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Research article

Berberine attenuates 5-fluorouracil-induced intestinal mucosal injury by modulating the gut microbiota without compromising its anti-tumor efficacy

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ABSTRACT

Background: 5-Fluorouracil (5-Fu), a prominent chemotherapeutic agent for colorectal cancer (CRC) treatment, is often associated with gastrointestinal toxicities, particularly diarrhea. Our previous study demonstrated that berberine (BBR) ameliorates 5-Fu-induced intestinal mucosal injury by modulating the gut microbiota in rats. Nevertheless, the precise molecular mechanism underlying BBR's protective effect on intestinal mucosa remains elusive, and its impact on the anti-tumor efficacy of 5-Fu warrants further investigation.

Methods: The effect of BBR on 5-Fu-induced intestinal mucosal injury was investigated using a tumor-bearing murine model, employing H&E staining, 16 S rDNA sequencing, transcriptome sequencing, Western blot analysis, cell experiments and constructing a pseudo-germ-free tumor xenograft model.

Result: Our findings demonstrate that BBR alleviates intestinal mucosal damage, reduces the levels of inflammatory factors (IL-6, TNF-α, and IL-1β), and inhibits epithelial cell apoptosis in 5-Fu-treated mice without compromising 5-Fu's anti-tumor efficacy. Moreover, 16 S rDNA

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sequencing indicated that BBR significantly increases the abundance of Akkermansia and decreases the abundance of pathogenic bacteria Escherichia/Shigella at the genus level. Mechanistically, transcriptome sequencing and Western blot analysis confirmed that BBR upregulates PI3K/AKT/mTOR expression in the intestinal mucosa. However, this effect was not observed in tumor tissues. Notably, BBR did not demonstrate a direct protective effect on 5-Fu-treated CCD841 and SW480 cells. Additionally, BBR had no effect on the PI3K/AKT/mTOR pathway in the intestinal tissue of the 5-Fu-treated mouse model with a depleted gut microbiota.

Conclusion: This study indicates that BBR alleviates 5-Fu-induced intestinal mucosal injury by modulating the gut microbiota and regulating the PI3K/AKT/mTOR signaling pathway without compromising the anti-tumor efficacy of 5-Fu.

1. Introduction

Colorectal cancer (CRC), the second most prevalent malignancy globally, significantly threat to human health [1]. 5-Fluorouracil (5-Fu), a primary chemotherapeutic agent for CRC [2], is associated with a high incidence of severe diarrhea (grades 3 and 4), affecting 14 %–47 % of patients undergoing treatment [3]. This adverse effect compromises treatment efficacy. Research indicates that 5-Fu disrupts gut microbiota, increases inflammation, and damages the intestinal mucosal barrier, leading to diarrhea [4–6]. These complications severely impact patients' quality of life and therapeutic outcomes, highlighting the urgent for comprehensive strategies to prevent and manage 5-Fu-induced diarrhea in CRC patients.

Recent advancements in treating chemotherapy-induced diarrhea have primarily focused on modulating the gut microbiota using substances such as Aquilariae Lignum Resinatum extract, Qingjie Fuzheng granules, and patchouli alcohol [7–9]. Nevertheless, the clinical utility of these substances is limited due to a lack of comprehensive research and rigorous clinical evaluation. Berberine (BBR), mainly derived from *Coptis* and *Phellodendron* species, is well-recognized for its efficacy in managing diabetes, arthritis, atherosclerosis, and ulcerative colitis [10–13]. Additionally, BBR alleviates irinotecan (CPT-11)- and dextran sulphate sodium (DSS)-induced intestinal mucosal inflammation by regulating the gut microbiota [14,15]. Clinical studies showed that BBR can prevent radiation-induced diarrhea, irritable bowel syndrome, and infections caused by *Escherichia coli* and *Vibrio cholerae* [16–18]. Recent research has also explored BBR's anti-tumor effects, revealing that it inhibits CRC by regulating gut microbiota-mediated lysophosphatidylcholine in vivo [19]. BBR also downregulation aquaporins (AQPs) 1, 3, and 5 expression and acts similarly to DNA methyltransferase (DNMT) inhibitors, thereby inhibiting HT29 cell proliferation [20,21]. Notably, a two-year regimen of oral BBR significantly reduced the recurrence rate of colorectal adenoma without any serious adverse events [22]. However, the efficacy of BBR in chemotherapy-induced diarrhea and its underlying mechanisms remain unclear, limiting its clinical application.

Our previous study demonstrated that BBR ameliorates 5-Fu-induced intestinal mucosal injury by modulating the gut microbiota [23]. Nevertheless, the precise molecular mechanism behind BBR's protective effect on intestinal mucosa remains elusive, and its impact on the anti-tumor efficacy of 5-Fu requires further investigation. This study aimed to elucidate the mechanism underlying of BBR's protective effect against 5-Fu-induced intestinal mucosal injury in tumor-bearing mice and assess its impact on the anti-tumor properties of 5-Fu.

2. Materials and methods

2.1. Medicinal materials

Berberine (purity>97 %, CSA: 141,433-60-6) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). 5-Fluorouracil injection was purchased from Southwest Pharmaceutical Co., Ltd (Chongqing, China; State Food and Drug Administration: H50020128). Purchased 5-Fluorouracil (purity≥99 %, powder, CAS: 51-21-8) from Sigma (Sigma-Aldrich, USA). Ciprofloxacin (purity>98 %, CAS: 85,721-33-1), Vancomycin (purity>95 %, CAS: 1404-90-6), Metronidazole (purity>95 %, CAS: 443-48-1), and Neomycin Sulphate (purity>97 %, CAS: 1405-10-3) were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China).

The male BALB/c mice, weighing approximately 18–22 g and aged 6–8 weeks, were purchased from Shanghai Sippe-Bk Lab Animal Co., Ltd. The mice were kept in a suitable environment at in the laboratory animal center of Zhejiang University of Traditional Chinese Medicine. The environment conditions maintained for the mice included a temperature of 20 ± 2 °C, a humidity of 50 ± 5 %, and 12 h light/dark cycle. During the study, the mice were provided with unrestricted access to food and water in accordance with the established institutional guidelines for animal care.

2.2. Animal experiments design

The 5-Fu-induced intestinal mucosal injury with tumor xenograft model was constructed after a one-week adaptability period. In short, we harvested CT-26 cells (2.5×10^5 in 150 μL of PBS) and injected them into the right axilla of 30 mice, separately. 10 days later, 30 mice were randomly assigned to the following groups (10 mice each): control, 5-Fu, and 5-Fu + BBR group. The oral dosage of BBR and the injection method of 5-Fu in mice were both based on previous research [14,24]. Mice from 5-Fu and 5-Fu + BBR group were injected intraperitoneally with 5-Fu (40 mg/kg) every 3 days, while control group mice were administered an equal amount of 0.9 %

saline. Additionally, mice in the 5-Fu + BBR group were given BBR (80 mg/kg/day) by a gavage for 12 days, while the mice in the other groups were given the same PBS on a daily basis.

To construct the pseudo-germ-free tumor xenograft model of 5-Fu-induced intestinal mucosal injury. Executing the quadruple antibiotic (ABX) method: Ciprofloxacin 0.2 g/L, Vancomycin 0.5 g/L, Metronidazole 1 g/L, Neomycin Sulphate 1 g/L. A total of 21 mice were randomly assigned to three groups: 5-Fu, 5-Fu + ABX, and 5-Fu + ABX + BBR group. Next, 14 mice from 5-Fu + ABX, 5-Fu + ABX + BBR group were administered antibiotic water for a duration of 10 days. Another 7 mice (5-Fu group) were given regular water after 7 days of adaptive feeding. After discontinuing the use of antibiotic water for three days, CT-26 cells were injected into each mouse. The subsequent experimental process and method followed the same procedures as the establishment of the 5-Fu-induced intestinal mucosal injury with tumor xenograft model.

2.3. Intestinal mucositis assessment and sample collection

Throughout the experiment, the general condition of the mice was monitored, including their weight, tumor volume, and diarrhea score. The diarrhea score was classified as follows: normal (score 0), formed stool (score 1), soft deformed (score 2), loose stools (score 3), and watery stools (score 4). Feces samples were collected from the mice the day before they were euthanized. To collect the feces, each mouse was placed individually in metabolic cages to allow for natural defecation. Once the stool sample was obtained, it was immediately stored in liquid nitrogen. Subsequently, the samples were transferred to −80 °C storage for further testing. Afterward, the mice were sacrificed and measured their colon length. Colon tissue and tumor tissue were collected for protein assay.

