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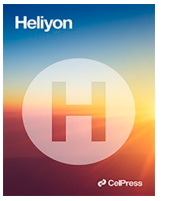

Research article

Ginger volatile oil inhibits the growth of MDA-MB-231 in the bisphenol A environment by altering gut microbial diversity

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ABSTRACT

Objective: To examine the impact of ginger volatile oil (GVO) on the growth of MDA-MB-231 breast cancer cells in the presence of bisphenol A (BPA) by modulating the diversity of gut microbiota. Methods: MDA-MB-231 breast cancer cells were injected subcutaneously into the right armpit of female BALB/c Nude (nu/nu) mice to create a triple negative breast cancer model. Thirty nude mice were randomly divided into 5 groups: control group (distilled water every day), BPA control group (distilled PEG-400+ DMSO + cyclodextrin every day), BPA + GVO (0.25 mL/kg) group, BPA + GVO (0.5 mL/kg) group, BPA + GVO (1 mL/kg) group, 6 mice in each group; The drug was given by gavage once a day for 4 weeks. At the end of the experiment, the changes of tumor mass and tumor volume were observed and compared in 5 groups of tumor-bearing mice. High-throughput sequencing (16S rRNA) was used to detect the changes of gut microflora in each group. Results: The volume and weight of breast cancer decreased in the low, medium and high dose groups of GVO. Among them, the difference between the high-dose group and the BPA group reached a significant level ($P < 0.05$). The species and abundance of gut flora decreased following BPA treatment, but increased after combined treatment of BPA with GVO. In the tumor control group, the ratio of Firmicutes(F) and Bacteroidea(B) respectively was 0.10:0.79 at the phylum level, while the ratio of BPA group further decreased (0.04:0.88). After feeding GVO, the number of Firmicutes and Bacteroidea increased, the F/B ratio increased, and the level of *Lactobacillus* and *alistipes* increased. In the BPA and GVO treatment group, the predominant gut microflora functions are cell membrane biogenesis, carbohydrate transport and metabolism. This is followed by amino acid transport and metabolism, and transcription function. After GVO administration, the Gram-positive bacteria (G^+) ratio had an increasing trend and the Gram-negative bacteria (G^-)ratio had a decreasing trend. Conclusion: The species and abundance of gut flora decreased following BPA treatment, but increased after combined treatment of BPA with GVO.

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1. Introduction

Bisphenol A (BPA) is the most widely used bisphenol compound, and products made from it can be found everywhere in daily life, such as plastic bottles, plastic toys, thermal paper, cosmetics, etc. In addition, BPA is also present in the air, with a concentration of 0.43 ng m^{-3} in the unit volume of air (i.e vapor and particle phase). It enters the human body through ingestion or skin contact [1–5]. BPA has varying degrees of harm to human development [6], the endocrine system [7,8], the nervous system [9,10], and the respiratory system [5,11]. Based on existing toxicology data, the European Food Safety Authority (EFSA) limits the daily intake of BPA to 4 mg/kg/day [12]. As an exogenous estrogen, BPA is also closely related to the incidence of breast cancer. In 1988, Epstein et al. [13] confirmed that estrogen can directly or indirectly stimulate the proliferation of human breast cancer cells and promote the occurrence of breast cancer in rodents. Breast cancer is the main cancer affecting women's health, and its incidence is increasing year by year [14]. In 2020, the number of breast cancer cases reached 2.26 million, surpassing lung cancer for the first time, accounting for 11.7 % of new cancer cases and signifying its burden as a public health problem threatening women's health [15]. Research has found that BPA promotes the migration and invasion of triple-negative breast cancer (TNBC) cells through estrogen-related receptor γ [16]. TNBC is a molecular subtype of breast cancer in which estrogen receptor (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (ERBB2) are all negative, accounting for 10%–20 % of invasive breast cancers [17]. The main treatment methods for early and postoperative TNBC are neoadjuvant chemotherapy containing anthracyclines and taxanes [14], which commonly cause acute and chronic adverse reactions, including nausea, vomiting, neurological disorders, infertility, and congestive heart failure, and patients have poor clinical outcomes [18,19]. Therefore, it is urgently needed to optimize the current treatment strategies for TNBC patients and find new treatment target drugs. Some medicinal and edible herbs may have novel anti-tumor mechanisms with minimal side effects. For example, previous studies have found that ginger has good anti-inflammatory and anti-tumor effects, providing the possibility of developing medicinal and edible drugs for the treatment of TNBC [20,21]. The microbiota, which is composed of a vast array of microorganisms that reside in our bodies, has been found to play a crucial role in maintaining our health and wellbeing. In recent years, the intestinal flora has shown exciting results in the development of tumorigenesis as well as in treatment [22–24]. Furthermore, the microbiota has been found to affect cancer prognosis. For example, patients with colon cancer who have a higher abundance of certain beneficial bacteria in their microbiota have been shown to have a better prognosis than those with lower levels [25]. These beneficial bacteria include *Bifidobacterium*, *Lactobacillus*, *Faecalibacterium prausnitzii*, *Akkermansia muciniphila*, etc. which can reduce the occurrence and development of colon cancer by inhibiting inflammatory reactions and reducing inflammation damage to the intestinal mucosa. They can also regulate immune responses, produce anti-tumor substances, and regulate the balance of intestinal microbiota, thereby reducing the promoting effect of harmful bacteria on colon cancer. These anticancer mechanisms of beneficial bacteria can also be beneficial for breast cancer prevention. Understanding the role of the microbiota in cancer development and prognosis could lead to the development of new strategies for cancer prevention and treatment. For example, targeting specific bacteria in the microbiota may help prevent the development of certain types of cancer or improve the effectiveness of cancer treatments [26]. The microbiota plays a crucial role in cancer prognosis and development, and further research in this area is needed to fully understand the mechanisms involved and to develop new strategies for cancer prevention and treatment. We chose the TNBC cell line MDA-MB-231 to conduct experiments and observe the effects of ginger oil on the proliferation of MDA-MB-231 cells and changes in intestinal microbial biodiversity in nude mice bearing MDA-MB-231 breast cancer under the influence of environmental estrogen BPA, laying a foundation for the development of new anti-TNBC drugs.

2. Materials and methods

2.1. Experimental animals

Thirty 5-week-old female Balb/c-nu/nu mice and three NOG mice were provided by Hunan Slake Jingda Laboratory Animal Co., Ltd., with a production license number of SCXK (Xiang):2019-0004. They were raised in the SPF barrier environment of the Experimental Animal Technology Center of Jiangxi University of Traditional Chinese Medicine, with a permit number of SYXK (Gan): 2017-0004. The experimental mice were given free access to water and food, with a 12-h light/dark cycle, temperature of 20–22 °C, and humidity of 45–65 %. The entire animal experiment was approved and supervised by the Ethics Committee of Experimental Animals of Jiangxi University of Traditional Chinese Medicine (ethical number: JZSYDWLL20200105), and the entire experimental process followed the 3R principle of experimental animals.

2.2. Instruments

CO_2 incubator (Binde, Germany), biological inverted microscope (SOPTOP), high-speed refrigerated centrifuge (Sigma, USA), one thousandth analytical balance (Sartorius, Germany), multi-function microplate reader (Thermo), PCR instrument (BD, USA), multi-function imager (analytikjena, Germany), ultrasonic cell disruptor (Ningbo Scientz Biotechnology Co., Ltd.) was used.

