Suppression of Berberine and Probiotics (in vitro and in vivo) on the Growth of Colon Cancer With Modulation of Gut Microbiota and Butyrate Production

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Background and Objective: An increasing number of evidence has revealed that the gut microbiome functions in immunity, inflammation, metabolism, and homeostasis and is considered to be crucial due to its balance between human health and diseases such as cancer, leading to the emergence of treatments that target intestinal microbiota. Probiotics are one of them. However, many challenges remain regarding the effects of probiotics in cancer treatment. Berberine (BBR), a natural extract of Rhizoma Coptidis and extensively used in the treatment of gastrointestinal diseases, has been found to have antitumor effects in vivo and in vitro by many recent studies, but its definite mechanisms are still unclear. This study aimed to explore the inhibitory effect of BBR and probiotics on the growth of colon cancer cells in vitro and in vivo, and the regulatory influence on the gut microbiome and butyrate production.

Methods: Colon cancer cell line HT29 was used to establish a xenograft model of nude mice and an in vitro model. A total of 44 nude mice and HT29 cells were divided into control, model, model + BBR, model + probiotics, and model + combination of BBR with probiotics (CBPs). Live combined Bifidobacterium, Lactobacillus, and Enterococcus powder (LCBLEP) was used as a probiotic preparation. LCBLEP was cultured in the liquid medium under anaerobic conditions (the number of viable bacteria should reach $1 \times 10^8$ CFU), and the supernatant was collected, and it is called probiotic supernatant (PS). Model + BBR and model + probiotics groups were treated with BBR and LCBLEP or PS for 4 weeks in vivo or 48, 72, and 96 h in vitro, respectively. Tumor volume or cell proliferation was measured. Gut microbiota was pyrosequenced using a 16S rDNA amplicon. HDAC1 mRNA level in HT29 cells and sodium butyrate (SB) expression in the serum of mice was detected by QPCR and ELISA.

Results: The treatment of BBR and CBP reduced the growth of neoplasms in mice to a different extent ($p > 0.05$), especially at 14 days. The inhibitory effect of LCBLEP on tumor growth was more significant, especially at 11–21 days ($p < 0.05$).
INTRODUCTION

So far, cancer remains a major global killer. Colorectal cancer (CRC) is the third most common cancer type (10.2%) and the second most fatal cancer worldwide (9.2%) (Bray et al., 2018). The exploration of cancer pathogenesis and related drugs has always been the focus of research. Recently, more and more attention has been paid to the development of tumor-related microenvironments and natural drugs.

The human microbiota consists of 10–100 trillion microbes including bacteria, viruses, protozoa, and fungi, and most harbor the gut microbiota. Probiotics were first defined by Lilly and Stillwell (1965), but its restriction was only some substances produced by bacteria. The exploration never ends. Recently, probiotics were found that the disorder of gut microbiota is responsible for many types of diseases. Therefore, the improvement in gut microbiota and its function of the gut microbiome have an influence on the intestinal barrier, digestion and metabolism, and immune responses. An increasing number of evidence has found that the disorder of gut microbiota is responsible for many types of diseases. Therefore, the improvement in gut microbiota contributed by probiotics has generated considerable interest. Because probiotics are derived from different foods or drugs, understanding probiotics would promote the development of food and pharmaceutical industries, such as commercial yogurt and prebiotic drinks.

Live combined *Bifidobacterium*, *Lactobacillus*, and *Enterococcus* powder (LCBLEP) is a commercial probiotic preparation (the brand name is Peifeikang) and is used widely in a clinic. Several research studies have demonstrated that these probiotics have the potential to reduce the enteropathogenic complications in patients with colon cancer undergoing surgery or inhibit the growth of the cancer cells through the creation of the integrity of intestinal mucosal, promotion of the immune, and production of antimetabolites, then preventing the progress of the CRC (Eslami et al., 2019; Bazireh et al., 2020; Sugimura et al., 2021).

Berberine (BBR), a natural plant alkaloid extracted from *Coptis chinensis* (Huanglian), has long been used to treat digestive diseases as an ancient antidiarrheal medication in China (Zhang Y. et al., 2020). In addition, BBR is also employed to treat...
metabolic and tumorous disorders, such as type 2 diabetes and malignancy (Zhang et al., 2014; Gong et al., 2020). Animal studies have shown that BBR significantly altered the microbiome of the intestine and microbe-related mechanisms through 16S rRNA gene sequencing (Kumar et al., 2015; Yang et al., 2017). Given the efficacy of BBR, it is considered a bacteriostatic agent and is used to treat the kinds of diseases with probiotics (Zhang Y. et al., 2020). However, it is unclear about the effect of the combination of BBR and probiotics on the growth of colon cancer and whether this efficacy is associated with the changes in the gut microbiome.