2.4. Cell culture

Human intestinal epithelial cells (CCD841) (1.0×10^4/well) were seeded in 96-well plates and incubated with various concentrations of 5-Fu (10 mg/L, 20 mg/L, 40 mg/L, 80 mg/L, and 160 mg/L) for a duration of 24 h. In addition, we observed the effects of incubation with 5-Fu (80 mg/L) combined with different concentrations of BBR (0 μM, 2 μM, 4 μM, 8 μM, 16 μM, and 32 μM) for 24 h on the proliferation of CCD841 using the Cell Count Kit-8 (CCK-8, BOSTER, AR119-10, China).

CCD841 and human colon cancer cells (SW480) were plated in 6-well plates at a density of 2×10^5 cells each, and subsequently incubated overnight at 37 °C in a 5 % CO2 environment. The cells were then separated into four different intervention groups: control, 5-Fu, 5-Fu + BBR (8 μM), and 5-Fu + BBR (16 μM). CCD841 and SW480 cells were exposed to 5-Fu (80 mg/L), 5-Fu + BBR (8 μM), and 5-Fu + BBR (16 μM) respectively for 24 h. After the incubation period, cellular proteins were extracted, and their expression was analyzed using Western blot. CCD841 and SW480 cells were purchased from Shanghai Jinyuan Biotechnology Co., LTD. CT-26 cell was obtained from Wuhan Punosai Life Technology Co., LTD.

2.5. Histopathological assessment

The fresh colon tissue was gathered for subsequent histological analysis. Brief steps: the colon tissue was soaked in 4 % para formaldehyde, dehydration, paraffin-embedded, sliced, and Hematoxylin and Eosin (H&E) staining. Finally, light microscopy was used to observe and evaluate any histological changes present in the tissue. Based on the criteria provided in Supplementary Table 1, the intestinal tissue damage was assessed.

2.6. Western blot

Proteins were extracted from tissues and cells (CCD841, SW480) using RIPA lysis buffer (Millipore Corp, USA) containing a protease (EpiZyme, China). After centrifugation, the supernatant was obtained (13,000 rpm, 10min, 4 °C). Protein loading volume (protein amount 15 μg) was determined by measuring protein concentration with the BCA kit (EpiZyme, China) and mixing with loading buffer 4:1 (Yamei, LT101S, China). Those proteins were then subjected to electrophoresis, and PVDF membrane transfer. Using 5 % skim milk to block, the PVDF membrane was infiltrated in primary antibodies at 4 °C overnight (all antibodies at 1:1000 dilutions): β-actin (Proteintech, 66009-1-Ig, USA), Occludin (Abcam, ab216327, UK), ZO-1 (Abcam, ab216880, UK), Bax (Abcam, ab32503, UK), Bcl-2 (ABcolonal, A11025, China), Cleaved Caspase-3 (Cell Signaling, 9664St, USA), IL-1β (Cell Signaling, 50794 S, USA), IL-6 (HUABIO, R1412-2, China), Tumor necrosis factor-α (TNF-α) (SAB, 41,504, USA), Anti-PI3K (Abcam, ab191606, UK), Anti-AKT (Cell Signaling, 9272 S, USA), Anti-Phospho-AKT (S473) (HUABIO, ET1607-73, China), Anti-mTOR (Abcam, ab32028, UK), Anti-Phospho-mTOR (S2481) (Servicebio, gb114489, China), cleaned with TBST (10 min, 3 times), incubated secondary antibodies at room temperature and cleaned with TBST (10 min, 3 times). Ultimately, Image Lab was used to analyze western blots visualized with enhanced chemiluminescence (ECL) detection reagents. The protein level was separately analyzed by the Image J software.

2.7. 16 S rDNA miseq sequencing

Fecal samples were collected from 4 mice in each group for 16 S rDNA Miseq sequencing. E.Z.N.A.®Stool DNA Kit was used to extract DNA from the stool, following the instructions provided by the manufacturer. As stated above, PCR amplification was performed using the V3+V4 region of the bacterial 16 S rDNA gene as the primers of extracted DNA. The resulting PCR products were confirmed with 2 % agarose gel electrophoresis using AMPure XT beads purified (Beckman Coulter Genomics,

Danvers, MA, USA) and Qubit quantified (Invitrogen, USA). To assess the quality and concentration of the amplicon pools, the

Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA) and Agilent 2100 Bioanalyzer (Agilent, USA) were respectively used. Finally, the libraries were sequenced using the NovaSeq PE250 platform.

2.8. Transcriptome sequencing and analyzing

Colonic total RNA for sequencing and analysis was set to LC-Bio Technology (Hangzhou, China). RNA from the colon tissues of mice in the 5-Fu and 5-Fu + BBR groups was isolated using TRIzol (Invitrogen, CA, USA). The purified RNA was treated with DNase and specifically captured mRNA containing PolyA (polyadenylate) through two rounds of total RNA purification (1 μg). The polyA RNA

