

Original Article

Pulsatilla decoction attenuates colorectal cancer progression in association with gut microbiota remodeling, T-cell immune restoration, and NF- κ B/JAK2/STAT3 signaling suppression

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Received May 21, 2026; Accepted July 30, 2026; Epub September 25, 2026; Published September 30, 2026

Abstract: Pulsatilla Decoction (PD) exhibits anti-inflammatory properties, but its impact on colorectal cancer (CRC) and the role of gut microbiota remain incompletely understood. This study investigated the antitumor effects of PD in a xenograft mouse model of HCT116 cells reconstituted with human peripheral blood mononuclear cells. We also analyzed corresponding changes in gut microbiota, T-cell immunity, inflammatory responses, and NF- κ B/JAK2/STAT3 signaling. Tumor-bearing mice were treated for three weeks with low-, medium-, or high-dose PD, or with 5-fluorouracil. Gut microbiota composition and function were analyzed using 16S rRNA and metagenomic sequencing. Immune cell populations, cytokines, and pathway-related proteins were assessed via flow cytometry, immunofluorescence, ELISA, RT-qPCR, and western blotting. To further evaluate the involvement of microbiota, we performed antibiotic pretreatment followed by fecal microbiota transplantation (FMT). PD effectively reduced tumor volume and weight without causing significant body weight loss. High-dose PD partially restored microbial diversity and community structure, increased *Akkermansia muciniphila*, and modulated inflammation- and metabolism-related microbial pathways. Furthermore, PD enhanced peripheral and intratumoral CD4⁺ and CD8⁺ T-cell responses, improved the splenic Treg/Th17 balance, decreased TNF- α , IL-6, and IL-17A, increased IL-10, and suppressed NF- κ B, JAK2, and STAT3 activation in tumor tissues. FMT from high-dose PD-treated donors partially transferred tumor inhibition, increased tumor CD8A and granzyme B, reduced Foxp3, and lowered NF- κ B, JAK2, and STAT3 levels in recipient mice not directly treated with PD. These findings suggest that PD inhibits CRC progression by coordinately modulating gut microbiota, T-cell immunity, inflammation, and NF- κ B/JAK2/STAT3 signaling, with the gut microbiota partially contributing to its antitumor effects.

Keywords: Pulsatilla decoction, colorectal cancer, gut microbiota, T cell immunity, NF- κ B/JAK2/STAT3 pathway

Introduction

Colorectal cancer (CRC) is a malignant tumor that originates from the epithelial cells of the colon or rectum. Most sporadic CRCs develop through a multistep adenoma-carcinoma sequence, characterized by the progressive accumulation of genetic and epigenetic alterations. Several factors influence CRC risk, including hereditary susceptibility, inflammatory bowel disease, smoking, alcohol consumption,

obesity, dietary patterns, and physical inactivity [1]. Current CRC management includes surgery, chemotherapy, radiotherapy, targeted therapy, and immune checkpoint blockade [2]. However, treatment efficacy remains limited by tumor heterogeneity, acquired resistance, treatment-related toxicity, and the poor response of most microsatellite-stable or mismatch-repair-proficient tumors to immune checkpoint inhibitors [3, 4]. For this reason, CRC treatment may benefit from strategies that can suppress tumor

growth, reduce inflammatory immune imbalance, and improve the intestinal microenvironment at the same time.

Gut microbiota dysbiosis is closely related to CRC initiation and progression. It can weaken intestinal barrier function, change microbial metabolites, increase inflammatory stimulation, and alter host immune responses [5, 6]. Conversely, commensal or probiotic taxa may suppress colorectal tumorigenesis by restoring microbial homeostasis and modulating host-microbe interactions, as demonstrated for *Lactococcus lactis* HkyuLL 10 via an alpha-mannosidase-dependent mechanism [7]. Among gut microbes, *Akkermansia muciniphila* is often studied because of its links with mucin metabolism, epithelial barrier function, and immune regulation. It has been considered a possible next-generation probiotic for CRC. However, its effects may differ according to disease stage, host background, bacterial viability, and experimental model. For this reason, CRC-related microbiota studies should include microbiome composition, microbial function, inflammatory changes, and immune responses [8, 9].

Chronic inflammation is another factor closely associated with CRC progression. Cytokines can activate signaling pathways that regulate tumor behavior and immune responses. Among these pathways, NF- κ B and JAK/STAT3 have been widely studied [10]. IL-6-JAK-STAT3 signaling contributes to tumor cell viability, expansion, metastatic infiltration, and remodeling of the immune milieu [11]. NF- κ B and STAT3 may further interact in the tumor microenvironment and help maintain persistent inflammation. Therefore, targeting the NF- κ B/JAK2/STAT3 axis may represent a rational strategy for limiting inflammation-associated CRC progression.

CRC progression is also closely related to abnormal T-cell immunity. CD8-positive T lymphocytes mediate direct cytolytic activity against neoplastic targets. Within the tumor microenvironment, CD4⁺ T cells participate in regulating immune activation and cellular differentiation [12]. The functions of Treg and Th17 cells in CRC are context-dependent. Th17-associated cytokines may sustain tumor-promoting inflammation in some settings, whereas Treg subsets can either curb excessive intestinal inflammation or dampen antitumor immu-

nity, depending on their phenotype and tissue location. Therefore, changes in Treg and Th17 cells should be interpreted together with cytokine profiles and the anatomical compartment examined [13, 14]. Accordingly, CD4⁺ and CD8⁺ T-cell responses, Treg/Th17 differentiation, and inflammatory cytokines should be evaluated together when assessing the immune effects of anti-CRC treatments.