2.3. Reagents

Reagents used in this study were HL15 (Hyclone, SH300048.01), fetal bovine serum (Fetal Bovine Serum) (S601S-500, Sera&Pro), trypsin (0457, Amresco), PBS (SH30256.01, Hyclone), Matrigel (356234, BD), β-Estradiol (E8875, Sigma).

2.4. Tested drugs and Preparation methods

Ginger volatile oil (GVO) obtained from XingGuo Jiushan ginger by steam distillation. Accurately draw 100 μl (Low dosage), 200 μl (middle dosage), 400 μl (high dosage) of the drug, added PEG-400 dropwise to 2 ml. Prepare bisphenol A, accurately weighed 2.7 mg, and after 40 μl DMSO was dissolved, add cyclodextrin solution (2-hydroxypropyl-β-cyclodextrin, 0.3 g/mL) dropwise to help dissolve, dilute to 13.5 ml. The dosage of each animal is 0.2 mg/ml.

2.5. Methods

2.5.1. Cell culture

MDA-MB-231 triple-negative breast cancer cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences and cultured in RPMI1640 medium containing 10 % fetal bovine serum. Index-growth phase cells were collected, and serum-free HL15 was resuspended to an appropriate density (5×10^7/ml, 1:1 added Matrigel) for subcutaneous tumor transplantation in mice.

2.5.2. Tumor cell transplantation

MDA-MB-231 cells in the rapid growth phase were transplanted subcutaneously under the bilateral axillae of female NOG mice, 3 mice per group. When the subcutaneous tumors of MDA-MB-231 in NOG mice grew to about 500 mm^3, the subcutaneous tumors were peeled off, and the fleshy tumor tissue was partially divided into $3 \times 3 \times 3$ mm tumor tissue blocks in the culture medium. The tumor tissue blocks were transplanted into the subcutaneous tissue under the axilla of Balb/c-nu/nu mice using surgical methods, with a total of 35 animals.

2.5.3. Randomized grouping

Before treatment, the weight of all animals was measured, and the tumor volume was measured using a Vernier caliper. A randomized grouping design was adopted to ensure that the tumor volumes were similar between different groups (except the experimental groups given the drug immediately after transplantation). Thirty mice with well-growing tumors were selected and grouped according to the tumor volume of the mice. 1.5.5 Drug Administration.

On the day of grouping, drug administration was started according to the experimental design plan. The mice were administered BPA dissolved in PEG, DMSO, and cyclodextrin (BPA group), or the same BPA solution combined with low, medium, and high GVO concentrations. The tumor control group was given PEG-400+ DMSO + cyclodextrin.

2.5.4. Experimental observation and data Collection

After subcutaneous transplantation of MDA-MB-231 cells, the growth of nude mice and the effect of drug treatment on the daily behavior of experimental mice were observed, including the activity, feeding and drinking status, body weight changes, eye appearance, nude mouse appearance, and other abnormal conditions. The observed clinical manifestations were recorded in the original data in a timely manner. After the start of GVO administration, tumor length (L) and short diameter (D) were measured twice a week using a Vernier caliper, and tumor volume (mm^3) was calculated as $1/2 \times$ (tumor length $\times$ tumor short $diameter^2$).

2.5.5. Termination of experiment and sample Collection

The experiment was terminated 24 h after the last drug administration. After anesthetizing the mice, the tumor status and photographs of the separated tumor mass were collected. The tumor was then weighed, and the tumor tissue was stored at −80 °C. Fecal samples were collected by taking the colon contents immediately after sacrificing the mice, and storing them in freezing tubes in liquid nitrogen, and then transferring them to a −80 °C freezer.

2.6. Obtaining and amplifying fecal genomic DNA

The SDS method was used to extract fecal genomic DNA, and the purity and concentration of DNA obtained by agarose gel electrophoresis were detected. Qualified samples took an appropriate amount of sample DNA in a centrifuge tube, diluted it with sterile water to 1 ng/μl, and used this as a template. Based on the selection of sequencing regions, PCR was performed using primers with barcodes, PCR mix system and high-fidelity enzyme from New England Biolabs (NEB), and primers for 16S V4 region (515F and 806R), which also include amplification of other regions such as 16S V3–V4/16S V4–V5/16SV5–V7; archaeal 16S V4–V5/archaeal 16S V8; 18S V9 and ITS2. The PCR products were detected by 2 % agarose gel electrophoresis, and then mixed equally according to the concentration of PCR products. After detecting the PCR products again by 2 % agarose gel electrophoresis, the target bands were recovered and TruSeq® DNA PCR-Free Sample Preparation Kit was used to construct libraries. The library was quantified and qualified by Qubit and Q-PCR before sequencing on NovaSeq 6000.

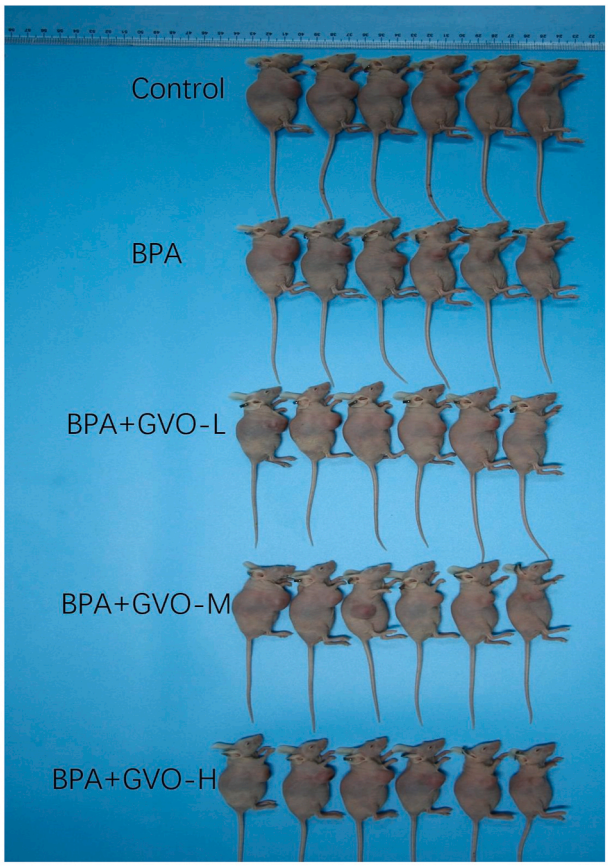
2.7. Processing of sequencing data sequencing

Data after sequencing were split into each sample data according to Barcode and PCR amplification primer sequences. FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) was used to splice the reads of each sample after removing the above primer sequences, obtaining the original Tags data (Raw Tags). Raw tags were assembled and strictly filtered [27] to obtain high-quality Clean Tags. Referring to the Tag quality control process of Qiime (V1.9.1, http://qiime.org/scripts/split_libraries_fastq.html), Tags data sets were

obtained by Tag truncation and length filtering, and then tags less than 75 % of the Tags length were further filtered out. The sequences of these Tags were compared with the species annotation database through (https://github.com/torognes/vsearch/) to remove chimeric sequences and obtain final effective data (Effective Tags).