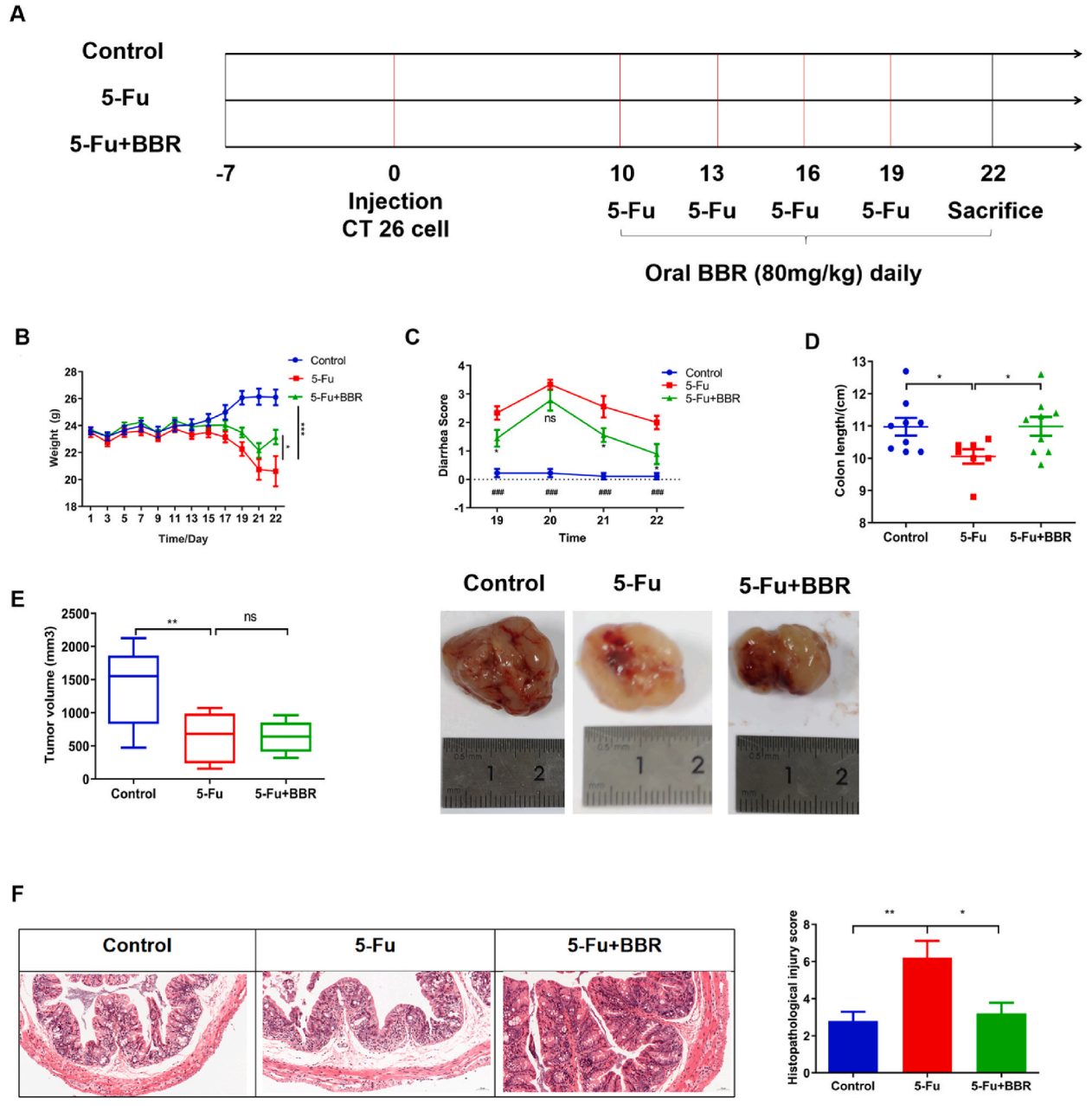

Fig. 1. BBR alleviated the intestinal mucosal damage by 5-Fu-induced without impairing anti-tumor effect of 5-Fu.
(A) Design of the experiment. (B) The weight of mice was recorded throughout the experiment to monitor any changes in body weight (n = 9 per group). (C) Diarrhea was evaluated at the end of 5-Fu treatment (D) Colonic length was measured to assess the protective effect of BBR against the shortening of the colon caused by 5-Fu. (E) 5-Fu significantly inhibited tumor growth, while the anti-tumor effect was not affected by BBR. (F) H&E staining was performed on colon tissue sections, and a histopathological injury score was assigned to assess the severity of colonic injury, All data as the mean ± SEM. ns $P > 0.05$, *$p < 0.05$, **$P < 0.01$, ***$P < 0.001$, $^{###}P < 0.001$.

was then fragmented and subjected to reverse transcription to generate cDNA. Two-strand synthesis was performed using *E. coli* DNA polymerase I (NEB, item No. M0209, USA) and RNase H (NEB, Item No. M0297, USA). The resulting cDNA was digested with UDG enzyme and subjected to PCR (Predegeneration for 3 min at 95 °C, Denaturation for 15 s at 98 °C, 8 cycles, annealing 15 s in 60 °C, and next 30sec to extension at 72 °C). Finally, following standard procedures, the 2 × 150bp paired-end sequencing (PE150) was performed on an Illumina Novaseq™ 6000 (LC-Bio Technology CO., Ltd., Hangzhou, China).

2.9. Immunohistochemistry staining

The paraffin blocks containing colon tissue were sectioned into slices of 4 μM thickness and underwent dewaxing. The slices were then immersed in citrate buffer (pH 6.0) for heat treatment. The sections were soaked in 3 % hydrogen peroxide for 5 min and rinsed in

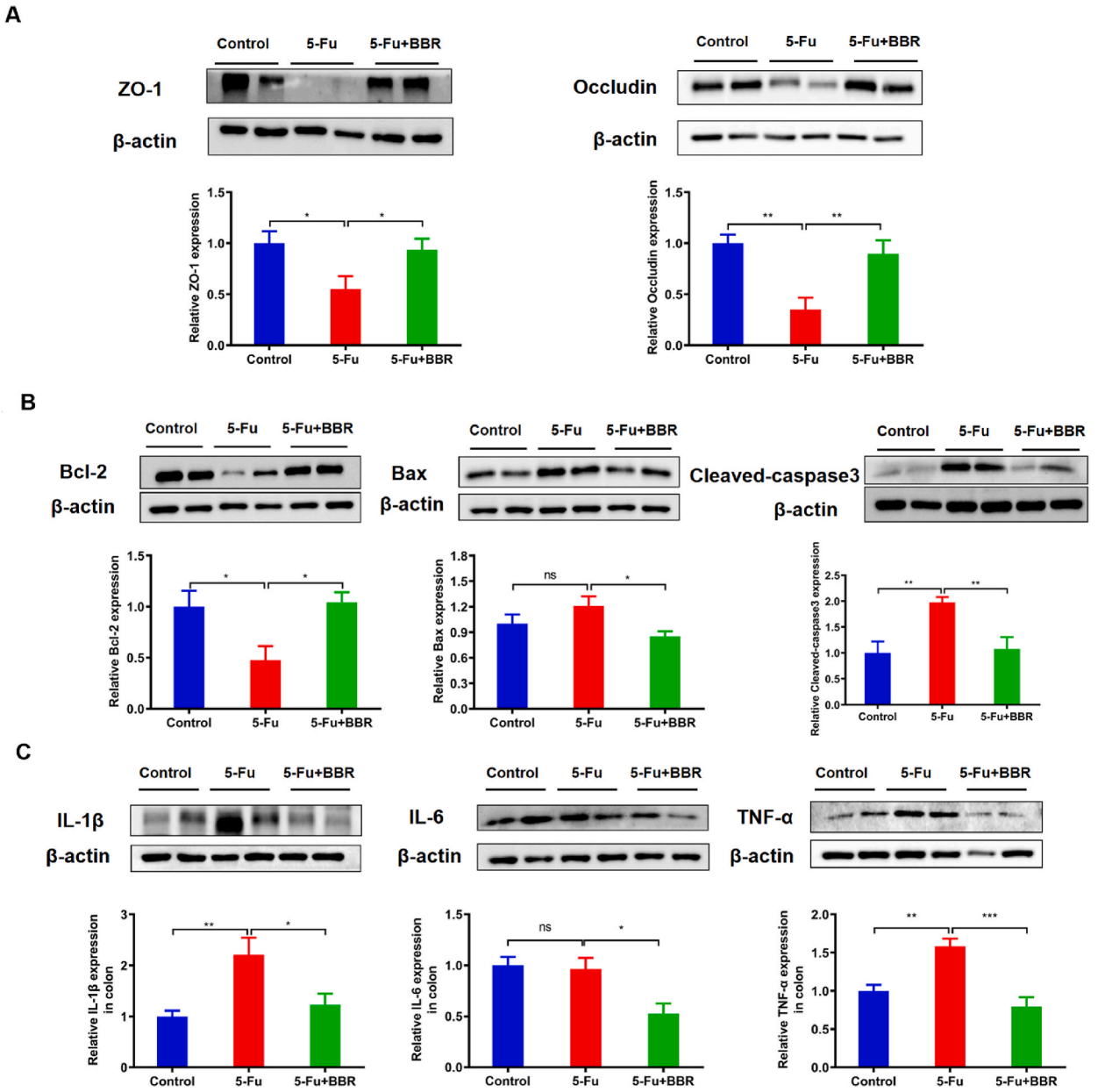

Fig. 2. BBR promoted the expression of tight junction proteins, inhibited the apoptosis of intestinal epithelial cells, and reduced inflammation in vivo.
(A) Levels of tight junction protein (Occludin, ZO-1). (B) Bcl-2, Bax, and Cleaved-caspase3 expressions were detected in colon tissue. (C) Expression of relevant inflammatory factors (IL-6, IL-1β, TNF-α). Full WB images are available in supplementary material 1. All data as the mean ± SEM (n = 5). ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

PBS (phosphate-buffered saline) for 2 min. Blocking: 3 % BSA (bovine serum albumin) or other blocking solution was added dropwise into the histochemical circle and incubated at room temperature for 30 min. Subsequently, anti-ZO-1 antibody and anti-Occludin (1:200; Abcam, USA) and secondary antibody incubation steps were performed. Finally, the slices were dehydrated with ethanol, sealed, and examined under a microscope.

