast Cancer Group and the Non-Breast Cancer Group ($\bar{x} \pm s$)

Flora name	BC group	Non-BC group	*t*	*P* value
Epsilonproteobacteria	0.09 ± 0.04	0.01 ± 0.04	8.12	.000
Acidobacteria_Gp6	0.00 ± 0.00	0.01 ± 0.01	-4.82	.000
norank_*Armatimonadetes*	0.05 ± 0.04	0.01 ± 0.01	5.04	.000
Anaerolineae	0.09 ± 0.07	0.01 ± 0.01	5.80	.000
Acidobacteria_Gp17	0.00 ± 0.00	0.02 ± 0.03	-3.70	.000
Nitrospira	0.01 ± 0.01	0.07 ± 0.08	-2.60	.000
Planctomycetia	0.01 ± 0.01	0.00 ± 0.00	5.09	.000
Acidobacteria_Gp16	0.00 ± 0.00	0.01 ± 0.01	-4.70	.000
Subdivision3	0.00 ± 0.00	0.00 ± 0.00	4.78	.000
unclassified_Actinobacteria	0.00 ± 0.00	0.01 ± 0.01	-4.09	.000
Spartobacteria	0.00 ± 0.00	0.00 ± 0.00	4.13	.000
Chlamydiia	0.00 ± 0.00	0.00 ± 0.00	4.50	.000
norank_Aminicenantes	0.00 ± 0.00	0.00 ± 0.00	4.86	.000
unclassified_Verrucomicrobia	0.00 ± 0.00	0.00 ± 0.00	4.46	.000
Ignavibacteria	0.00 ± 0.00	0.00 ± 0.00	3.60	.001
Acidobacteria_Gp10	0.00 ± 0.00	0.02 ± 0.02	-3.36	.001
Acidobacteria_Gp4	0.01 ± 0.02	0.05 ± 0.06	-3.28	.002
Deferribacteres	0.01 ± 0.01	0.00 ± 0.00	3.18	.002
Gammaproteobacteria	15.07 ± 20.16	3.79 ± 4.96	2.98	.004
Gemmatimonadetes	0.01 ± 0.01	0.02 ± 0.02	-3.04	.004
Actinobacteria	6.00 ± 5.35	12.67 ± 11.26	-2.93	.005
Deltaproteobacteria	0.44 ± 0.58	0.14 ± 0.16	2.76	.008
Flavobacteriia	0.02 ± 0.02	0.01 ± 0.03	2.59	.012
Holophagae	0.00 ± 0.00	0.00 ± 0.00	-2.55	.013
Deinococci	0.00 ± 0.00	0.00 ± 0.00	2.39	.020

Table 12. Difference Analysis of the Class Level Flora Between the BCAD Group and the BCWAD Group ($\bar{x} \pm s$)

Flora name	BCAD Group	BCWAD Group	*t*	*P* value
Cytophagia	0.00 ± 0.00	0.01 ± 0.00	-3.11	.000
unclassified_Bacteria	0.09 ± 0.10	0.29 ± 0.27	-2.70	.015
Epsilonproteobacteria	0.07 ± 0.04	0.11 ± 0.03	-2.42	.023
Synergistia	0.00 ± 0.00	0.01 ± 0.02	-2.45	.028
Flavobacteriia	0.01 ± 0.02	0.03 ± 0.03	-2.13	.044
Acidobacteria_Gp6	0.00 ± 0.00	0.00 ± 0.00	-2.17	.048

Table 13. Difference Analysis of the Class Level Flora Between the HAD Group and the HWAD Group ($\bar{x} \pm s$)

Flora name	HAD Group	HWAD Group	*t*	*P* value
Planctomycetia	0.00 ± 0.00	0.00 ± 0.00	2.40	.031

Figure 9. Results of the Class Level Difference Analysis Between the BCAD Group and the BCWAD Group, and Between the HAD Group and the HWAD Group

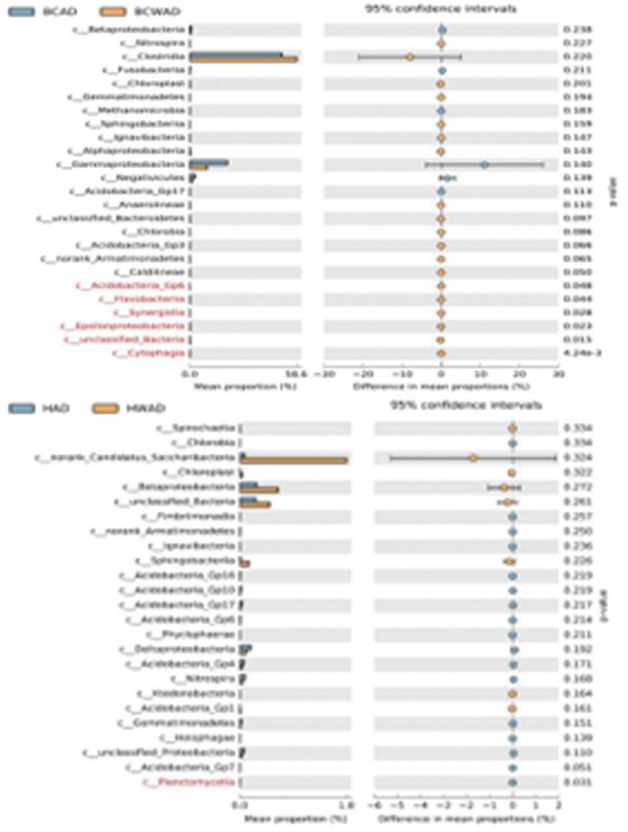


Figure 10. Relative Abundance of the Intestinal Flora in the Four Groups at the Order Level

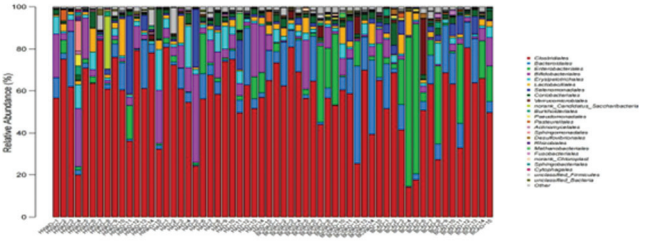


Table 14. Difference Analysis of the Order Level Flora Between the Breast Cancer Group and the Non-Breast Cancer Group ($\bar{x} \pm s$)