**MATERIALS AND METHODS**

**Animals and Reagent**

Human colonic cancer cell line HT29 (JNO-21409) was obtained from Guangzhou Genio Biological Technology Co., Ltd. BALB/c nude mice aged 4–5 weeks were collected from Guangdong Yaokang Biotechnology Co., Ltd. Fetal bovine serum was purchased from Gibco (10099-141). BBR was purchased from Northeast Pharmaceutical Group Shenyang First Pharmaceutical Co. Ltd. Live combined Bifidobacterium (BNCC232112), Lactobacillus (BNCC336974) and Enterococcus (BNCC192631) powder (LCBLEP), MRS medium dry powder (0016), and Intestinal Bacterial Enrichment Broth (EE broth, BNCC336974) powder (LCBLEP), MRS medium dry powder (BNCC336974), and enterococcus (BNCC192631) powder (LCBLEP). The colon cancer cell line HT29 was conventionally purchased from Guangzhou Leisha Biological Technology Co., Ltd. Nucleic acid extraction or purification reagent, Agencourt AMPure XP60ml Kit, Qubit dsDNAHS Assay Kit, NovaSeq 6000 S4 Reagent Kit (300 cycle) were purchased from Guhe of China (GHFDE100), Beckman Coulter (A63881), Life tech (Q32851), and Illumina (20012866), respectively. Mouse sodium butyrate (SB) ELISA Kit was from MEIMIAN Biotechnology Co., Ltd (MM-46099M1). A total number of three HDAC inhibitors, such as panobinostat (LBH589), sodium butyrate, trichostatin A (TSA), were obtained from Beyotime (Wuhan, China).

**Establishment of a Xenograft Model of Colon Cancer in Nude Mice**

Animal feeding and management were carried out in accordance with "laboratory animal environment and facilities" (GB 14925-2010). The colon cancer cell line HT29 was conventionally cultured, and the cells were collected after digestion with trypsin until 80–90% of the culture flask was filled and resuspended with PBS to 2–5 × 10⁶/ml. The cell suspension with 0.2 ml was inoculated subcutaneously on the back of nude mice for 4 weeks. Tumor growth was observed weekly and tumor size was measured. All animals’ performances were conducted in accordance with animal ethics.

**Treatment of Berberine and Probiotics in vitro and in vivo**

**In vitro**

After the mixed probiotics (Bifidobacterium, Lactobacillus acidophilus, and Enterococcus faecalis) were cultured in the liquid medium under anaerobic conditions (the number of viable bacteria should reach 1 × 10⁶ CFU), the culture supernatant was collected, and it is probiotic supernatant (PS). HT29 cells were treated with 120 μmol/L, 280 μmol/L, 420 μmol/L BBR, 22.1 μmol/L cetuximab, and 25, 50, and 75% PS (diluted with culture medium) for 48, 72, 96 h, respectively. Morphological changes were observed using an inverted phase-contrast microscope (BX53M, Olympus) (10 × 20). The cell proliferation rate of each group was detected by methythiazolyldiphenyl-tetrazolium bromide (MTT).

**In vivo**

A number of 44 BALB/c nude mice (half male and half female) were randomly divided into five groups, namely, normal control (NC group, n = 8), model group (n = 9), model + BBR (n = 9), model + LCBLEP (Model + LCBLEP, n = 9), and model + Combination of BBR with LCBLEP (Model + CBP, n = 9). In the model group, the xenograft model was constructed according to the above method. In the Model + BBR and Model + LCBLEP groups, the mice were, respectively, treated with BBR (78 mg/kg) and LCBLEP (7.8 × 10⁶ CFU/kg) by gavage at the beginning of modeling. A normal control group was injected subcutaneously with the same volume of normal saline. At the end of modeling, the feces of mice were collected for gut microbiome detection. Then, the nude mice were anesthetized by intraperitoneal injection of chloral hydrate to obtain tumor tissues and then sacrificed through cervical dislocation. There was no abnormal death of animals in the process of modeling.

**Detection of Cell Proliferation by CCK-8 or Methythiazolyldiphenyl-Tetrazolium Bromide (MTT)**

After the cells that were plated into 96-well plates were cultured, MTT solution was added to each well (final concentration 0.5 mg/ml) and incubated at 37°C for 4 h. The culture supernatant was removed from the well, and 150 μl DMSO was added. OD values were detected at 490-nm wavelength. Alternatively, the wells were added with 100 μl of CCK-8 solution (CK04-500, Dojindo, Japan) to each well and cultured at 37°C for 2 h, and then, OD values were detected at 450-nm wavelength.

**ELISA Assay of Sodium Butyrate in Mouse Serum**

The mice were killed through disconnecting cervical vertebra and the corresponding serum samples were obtained. In total, 10 μl sample with 40 μl sample diluent were added into each well of the enzyme plate which has been precoated with mice SB-specific monoclonal capture antibody. Then, 100 μl horseradish peroxidase (HRP)-labeled mice SB antibody was added and incubated at 37°C for 1 h, and the plate was washed 3 times. Color developer solution was added at 100 μl/well and incubated at room temperature for 15 min. After 50 μl/well stop buffer was added for 5 min, OD values were detected at 450-nm wavelength.

**DNA Extraction**

Feces from nude mice were collected and stored in a special sample preservation solution. Nucleic acid extraction reagent
(Guhe Biological Co., Ltd., Hangzhou, China, GHFDE100) was used for DNA extraction, and the NanoDrop luminance meter (Thermo Fisher Scientific, Waltham, MA, United States) was used to determine the concentration and quality of DNA.