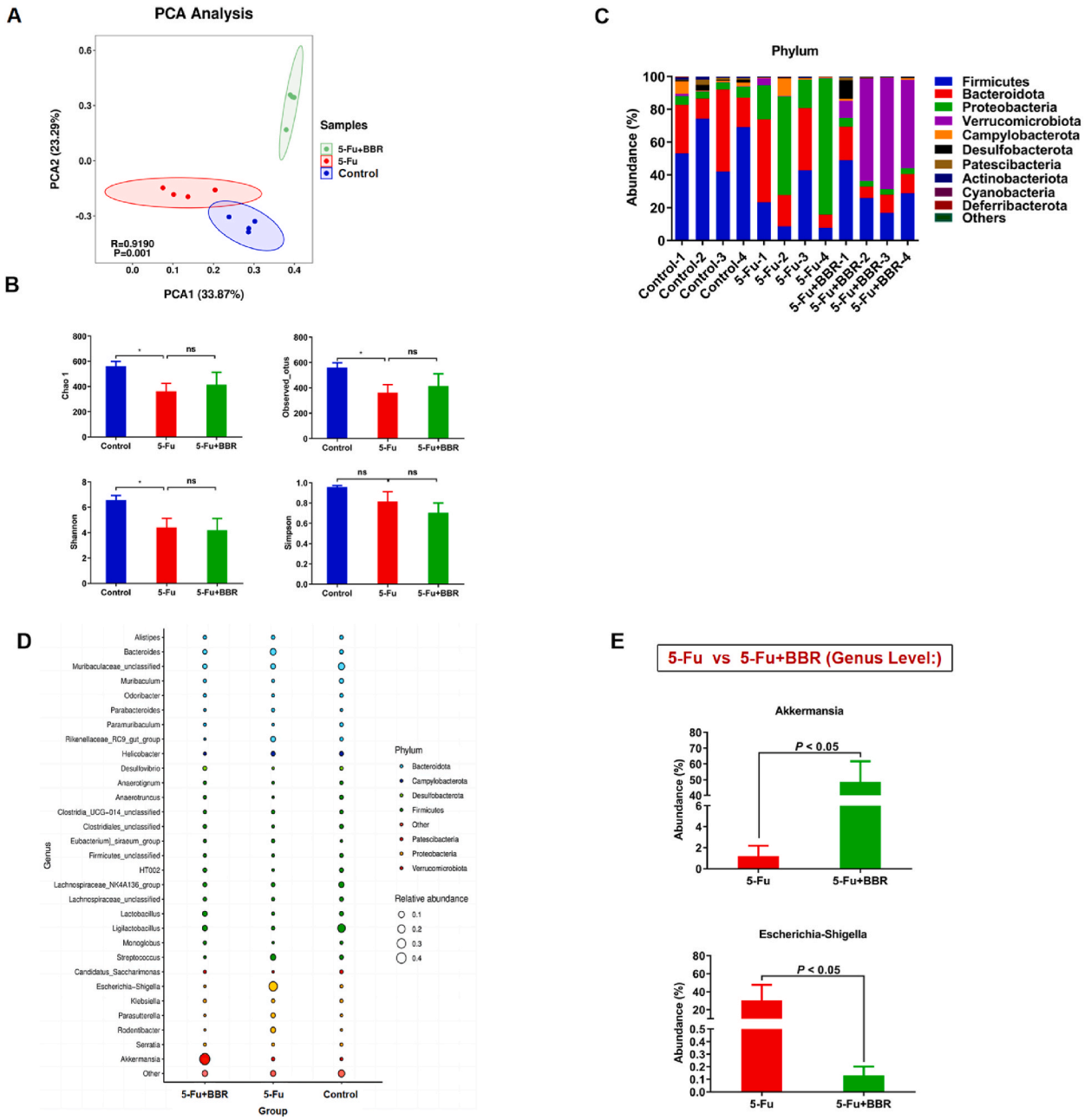

Fig. 3. BBR altered gut microbiota in the tumor xenograft model mice of 5-Fu-caused intestinal mucositis.
(A) Comparison of the intestinal microorganisms' Principal Component Analysis (PCA) among three groups. (B) Chao 1, Shannon, Observed OTUs and Simpson index analysis. (C) The differences in relative richness of intestinal microorganisms among three groups at phylum levels. (D) Bubble plots showed the relative abundance of bacterial genera. The different colors stood for the different phylum. The bubble size represented the relative richness of the Genus. (E) The abundance of Akkermansia and Escherichia-shigella. Significant differences were observed between two groups. ns $P > 0.05$, *$P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

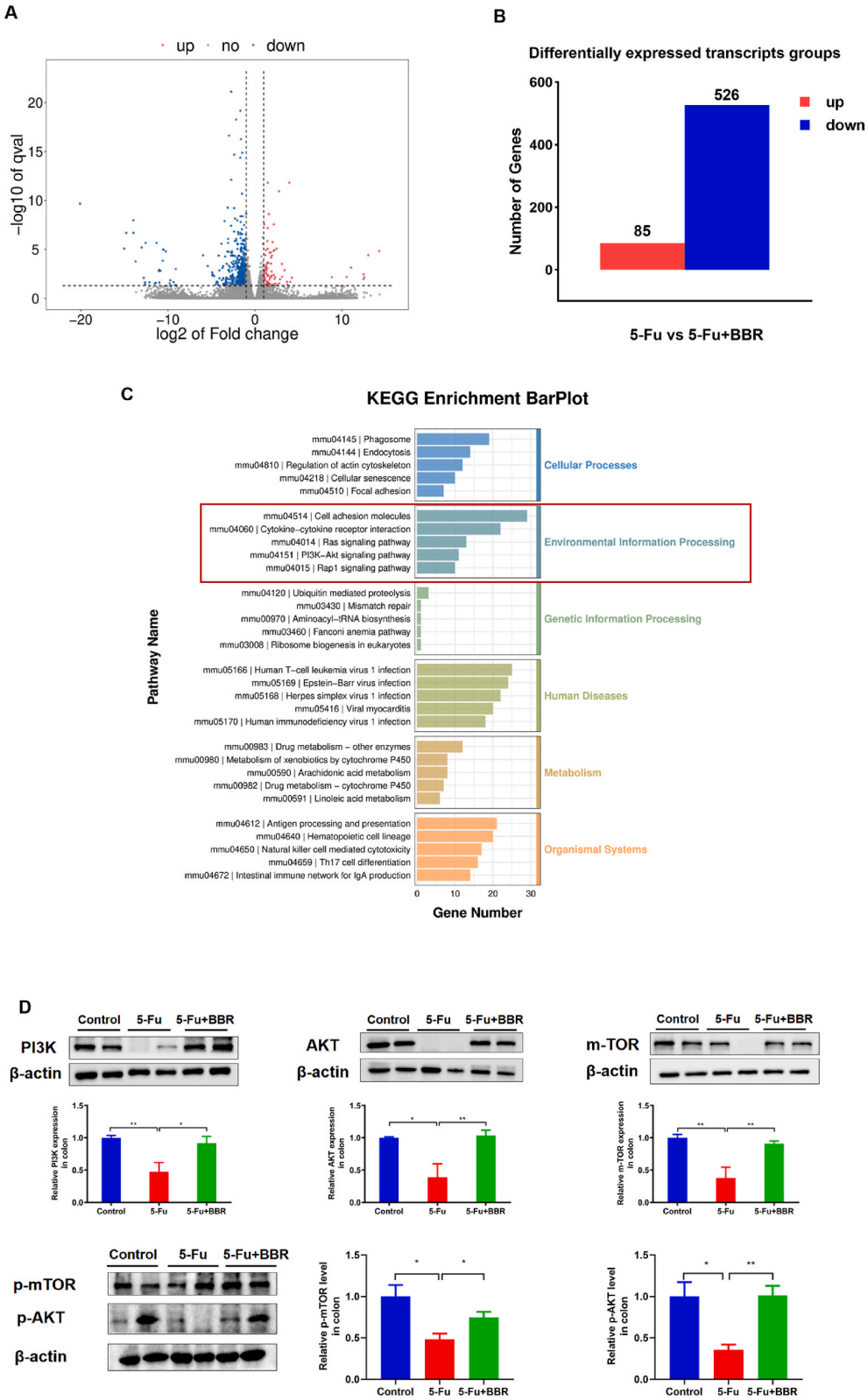

Fig. 4. The regulatory impact of BBR on the PI3K/AKT/mTOR signaling pathway in both intestinal mucosal and tumor tissue in vivo. Transcriptome sequencing analysis of colon tissues (A) Up- and down-regulation of colonic gene expression in two groups of mice. (B) In 5-Fu vs BBR, 85 up-regulated and 526 down-regulated. (C) We observed PI3K/AKT signaling pathway in the environment information processing from

KEGG Enrichment Barplot. (D, E) Expression of PI3K, AKT, p-AKT, mTOR and p-mTOR in colon tissue and tumor tissue separately. Full WB images are available in supplementary material 2. All data as the mean ± SEM (n = 5). ns P > 0.05, *P < 0.05, **P < 0.01.