Flora name	BC group	Non-BC group	*t*	*P* value
Desulfuromonadales	0.00 ± 0.00	0.00 ± 0.00	4.44	.000
Campylobacterales	0.09 ± 0.04	0.01 ± 0.04	8.12	.000
Methylophilales	0.02 ± 0.02	0.00 ± 0.01	4.66	.000
Aeromonadales	0.01 ± 0.01	0.00 ± 0.00	6.00	.000
Syntrophobacterales	0.01 ± 0.01	0.00 ± 0.00	5.20	.000
Anaerolineales	0.09 ± 0.07	0.01 ± 0.01	5.80	.000
Oceanospirillales	0.03 ± 0.03	0.00 ± 0.00	5.40	.000
Nitrosomonadales	0.00 ± 0.00	0.00 ± 0.01	-4.29	.000
Alteromonadales	0.01 ± 0.01	0.00 ± 0.00	5.23	.000
Thermales	0.00 ± 0.00	0.00 ± 0.00	4.01	.000
Nitrospirales	0.01 ± 0.01	0.07 ± 0.08	-4.04	.000
Planctomycetales	0.01 ± 0.01	0.00 ± 0.00	5.14	.000
Thiotrichales	0.01 ± 0.01	0.00 ± 0.00	4.02	.000
Desulfobacterales	0.00 ± 0.00	0.00 ± 0.00	5.20	.000
Chlamydiales	0.00 ± 0.00	0.00 ± 0.00	4.50	.000
Solirubrobacterales	0.00 ± 0.00	0.01 ± 0.01	-4.41	.000
Myxococcales	0.00 ± 0.00	0.03 ± 0.04	-4.29	.000
norank_*Armatimonadetes*	0.05 ± 0.04	0.01 ± 0.01	5.04	.000
norank_*Aminicenantes*	0.00 ± 0.00	0.00 ± 0.00	4.86	.000
Ignavibacteriales	0.00 ± 0.00	0.00 ± 0.00	3.60	.001
Candidatus_Brocadiales	0.00 ± 0.00	0.00 ± 0.00	3.25	.002
Deferribacterales	0.01 ± 0.01	0.00 ± 0.00	3.18	.002
Bifidobacteriales	3.59 ± 5.05	9.95 ± 10.23	-3.05	.003
Gemmatimonadales	0.01 ± 0.01	0.02 ± 0.02	-3.04	.004
Enterobacteriales	14.12 ± 19.85	2.98 ± 4.75	2.99	.004

Table 15. Difference Analysis of the Order Level Flora Between the BCAD Group and the BCWAD Group ($\bar{x} \pm s$)

Flora name	BCAD Group	BCWAD Group	*t*	*P* value
Cytophagales	0.00 ± 0.00	0.01 ± 0.00	-3.11	.004
Desulfuromonadales	0.00 ± 0.00	0.01 ± 0.00	-3.12	.006
norank_*Deltaproteobacteria*	0.00 ± 0.00	0.00 ± 0.00	-2.88	.010
unclassified_Bacteria	0.09 ± 0.10	0.29 ± 0.27	-2.70	.015
Campylobacterales	0.07 ± 0.04	0.11 ± 0.03	-2.42	.023
Bdellovibrionales	0.02 ± 0.02	0.03 ± 0.02	-2.37	.025
Synergistales	0.00 ± 0.00	0.01 ± 0.02	-2.45	.028
Gammaproteobacteria_incertae_sedis	0.01 ± 0.01	0.01 ± 0.01	-2.13	.043
Flavobacteriales	0.01 ± 0.02	0.03 ± 0.03	-2.13	.044
norank_*Acidobacteria_Gp6*	0.00 ± 0.00	0.00 ± 0.00	-2.17	.048
Acidimicrobiales	0.01 ± 0.01	0.02 ± 0.01	-2.06	.049

Table 16. Difference Analysis of the Order Level Flora Between the HAD Group and the HWAD Group ($\bar{x} \pm s$)

Flora name	HAD Group	HWAD Group	*t*	*P* value
Nitrosomonadales	0.01 ± 0.01	0.00 ± 0.00	2.40	.027
Planctomycetales	0.00 ± 0.00	0.00 ± 0.00	2.40	.031
Rhodocyclales	0.08 ± 0.07	0.04 ± 0.04	2.21	.038

including *Enterobacteriales*, *Nitrosomonadales*, *Gemmatimonadales*, and *Nitrospirales*, etc. In the non-BC group, the abundance of 19 species of bacteria (including *Nitrosomonadales*, *Nitrospirales*, *Solirubrobacterales*, *Myxococcales*, *Bifidobacteriales*, and *Gemmatimonadales*, etc,) is higher than that found in the BC group, while other floras are all lower than that of the BC group ($P_{average} < .05$). See Table 14 (only the 25 bacteria with the lowest *P* values are listed).

2) Compared to the BCWAD group, the abundance of 11 species of bacteria including *Cytophagales*, *Desulfuromonadales*, and *Campylobacterales* is lower in the BCAD group. The difference is statistically significant ($P_{average} < .05$). See table 15, and Figure 11.

3) Compared to the HWAD group, the abundance of 3 species of bacteria including *Nitrosomonadales*, *Planctomycetales*, and *Rhodocyclales* is higher in the HAD group. The difference is statistically significant ($P_{average} < .05$). See Table 16 and Figure 11.

Family level analysis: 1) Analysis of all specimens from the BC and the non-BC groups show that there are statistical differences between the two groups in 95 species of bacteria

Figure 11. Results of the Order Level Difference Analysis Between the BCAD Group and the BCWAD Group, and Between the HAD Group and the HWAD Group