**Sample Amplification and Electrophoresis**

Nuclease-free water was used to dilute the primers to 1 µm and gDNA to 5 ng/µl. The primers (F: Illumina_uni_sequence-Read1_sequence_GTGCCAGCMGGCGGTATAA, R: Illumina_uni_sequence_(barcode)_read2_sequence_GGACTACHVGGGTWTCTAAT) used for PCR had been fused with the V4 universal primers of Illumina sequencing platform. A total of 50 µl PCR reaction system was prepared using Phusion High-Fidelity PCR Master Mix with HF buffer, and PCR amplification was performed. Then, PCR products were detected by 1% agarose gel electrophoresis with a sample loading of 2 µl.

**Purification of Magnetic Beads**

Totally, 0.85x AMPure XP Beads were added to the remaining PCR products and mixed for at least 10 times. The PCR tubes were placed at room temperature for 5 min and then placed on the magnetic bead plate until the liquid became clear (about 5 min). The PCR tubes were mixed with 200 µl of 80% alcohol and washed for 30 s, and then, the liquid was carefully sucked out and discarded. The PCR tubes on the magnetic bead plates were dried until the beads were cracked completely (about 5 min). About 17 µl of nuclease-free water was added to the dried beads, and the beads were blended. The PCR tubes were put on the magnetic bead plates for separation, and after the liquid became clear, 15 µl liquid was transferred into the new PCR tube.

**Pyrosequencing Using 16S rDNA Amplicon**

Forward primer 515F (5′-GTGCCAGCMGGCGGTATAA-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used for PCR amplification of V4 region of bacterial 16S rRNA gene. Barcode was synthesized into the sequence using a 7-bp specific sequence. A total of 50 µl PCR reaction system was performed using Phusion High-Fidelity PCR Master Mix with HF buffer, and PCR amplification was performed by the computer. The relative expression of the HDAC1 gene was calculated as follows: 

$$2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct_{	ext{Test}} - \Delta Ct_{	ext{Control}})}$$

**Sequence Analysis**

The data of each sample from the original data according to the barcode sequence and primer sequence were split. After the barcode and primer sequences were cut off, Vsearch v2.4.4 was used to splicing the reads of each sample to obtain the original Tags data (Raw Tags). Simultaneously, the control and filtration of the sequence quality were performed. The curves for screening low-quality sequences were as follows: the sequences less than 150 bp, the average mass value less than 20, the sequences containing unclear bases, and the mononucleotide repeats containing > 8 bp were screened; the chimicer sequences were also removed and the final valid data (Effective Tags) were obtained.

**Statistics Analysis**

Quantitative Insights Into Microbial Ecology (QIIME) software was used to calculate the alpha diversity index of operational taxonomic unit (OTU) level, including Chao1, ACE, PD_whole_tree, Shannon, and Simpson, then, the curve of ranked abundance was formed, and the dilution curve was drawn. The difference analysis of the alpha diversity index between the groups was used to compare OTU abundance and evenness between the samples. The beta diversity analysis was performed using QIMME software to calculate the UniFrac distance measure (Lozupone and Knight, 2005; Lozupone et al., 2007). Principal component analysis (PCA), principal coordinate analysis (PCoA), and non-metric multidimensional scaling (NMDS) maps were drawn for beta diversity analysis of microbial flora structure of different samples.

The t-test and the Monte Carlo permutation test were used to draw a box plot to compare UniFrac distance differences between groups. The Kruskal method of the R stats package was used to compare the differences in taxonomic phylum, class, order, family, and genus between samples and groups. In LDA effect size (LEfSe) analysis, the LEfSe default setting was used to detect the differences in classification units between the groups. In random forest analysis, the R package default setting was used to compare the differences between groups. ANOVA was used to compare tumor volumes and cellular proliferation inhibition rate between groups, and least significant difference (LSD) was served to perform the multiple comparisons. p-value < 0.05 was considered to be statistically significant.

**RESULTS**

**Berberine and Probiotics Reduced the Growth of HT29 Cells and Colon Cancer in Nude Mice**

The cultured HT29 cells were treated with BBR or PS with different concentrations, the diverse cellular
morphology was observed compared with the control (Figure 1A), and cell growth appeared to be significantly inhibited. We further found that the inhibition of BBR on cell proliferation was concentration-dependent which was not significant in the cells with PS using MTT methods (Figure 1B). The suppression of 75% PS on the proliferation was the most significant. These results revealed that both BBR and probiotics could inhibit the growth of colon cancer cells in vitro.
As shown in Figures 1C,D and Table 1, the tumor of the model began to increase in size by 5 days. The treatment of BBR and combination of BBR with probiotics (CBP) reduced the growth of neoplasms to a different extent, especially at 14 days; however, no statistical difference was observed compared with the model. Interestingly, the inhibitory effect of LCBLEP on the tumor growth was obvious, especially at 11—21 days, there was a difference in statistics between LCBLEP and the model group.