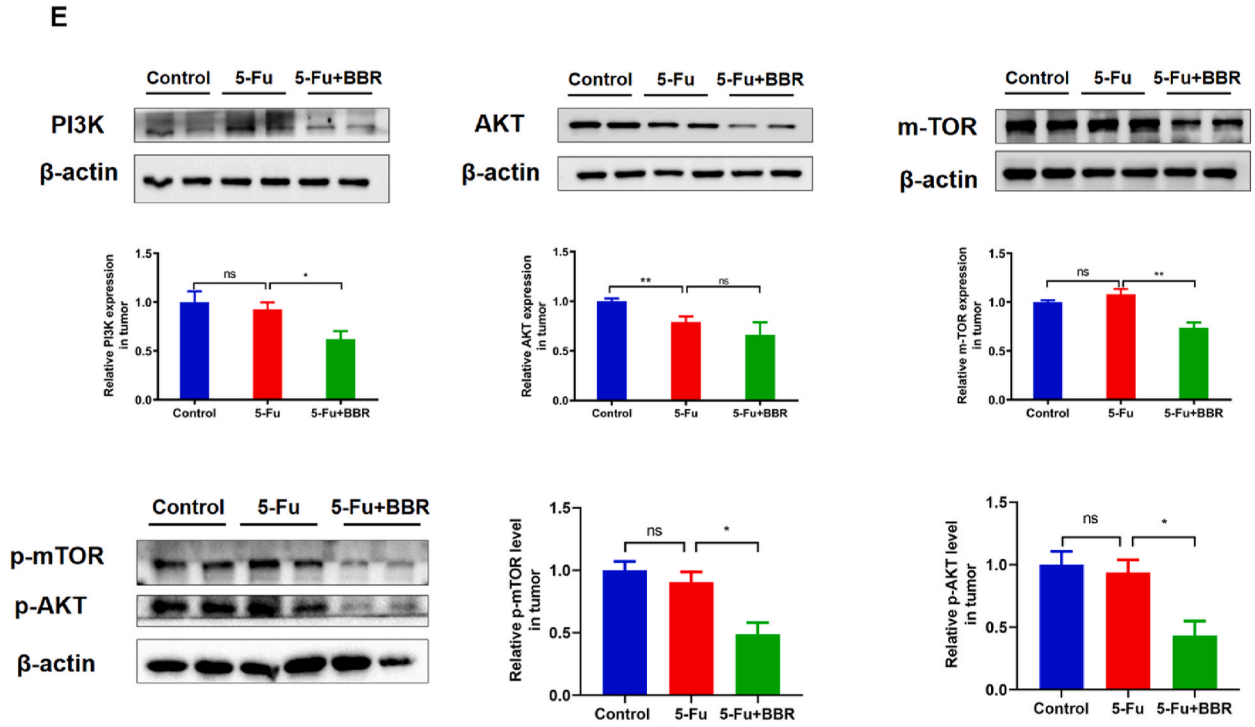

Fig. 4. (*continued*).

2.10. Statistical analysis

In our experiment, all experimental data underwent rigorous evaluation using the SPSS 23.0 software (IBM, Chicago, IL, USA). By using the GraphPad Prism 8.0 software (La Jolla, CA, USA) to perform a *t*-test analysis on all data, we display all results graphically. Non-parametric tests were employed to analyze significant differences between groups when the data were not normally distributed or the variance was unequal. All data as the mean ± SEM. The value $P < 0.05$ was considered to be statistically significant.

3. Result

3.1. BBR alleviates 5-fu-induced intestinal mucosal injury

A tumor-bearing murine model was developed to investigate the effect of BBR on 5-Fu-induced intestinal mucositis. Fig. 1A illustrates the experimental design and workflow. Compared to the control group, mice treated with 5-Fu exhibited significant weight loss, increased diarrhea scores, and reduced colon length ($P < 0.001$). In contrast, BBR treatment alleviated these effects ($P < 0.05$) (Fig. 1B–D). Notably, 5-Fu significantly reduced tumor volume ($P < 0.01$), and this effect was not altered by BBR treatment (Fig. 1E). Additionally, histopathological examination using H&E staining revealed significantly higher levels of intestinal mucosal damage in the 5-Fu group compared to the other two groups ($P < 0.05$) (Fig. 1F). These results indicate that BBR can ameliorate 5-Fu-induced intestinal mucosal damage without compromising its anti-tumor properties.

3.2. BBR increases the expression of tight junction proteins while suppressing intestinal epithelial cell apoptosis and the inflammatory response

Tight junction proteins, such as ZO-1 and Occludin, are essential for maintaining mucosal barriers integrity. Our study revealed that 5-Fu significantly reduced intestinal Occludin and ZO-1 levels ($P < 0.05$), an effect that was significantly reversed by BBR ($P < 0.05$) (Fig. 2A). Meanwhile, to further clarify the effect of BBR on the intestinal mucosal barrier function impairment caused by 5-Fu, immunohistochemical analysis was performed to observe the status of Occludin and ZO-1. It was found that BBR significantly inhibited the decrease in the expression of ZO-1 and Occludin induced by 5-Fu (Supplementary Fig. 1). Addtionally, as shown in Fig. 2B, the expression levels of Bax and cleaved caspase-3 were significantly higher in the 5-Fu group compared to the control group. While Bcl-2 exhibited the opposite pattern ($P < 0.01$). BBR treatment decreased the protein levels of Bax and cleaved caspase-3 while increasing Bcl-2 levels in colon tissues ($P < 0.05$). The study also assessed inflammation levels in colon tissues, finding that 5-Fu