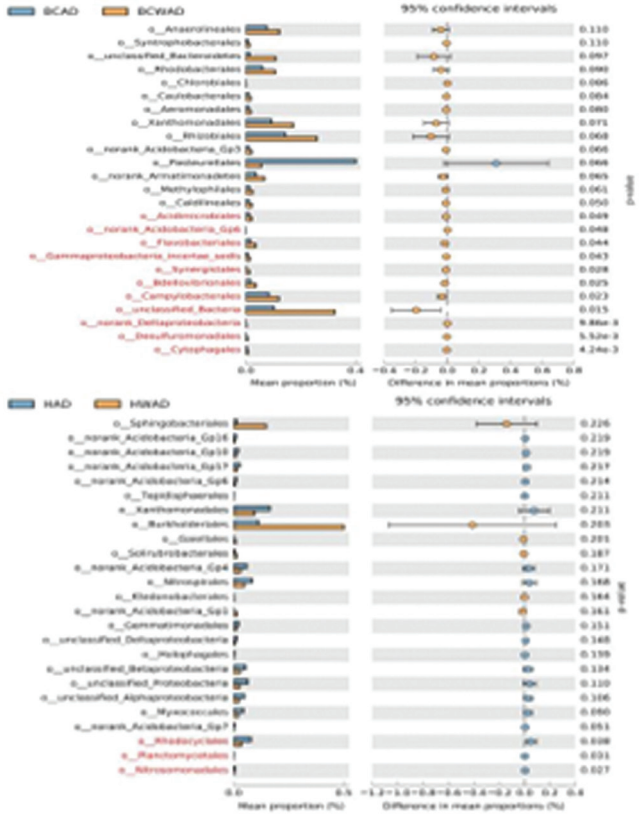
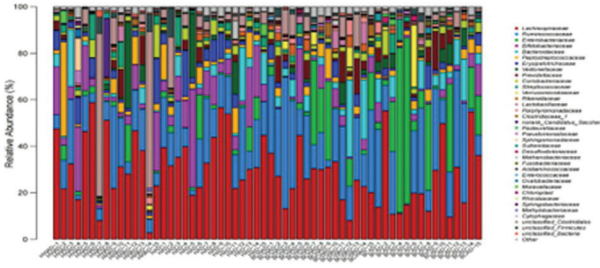


Figure 12. Relative Abundance of the Intestinal Flora in the Four Groups at the Family Level



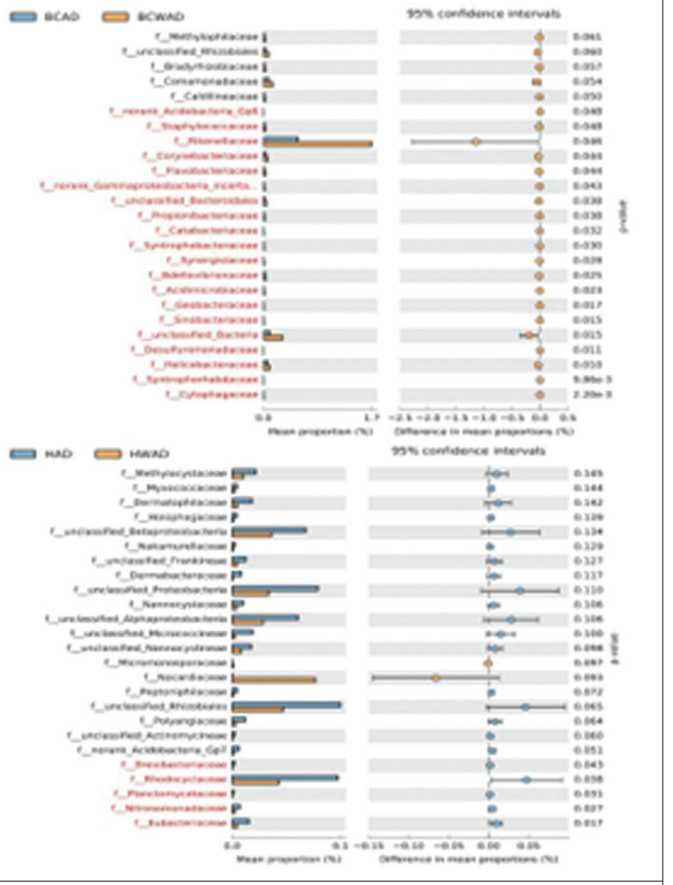
including *Helicobacteraceae*, *Staphylococcaceae*, *Aeromonadaceae*, and *Gemmatimonadaceae*, etc. In the non-BC group, the abundance of 19 species of bacteria (including *Nitrosomonadaceae*, *Nitrospiraceae*, *Labilitrichaceae*, and *Conexibacteraceae* etc.) are higher than that of the BC group, while other floras are all lower than that found in the BC group ($P_{average} < .05$). See table 17 (only the 25 bacteria with the lowest P values are listed).

2) Compared to the BCWAD group, the abundance of 20 species of bacteria (including *Cytophagaceae*, *Syntrophorhabdaceae*, *Helicobacteraceae*, and *Desulfuromonadaceae*, etc.) are lower in BCAD group. The difference is statistically significant ($P_{average} < .05$). See Table 18 and Figure 13.

Table 18. Difference Analysis of the Family Level Flora Between the Breast Cancer Group and the Non-Breast Cancer Group ($\bar{x} \pm s$)

Flora name	BC group	Non-BC group	*t*	*P* value
Helicobacteraceae	0.07 ± 0.04	0.01 ± 0.04	7.07	.000
Geobacteraceae	0.00 ± 0.00	0.00 ± 0.00	4.21	.000
Syntrophobacteraceae	0.01 ± 0.01	0.00 ± 0.00	5.50	.000
Staphylococcaceae	0.02 ± 0.02	0.00 ± 0.01	4.06	.000
Methylophilaceae	0.02 ± 0.02	0.00 ± 0.01	4.66	.000
Aeromonadaceae	0.01 ± 0.01	0.00 ± 0.0	6.10	.000
Anaerolineaceae	0.09 ± 0.07	0.01 ± 0.01	5.80	.000
Oceanospirillaceae	0.03 ± 0.03	0.00 ± 0.00	5.42	.000
Nitrosomonadaceae	0.00 ± 0.00	0.00 ± 0.01	-4.29	.000
Alteromonadaceae	0.01 ± 0.01	0.00 ± 0.00	5.23	.000
Thermaceae	0.00 ± 0.00	0.00 ± 0.00	4.01	.000
Nitrospiraceae	0.01 ± 0.01	0.07 ± 0.08	-4.04	.000
Demequinaceae	0.00 ± 0.00	0.00 ± 0.00	4.88	.000
Cellulomonadaceae	0.01 ± 0.01	0.00 ± 0.00	5.63	.000
Planctomycetaceae	0.01 ± 0.01	0.01 ± 0.01	5.14	.000
Labilitrichaceae	0.00 ± 0.00	0.00 ± 0.00	-4.21	.000
Conexibacteraceae	0.00 ± 0.00	0.01 ± 0.01	-4.19	.000
Syntrophaceae	0.00 ± 0.00	0.00 ± 0.00	3.98	.000
Thiotrichaceae	0.00 ± 0.00	0.00 ± 0.00	3.75	.000
Campylobacteraceae	0.02 ± 0.01	0.00 ± 0.01	5.32	.000
Beijerinckiaceae	0.00 ± 0.00	0.03 ± 0.05	-3.73	.000
unclassified_*Chlamydiales*	0.00 ± 0.00	0.00 ± 0.00	4.50	.000
norank_*Armatimonadetes*	0.05 ± 0.04	0.01 ± 0.01	5.04	.000
norank_*Spartobacteria*	0.00 ± 0.00	0.00 ± 0.00	4.13	.000
norank_*Aminicenantes*	0.00 ± 0.00	0.00 ± 0.00	4.86	.000

Figure 13. Results of the Family Level Difference Analysis Between the BCAD Group and the BCWAD Group, and Between the HAD Group and the HWAD Group



3) Compared to the HWAD group, the abundance of 4 species of bacteria (*Nitrosomonadaceae*, *Planctomycetaceae*, *Rhodocyclaceae*, and *Brevibacteriaceae*) in addition to *Eubacteriaceae*, is higher in the HAD group. The difference is statistically significant ($P_{average} < .05$). See Table 19 and Figure 13.