2.8. Analysis of gut microbiome diversity

Uparse software (version 7.0.1090 http://www.drive5.com/uparse/) was used for OTU(Operational Taxonomic Units) clustering



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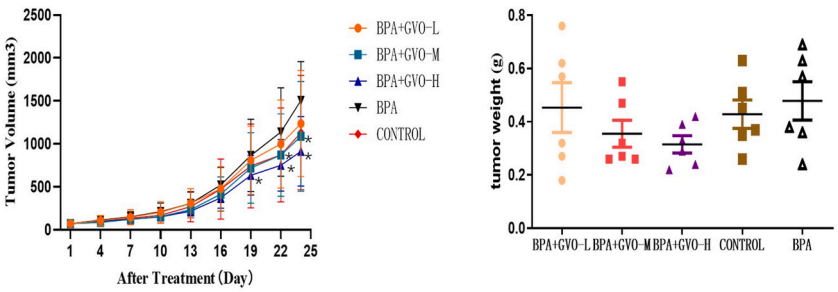

B

Fig. 1. A Effect of GVO on MDA-MB -231 breast cancer-bearing nude mice in BPA environment. B Effect of GVO on tumor volume and weight of MDA-MB-231 breast cancer bearing nude mice under the environment of Bisphenol A. Note: * Compared with bisphenol a group, the difference is significant (<0.05) BPA + GVO-L is a low-dose of GVO + BPA, BPA + GVO-M is a medium-dose of GVO + BPA, BPA + GVO-H is a high-dose of GVO + BPA, CON is the control group, and BPA is the bisphenol A group.

analysis of all samples, and biological statistical analysis was performed on OTUs. Species Venn diagram (R language (version 3.3.1)) was constructed; dilution curves and Rank-Abundance curves were created using R language tools. Alpha diversity analysis of samples was performed using mothur (version v.1.30.2 https://mothur.org/wiki/calculators/) software. Beta diversity analysis was performed using Qiime (Version 1.9.1 http://qiime.org/install/index.html). PCoA statistics using R language (version 3.3.1) were used to analyze principal coordinate analysis to study the similarity or difference of sample community composition. All statistical analysis was provided by Shanghai Mejile Biomedical Technology Co., Ltd.

2.9. Statistical methods

SPSS20.0 statistical software was used for statistical analysis of experimental results. Experimental data were expressed as mean ± standard deviation (x ± s). One-way analysis of variance was used to compare differences between groups, and LSD method was selected for multiple comparisons between groups. $P < 0.05$ indicates statistically significant difference. GraphPad Prism 8.0.1 was used for plotting. The software for bacterial community statistics was provided by Shanghai Mejile Biomedical Technology Co., Ltd.

3. Result

3.1. Inhibitory effects of tumors

The volume and weight of breast cancer decreased in the low, medium and high dose groups of GVO. Among them, the difference between the high-dose group and the bisphenol a group reached a significant level ($P < 0.05$), Fig. 1A and B.

3.2. Comparison of species abundance and alpha diversity of intestinal microecological samples (Alpha diversity)

The fecal OUT species diversity analysis shown in Fig. 2 shows that there are a total of 22 phyla, 48 classes, 93 orders, 150 families, 260 genera, 429 species, and 1004 OUTs. The tumor group has 628, the BPA group has 474, and the low, medium, and high doses of ginger oil are 568, 652, and 496 respectively. Based on the clustering of common and specific OTUs among different samples (groups), the Venn diagram of OTUs was obtained, showing that there were 276 OTUs common to 5 groups, while the number of specific OTUs for each treatment group was 59 for low-dose ginger oil, 83 for medium-dose ginger oil, 37 for high-dose ginger oil, 37 for BPA, and 139 for tumor control group. Fig. 2 shows the fecal OUT species diversity analysis of ginger oil on MDA-MB-231 breast cancer tumors in the presence of BPA. Each ellipse represents a sample processing group in the figure, and the numbers overlapping the ellipses represent the number of common OTUs between processing groups. The values without overlapping parts represent the number of specific OTUs for processing groups. Sobs index (Fig. 3A),Ace index (Fig. 3B) and Chao index (Fig. 3C) are indices used in ecological statistics to estimate the number of OTUs in a community. The higher the Sobs index 、 Chao index and Ace index, the more bacteria species are present in the sample. From Fig. 3, it can be seen that bacterial species decreased after inoculation of tumors, while the ginger oil treatment increased the diversity of intestinal bacteria. Shannon (Fig. 3D) and Simpson (Fig. 3E) indices can be used to

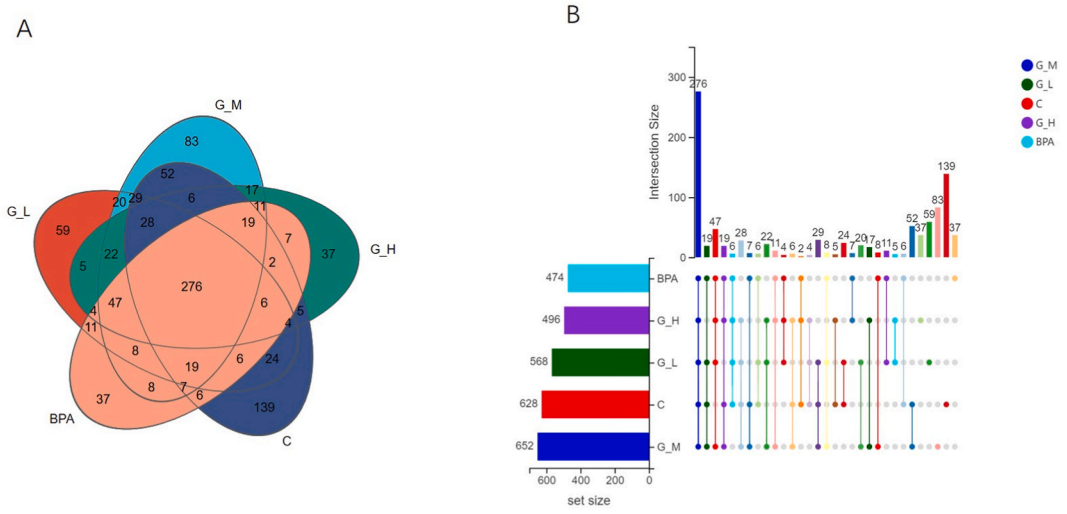

Fig. 2. Analysis of species diversity of MDA-MB-231-bearing breast cancer tumor feces OTU in BPA environment by GVO. Note:2A: Different colors represent different groups (or samples), the number of overlapping parts represents the number of species shared by multiple groups, and the number of non-overlapping parts represents the number of species unique to the corresponding group. 2B: The UpSet Wayne diagram mainly uses the form of connection combined with the histogram to show the common and unique element information of different grouping forms. The results are clear and easy to understand, and are especially suitable for the case of more grouping.