Berberine and Probiotics Affected Richness and Evenness in the Gut Microbiota

As observed in Figures 1E,F, the growth of colon cancer cells had an influence on the abundance of intestinal microbiota. Compared with the normal control, the richness and evenness of gut microbiota in the model nude mice were decreased, which was affected by the treatment of BBR and LCBLEP. The supplement of probiotics (LCBLEP) significantly increased both the richness and evenness, whereas the effects of alone use of the BBR and the CBP on the enhancement of richness and evenness were not obvious.

Berberine and Probiotics Significantly Improved the Composition of Gut Microbiota in Nude Mice

As shown in Figure 2A, the gut microbiota was analyzed according to the classification of phylum, class, order, family, and genus. At the level of phylum, the relative abundance of Firmicutes, Verrucomicrobia, and Clostridia was significantly increased in the xenograft model of colon cancer in nude mice. On the contrary, the abundance of Bacteroidetes and Proteobacteria was decreased in the model, which was improved by the treatment of BBR instead of LCBLEP. The addition of probiotics (LCBLEP) significantly increased both the richness and evenness, whereas the effects of alone use of the BBR and the CBP on the enhancement of richness and evenness were not obvious.

Table 1

| Days | Groups | 0 day | 5 days | 9 days | 11 days | 14 days | 16 days | 21 days |
|------|--------|-------|--------|--------|---------|---------|---------|---------|
|      | Group A (n = 8) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
|      | Group B (n = 9) | 53.32 ± 14.01 | 182.00 ± 75.18 | 226.62 ± 85.33 | 494.47 ± 219.56 | 828.44 ± 426.19 | 1043.50 ± 458.30 | 1388.74 ± 644.50 |
|      | Group C (n = 9) | 68.64 ± 19.13 | 186.57 ± 120.31 | 270.69 ± 200.13 | 440.94 ± 300.95 | 701.82 ± 532.70 | 911.07 ± 648.90 | 1108.13 ± 711.59 |
|      | Group D (n = 9) | 45.99 ± 16.90 | 125.60 ± 49.16 | 163.40 ± 54.12 | 266.65 ± 107.86* | 420.71 ± 131.57* | 579.12 ± 180.78* | 773.03 ± 261.63* |
|      | Group E (n = 9) | 51.54 ± 23.46 | 149.65 ± 100.75 | 231.37 ± 155.80 | 426.63 ± 339.11 | 639.99 ± 550.40 | 835.55 ± 716.93 | 1093.91 ± 903.57 |

Group A, normal control; Group B, model of colon cancer; Group C, model with berberine (BBR); Group D, model with probiotics preparation (LCBLEP); Group E, model with CBP. *P<0.05 vs. Group B.

Dysregulated Diversity of Intestinal Microbiota Was Modulated by Berberine and Probiotics

Alpha diversity, an analysis of species diversity in a single sample, is calculated and assessed by the richness and diversity of the microbiome through a series of statistical indices. The diversity index measures the heterogeneity of a community. So far, commonly used measure indictors for alpha diversity include Chao1, Shannon, and Simpson. Chao1 is used to measure community
FIGURE 2 | (A) Histograms of relative abundance of species composition at phylum, class, order, family, and genus level, respectively. (B) Clustering heat maps based on genus level were performed to reveal the similarity between samples and the similarity of community composition at the genus level. (C) Box diagram of alpha diversity was analyzed through assessing the species richness and community diversity of the microbiome by Shannon, Simpson, and Chao1. (D) The beta diversity was used to compare the differences between multiple groups of samples by the multivariate statistical method including principal component analysis (PCA), principal coordinate analysis (PCoA), and non-metric multidimensional scaling (NMDS). The horizontal and vertical box diagrams were the distribution of the values of the different groups on the first and the second principal coordinates. Each dot represented a sample, the same color was from the same group, and distance reflected sample similarity.
Berberine and Probiotics Significantly Affected the Biomarker of the Gut Microbe in Nude Mice With Colon Cancer

Biomarkers, namely, the dominant species with significant difference between groups, were screened through LEfSe analysis. As shown in Figure 3A, the biomarkers in the model group included Psychrobacter, Moraxellaceae, Odoribacteraceae, and Odoribacter. Verrucomicrobiacea, Verrucomicrobiales, Verrucomicrobia, Verrucomicrobia, and Akkermansia, were the primary biomarkers in the group treated with BBR. On the contrary, Clostridia, Firmicutes, Clostridiales, and Coprococcus were the markers in the mice intervened with LCBLEP. However, the biomarkers in the group treated with CBP were significantly different from the other groups, including Rikenellaceae, Bacilli, Lactobacillales, Anaeroplasmataceae, Anaeroplasmatales, Tenericutes, Mollicutes, Ruminococcus, Lactobacillaceae, Lactobacillus, and Rikenella.