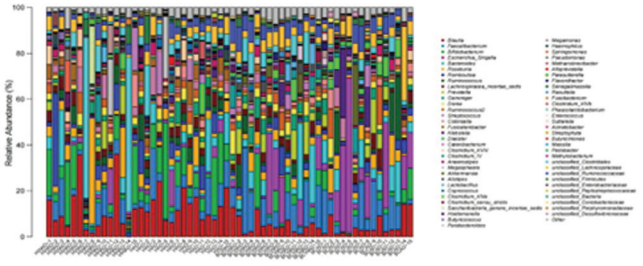
Table 19. Difference Analysis of the Family Level Flora Between the BCAD Group and the BCWAD Group ($\bar{x} \pm s$)

Flora name	BCAD Group	BCWAD Group	*t*	*P* value
Cytophagaceae	0.00 ± 0.00	0.00 ± 0.00	-3.45	.002
Syntrophorhabdaceae	0.00 ± 0.00	0.00 ± 0.00	-2.88	.010
Helicobacteraceae	0.06 ± 0.04	0.09 ± 0.02	-2.81	.010
Desulfuromonadaceae	0.00 ± 0.00	0.00 ± 0.00	-2.92	.011
unclassified_Bacteria	0.09 ± 0.10	0.29 ± 0.27	-2.70	.015
Sinobacteraceae	0.00 ± 0.00	0.01 ± 0.00	-2.60	.015
Geobacteraceae	0.00 ± 0.00	0.00 ± 0.00	-2.61	.017
Acidimicrobiaceae	0.01 ± 0.01	0.01 ± 0.01	-2.41	.023
Bdellovibrionaceae	0.02 ± 0.02	0.03 ± 0.02	-2.37	.025
Synergistaceae	0.00 ± 0.00	0.01 ± 0.02	-2.45	.028
Syntrophobacteraceae	0.00 ± 0.00	0.01 ± 0.01	-2.31	.030
Catabacteriaceae	0.00 ± 0.00	0.01 ± 0.01	-2.37	0.032
Propionibacteriaceae	0.01 ± 0.01	0.02 ± 0.01	-2.18	0.038
unclassified_*Bacteroidales*	0.02 ± 0.02	0.04 ± 0.04	-2.20	0.038
norank_*Gammaproteobacteria*	0.01 ± 0.01	0.01 ± 0.01	-2.13	0.043
Flavobacteriaceae	0.01 ± 0.02	0.03 ± 0.03	-2.13	0.044
Corynebacteriaceae	0.03 ± 0.04	0.06 ± 0.04	-2.11	0.044
Rikenellaceae	0.54 ± 0.76	1.68 ± 1.93	-2.14	0.046
Staphylococcaceae	0.01 ± 0.02	0.03 ± 0.02	-2.08	0.048
norank_*Acidobacteria_Gp6*	0.00 ± 0.00	0.00 ± 0.00	-2.17	0.048

Table 20. Difference Analysis of the Family Level Flora Between the HAD Group and the HWAD Group ($\bar{x} \pm s$)

Flora name	HAD Group	HWAD Group	*t*	*P* value
Eubacteriaceae	0.00 ± 0.00	0.01 ± 0.01	-2.65	.013
Nitrosomonadaceae	0.01 ± 0.01	0.00 ± 0.00	2.4	.027
Planctomycetaceae	0.00 ± 0.00	0.00 ± 0.00	2.40	.031
Rhodocyclaceae	0.08 ± 0.07	0.04 ± 0.04	2.21	.038
Brevibacteriaceae	0.00 ± 0.00	0.00 ± 0.00	2.19	.043

Figure 14. Relative Abundance of the Intestinal Flora in the Four Groups at the Genus Level



Genus level analysis: 1) Analysis of all specimens from the BC and the non-BC groups show that there are statistical differences between the two groups in 177 species of bacteria including *Geobacter*, *Flavobacterium*, *Simplicispira*, *Tessaracoccus*, and *Geoalkalibacter*, etc. In the non-BC group, the abundance of 63 species of bacteria (including *Aridibacter*, *Nitrosomonas*, *Ferribacterium*, *Nitrospira*, etc,) are higher than that of the BC group, while other floras are all lower than that of the BC group ($P_{average} < .05$). See table 21 (only the 25 bacteria with the lowest *P* values are listed).

2) Compared to the BCWAD group, the abundance of 34 species of bacteria (*Runella*, *Roseomonas*, *Syntrophorhabdus*, and *Desulfuromonas*, etc.) are lower in the BCAD group. The difference is statistically significant ($P_{average} < .05$). See table 22, figure 15 (only the 25 bacteria with the lowest *P* values are listed).

3) Compared to the HWAD group, the abundance of 15 species of bacteria (including *Nitrosomonas*, *Limnohabitans*, *Eggerthella*, and *Ferruginibacter*, etc.) in addition to *Eubacterium*, is higher in the HAD group. The difference is statistically significant ($P_{average} < .05$). See Table 23 and Figure 15.