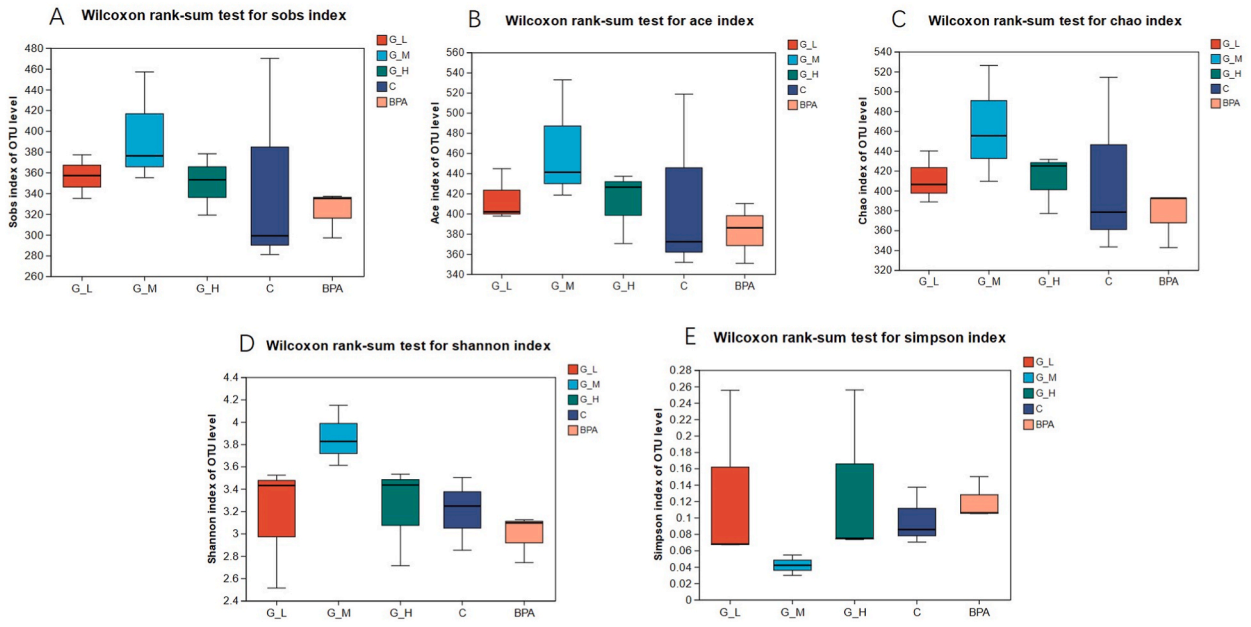

Fig. 3. Changes of Alpha index of intestinal microflora in tumor-bearing mice and treated with GVO. A is the Sobs index, B is the Ace index, C is the Chao index, D is the Shannon index, and E is the Simpson index.

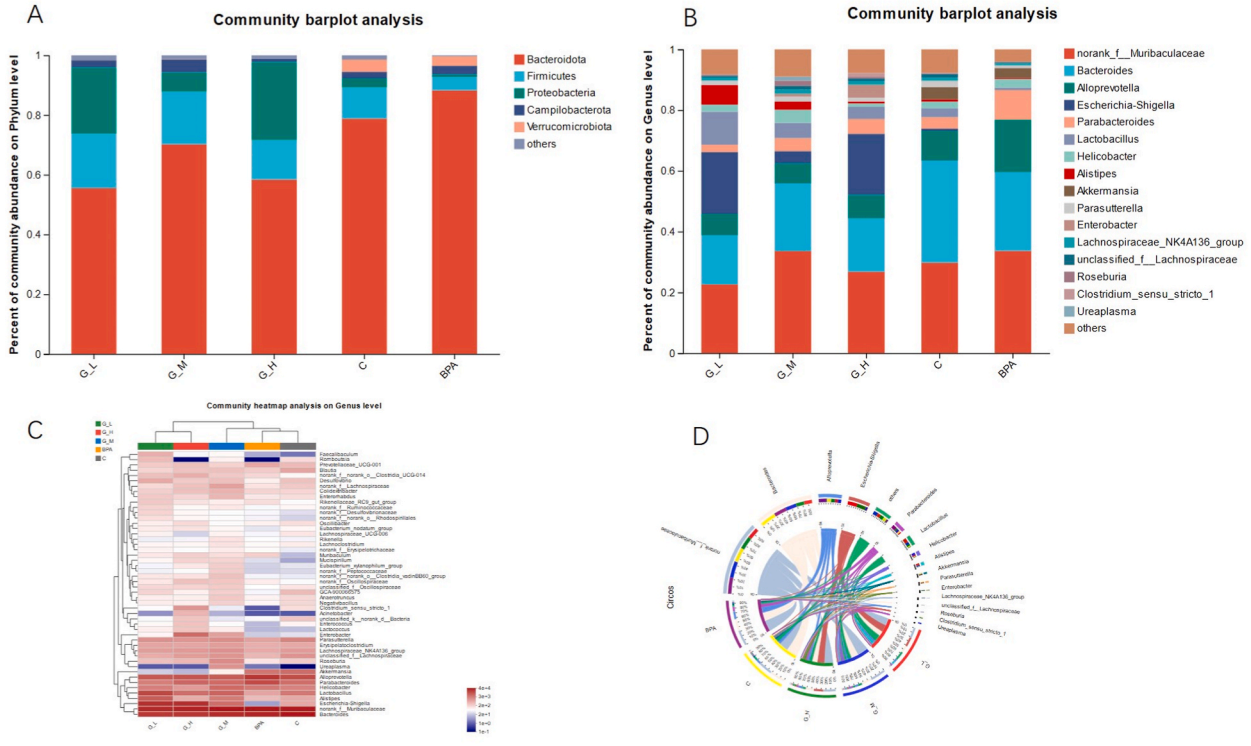

Fig. 4. of community abundance on phylum and genus level in MDA-MB-231 bearing mice and GVO treated mice. A: Phylum-level relative abundance bar chart, B:Genus -level relative abundance bar chart, C: Community heatmap analysis on genus level, D: The distribution proportion of dominant species in each (or group) sample is reflected by the 4D visual circle diagram, And the distribution proportion of dominant species in different samples (groups).

indicate the abundance of a community, where a larger Shannon index represents higher biodiversity while a larger Simpson index indicates lower biodiversity. Combining these two indicators, it can be seen that the abundance of intestinal bacterial communities in nude mice treated with bisphenol A decreases, while it increases after being given ginger oil.

3.3. Relative abundance of intestinal bacterial species in each group

Using the phylum-level relative abundance bar chart as an example (see Fig. 4A), we detected 11 bacterial phyla in mouse fecal samples after consuming ginger oil, including Bacteroidota, Proteobacteria, Firmicutes, Campilobacterota, Desulfobacterota, Actinobacteriota, Deferribacteres, and Verrucomicrobiota. Bacteroidetes and Firmicutes accounted for over 75 % of total bacteria in all five fecal samples. In the tumor control group, Bacteroidota accounted for 79 %, which increased to 88 % in the BPA group, but decreased to 55 %, 70 %, and 58 % respectively after being fed ginger oil. Firmicutes were only 10 % in the tumor control group, dropping to 4 % in the BPA group but increasing to 18 %, 18 %, and 13 % after being given ginger oil. In normal nude mice, Firmicutes accounted for 60 % and Bacteroidota accounted for 37 % [28]. The number of Verrucomicrobiota in the ginger oil treatment group decreased significantly compared to that in the BPA and tumor control groups. Fig. 4B shows the structure of intestinal bacterial communities in fecal samples from nude mice at the genus level. A total of 16 genera were involved, including norank_f_Muribaculaceae, Bacteroides, Alloprevotella, Escherichia, Shigella, Parabacteroides, Lactobacillus, Helicobacter, Alistipes, Akkermansia, Parasutterella, Enterobacter, Lachnospiraceae_NK4A136_group, unclassified_f_Lachnospiraceae, Roseburia, and Clostridium_sensu_stricto_1. Based on the species abundance information and related annotations at the genus classification level of all processing groups, a heat map (Fig. 4C) was drawn. The tumor control group and the BPA group clustered together, then combined with the medium dose of ginger oil, while the low and high doses of ginger oil formed their own clusters, showing some differences. Fig. 4D visualizes the dominant species distribution in each sample (The distribution proportion of dominant species in each (or group) sample is reflected by the 4D visual circle diagram, And the distribution proportion of dominant species in different samples (groups).