Next, the biomarkers were selected between groups according to the species classification level (Figure 3B). In phylum, the dominant biomarkers included Bacteroidetes, Firmicutes, Tenericutes, and Verrucomicrobia. These species were dysregulated in the nude mouse model of colon cancer but modulated by BBR or LCBLEP. BBR significantly promoted the relative abundance of Bacteroidetes but inhibited the level of Firmicutes, which was consistent with that of CBP. In class, the relative abundance of Bacteroidia and Mollicutes was increased by BBR, with decreased Clostridia. The treatment of CBP obviously promoted the relative level of Mollicutes but decreased the abundance of Verrucomicrobia. Similarly, these dominant species in the order were Bacteroidales, Lactobacillales, Clostridiales, Campylobacterales, Anaeroplasmatales, Mol_RF39, and Verrucomicrobiales. The abundance of Anaeroplasmatales was dramatically increased by the CBP, with decreased Verrucomicrobiales. In family, these biomarkers reduced by BBR included Porphyromonadaceae, Rikenellaceae,
and Odoribacteraceae, as well as Lactobacillaceae and Anaeroplasmataceae were increased. Compared with the model, reduction of Porphyromonadaceae, Odoribacteraceae, Verrucomicrobiaceae, and Moraxellaceae by probiotics was found, with increased Anaeroplasmataceae. Surprisingly, no influence of BBR or LCBLEP on the markers including Bacteroidaceae, Paraprevotellaceae, and Helicobacteraceae was observed in contrast to the model group. Similarly, the effect of BBR or LCBLEP on the biomarkers in genus involving Bacteroides, Prevotella, and Proteus was also not observed compared with the model. BBR decreased the abundance of Parabacteroides, Rikenella, Odoribacter, Coprococcus, Ruminococcus, and Psychrobacter, with increased Anaeroplasma and Lactobacillus, whereas the relative level of Parabacteroides, Odoribacter, Ruminococcus, Akkermansia, and Psychrobacter was reduced by treatment of LCBLEP compared to the model group. Besides, we found that the relative abundance of Moraxellaceae and Psychrobacter was dramatically high and dominant biomarkers in the gut of colon cancer.

Whether the grouping of these markers was meaningful? Analysis of similarities (ANOSIM) was used to test whether the difference between groups was significantly greater than the difference within groups. As shown in Figure 4A, the difference between groups was higher than that within groups ($R = 0.194$, $p < 0.05$). However, what were the predominant species used for classification. A random forest classification tree was adopted. As revealed in Figure 4B, Peptococcaceae was the dominant microbe playing important role in the grouping, followed by Acinetobacter and Rikenella. How effective were these major biomarkers in a grouping? We found that these biomarkers demonstrated some accuracy in the diagnosis of all groups (all AUC > 0.85) (Figure 4C).

In this study, we performed an evolutionary tree of species by GraPhlan to find the dominant species more visually in each group (Figure 4D). Odoribacteraceae, Dehalobacteriaceae, and Proteobacteria were the predominant intestinal microbes in colon cancer, whereas only Verrucomicrobiaceae was dominant in the gut treated by BBR. The main species in the gut treated by LCBLEP were Desulfovibrionaceae, Ruminococcaceae, and Lachnospiraceae, which was partly shared with that of the intestine intervened by CBP where Anaeroplasmataceae, Coriobacteriaceae, and Rikenellaceae were also predominant.

Next, we established box diagrams of significant differences for function prediction with KEGG level 3 of species between groups at the genus level. As revealed in Figure 5B, compared with the control, the metabolisms in the gut of nude mice implanted with colon cancer cells were aberrantly active except for indole alkaloid biosynthesis and alpha-linolenic acid metabolism ($p < 0.05$), which was significantly repressed by the intervention of the LCBLEP. However, the treatment of BBR instead of LCBLEP inhibited the biosynthesis of stilbenoid diarylheptanoid and gingerol. Interestingly, the influence of LCBLEP on the metabolisms of gut microbe was similar to that of CBP.

Another analysis of the metabolic function of gut microbiota was performed through the comparison of phenotypic classification based on BugBase (an online 16S function prediction tool), and the results are shown in Figure 5C. These phenotypes included Gram-positive, Gram-negative, biofilm formation, pathogenicity, mobile elements, oxygen requirements, and oxidative stress tolerance, involving anaerobic bacteria, aerobic bacteria, and facultative bacteria. We found that Gram-negative bacteria, potentially pathogenic bacteria, facultative bacteria, and anaerobic bacteria were decreased in the gut of nude mice with colon cancer compared to the control ($p < 0.05$), whereas Gram-positive bacteria, stress tolerance, mobile elements, aerobic bacteria, and biofilm formation were upregulated ($p < 0.05$). On the contrary, BBR upregulated the relative abundance of Gram-negative bacteria, potential pathogenic bacteria, facultative bacteria, aerobic bacteria, and biofilm formation and downregulated the level of Gram-positive bacteria, mobile elements, and anaerobic bacteria. These effects of BBR were opposite to that of LCBLEP. However, the influence of CBP on the phenotype of intestine microbe was different from that of BBR or LCBLEP. CBP partly neutralized the effect of BBR and LCBLEP, such as gram-negative and -positive bacteria, aerobic bacteria, anaerobic bacteria, and biofilm formation. Interestingly, significant upregulation of facultative bacteria and downregulation of mobile elements were found in the gut treated with CBP, compared to that of BBR or LCBLEP. Besides, BBR, LCBLEP, and CBP inhibited the relative level of stress tolerance, especially LCBLEP and CBP.