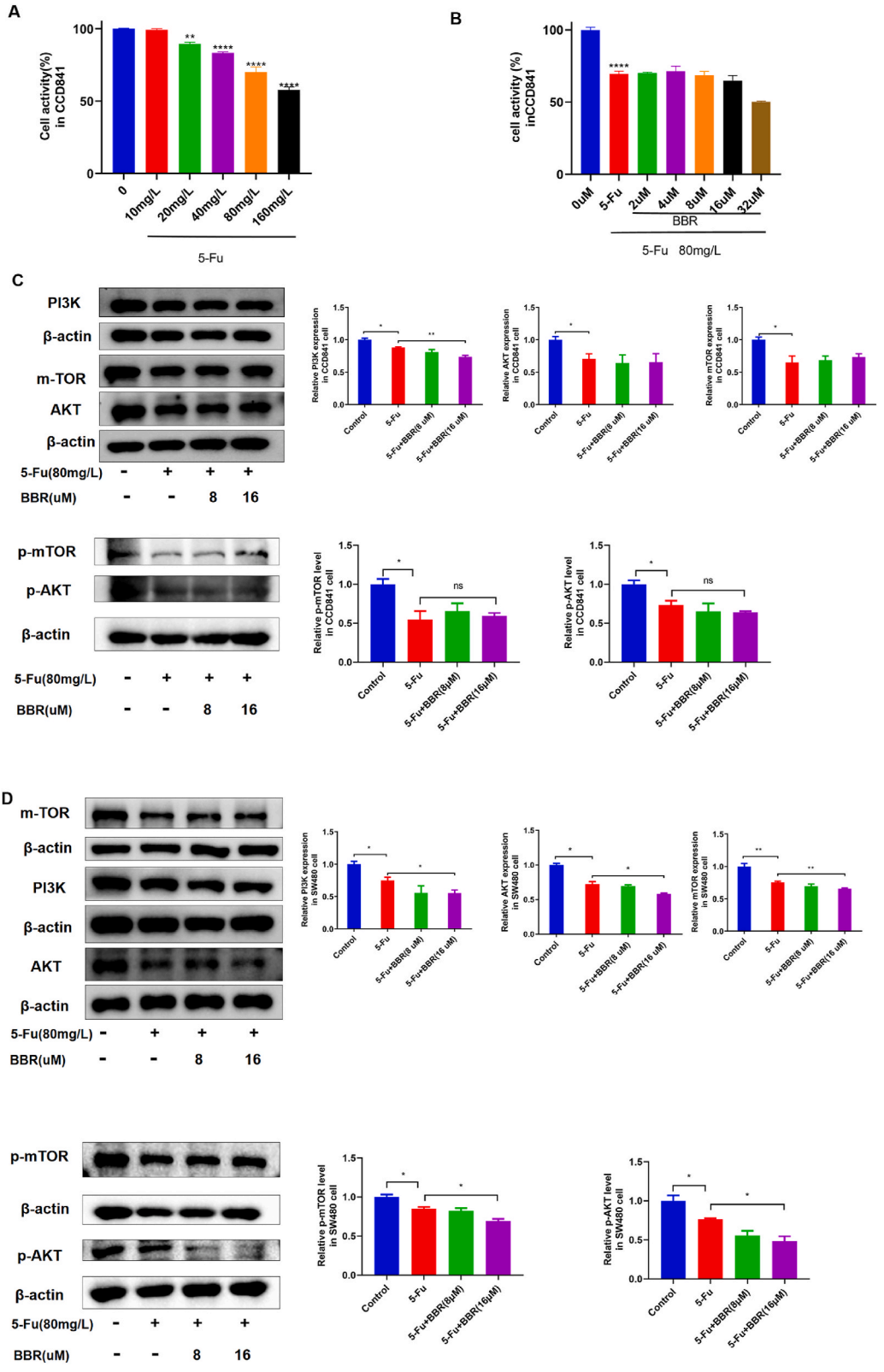

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Fig. 5. Regulatory effect of BBR on the PI3K/AKT/mTOR in CCD841 and SW480 cells.
(A) Different concentrations of 5-Fu inhibited the proliferation of CCD84. (B) Effects of 5-Fu combined with different concentrations of BBR on CCD841 cell proliferation was measured by CCK-8 assay. Experiments with the CCD841 and SW480 cells in four groups (C) After treatment with 5-Fu or 5-Fu + BBR, the expression of PI3K/AKT/mTOR and its phosphorylation in CCD841 was observed. (D) Also detected PI3K/AKT/mTOR and phosphorylation expression in SW480. Experiment was repeated three times individually. Full WB images are available in supplementary material 3. All data as the mean ± SEM, the experiment was repeated three times. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

upregulated inflammatory factors such as IL-1β and TNF-α ($P < 0.01$). Conversely, BBR downregulated the expression of IL-6, TNF-α, and IL-1β ($P < 0.05$) (Fig. 2C). These results demonstrate that BBR attenuates intestinal mucosal injury by repairing tight junctions, inhibiting intestinal epithelial cell apoptosis, and reducing inflammation.

3.3. BBR ameliorates 5-fu-induced intestinal flora dysbiosis

Changes in the gut microbiome were investigated using 16 S rDNA MiSeq sequencing, with data analyzed at the phylum and genus levels. Principal component analysis (PCA) (Fig. 3A) revealed distinct cluster distributions among the three groups of mice based on the proportions of the first principal component axis (PCA1) and PCA2, which accounted for 33.87 % and 23.29 % of the variation, respectively. Collectively explaining 57.16 % of the variation in bacterial communities. Community richness was assessed using indices such as Chao 1, Shannon, observed OTUs, and Simpson's diversity. Results indicated significant reductions in these indices, except for Simpson's index, following 5-Fu treatment (Fig. 3B).

Diversity analysis further examined the relative abundance and composition of intestinal flora at the phylum level in the three groups of mice. Treatment with 5-Fu significantly increased the relative abundance of Proteobacteria while decreasing Firmicutes abundance compared to the control group (Fig. 3C). BBR effectively reversed the 5-Fu-induced alterations in *Firmicutes* and *Proteobacteria* while enhancing the relative abundance of *Verrucomicrobiota*. At the genus level, our analysis revealed that BBR stimulated the growth of *Akkermansi* and inhibited *Escherichia/Shigella* ($P < 0.05$) (Fig. 3D and E). These findings suggest that BBR may play a pivotal role in ameliorating 5-Fu-induced intestinal mucosal inflammation by modulating the composition of the intestinal flora.

3.4. Effect of BBR on the PI3K/AKT/mTOR signaling pathway in intestinal epithelial tissues and tumors

Transcriptome sequencing and subsequent analysis identified differentially expressed genes (DEGs) following treatment with BBR or 5-Fu. As shown in Fig. 4A, genes with $P < 0.05$ and $|\log FC| > 1$ were considered statistically significant. Compared to the 5-Fu + BBR group, the blue scatters on the left represent downregulated genes, while the red scatters on the right represent upregulated genes. A total of 611 DEGs were identified: 85 upregulated and 526 downregulated. In contrast, compared to the 5-Fu group, BBR upregulated 526 genes and downregulated 85 genes. (Fig. 4B). Notably, the PI3K/AKT signaling pathway showed significant differences, being implicated in environmental information processing (Fig. 4C). BBR significantly upregulated 9 genes in this pathway (Supplementary Fig. 2). Building on insights from the KEGG enrichment bar plot, the potential regulation of the PI3K/AKT signaling pathway by BBR was further investigated. Previous studies have confirmed that PI3K regulates the G1 cell cycle to promote the proliferation of intestinal epithelial cells [25–27]. Western blot analysis assessed the expression of PI3K, AKT, and mTOR in tumor and colon tissues. As shown in Fig. 4D, BBR supplementation reversed the loss of PI3K/AKT/mTOR signaling pathway expression induced by 5-Fu treatment ($P < 0.05$). In tumor tissue (Fig. 4E), BBR significantly inhibited the expressions of PI3K ($P < 0.05$) and mTOR ($P < 0.01$) compared to the other groups. Notably, AKT expression in tumor tissue was attenuated by 5-Fu but remained unaffected by BBR ($P < 0.01$). Additionally, the levels of p-AKT and p-mTOR expression in the 5-Fu + BBR group showed a significant decrease compared to those in the control group. These preliminary findings suggest that BBR reverses the 5-Fu-mediated suppression of PI3K/AKT/mTOR protein expression without compromising its anti-tumor efficacy.

3.5. BBR inactivates the PI3K/AKT/mTOR pathway proteins in CCD841 and SW480 cells

To further investigate the effect of 5-Fu and BBR co-treatment on the PI3K/AKT/mTOR signaling pathway, a CCK-8 assay was employed to assess the inhibitory effect of various concentrations of 5-Fu on CCD841 cell proliferation (Fig. 5A). A 5-Fu concentration 80 mg/L, with a 70 % inhibition rate, was selected to combine with the different concentrations of BBR (Fig. 5B). CCD841 and SW480 cells were utilized to determine whether BBR activates the PI3K/AKT/mTOR axis in vitro. Notably, the PI3K/AKT/mTOR axis was significantly inactivated by 5-Fu compared to the negative control. In CCD841 cells (Fig. 5C), no significant differences were observed in AKT and mTOR expression between the 8 μM or 16 μM BBR groups and the 5-Fu group. Analogously, no significant variation was noted in phosphorylated AKT/mTOR levels between these groups. Interestingly, in SW480 cells, BBR at 16 μM significantly reduced PI3K, AKT, and mTOR expression levels compared to 5-Fu-treated cells ($P < 0.05$), similar to phosphorylated AKT/mTOR expression (Fig. 5D). Based on these findings, We hypothesized that gut microbiota plays an indispensable role in BBR activating the PI3K/AKT/mTOR signaling pathway to ameliorate intestinal mucosal injury.

3.6. Antibiotic intervention indicated that BBR fails to activate the PI3K/AKT/mTOR pathway in cases of deficient intestinal flora

To elucidate the pivotal role of intestinal flora, a pseudo-sterile mouse model was established to investigate whether BBR triggers

the colonic PI3K/AKT/mTOR signaling pathway in the absence of intestinal microbiota (Fig. 6A). ABX administration conspicuously reversed weight loss, colon shortening, increased diarrhea scores, and mucosal structure damage induced by 5-Fu compared to the 5-Fu group ($P < 0.01$) (Fig. 6B–D). Furthermore, ABX administration affected the anti-tumor effect of 5-Fu (Fig. 6E). H&E staining and histopathological injury scoring revealed severe damaged to the intestinal mucosa caused by 5-Fu, while ABX-treated mice showed significantly less damage ($P < 0.05$) (Fig. 6F). Additionally, ABX treatment increased Occludin protein expression ($P < 0.01$) and reduced the Bax/Bcl-2 ratio ($P < 0.05$) compared to the 5-Fu group (Fig. 6G). The role of BBR in repairing the intestinal mucosal barrier may be lost or overridden by ABX in the absence of intestinal flora. However, it is worth noting that no significant differences were observed in the levels of PI3K, AKT, p-AKT, mTOR, and p-mTOR among the three groups in colon tissues (Fig. 6H). These results suggest that ABX administration mitigated 5-Fu-induced intestinal injury and compromised the anti-tumor activity of 5-Fu. It is noteworthy that BBR did not exhibit a direct effect on the PI3K/AKT/mTOR signaling pathway after ABX intervention.