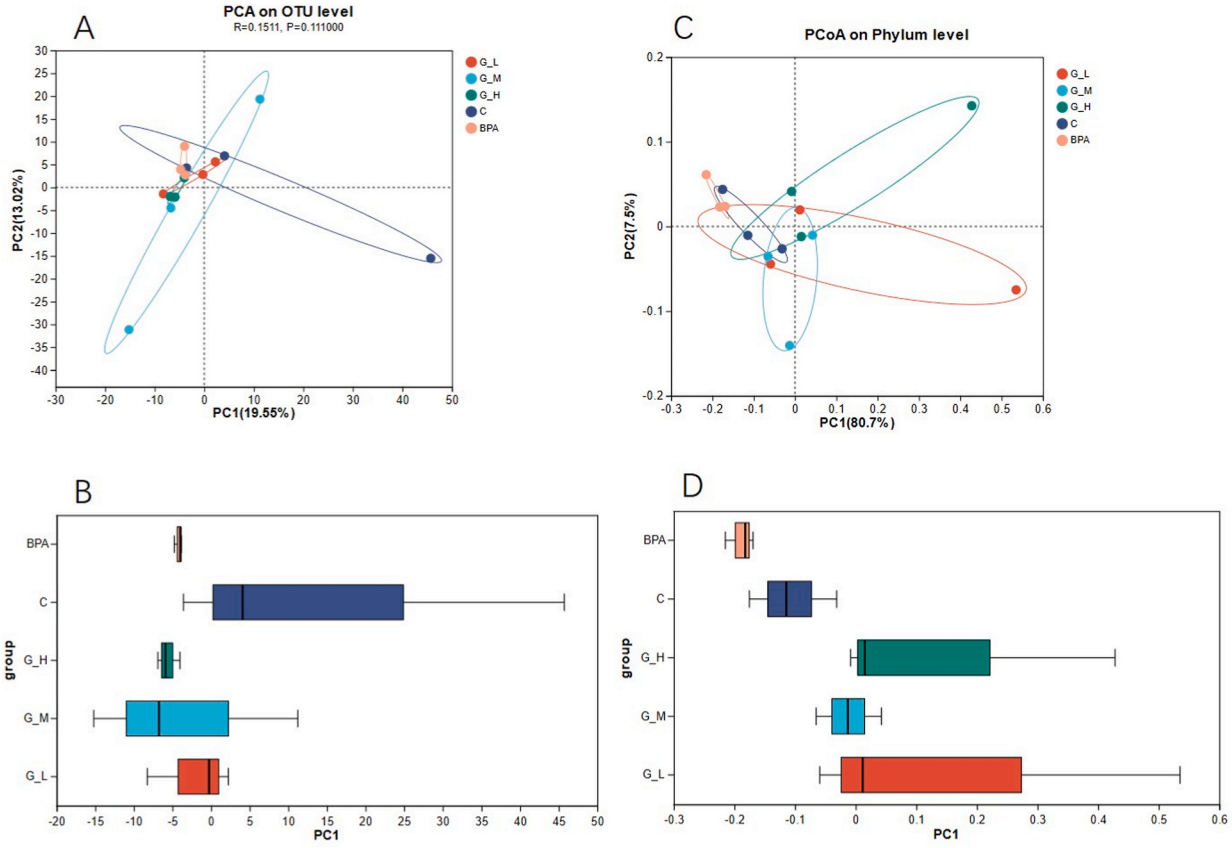

Fig. 5. Comparison of community composition between MDA-MB-231 bearing mice and GVO treated samples in different groups. A:represents the PCA plot, with the X-axis and Y-axis representing the two selected principal component axes, and the percentage representing the explanatory value of the principal component for the differences in sample composition; Points of different colors or shapes represent samples of different groups, and the closer the two sample points are, the more similar the species composition of the two samples is; B is a PCA box plot, with different colors representing different sample groups. The box plot in the graph represents the distribution dispersion of different groups of samples on the PC1 axis. C represents PCoA analysis, i.e. principal coordinate analysis, and D represents the PCoA box plot.

3.4. Comparative analysis of species diversity between groups

Beta diversity analysis was used to assess the similarity or difference in the composition of microbial communities between different sample groups following treatment with BPA, ginger oil, and tumor control. The results showed that there were differences in the colony composition of these groups to some extent, Fig. 5A,B,C,D.

3.5. Differences in species diversity between groups

Dominant phyla (Fig. 6A) and genera (Fig. 6B) were analyzed for significant differences in abundance between fecal samples from nude mice in five different groups: BPA, tumor control (C), and low, medium, and high doses of ginger oil. The results showed that Proteobacteria and Proteobacteria had significant differences ($P \leq 0.05$) at the phylum level, while Enterobacter, Roseburia, Negativibacillus, Acinetobacter, norank_f__Peptococcaceae, Candidatus_Saccharimonas, Herbaspirillum, Anaeroplasma, and Anaerovorax had significant or extremely significant differences (one-way ANOVA, $P < 0.05$, $P < 0.01$) at the genus level. Differential microbial species among the five different groups were identified using LEfSe analysis (LDA threshold = 2). The Anaeroplasma genus of the Acholeplasmataceae family and Acholeplasmatales order were enriched in the BPA group, while Bacilli, Saccharimonadia, Saccharimonadaceae, Saccharimonadales, Candidatus_Saccharimonas, Roseburia, Peptococcales, Peptococcaceae, norank_f__Peptococcaceae, Negativibacillus, Gammaproteobacteria, Proteobacteria, Enterobacter, Anaerovorax, Acinetobacter, Pseudomonadales, Moraxellaceae, Herbaspirillum, and Oxalobacteraceae were enriched in the low, medium, and high doses of ginger oil groups (Fig. 6C and D).

3.6. Functional Prediction and analysis of phenotypic contributions

PICRUSt was used to predict the functional contributions of OTUs by annotating them with COG functions based on their corresponding greengene IDs. The results showed that cell membrane biogenesis, carbohydrate transport, and metabolism were the most prominent functional categories in both the BPA and ginger oil treatment groups, followed by amino acid transport and metabolism