Berberine and Probiotics Regulated Butyrate Production in Nude Mice With Colon Cancer


decaylase Type 1 Expression

In the process of colon cancer, although amino acids and other bacterial metabolites increased, the production of butyrate was decreased. In fact, many beneficial species that maintain intestinal homeostasis by producing butyrate, including Bifidobacterium, Roseburia, and Faecalibacterium prausnitzii, were reduced in patients with CRC (Fukuda et al., 2011). Butyrate, a regulator of epigenetic modifications, is responsible for downregulating the acetylation of histones and is considered a carcinostatic agent. We observed that level of histone deacetylase type 1 (HDAC1) mRNA was reduced in the HT29 cells treated with BBR or PS compared with the control ($p < 0.05$), whereas this phenomenon was not found in the cells with cetuximab treatment (Figure 6A).
FIGURE 4 | (A) Species biomarkers were tested through analysis of similarities (ANOSIM) which was used to detect whether the difference between groups was significantly greater than the difference within groups, so as to judge whether the grouping was meaningful. R-value was used to compare whether there are differences between groups. If R-value was between (−1, 1) and greater than 0, the difference between groups was greater than that within groups. R-value < 0 indicated the inter-group difference was smaller than the intra-group difference. (B) Random forest classification tree with high accuracy was performed to effectively classify and predict the grouped samples. (C) The abscissa was the level of importance, and the ordinate was the species name in the order of importance. Receiver operating characteristic (ROC) curves were drawn at genus level. (D) GraPhlan figure, a map of sample communities in the evolutionary tree of species, was used to easily observe the dominant species.
Interestingly, the decreased level of HDAC1 mRNA in the BBR treatment group was lower than that of PS or BBR combined with PS ($p < 0.05$), which brought us a clue that BBR might upregulate or increased some products leading to suppression of HDACs such as HDAC1. As shown in the serum of mice (Figure 6B), the mice treated with BBR revealed a significantly increased
concentration of sodium butyrate (SB) in serum compared with
the model (p < 0.05) followed by the combination of BBR with
LCBLEP. Next, we selected three kinds of HDAC inhibitors,
LBH589, sodium butyrate, and trichostatin A. Our observation
found that the inhibitory effect of SB on the proliferation of
HT29 cells was stronger than LBH and TSA, and this effect was
time-dependent (Figure 6C).

DISCUSSION

Colorectal cancer is one of the most common types of cancer
and the third leading cause of cancer-related death (Fitzmaurice,
2018). The human colon is a complex microbial ecosystem.
Intestinal epithelial cells with high regeneration pressure are
frequently in contact with nutrients and microbiota and are
prone to malignant transformation. Extensive studies have
revealed the key role of the microbiome in colon tumorigenesis
(Roberti et al., 2020; Yang et al., 2022). Dysbiosis of gut
microbe has been demonstrated to be beneficial to the risk
of tumor formation (Garrett, 2019; Molska and Regula, 2019;
Nejman et al., 2020; Pothuraju et al., 2021). In 1975, researchers
first discovered the association between gut flora and CRC
(Weisburger et al., 1975). Antibiotics in CRC transplanted mice
not only resulted in decreased clostridium but also repressed
cancer cell proliferation and overall tumor growth (Yamaoka
et al., 2018; Oh et al., 2019). Compared with healthy people,
patients with CRC had less diversity and significantly decreased
abundance of beneficial bacteria in their gut. These dysregulated
microbes can activate chronic inflammation releasing various
cytokines and producing a lot of exotoxins and endotoxins
directly or indirectly inducing DNA damage, genomic instability,
tumorigenesis, and adenocarcinoma invasion (Irrazabal et al.,
2020; Loke et al., 2020; Abu-Ghazaleh et al., 2021). In our
study, we found that obvious dysbiosis has existed in the gut
of nude mice with colon cancer cells. Therefore, these pieces of
evidence have suggested that regulation of intestine microbe is
an important strategy to prevent and cure the occurrence and
development of colon cancer.

Increasingly extensive clinical and experimental data
suggest that some Chinese herbal extracts play a therapeutic
role by regulating the abundance of intestinal microbiota.
BBR, an alkaloid extracted from Rhizoma Coptidis, is an
anti-inflammatory drug used to treat infections in the digestive
tract. Recent researches have demonstrated its potent antitumor
activity (Liu et al., 2020; Song D. et al., 2020; Zhang Q. et al.,
2020). Interestingly, BBR has been found to regulate the intestinal
microbe of rats fed with a high-fat diet, thus improving their
metabolic status (Sun et al., 2016), which is similar to that of probiotics. Probiotics, now widely regarded as biological therapies, have a variety of biological benefits to host health, including anti-bacterial activity, regulating the immune system, inhibiting colitis, and preventing CRC. In patients with CRC, direct supplementation of probiotics can promote the effect of CRC-related therapies through the regulation of intestinal microbiome (Molska and Regula, 2019; Fong et al., 2020; Torres-Maravilla et al., 2021). This suggests that mediation of dysbacteriosis may be one of the new antitumor mechanisms of BBR (Habtemariam, 2020). However, it is not known whether the combination of BBR and probiotics has a synergistic effect on the inhibition of colon cancer growth.