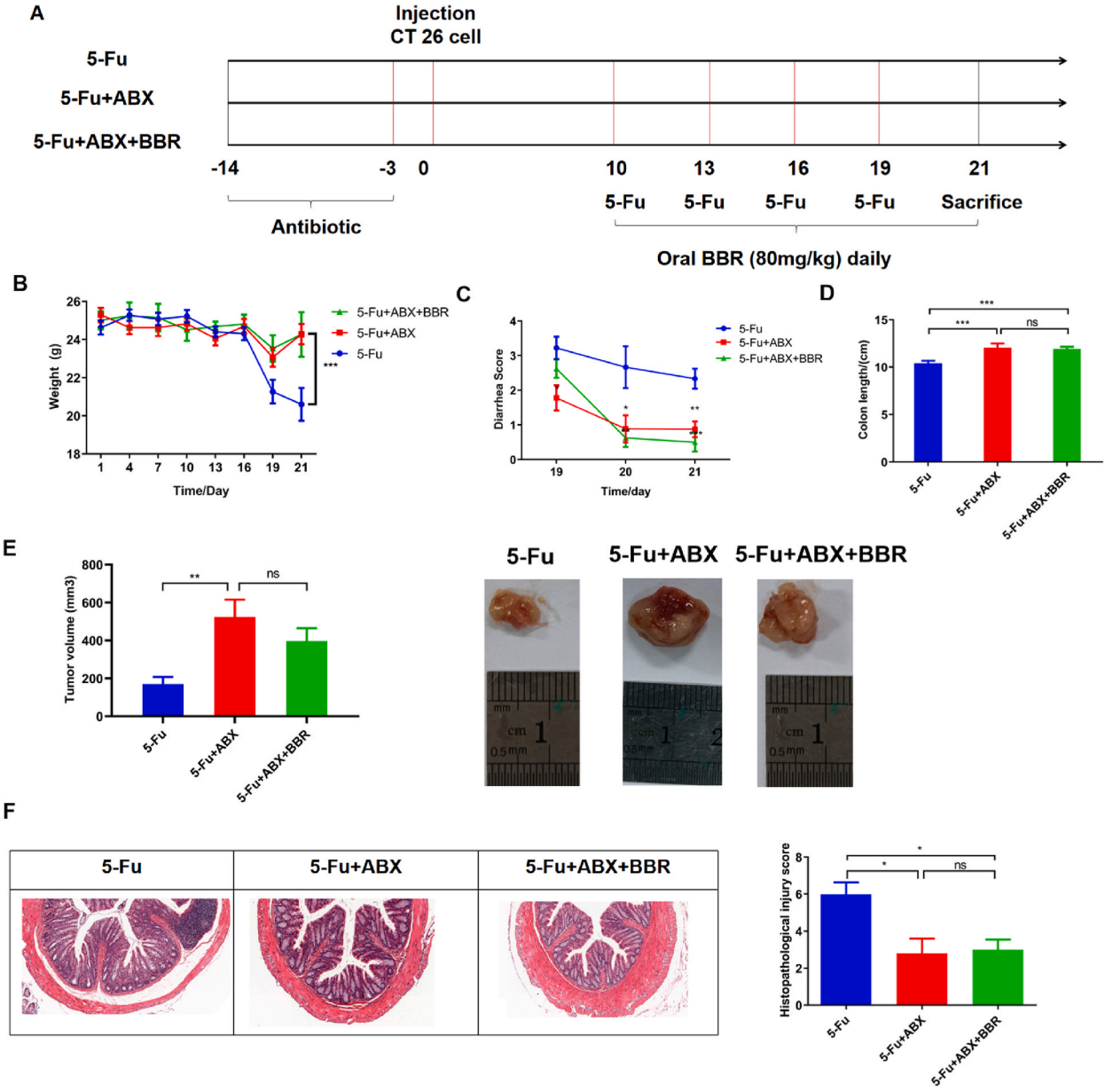

Fig. 6. The effect of BBR on intestinal mucosal damage in mice following an antibiotic intervention.
(A)Experimental design. The general condition: (B) weight, (C) diarrhea scores, (D) colon length, and (E) tumor volume was recorded. (F) H&E staining and histopathological injury score of colon tissue section. (G) Observation of Occludin and Bax/Bcl-2 expression. (H) In the colon tissue, levels of PI3K, AKT, p-AKT, mTOR, and p-mTOR. Full WB images are available in supplementary material 4. All data as the mean ± SEM (n = 6). ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

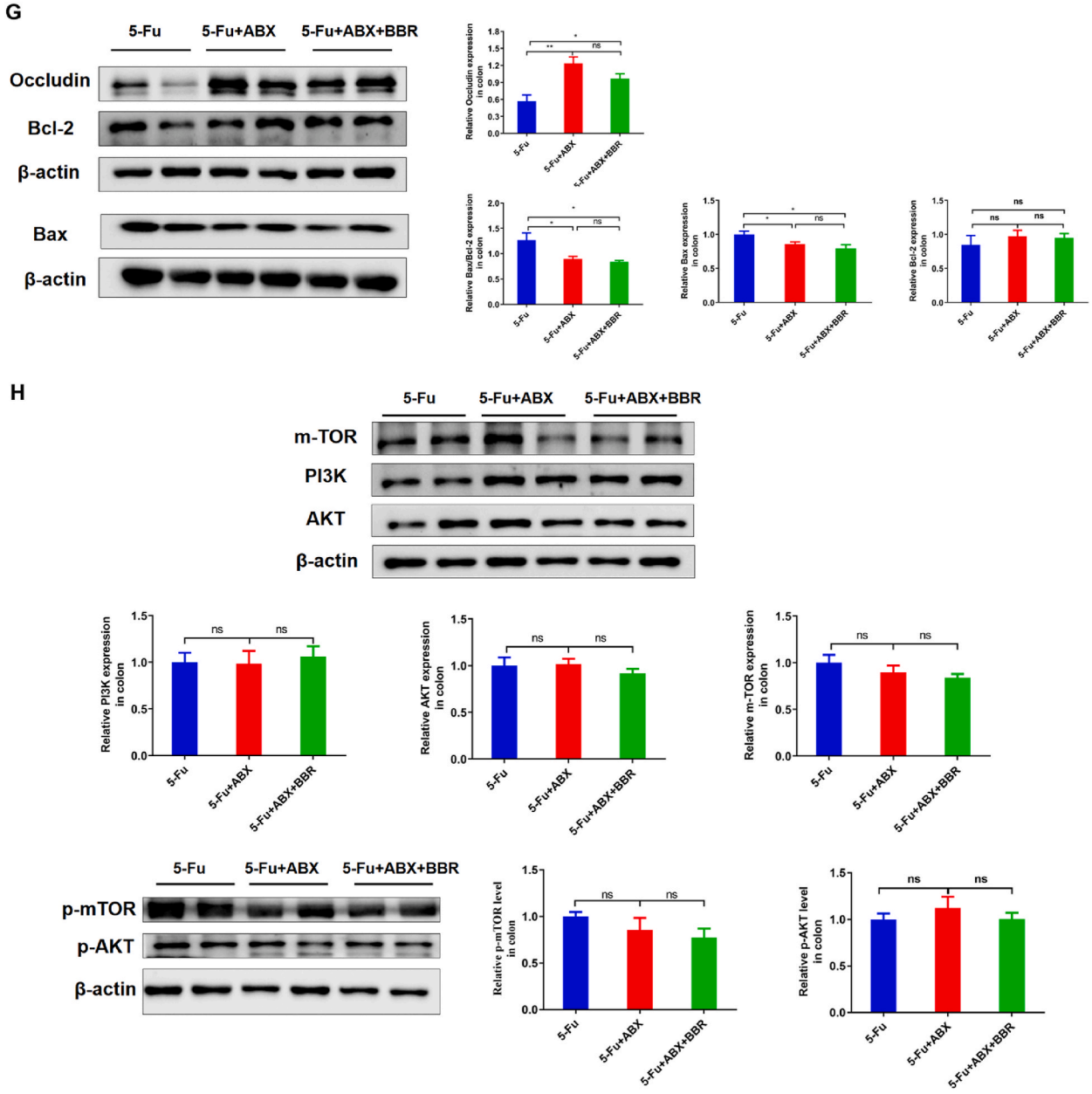

Fig. 6. (*continued*).