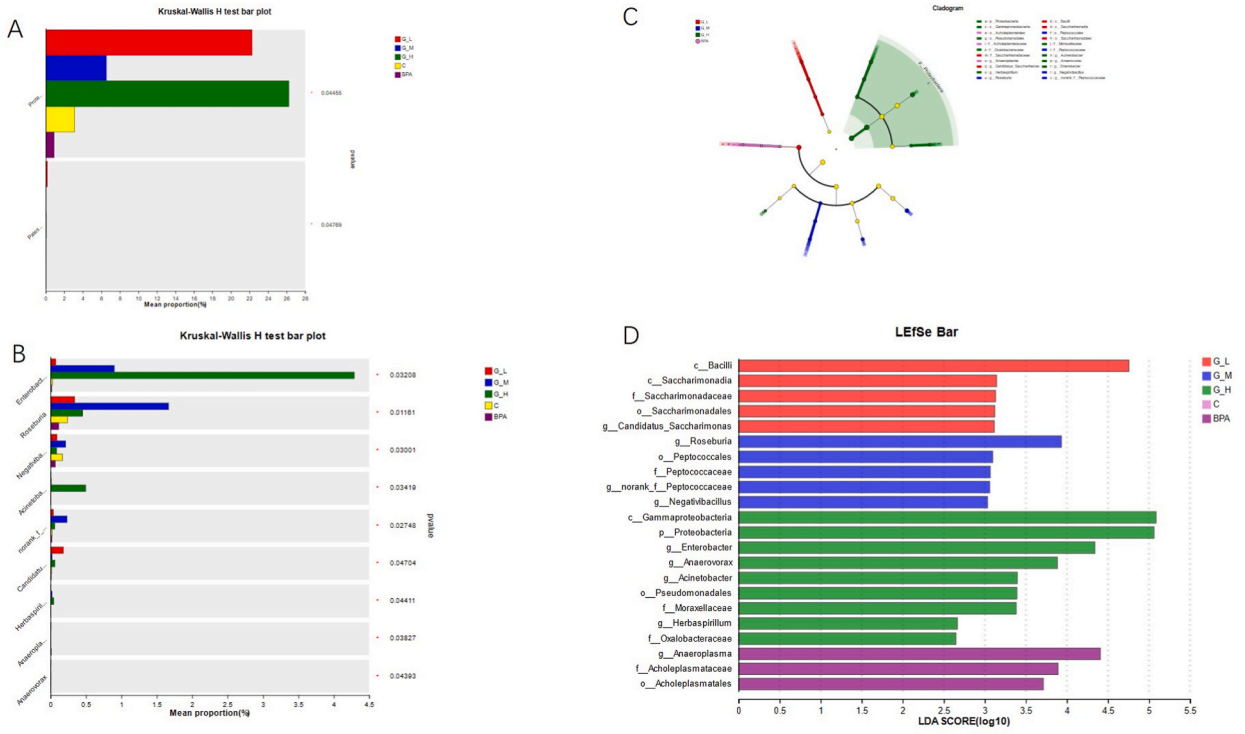

Fig. 6. Inter group difference test of species diversity in MDA-MB-231 bearing mice and GVO treated groups. A is a multi group comparison chart, B is a multi species difference test column chart, the Y-axis represents the name of species at a certain Taxonomy level, the X-axis represents the average relative abundance in different groups of species, and the columns with different colors represent different groups; On the far right is the P-value, * $0.01 < P \leq 0.05$, * * $0.001 < P \leq 0.01$, * * *$P \leq 0.001$. C is the LEfSe multi-level species hierarchy tree diagram, with different colored nodes representing microbial groups that are significantly enriched in the corresponding groups and have a significant impact on inter group differences; The light yellow nodes represent microbial groups that do not show significant differences in different groups or have no significant impact on inter group differences. If the number of significantly different species is ≤ 50, the legend position is displayed as one column on the right. If the number of significantly different species is > 50, the legend position is displayed as two columns on the right.

and transcription. These functional categories were more prevalent than in normal groups. Firmicutes and Actinobacteria were the most common Gram-positive (G^+) bacteria in tumor-bearing nude mice (Fig. 7A), with Firmicutes accounting for 0.16 in the tumor control group (C), 0.07 in the BPA group, and 0.23, 0.26, and 0.18 in the low, medium, and high doses of ginger oil groups, respectively. The abundance contribution of Actinobacteria was 0.004 in all groups. Differences in composition were observed between the groups, as shown in Fig. 7B. The G^+ BPA group had the lowest contribution to the entire microbial community, while the proportion of G^+ in the BPA group was also low, but increased after being given ginger oil. The Gram-negative (G^-)bacteria at the phylum level in tumor-bearing nude mice originated from Bacteroidota, Proteobacteria, Verrucomicrobiota, and Campilobacterota. Bacteroidota had the greatest impact, accounting for 0.68 in the tumor control group (C), 0.80 in the BPA group, and 0.54, 0.65, and 0.60 in the low, medium, and high doses of ginger oil groups, respectively, followed by Proteobacteria, which accounted for 0.04 in the tumor control group (C), 0.01 in the BPA group, and 0.2, 0.06, and 0.19 in the low, medium, and high doses of ginger oil groups, respectively (Fig. 7C). Fig. 7D shows the differences in the relative abundance of Gram-negative bacteria among the various groups, with the BPA group having the highest proportion of G^-, which decreased after being given ginger oil.

Fig. 7 shows the contributions of phenotypic properties of species and the differences between groups after treatment with BPA and ginger oil.

4. Discussion

The structure and composition of the gut microbiota can influence the occurrence, development, and prognosis of tumors. Some beneficial bacteria in the gut microbiota include *Bifidobacterium, Lactobacillus, Faecalibacterium prausnitzii, and Akkermansia muciniphila*. These bacteria can reduce the occurrence and development of colon cancer by inhibiting inflammatory reactions and reducing inflammation damage to the intestinal mucosa. They can also regulate immune responses, produce anti-tumor substances, and regulate the balance of the gut microbiota. These bacteria can also play an anti-tumor role in breast cancer. For example, *bifidobacterium* can regulate the balance of the gut microbiota and enhance immune function. Studies have shown that the presence of *bifidobacterium* can inhibit the growth and spread of breast cancer cells and suppress tumor development by regulating the gut microbiota and immune system [29,30]. *Lactobacillus* is a common lactic acid bacterium with antibacterial and immune-regulating effects. Research has found that *Lactobacillus* can inhibit the development of breast cancer by regulating the gut microbiota and immune system, and it can enhance the efficacy of chemotherapy drugs [31]. *Faecalibacterium prausnitzii* is a gut probiotic with anti-inflammatory and immune-regulating effects. Research has found that a decrease in the abundance of *Faecalibacterium prausnitzii* is associated with the occurrence and development of breast cancer. Its presence can inhibit the development of breast cancer by suppressing inflammatory reactions and regulating the immune system [32,33]. *Akkermansia muciniphila* is a gut mucosal bacterium that regulates the gut mucosal barrier and has anti-inflammatory effects. Research has found that the presence of *Akkermansia muciniphila* can inhibit the growth and spread of breast cancer cells and suppress tumor development by regulating the gut microbiota and mucosal barrier [34]. Therefore, studying changes in the gut microbiota is helpful for understanding the occurrence, development, and treatment of tumors.

The impact of exogenous estrogen BPA on breast cancer in women has been confirmed by numerous studies. However, BPA is still widely used in various daily products, causing persistent harm. In addition to finding alternative substitutes for BPA and reducing its use, therapeutic strategies should also be developed to target the proliferation of triple-negative breast cancer caused by BPA and identify new treatment targets.