Table 21. Difference Analysis of the Genus Level Flora Between the Breast Cancer Group and the Non-Breast Cancer Group ($\bar{x} \pm s$)

Flora name	BC group	Non-BC group	*t*	*P* value
Geobacter	0.00 ± 0.00	0.00 ± 0.00	3.97	.000
Flavobacterium	0.02 ± 0.02	0.00 ± 0.01	4.46	.000
Simplicispira	0.02 ± 0.02	0.00 ± 0.00	5.52	.000
Tessaracoccus	0.01 ± 0.02	0.00 ± 0.00	4.75	.000
Geoalkalibacter	0.00 ± 0.00	0.00 ± 0.00	3.80	.000
Sedimenticola	0.01 ± 0.01	0.00 ± 0.00	5.24	.000
Aerococcus	0.00 ± 0.00	0.00 ± 0.00	5.44	.000
Staphylococcus	0.02 ± 0.02	0.00 ± 0.01	4.06	.000
Thermomonas	0.08 ± 0.08	0.00 ± 0.00	5.60	.000
Aeromonas	0.01 ± 0.01	0.00 ± 0.00	6.74	.000
Luteolibacter	0.01 ± 0.01	0.00 ± 0.00	5.81	.000
Acidovorax	0.05 ± 0.04	0.00 ± 0.02	4.79	.000
Thermovirga	0.00 ± 0.00	0.00 ± 0.00	3.78	.000
Aridibacter	0.00 ± 0.00	0.01 ± 0.01	-4.43	.000
Phreatobacter	0.01 ± 0.01	0.00 ± 0.00	5.17	.000
Tissierella	0.00 ± 0.00	0.00 ± 0.00	5.08	.000
Pseudoxanthomonas	0.01 ± 0.01	0.00 ± 0.00	4.52	.000
Marinobacterium	0.03 ± 0.03	0.00 ± 0.00	5.42	.000
Nitrosomonas	0.00 ± 0.00	0.00 ± 0.01	-4.00	.000
Tolumonas	0.00 ± 0.00	0.00 ± 0.00	4.67	.000
Bosea	0.01 ± 0.01	0.00 ± 0.01	3.98	.000
Marinobacter	0.01 ± 0.01	0.00 ± 0.00	5.23	.000
Meiothermus	0.00 ± 0.00	0.00 ± 0.00	4.01	.000
Ferribacterium	0.00 ± 0.00	0.01 ± 0.02	-3.69	.000
Nitrospira	0.01 ± 0.01	0.07 ± 0.08	-4.04	.000

Table 22. Difference Analysis of the Genus Level Flora Between BCAD Group and the BCWAD Group ($\bar{x} \pm s$)

Flora name	BCAD Group	BCWAD Group	*t*	*P* value
Runella	0.00 ± 0.00	0.00 ± 0.00	-3.67	.002
Roseomonas	0.00 ± 0.00	0.00 ± 0.00	-3.19	.005
Syntrophorhabdus	0.00 ± 0.00	0.00 ± 0.00	-2.88	.010
Desulfuromonas	0.00 ± 0.00	0.00 ± 0.00	-2.92	.011
Bradyrhizobium	0.00 ± 0.00	0.01 ± 0.01	-2.47	.021
Bdellovibrio	0.01 ± 0.02	0.03 ± 0.02	-2.45	.021
Geobacter	0.00 ± 0.00	0.00 ± 0.00	-2.48	.022
Ilumatobacter	0.01 ± 0.01	0.01 ± 0.01	-2.41	.023
Flavobacterium	0.01 ± 0.01	0.02 ± 0.02	-2.36	.026
Ohtaekwangia	0.00 ± 0.00	0.00 ± 0.00	-2.36	.028
Simplicispira	0.01 ± 0.02	0.03 ± 0.02	-2.29	.030
Catabacter	0.00 ± 0.00	0.01 ± 0.01	-2.37	.032
Aquabacterium	0.00 ± 0.00	0.00 ± 0.00	-2.26	.033
Ancalomicrobium	0.00 ± 0.01	0.01 ± 0.01	-2.23	.034
Sphingosinicella	0.00 ± 0.00	0.00 ± 0.00	-2.20	.040
Tessaracoccus	0.01 ± 0.01	0.01 ± 0.01	-2.16	.040
Geoalkalibacters	0.00 ± 0.00	0.00 ± 0.00	-2.19	.041
Sedimenticola	0.01 ± 0.01	0.01 ± 0.01	-2.13	.043
Brevundimonas	0.01 ± 0.01	0.01 ± 0.01	-2.12	.044
Corynebacterium	0.03 ± 0.04	0.06 ± 0.04	-2.11	.044
Proteocatella	0.00 ± 0.00	0.00 ± 0.00	-2.10	.046
Alistipes	0.54 ± 0.76	1.68 ± 1.93	-2.14	.046
Aerococcus	0.00 ± 0.00	0.00 ± 0.00	-2.10	.046
Haliscomenobacter	0.00 ± 0.00	0.00 ± 0.00	-2.15	.047
Staphylococcus	0.01 ± 0.02	0.03 ± 0.02	-2.08	.048

Table 23. Difference Analysis of the Genus Level Flora Between HAD Group and the HWAD Group ($\bar{x} \pm s$)

Flora name	HAD Group	HWAD Group	*t*	*P* value
Eubacterium	0.00 ± 0.00	0.01 ± 0.01	-2.83	.012
Nitrosomonas	0.01 ± 0.01	0.00 ± 0.00	2.81	.013
Limnohabitans	0.00 ± 0.00	0.00 ± 0.00	2.67	.016
Eggerthella	0.16 ± 0.14	0.06 ± 0.06	2.64	.016
Ferruginibacter	0.00 ± 0.00	0.00 ± 0.00	2.60	.019
Coprococcus	1.07 ± 1.04	0.37 ± 0.40	2.41	.027
Ralstonia	0.00 ± 0.01	0.00 ± 0.00	2.39	.031
Paraprevotella	0.07 ± 0.09	0.02 ± 0.02	2.35	.033
Klebsiella	0.88 ± 1.31	0.10 ± 0.12	2.28	.039
Brevibacterium	0.00 ± 0.00	0.00 ± 0.00	2.19	.043
Tessaracoccus	0.00 ± 0.00	0.00 ± 0.00	2.17	.043
Anaerotruncus	0.00 ± 0.00	0.00 ± 0.00	2.15	.044
Propioniciclava	0.00 ± 0.00	0.00 ± 0.00	2.21	.045
Aridibacter	0.01 ± 0.01	0.00 ± 0.00	2.11	.047
Gordonibacter	0.01 ± 0.01	0.01 ± 0.01	2.06	.049
Anaerococcus	0.00 ± 0.00	0.00 ± 0.00	2.14	.049

Figure 15. Results of the Genus Level Difference Analysis Between the BCAD Group and the BCWAD Group, and Between the HAD Group and the HWAD Group