Live combined *Bifidobacterium*, LCBLEP, is an oral probiotic preparation widely used in a clinic. Consistent with the previous study, our observation has found that, although there is no difference in statistics for BBR, both BBR and probiotics can inhibit the growth of colon cancer cells to some extent, suggesting that the suppressive effect of BBR is lower than that of probiotics. Further study has found that, although the treatment of the BBR on the enhancement of richness and evenness was not obvious in contrast to that of the LCBLEP, composition of microbe at each classification level can be modulated by BBR in which the abundance of *Bacteroidetes* and *Proteobacteria* is significantly increased. In our study, we found dramatically decreased *bacteroides* in the gut of colon cancer mice, which was increased by BBR and LCBLEP, especially CBP. These suggest that *Bacteroides* is a beneficial bacterium that suppresses the growth of colon cancer. Some studies have demonstrated that *Bacteroidetes* can regulate the production of E-cadherin, nuclear factor-κB, and STAT3, and its abundance is decreased in CRC (Hwang et al., 2020; Jiang et al., 2020; Zhang W. et al., 2020).

In addition, we observed that BBR significantly increased the abundance of *Roseburia* which was dramatically reduced in CRC (Fukuda et al., 2011), suggesting that *Roseburia* is one of the beneficial species that maintain intestinal flora homeostasis by producing butyrate, and this efficacy is further enhanced by the CBP. It means that the improvement of intestinal dysbiosis by a combination of BBR with probiotics is stronger than that by BBR and probiotics alone. Just because of this, both BBR and probiotics can improve the alpha biodiversity and beta biodiversity in the gut with colon cancer cells. Once the gut microbiota remains in dysbiosis, the diversity of beneficial symbiotic bacteria is reduced, resulting in the production of various bacteriotoxins or increased exposure of colon epithelial cells to carcinogens (Meng et al., 2018). Recent research also reveals that BBR can reverse the structural and numerical changes in the intestine microbiota under pathological conditions (Habtemariam, 2020). Consistent with our study, BBR and LCBLEP can alter the biomarkers in the gut, especially the CBP, suggesting that the combined BBR and probiotics had a greater regulatory effect on the bacterial community disorder than BBR and probiotics alone.

Many studies have shown that obesity is considered a risk factor for CRC (Chen et al., 2021), and recent research suggests that an increased intestinal *Firmicutes/Bacteroides* ratio is the hallmark of obesity (Kim et al., 2021). We speculated that regulation of the *Firmicutes/Bacteroides* ratio may be a strategy to control the growth of colon cancer. Our results demonstrated that the increased abundance of *Firmicutes* and decreased *Bacteroides* existed in the model planted with colon cancer, which was reversed by BBR and CBP. Besides, some pro-inflammatory bacteria such as *Ruminococcus*, *Peptococcaceae*, *Lactobacillus*, and anti-inflammatory *Bifidobacterium* exist within the gut (Kim et al., 2021). Decreased *Ruminococcus* and increased *Lactobacillus* were observed in the mice treated with BBR, suggesting that BBR can regulate the proportion of inflammatory bacteria. *Clostridiales*, *Bifidobacterium*, and *Lachnospiraceae* are beneficial bacterial taxa (Zhou et al., 2021), and both LCBLEP and CBP promoted the relative abundance of *Clostridiales* and *Lachnospiraceae*. These data suggest that BBR and LCBLEP mediate the dysbacteriosis in the gut of nude mice with colon cancer, but the effect of the combination of BBR with probiotics may be more potent.

In addition, we found other bacteria increased by BBR, such as *Roseburia*, and thus, we considered that *Roseburia* is also useful in suppression of colon cancer. An increasing number of evidence reveals that anti-cancer short-chain fatty acids (SCFAs) were produced by beneficial bacteria including *Eubacterium*, *Roseburia*, and *Rikenella* (Shi et al., 2017; Zheng et al., 2020), and these bacteria were reduced in patients with CRC (Wang et al., 2012). Similarly, reduced *Lachnospira* was observed in the gut of colon mice or patients with cancer (Clos-Garcia et al., 2020), which was increased by BBR and LCBLEP but not CBP. However, CBP can dramatically increase the abundance of *Adliccreutzia*, *Lactobacillus*, and *Rikenella* families, compared with that of BBR and LCBLEP. These results have concluded that the combination of BBR and probiotics has a greater effect on the regulation of bacterial dysregulation in the process of inhibiting colon cancer growth.