Gut flora closely affects the physiological and pathological process of the host by forming a micro ecosystem with the host. In recent years, the research on the relationship between gut flora and tumor has received attention. Our study found that BPA significantly reduced the number of fecal OUTs, decreased the richness of the population, and increased after eating ginger oil. At the phylum level, BPA increased the amplitude of *Bacteroidea*, decreased the abundance of *Firmicutes*, and reduced the ratio of F/B. Beta diversity analysis showed that BPA was different from the colony composition of tumor group and treatment group to a certain extent. In the genus level, *Lactobacillus* and *alistipes* in BPA group decreased significantly compared with tumor group C, and increased significantly after taking ginger oil. *Alistipes*, a genus of bacteroidea, is gram-negative, obligatory anaerobic, does not exercise, can produce indole, cannot reduce nitrate, does not hydrolyze arginine and urea, and the end products of glucose metabolism are succinic acid and a small amount of acetic acid, propionic acid [35]. It has been proved that the genus can play a beneficial role in cancer immunotherapy by regulating the tumor microenvironment. Therefore, a form of anti-cancer therapy is actually manipulating the tumor microenvironment [36,37]. Manipulating the tumor microenvironment by inducing tumor related bone marrow cells to produce tumor necrosis factor (TNF) can eventually lead to tumor eradication. It can be seen that the effect of immunotherapy depends on the existence of microbiota. Intragastric administration of LPS to MC38 tumor bearing mice can reconstruct the expression of TNF. *Alistipes* and TLR4 induce the production of TNF. The recovery of TNF is due to pro-inflammatory Gram-negative bacteria a *Shahii* binds with TLR4 to initiate the expression of TNF [31]. *Lactobacillus* also reported anti-tumor proliferation [38,39]. Li, Q. et al. reported that *Lactobacillus* regulates gut microbiota and produces anticancer metabolites to prevent colorectal cancer [40]. Therefore, GVO may inhibit the proliferation of MDA-MB-231 by up regulating *Lactobacillus* and *aristipes*, and may become a new target of GVO for anti-tumor. LPS is known as endotoxin and is the main pathogenic component of gram-negative bacteria, which can cause strong immune reactions, leading to changes in morphology, metabolism, and gene expression in almost all eukaryotic cells, stimulating uncontrolled expression of host cell cytokines, causing inflammatory infections or triggering immune responses. In the BPA group, G^-bacteria mainly increased the abundance of Bacteroidota and Campilobacterota, while GVO can significantly down-regulate the abundance of Bacteroidota and up-regulate the abundance of Proteobacteria, which can also become a target for GVO in anti-BPA-induced MDA-MB-231. Ginger volatile oil can affect the proliferation of MDA-MB-231 under BPA environment by influencing the diversity and abundance of gut microbiota and affecting certain groups of bacteria. Different compositions of gut microbiota may lead to individual differences in

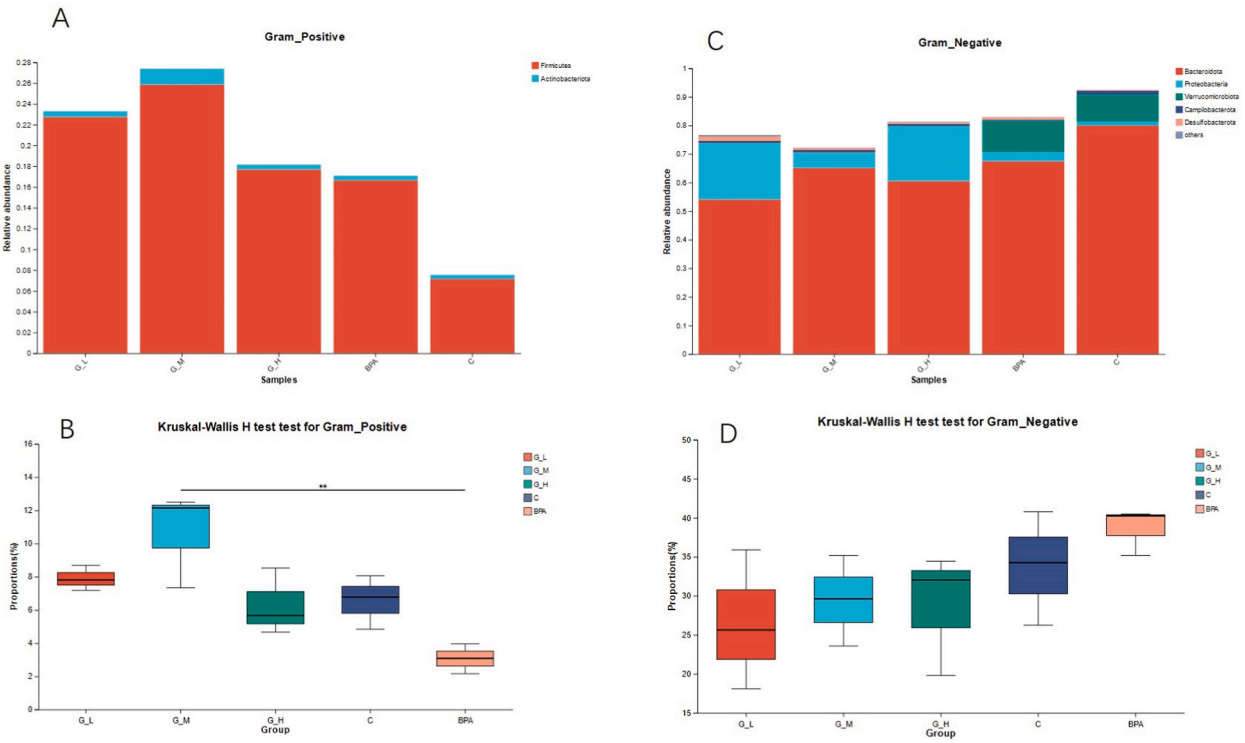

Fig. 7. Contribution of gram attribute phenotype of each group of species and test of differences between groups after MDA-MB-231 bearing mice and GVO treatment. A is the result of phenotypic contribution analysis of species Gram-positive bacteria, C is the result of phenotypic contribution analysis of species Gram-negative bacteria, the abscissa is the grouping name, different color legends represent different species, and the ordinate is the contribution of different species in the sample to this phenotype. B is the phenotype comparison chart of Gram-positive bacteria. The abscissa represents the group name or sample name, the ordinate represents the percentage of relative abundance of a certain phenotype of different samples, and different colors represent different groups. On the far right is the P-value, * $0.01<P \leq 0.05$, * * $0.001<P \leq 0.01$, * * *$P \leq 0.001$.

anti-tumor immunity, and such differences may affect systemic immunity, thereby affecting the effect and side effects of tumor immunotherapy by regulating host immunity, and intervening in gut microbiota (such as probiotics and fecal microbiota transplantation), which is expected to become an important means of assisting tumor immunotherapy.

5. Conclusion

GVO can affect the proliferation of MDA-MB-231 in BPA environment by affecting the abundance of intestinal microbial diversity and a certain kind of flora. Due to the different composition of gut flora, it may lead to the difference of anti-tumor immunity between individuals. This difference in gut flora may affect systemic immunity, and then affect the effect and toxic side effects of tumor immunotherapy by regulating host immunity, Intervention of gut flora (such as probiotics, fecal bacteria transplantation and other methods) is expected to become an important means of adjuvant tumor immunotherapy.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Animal studies were approved by the Committee on the Ethics of Animal Experiments of the Jiangxi University of Chinese Medicine.