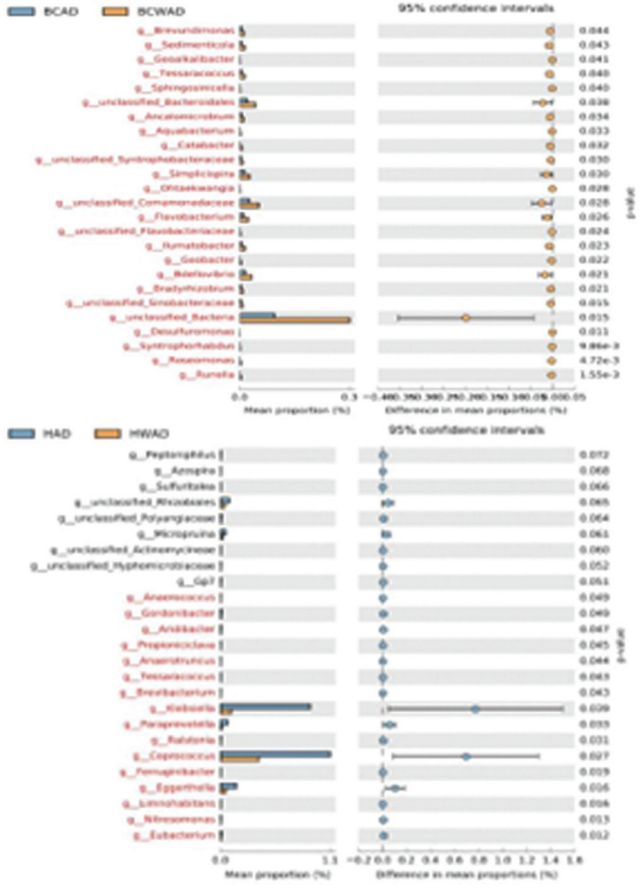
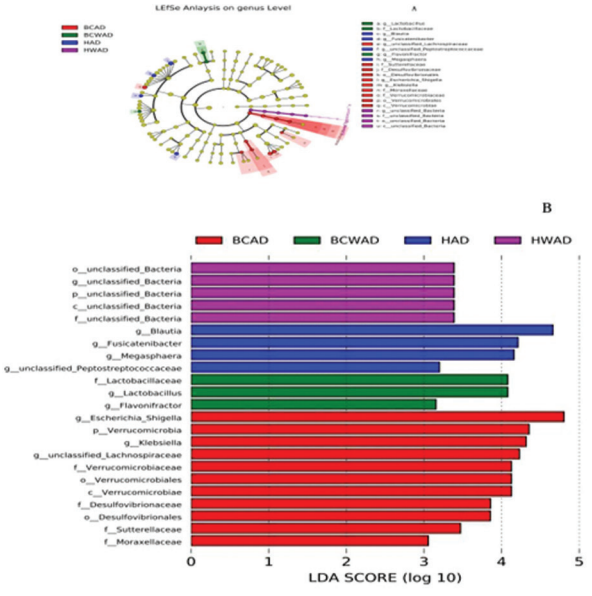


Figure 16. Structural Differences and Functional Flora of the Four Groups



Analysis of important bacteria in each group of samples. Linear discriminant analysis Effect Size (LEfSe) differential species discriminant analysis is adopted in order to further analyze the composition of the intestinal flora and the abundance of each component in each group for each taxonomy level of phylum, class, order, family and genus, and at the same time, to screen out the intestinal microorganisms that play an important role in each group. LefSe is a statistical method that is used to find the difference characteristics and significance test. The software first uses non-parametric coefficients of Kruskal-Wallis (KW) sum-rank test to detect the characteristics of significant differences in abundance between groups. If there are related subgroups between the groups, (unpaired) Wilcoxon rank-sum test is further used to check the consistency of the difference features of the previous step in the subgroups. Finally, LDA discriminant analysis is used to estimate the impact of these difference features on the difference between the groups.

The data obtained by LefSe reflect the branching diagram of the differences in the intestinal flora structure among each group (see figure 16), which shows that the microbial communities of the four groups of samples have relatively large differences at each taxonomic level. According to the LDA value, the effective intestinal microorganisms in the BCAD group are respectively unclassified-*Lachnospiraceae*, *Sutterellaceae*, *Desulfovibrionales*, *Escherichia-Shigella*, *Klebsiella*, *Moraxellaceae*, and *Verrucomicrobiales*. The effective intestinal microorganisms in the BCWAD group are *Lactobacillus* and *Flavonifractor*. The effective intestinal microorganisms in the HAD group are *Blautia*, *Fusicatenibacter*, and *Megasphaera*. The effective intestinal microorganisms in the HWAD group are unclassified-Bacteria (unclassified-class, unclassified-order, unclassified-family, and unclassified-genus) respectively, and a total of 21 species have an important influence on sample partitioning. The remaining unlisted effective flora meeting the biomarker screening criteria of LefSe program are shown in Figure16. Due to species diversity, only the LDA values at the genus level are listed (Figure 16).

DISCUSSION

Development and advantages of high-throughput sequencing of intestinal microbes

In the past, human studies of microorganisms have been largely based on laboratory cultures, but most microorganisms in the environment are generally not available using conventional laboratory culture methods. This poses a challenge in understanding the function of these microorganisms, and a large amount of effective microbial information is often lost in the process of laboratory culture, making it difficult to obtain complete and accurate picture of the microbial situation.[33] Therefore, Handelsman et al. first proposed metagenome in 1998 and pointed out the concept of the sum of the genetic material of all microorganisms in the environment, i.e., the total genetic material in an environmental sample should be studied during the research

process.[34] Metagenomics does not require isolation, culture and purification of microorganisms, and it overcomes the problem that the microorganism is difficult to cultivate, which provides a new way for us to know and understand microorganisms that cannot be cultured.

The research method based on high-throughput sequencing is the latest method for microbial research. High-throughput sequencing technology is an open system that can accurately obtain OTU of a specific gene, the number of the individuals, and the larger piece of information of DNA, and accurately study composition, structure, and evolutionary relationships of microorganisms.[35] High-throughput sequencing technology provides a large amount of data and information for the study of intestinal microbes, but mining, screening, and extracting valuable data from the large volume of data produced are the main problems of this technology. Metagenomics studies can compare the differences between different groups in terms of sequence composition, species diversity, and functional composition, while the comparison of microbial structure and diversity can be done on the basis of 16S. For microbes with low diversity, we can use genomic reconstruction from genomic data sets.[36] At present, high-throughput sequencing has become a powerful tool for the detection and identification of the intestinal flora. [37-40,77]

Changes of the intestinal flora in the Breast Cancer and the non-Breast Cancer population

In this study, rank abundance curve and Alpha index dilution curve are used to evaluate the uniformity of species richness. The results show that the sequencing amount of each group of samples in the experiment is sufficient to cover the maximum range of bacterial species, and the desired sequencing depth is reached. The obtained sequencing data are reliable and the results are accurate.