Healthy gut microbiota is described by metabolic function. Antimicrobial peptides and immunomodulatory compounds produced by the beneficial microbiome can affect the mucosal immune system, regulating their anticancer effects. Microbiota and its related metabolites are not only closely related to carcinogenesis by inducing inflammation and immune disorders, leading to genetic instability (Kompella and Vasquez, 2019; Tan et al., 2021). Therefore, the metabolic pathways with the highest abundance including replication, recombination, and DNA repair were observed in our study. These active metabolic functions were found in the gut of colon cancer mice, including glycosaminoglycan degradation, glycosyltransferases, lipopolysaccharide biosynthesis, lipid biosynthesis proteins, N-glycan biosynthesis, etc., and these were reversed by LCBLEP or CBP instead of BBR which only downregulated the stilbenoid diarylheptanoid and gingerol biosynthesis. However, indole alkaloid biosynthesis and alpha-linolenic acid metabolism were reduced in colon cancer and mediated by both BBR, LCBLEP, or CBP. These data suggest that the effects of probiotics on the metabolic function of microbe in the colon cancer mice are higher than that of the BBR.

However, the phenotypic effects of BBR on the metabolic functions of bacterial community are very significant. Our
observations reveal that BBR can obviously promote the abundance of microbiota including G+ bacteria, facultatively anaerobic bacteria, aerobic bacteria and repress the G− bacteria, mobile elements, and anaerobic bacteria, compared with the model. Bacteroidetes are G- bacteria and Firmicutes are G +, combining with the effect of BBR on the other bacteria including Verrucomicrobiaceae, Akkermansia, and Enterobacteriaceae, etc. These indicate that BBR has a broad spectrum of regulation on bacterial species.

Biofilm formation can destroy the mucus layer of the colon, strengthening cytotoxicity or genotoxicity through enhancement of colonic epithelial invasion, inflammation, and abnormal immune response (Dejea and Sears, 2016). These phenomena eventually result in malignant proliferation and colorectal cancer. In our study, although BBR increased the abundance of biofilm formation, LCBLEP and CBP significantly decreased the level, suggesting that the combination of BBR with probiotics has an effect on the metabolic function of gut microbiome in nude mice with colon cancer.

An increasing number of extensive evidence has demonstrated that metabolites of gut microbes play vital roles in maintaining healthy intestinal homeostasis and preventing colon carcinogenesis. Butyrate can serve as a fuel source for intestinal epithelial cells (Donohoe et al., 2011) and have anti-inflammatory effects. Bifidobacterium, Roseburia, and Faecalibacterium prausnitzii are beneficial species for maintaining the homeostasis of the intestinal microbe by producing butyrate, which can be mediated by BBR. These suggest that sodium butyrate upregulated by BBR changes the patterns of histone modification of some genes participating in the inhibition of cancer growth. However, less research has been done on the relationship between the microbiome and epigenetic changes in CRC. Some study has found that butyrate induced the expression of cell cycle regulation genes (CCND3 and CDKN1A) in intestinal cells. Infection with L. monocytogenes causes H3K18 deacetylation of many genomic proteins in colon cells, including SMAD1, IRF2, SMARCA2, and CXCL12 (Sabit et al., 2019). Reduced butyrate can disrupt intestinal barrier function, causing immune dysregulation and leading to cell proliferation, which leads to the development of CRC. In this case, reducing the abundance of Clostridium in the intestinal microbe and increasing butyric-producing bacteria may be a treatment strategy for CRC. Furthermore, our results have also found that probiotic was inferior to BBR in increasing butyrate production, suggesting that BBR inhibits the growth of colon cancer cells through the regulation of SB production and HDAC1 expression.

In conclusion, BBR and probiotics can reduce the growth of colon cancer cells, with more potent effect of the latter. Besides, BBR and probiotics can mediate the composition, structure, abundance, biological diversity, and metabolic function of gut microbiome in nude mice with colon cancer, which is more significant in the mice treated with the combination of BBR with probiotics. Therefore, BBR can also be used as a regulator of intestinal microbiome similar to the probiotics. However, the influence of combination of the two on the growth of neoplasm in nude mice is not obvious, suggesting that the combination of BBR and probiotics has no advantage in inhibiting tumor growth compared with drug alone. Besides, BBR instead of probiotics can significantly increase the level of SB production inhibiting the HDAC1 expression, and the inhibitory effect of SB on the growth of colon cancer cells was stronger than LBH and TSA. Based on the current research results, we believe that the mechanism of BBR and probiotics inhibiting the growth of colon cancer cells should be consistent. BBR inhibits the growth either through its direct cytotoxic effects or by increasing SB production, while the mechanisms by which probiotics inhibit the growth of cancer cells are likely to work through something other than increased SB production, such as production of SCFAs (Zheng et al., 2020), regulation of macrophages, or relative signaling pathways (Fan et al., 2021), etc.

Unfortunately, no drug positive control for in vivo experiments was performed in our design. In addition, the comparison between LCBLEP and other control probiotic strains with proven anti-colon cancer effects, and the performance of cytotoxicity assay of probiotics on normal cells and other different cancer cell lines are our negligence in this study, which will be considered in further research.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

ETHICS STATEMENT

The animal study was reviewed and approved by the Laboratory Animal Ethics Committee, Shenzhen University Health Science Center.

AUTHOR CONTRIBUTIONS

CH was responsible for the research protocol design and collection of data. YS was responsible for the verification of manuscripts. S-RL, Z-XC, and H-FL were responsible for figure making and layout. W-ZS was responsible for the procurement of reagents or materials. All authors contributed to the article and approved the submitted version.

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