4. Discussion

5-Fu remains an essential component in CRC treatment, significantly extending patient survival time [28]. However, its use can lead to severe complications, particularly diarrhea [29]. Clinical studies have confirmed that 5-Fu increases the abundance of harmful gut bacteria, resulting in intestinal mucosal damage and consequent diarrhea [30,31]. In contrast, BBR exhibits minimal systemic absorption and primarily accumulates in the gut, where it significantly regulates intestinal flora [32,33]. Previous studies demonstrated that BBR can modulate the intestinal flora in rats with 5-Fu-induced intestinal mucosal injury [23]. This study presents, for the first time, evidence that BBR protects against intestinal mucositis by modulating the PI3K/AKT/mTOR pathway, which is associated with intestinal flora. Importantly, our findings indicate that BBR does not compromise the anti-tumor efficacy of 5-Fu.

Berberine has been widely used in clinical practice due to its antibacterial, antiprotozoal, antidiarrheal, and anti-trachoma activities [34]. This study has demonstrated that BBR significantly ameliorates intestinal barrier dysfunction, reducing inflammation and the inhibiting intestinal epithelial cell apoptosis in a tumor-bearing murine model of 5-Fu-induced intestinal mucositis. Importantly, the anti-tumor efficacy of 5-Fu remained unaffected by BBR treatment. Additionally, 16 S rDNA sequencing revealed that BBR

administration significantly impacted OTU numbers, taxa richness, and microbiome diversity, notably increasing the relative abundance of the *Verrucomicrobiota*. BBR also decreased the abundance of *Proteobacteria* and *Bacteroidota* at the phylum level. At the genus level, BBR increase the abundance of *Akkermansi*a and *Ligilactobacillus* while suppressing pathogenic bacteria, particularly *Escherichia/Shigella*. Remarkably, *Akkermansia* abundance increased by 50 % following BBR treatment. Previous studies have shown that augmenting *Akkermansia* can effectively ameliorate chemotherapy-induced intestinal mucositis and enhance the anti-tumor efficacy of FOLFOX [35–37]. Another study also observed an enrichment of *Paracoides* and *Escherichia/Shigella* in tumorous mice [38–40]. Building on our previous research, this study provides further evidence that BBR significantly inhibits *Escherichia/Shigella* and enriches *Akkermansia* bacteria in mice. Consequently, we propose that the amelioration of 5-Fu-induced intestinal mucosal injury by BBR may be related to the augmentation of *Akkermansia.*

To further elucidate the underlying mechanisms, transcriptome sequencing was conducted to investigate gene expression differences between the 5-Fu group and the 5-Fu + BBR group. The analysis revealed several signaling pathways, including PI3K/AKT, Ras, and Rap1. Previous studies have confirmed that *Akkermansia* inhibits the inflammatory response by increasing the levels of short-chain fatty acids (SCFAs) [41]. Moreover, *Akkermansia* regulates immune homeostasis and activates the PI3K/AKT signaling pathway, attenuating colitis through the secretion of threonyl-tRNA synthetase (AmTARS) [42,43]. Furthermore, studies have shown that the activation of the PI3K/AKT signaling pathway enhances recovery from intestinal injury, supporting our research direction [44]. Similarly, transcriptome analysis and Western blot demonstrated that BBR significant regulates the PI3K/AKT/mTOR signaling pathway. This study provides evidence for the enrichment of *Akkermansia* and the stimulation of the PI3K/AKT/mTOR signaling pathway in colon tissue following BBR treatment. In contrast, BBR inhibited the protein expression of PI3K/AKT/mTOR in tumor tissues. Based on these results, we initially proposed that BBR may exert an indirect regulatory effect on the PI3K/AKT/mTOR signaling pathway by modulating the composition and activity of the gut microbiota.

To further validate the role of gut microbiota in regulating the PI3K/AKT/mTOR signaling pathway normal intestinal epithelial cells (CCD841) and human tumor cells, (SW480) were selected to investigate BBR's direct effect on this pathway. Interestingly, BBR did not directly influence the PI3K/AKT/mTOR signaling pathway in CCD841 cells. However, in SW480 cells, BBR exhibited a synergistic effect when combined with 5-Fu. In CCD841 cells, the potent inhibitory effect of 5-Fu may limit efficacy of BBR. Conversely, in tumor cells, BBR further suppressed the PI3K/AKT/mTOR signaling pathway, likely due to the pathway's overexpression in these cells [45].

A gut microbiome-depleted mouse model was developed to further investigate the regulatory effects of BBR on the PI3K/AKT/mTOR signaling pathway in the absence of gut microbiota. Previous studies have indicated that antibiotics significantly alleviate intestinal mucositis but abolish the anti-tumor effect of 5-Fu [46,47]. In our study, ABX effectively mitigated the intestinal mucosal damage induced by 5-Fu, though it also compromised the anti-tumor potential of 5-Fu. Unlike ABX, BBR, while not reaching statistical significance between the ABX and ABX + BBR groups, reduced tumor size. indicating a potential synergistic anti-tumor effect and suggesting that BBR may attenuate ABX's impact on tumors. Additionally, no statistically significant differences were observed in the expression of PI3K/AKT/mTOR proteins among the three groups of mice, suggesting that the BBR-regulated gut microbiota plays a pivotal role in regulating the PI3K/AKT/mTOR signaling pathway, which aligns with our initial hypothesis.

5. Conclusion

Compelling evidence indicates that BBR significantly ameliorates 5-Fu-induced intestinal mucositis. The underlying mechanism involves activating the PI3K/AKT/mTOR signaling pathway through alterations in gut microbiota composition, thereby inhibiting intestinal epithelial cell apoptosis. Using a gut microbiota-depleted model and conducting in vitro experiments, the study further validated that BBR-induced changes in the gut microbiota can activate the PI3K/AKT/mTOR signaling pathway in colonic tissue without compromising the anti-tumor efficacy of 5-Fu. Notably, while ABX repairs chemotherapy-induced intestinal mucosal damage, it also diminishes the anti-tumor efficacy of 5-Fu. Therefore, BBR shows greater potential in managing chemotherapy-induced diarrhea compared to antibiotics. These findings offer novel insights into the potential application of BBR for preventing chemotherapy-induced intestinal injury.

Ethics approval and consent to participate

All experimental procedures involving the animals were conducted in accordance with ethical standards and were approved by the Animal Experimentation Ethics Committee of Zhejiang Chinese Medical University. (No.20210927–09). CCD841 and SW480 cells were purchased from Shanghai Jinyuan Biotechnology Co., LTD. CT-26 cell was obtained from Wuhan Punosai Life Technology Co., LTD.

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Data availability statement

Therefore, all public data have been uploaded to the SRA database.

16 S rDNA sequencing raw data: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA890779, Transcriptome raw data: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE215201.

CRediT authorship contribution statement

Changhong Wu: Writing – original draft, Validation, Methodology, Data curation, Conceptualization. **Jie Yang:** Visualization, Supervision, Data curation. **Chenxiao Ye:** Writing – original draft, Methodology, Data curation, Conceptualization. **Hui Wu:** Software, Investigation, Formal analysis. **Wenxi Shu:** Validation, Resources, Methodology. **Rongrong Li:** Supervision. **Sihan Wang:** Investigation. **Yi Lu:** Software. **Haitao Chen:** Writing – review & editing, Project administration, Data curation, Conceptualization. **Zewei Zhang:** Supervision, Project administration, Funding acquisition. **Qinghua Yao:** Writing – review & editing, Supervision, Project administration, Funding acquisition, 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34528.

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