In terms of diversity of the intestinal flora, the Chao Index, the Ace index, and Coverage index of the BC patients are statistically different from those of the non-BC patients ($P < .05$), while there is no statistical significance between the Shannon index, the Simpson index and the Shannon-even index ($P > .05$). In general, there are differences in the intestinal flora diversity between the BC and the non-BC populations, which is similar to the results obtained in other studies.[41] The study has found that although the abundance of the intestinal flora in the BC patients is increased when compared with the non-BC patients, the diversity is decreased, which to some extent confirms the previous study which suggests that the BC patients have reduced the intestinal flora diversity,[42,43] and reflects the reliability of the experimental results.

In terms of the intestinal flora community, there are differences between the BC patients and the non-BC patients at different levels of family and genus ($P < .05$), as seen in different bacteria groups such as *Nitrospirae* (including its class, order, family and genus), *Firmicutes* (including *Lactobacillus*, *Clostridium*, *Eubacter*, etc.), *Bacteroidetes* (including its class, order, family and genus, Flavobacteria, Phagocytes etc.), *Actinobacteria* (including its class, order, family and genus, *Acidobacteria Bifidobacteria*, etc.). With the layer-by-layer classification, the number of bacterial categories gradually increases, and simultaneously, the number of different bacterial species gradually increases.[44,45] Among them, Bacteroidetes and Firmicutes are the two dominant bacteria in human intestinal tract. The study indicates that the BC patients have higher *Bacteroidetes* than the non-BC patients, and have lower *Firmicutes* than the non-BC patients, similar to the results obtained in other studies.[46,47] Higher levels of Bacteroidetes and lower levels of *Firmicutes* can lead to body weight gain, and obesity is a risk factor for various cancers, including the BC.[48] However, there are also contradictory findings suggesting that obese people have more *Firmicutes* and fewer *Bacteroidetes*.[49-51] These differences are most likely due to different living environments, diet, physical activity, and socioeconomic impacts.[52] The imbalance of *Firmicutes* and *Bacteroidetes* has broken the balance of normal flora in the intestinal tract and caused the occurrence and development of the BC,[53,54] which is also confirmed to some extent by this study.

Changes in the intestinal flora of people with anxiety and depression

Changes in the intestinal flora of people without the BC with anxiety and depression. Compared to the non-BC group without anxiety and depression (HWAD group), the non-BC group with anxiety and depression (HAD group) has statistical differences in terms of the diversity of the intestinal flora ($P < .05$) in the Chao index, the Shannon index, the Shannon-even index, and the Simpson index. However, there is no difference in the Ace index and the Coverage index ($P > 05$), indicating that people with negative emotions do have changes in their intestinal flora. Such changes mainly relay on the microbe-gut-brain axis. The microbe-gut-brain axis is a complex multi-organ bidirectional signaling system between the microbe and the brain, and plays a fundamental role in the homeostasis development and metabolism of the host.[55] The results of this study are similar to those of other studies conducted on Chinese population,[56] but contrary to the findings of studies based on populations of the United States[57] and Norway, [58] etc. We suspect that this may be due to differences in geography, ethnicity, diet, and culture. On the other hand, dysregulation of intestinal flora environment in the body can affect human behavior and cognition through brain-gut-microbial axis, resulting in the occurrence of anxiety and depression.[59-62] The possible mechanism is that several of the peptides and their receptors expressed in the human gut are also widely expressed along the microbe-gut-brain axis and assist in the signaling, and these peptides have clearly been shown to play a role in the neurobiology of anxiety and depression.[63,64] The next step for researchers is to focus on these peptides while studying changes in the intestinal flora to explore the underlying mechanisms and connections.

In terms of the community of the intestinal flora, compared with the non-BC group without anxiety and depression, the non-BC group with anxiety and depression has Planctomycetes differences (the abundance of bacteria in the HAD group is greater than that in the HWAD group, $P < .05$) at the levels of phylum and class, and has differences in *Eubacterium*, *Coprococcus*, *Klebsiella*, and other 16 bacterial groups at the genus level (except *Eubacillus*, the abundance of the HAD group is higher than that of the HWAD group, $P < .05$). In this study, except for *Eubacteriae*, there is no difference in other beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus*, and this result is not aligned with the findings of other researchers which suggest that people with anxiety and depression have less *Bifidobacterium* and *Bacteroidetes*.[28,,65-67] In terms of intestinal microbes, anxiety and depression are mainly associated with *Blautia*, *Fusicatenibacter*, and *Megaflococcus*, while the intestinal flora without anxiety and depression is mainly associated with unclassified_bacteria. There are differences between the two groups, but the effects of *Bacteroidetes* in the intestinal tract are different from those previously reported in the non-BC populations. Intestinal flora is a complex group, which is in a constantly changing state during the growth of the human body;[68,69] and different social and psychological environments in different regions can cause differences in the intestinal flora. In addition, the small sample size included in this experiment may also be the reason for the inconsistent results. The research team will expand the sample size in the following study to explore this experimental result.

Changes in the intestinal flora due to anxiety and depression in the BC patients. Compared to the BC group without anxiety and depression (BCWAD group), the BC group with anxiety and depression (BCAD group) has differences in the Chao index, the Shannon index, the Coverage index, and the Shannon-even index in terms of the diversity of the intestinal flora, ($P < .05$). However, there is no difference in the Ace index and Simpson index ($P > .05$), indicating that the diversity of the intestinal flora in the BC patients with anxiety and depression is different, particularly with respect to the decrease in the degree of the intestinal flora diversity in the BC patients,[70,71] which somewhat corroborates to previous studies, i.e., the decrease in the diversity of the intestinal flora may cause people to have negative emotions.[72] Despite the differences, the specific theory of changes in intestinal microbial richness and diversity in the BC patients with anxiety and depression is still unclear and needs further study,[73] which is what we're going to focus on next. However, there is no difference between the two groups in terms of bacteria such as *Bifidobacterium* and *Lactobacillus* ($P > .05$), which is both similar as well as different from other studies.[74,75]

In terms of the community of the intestinal flora, there are differences between the groups with and without anxiety and depression in different bacterial groups, such as unclassified_Bacteria (including class, order, family, and genus), *Synergistetes* (including class, order, family and genus), *Cytophagia*, and *Epsilonproteobacteria*, etc. With the adoption of layer-by-layer classification, the number of bacterial categories have also increased and there are more and more different bacterial species.