CRediT authorship contribution statement

Liming Luo: Project administration, Data curation. **Yuran Chen:** Data curation. **Qiuting Ma:** Writing – original draft, Methodology. **Yun Huang:** Writing – original draft, Software. **Lei Xu:** Writing – original draft, Data curation. **Kun Shu:** Validation, Data curation. **Zhongfa Zhang:** Investigation, Data curation, Conceptualization. **Zhiyong Liu:** Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] M. Ali, et al., Testing baby bottles for the presence of residual and migrated bisphenol A, Environ. Monit. Assess. 191 (1) (2018) 7.
[2] R.P. Huang, et al., Bisphenol A concentrations in human urine, human intakes across six continents, and annual trends of average intakes in adult and child populations worldwide: a thorough literature review, Sci. Total Environ. 626 (2018) 971–981.
[3] N. von Goetz, et al., Including non-dietary sources into an exposure assessment of the European Food Safety Authority: the challenge of multi-sector chemicals such as Bisphenol A, Regul. Toxicol. Pharmacol. 85 (2017) 70–78.
[4] Huang, et al., Bisphenol A concentrations in human urine, human intakes across six continents, and annual trends of average intakes in adult and child populations worldwide: a thorough literature review, ENCE OF THE TOTAL ENVIRONMENT 626 (2018) 971–981.
[5] Wenlong Liu, Yujie Wang, Ye Liu, et al., Comparative assessment of human exposure to phthalate esters and bisphenol A from different indoor dust, J. Shanghai Univ. (Engl. Ed.) 25 (2) (2019) 282–292.
[6] K. Berger, et al., Association of prenatal urinary concentrations of phthalates and bisphenol A and pubertal timing in boys and girls, Environ. Health Perspect. 126 (9) (2018) 97004.
[7] E.M. Bell, et al., Concentrations of endocrine disrupting chemicals in newborn blood spots and infant outcomes in the upstate KIDS study, Environ. Int. 121 (Pt 1) (2018) 232–239.
[8] X. Sun, et al., Maternal exposure to bisphenol A and anogenital distance throughout infancy: a longitudinal study from Shanghai, China, Environ. Int. 121 (Pt 1) (2018) 269–275.
[9] M. Desai, et al., In vivo maternal and in vitro BPA exposure effects on hypothalamic neurogenesis and appetite regulators, Environ. Res. 164 (2018) 45–52.
[10] Z.H. Liu, et al., Early developmental bisphenol-A exposure sex-independently impairs spatial memory by remodeling hippocampal dendritic architecture and synaptic transmission in rats, Sci. Rep. 6 (2016) 32492.
[11] L. Mahemuti, et al., Bisphenol A induces DSB-ATM-p53 signaling leading to cell cycle arrest, senescence, autophagy, stress response, and estrogen release in human fetal lung fibroblasts, Arch. Toxicol. 92 (4) (2018) 1453–1469.
[12] J.H. Park, et al., Risk assessment based on urinary bisphenol A levels in the general Korean population, Environ. Res. 150 (2016) 606–615.
[13] F.H. Epstein, M. Clemons, P. Goss, Estrogen and the risk of breast cancer, N. Engl. J. Med. 344 (4) (2001) 276.
[14] A.G. Waks, E.P. Winer, Breast cancer treatment: a review, JAMA 321 (3) (2019) 288–300.
[15] C.E. DeSantis, et al., Breast cancer statistics, CA Cancer J Clin 69 (6) (2019) 438–451.
[16] X.L. Zhang, et al., Bisphenol A increases the migration and invasion of triple-negative breast cancer cells via oestrogen-related receptor gamma, Basic Clin. Pharmacol. Toxicol. 119 (4) (2016) 389–395.
[17] P. Kumar, R. Aggarwal, An overview of triple-negative breast cancer, Arch. Gynecol. Obstet. 293 (2) (2016) 247–269.
[18] S.M. Reddy, et al., Long-term survival outcomes of triple-receptor negative breast cancer survivors who are disease free at 5 years and relationship with low hormone receptor positivity, Br. J. Cancer 118 (1) (2018) 17–23.
[19] S. Al-Mahmood, et al., Metastatic and triple-negative breast cancer: challenges and treatment options, Drug Deliv Transl Res 8 (5) (2018) 1483–1507.
[20] M.M. Zhang, et al., Identification of the active substances and mechanisms of ginger for the treatment of colon cancer based on network pharmacology and molecular docking, BioData Min. 14 (1) (2021) 1.
[21] L. Zhao, et al., Efficacy based ginger fingerprinting reveals potential antiproliferative analytes for triple negative breast cancer, Sci. Rep. 10 (1) (2020) 19182.
[22] Shahid Riaz Rajoka Muhammad, et al., Gut microbiota targeted nanomedicine for cancer therapy: challenges and future considerations, in: Trends in Food Science & Technology, prepublish, 2020, https://doi.org/10.1016/j.tifs.2020.10.036.
[23] Stefania Gori, et al., Gut microbiota and cancer: how gut microbiota modulates activity, efficacy and toxicity of antitumoral therapy, Crit. Rev. Oncol.-Hematol. 143 (2019) C, https://doi.org/10.1016/j.critrevonc.2019.09.003.
[24] Greenhill Claire, Gut microbiota: anti-cancer therapies affected by gut microbiota, Nat. Rev. Gastroenterol. Hepatol. 11 (2014) 1, https://doi.org/10.1038/nrgastro.2013.238.
[25] Xin Guo, The Impact of Zheng Weiqin's Experience Formula on Intestinal Microbiota in Postoperative Patients with Colorectal Cancer [D], Hunan University of Traditional Chinese Medicine, 2022.
[26] B. Aja, A. Hina, M. Karan, et al., Effect of probiotics and gut microbiota on anti-cancer drugs: mechanistic perspectives, Biochimica et biophysica acta. Reviews on cancer 1875 (1) (2020) 188494.
[27] N.A. Bokulich, et al., Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing, Nat. Methods 10 (1) (2013) 57–59.
[28] Yu Zhang, Chunling Xiao, Dan Yang, et al., PM_ (2.5) changes in intestinal flora composition after exposure to tumor bearing nude mice [j], Chin. J. Microbiol. 30 (11) (2018) 1247–1251.
[29] Y. Liu, et al., Bifidobacterium can mitigate intestinal immunopathology in the context of CTLA-4 blockade, Proc Natl Acad Sci U S A 117 (27) (2020) 16057–16066.
[30] Y. Wang, et al., Bifidobacterium adolescentis exerts anti-proliferative effects on HCT116 colorectal cancer cells, Cells 8 (12) (2019) 1604.
[31] A.D.M.D. Leblanc, et al., Effects of milk fermented by Lactobacillus helveticus R389 on a murine breast cancer model, Breast Cancer Res. 7 (4) (2005) R477–R486, https://doi.org/10.1186/bcr1032.
[32] J. Ma, et al., Alter between gut bacteria and blood metabolites and the anti-tumor effects of Faecalibacterium prausnitzii in breast cancer, J. Clin. Oncol. 38 (15_suppl) (2020) e12575, https://doi.org/10.1200/JCO.2020.38.15_suppl.e12575.
[33] Andrew D. Fruge, et al., Fecal Akkermansia muciniphila is associated with body composition and microbiota diversity in overweight and obese women with breast cancer participating in a presurgical weight loss trial, J. Acad. Nutr. Diet. 120 (4) (2020) 650–659, https://doi.org/10.1016/j.jand.2018.08.164.
[34] P.D. Cani, et al., Akkermansia muciniphila: paradigm for next-generation beneficial microorganisms, Nat. Rev. Gastroenterol. Hepatol. 19 (2022) 625–637.
[35] B.J. Parker, et al., The genus alistipes: gut bacteria with emerging implications to inflammation, cancer, and mental health, Front. Immunol. 11 (2020) 1–15.
[36] X. Xu, X. Zhang, Effects of cyclophosphamide on immune system and gut microbiota in mice, Microbiol. Res. 171 (2015) 97–106.
[37] N. Lida, et al., Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment 342 (2013) 967–970.

[38] B. Hgna, et al., Lactobacillus acidophilus and Bifidobacterium longum exhibit antiproliferation, anti-angiogenesis of gastric and bladder cancer: impact of COX2 inhibition - ScienceDirect, PharmaNutrition 14 (2020) 1–10.
[39] H. Maroof, Z.M. Hassan, A. Mobarez, Immune-modulation by Lactobacillus acidophilus in murine model of breast cancer induces enhanced systemic anti-tumour immune responses 32 (6) (2012) 1353–1359.
[40] Q. Li, et al., Lactobacillus gallinarum modulates the gut microbiota and produces anti-cancer metabolites to protect against colorectal tumourigenesis, Gut 69 (2021) 7–34.