The abundance of bacteria in the groups with anxiety and depression is found to be lower than that without anxiety and depression. Interestingly, the intestinal flora of people with anxiety and depression are abundant in *Sutterellaceae*, *Escherichia-Shigella*, *Klebsiella*, and *Verrucomicrobiales*, etc., while the intestinal flora without anxiety and depression are abundant in *Lactobacillus* and *Flavonifractor*. There are differences between the two groups. In addition to *Klebsiella*, which is known to be associated with various infections and negative emotions,[29,76] *Escherichia-Shigella* is associated with cognitive impairment and peripheral inflammation in cerebral amyloidosis.[77] However, this study shows that both types of the intestinal bacteria in the BC patients with anxiety and depression have significant advantages, and *Escherichia-Shigella* has the greatest effect between groups. Whether *Escherichia-Shigella* regulates inflammation in the body to promote or aggravate anxiety and depression in the BC patients is worthy of further thinking and exploration, and can be the focus of our next research.

Above all, the results of this study reveal the complex relationship between gut microbiota and BC, as well as negative emotions (anxiety and depression). The following are the practical implications and potential impacts of the study results on patient care: (1) Gut Microbiota Diversity in BC Patients: We observed a significant decrease in gut microbiota diversity in BC patients, consistent with findings from other studies. This discovery suggests that maintaining or improving gut microbiota diversity in BC patients may contribute to their overall health. Therefore, patient care plans may include dietary and lifestyle interventions to support gut health. (2) Negative Emotions and Gut Microbiota: Our study found associations between anxiety and depression and alterations in gut microbiota. This underscores the critical connection between mental health and gut health. When caring for BC patients, it is essential not only to address their physical well-being but also to focus on their mental health. Psychological support and therapy may have a positive impact on improving gut microbiota and alleviating symptoms of anxiety and depression. (3) Specific Microbes in the Microbiota: Our research identified certain microbial communities that significantly increased in BC patients, such as *Escherichia-Shigella*. These microbes may be linked to the patients' physical health and negative emotions. Therefore, further research into the roles of these microbes and potential therapeutic interventions is crucial for enhancing patients' quality of life. (4) Personalized Care: Based on the findings related to gut microbiota, future care can be more personalized. Tailored care plans, including dietary recommendations, probiotics and prebiotics usage, and emotional management support, can be developed based on the unique gut microbiota characteristics of individual BC patients. In summary, this study provides a novel perspective

on the comprehensive care of BC patients, emphasizing the connection between mental health and gut health. Future care should be holistic, addressing overall well-being, and leveraging the research on gut microbiota to improve patients' quality of life. This also offers valuable directions for further research and treatment.

Although the sequencing results have confirmed that there are significant differences in the intestinal flora of each group, and that an imbalance of the flora occurred in the BC patients and those with anxiety and depression, the existing research still cannot specifically explain the causal relationship between the experimental results. Therefore, the existence of different intestinal flora for the BC patients and people with negative mood will become the research focus of our research team in the next step, in order to find out the possible intestinal flora causing BC and anxiety and depression.

In our study, we discussed the practical implications of our findings for patient care[78], including dietary and lifestyle interventions, psychological support, and personalized care plans. However, it is essential to acknowledge the potential challenges that healthcare providers and patients may face when translating these interventions into practice. In conclusion, while our study sheds light on promising interventions, it is essential to recognize and address the real-world challenges that may arise during their implementation. Tailoring interventions to the unique needs and circumstances of BC patients and fostering collaboration between healthcare providers and support systems can help overcome these challenges and improve patient outcomes.

CONCLUSION

Based on the 16S rRNA sequencing technology, the analysis of the distribution of the intestinal flora in the four groups has confirmed that there are differences in the diversity and structure of the intestinal flora in the breast cancer patients and people with anxiety and depression. Analysis of the non-breast cancer population and the breast cancer patients shows that the diversity of the intestinal flora in the breast cancer patients decreases, and the abundance of *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Nitrospirobacteria*, and *Spomonas* unclassified flora decrease. Analysis of the non-breast cancer group with and without anxiety and depression shows that the group with anxiety and depression tends to report an increase in the diversity of the intestinal flora. Except for *Eubacterium*, in the anxiety and depression group, the abundance of other different bacteria such as *Nitrosomonas*, *Planctomycetaceae*, and *Rhodocyclales* increase. The main acting bacteria in the group with anxiety and depression are *Blautia*, *Fusicatenibacter*, and *Macrococci*, while the main acting bacteria in the group without anxiety and depression are unclassified. Analysis of the breast cancer with and without anxiety and depression indicates that in the anxiety and depression group, the diversity of the intestinal flora is significantly decreased, and the abundance of different bacteria decrease. The major intestinal flora with anxiety and depression are unclassified-*Lachnospiracea*, *Sutterellaceae*, *Desulfovibrionales*, *Escherichia-Shigella*, *Klebsiella*, *Moraxellaceae*, and *Verrucomicrobiales*, while the main intestinal flora without anxiety and depression are *Lactobacillus* and *Flavonifractor*. The breast cancer patients with anxiety and depression shows further decline on the basis of decreased intestinal flora diversity in breast cancer. On the basis of the decrease of the abundance of the breast cancer flora, there are more differences in the abundance of the flora. It suggests that the intestinal flora of the breast cancer patients with anxiety and depression have further changes.

This study analyzes the possible influence of the intestinal flora on the occurrence and development of the breast cancer and anxiety and depression, so that it is possible to monitor the intestinal flora, prevent and even treat breast cancer and negative emotions, and to provide a preliminary basis for the further study of the different bacterial species related to anxiety and depression in the breast cancer. However, all the research objectives achieved in this study are linked to the same Three-A hospital, and since the intestinal flora may be affected by dietary conditions, the study findings cannot be generalized to represent all people. Therefore, the next step of our research will aim to recruit a large sample, conduct multi-area large sample experiment, make an in-depth analysis of the internal relationship and influencing mechanism between the intestinal flora and the breast cancer and anxiety and depression.

ETHICS APPROVAL

This study was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College, and all the study subjects signed informed consent.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

AUTHOR' CONTRIBUTION

Conception and design of the research: Chunfang Liu and Xiuchuan Li; Acquisition of data: Tingting Yang; Analysis and interpretation of the data: Hongming Ji; Statistical analysis: Shan Wang; Writing of the manuscript: Chunfang Liu; Critical revision of the manuscript for intellectual content: Xiuchuan Li.

FUNDING

This study was supported by the Natural Science Research Project of Anhui Educational Committee, No. KJ2020A0569; Natural Science Key Foundation of Bengbu Medical College, No. BYKY2019068ZD; Science Research Project of Bengbu Medical College, No. BYKF1784.

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