PD is composed of *Pulsatilla chinensis*, *Coptis chinensis*, *Phellodendron chinense*, and *Fraxinus rhynchophylla*. Studies on experimental colitis have shown that PD can modulate gut microbiota-related short-chain fatty acids, preserve intestinal barrier integrity, and regulate autophagy-associated inflammatory signaling [15, 16]. In addition, the n-butanol extract of PD was shown to alleviate mucosal inflammation and correct local immune imbalance by regulating mitochondria-associated type I interferon signaling and mitochondrial function [17]. Since persistent inflammation is associated with colorectal carcinogenesis [18], PD may have potential value in CRC by improving the intestinal inflammatory microenvironment. In addition, modified PD was reported to alleviate 5-fluorouracil-induced intestinal mucositis through changes in the TLR4/MyD88/NF- κ B pathway and gut microbiota, suggesting a possible role of this formula in regulating intestinal inflammation during cancer-related treatment [19]. Nevertheless, whether PD attenuates CRC progression through coordinated regulation of the gut microbiota, T-cell immunity, inflammatory cytokines, and NF- κ B/JAK2/STAT3 signaling remains unclear.

In this study, an HCT116 subcutaneous xenograft model was established in hACE2 knock-in BALB/c nude mice reconstituted with human peripheral blood mononuclear cells. The study evaluated the effects of PD on tumor burden, the structure and functional characteristics of the gut microbiota, T-cell-related immune markers in peripheral blood and the spleen, local immune status within the tumor, inflammatory factors, and NF- κ B/JAK2/STAT3 signaling in tumor tissue. Building on these findings, we further conducted experiments combining antibiotic pretreatment with fecal microbiota transplantation (FMT). We transplanted fecal microbiota derived from either the Model group or the PD-H group into recipient mice that had not directly received PD. By comparing tumor bur-

den, local tumor expression of CD8A, Granzyme B, and Foxp3, as well as levels of NF- κ B/JAK2/STAT3-related proteins between the ABX+FMT-Model group and the ABX+FMT-PD group, we assessed whether fecal microbiota from PD-H donors could transfer specific antitumor and local immunomodulatory phenotypes. This provides preliminary experimental evidence for the gut microbiota's involvement in the antitumor effects of PD.

Materials and methods

Cell culture

The HCT116 cell line was obtained from Wuhan Pricella Biotechnology Co., Ltd. (CL-0096). Cells were cultured in HCT116 Cell Complete Medium (Pricella, CM-0096) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Animals and tumor model establishment

SPF-grade male hACE2 knock-in BALB/c nude mice (Foxn1^{nu/nu}, Ace2^{em1(hACE2)/Y}; n=78), aged 8-12 weeks, were purchased from Guangzhou Mingxun Biotechnology Co., Ltd. (production license No. SCXK [Guangdong] 2024-0070). The mice were housed in an SPF-grade barrier facility in individually ventilated cages. Environmental conditions were maintained at 22-25°C, 50%-65% relative humidity, and a 12-h light/dark cycle, with ad libitum access to food and water.

Following a 7-day acclimatization period, each mouse received 1×10^7 human PBMCs (Procell, CP-H158) suspended in 200 μ L of sterile PBS via tail-vein injection. Fourteen days after PBMC injection, HCT116 cells were inoculated to establish a PBMC-reconstituted HCT116 xenograft model. All mice, except those in the blank control group, were subcutaneously injected with 1×10^6 HCT116 cells in 100 μ L into the right axillary region. Mice in the blank control group received an equal volume of sterile PBS at the same site. After inoculation, tumor volumes were regularly calculated based on measurements of the longest and shortest tumor diameters. This study received approval from the Ethics Committee of Jiangsu Huachuang Yutong Medical Laboratory Co., Ltd. (approval No. HC25032-P001-01).

Drug administration

Random allocation resulted in six experimental groups of nine mice each: Control, Model, PD-L, PD-M, PD-H, and 5-FU. Healthy control animals without implanted tumors received sterile saline by oral gavage. Tumor-bearing mice in the Model group received sterile saline. Tumor-bearing mice in the PD-L, PD-M, and PD-H groups received PD by oral gavage at 4, 8, and 16 g/kg/day, respectively [20]. Tumor-bearing mice in the 5-FU group received 5-fluorouracil at 50 mg/kg by intraperitoneal injection once weekly as the positive control. The treatment lasted three weeks.

Antibiotic pretreatment and fecal microbiota transplantation

To evaluate whether the gut microbiota modulated by PD contributes to its antitumor effects, mice were randomly divided into four groups (n=6 per group): Model, PD-H, ABX+FMT-Model, and ABX+FMT-PD. Mice in the Model group received an equal volume of vehicle by gavage, while those in the PD-H group received PD at 16 g/kg/day by gavage. Mice in the ABX+FMT-Model and ABX+FMT-PD groups did not directly receive PD. Instead, after antibiotic pretreatment, they received fecal microbiota from donor mice in the Model and PD-H groups, respectively.

The antibiotic pretreatment protocol was based on a previously published study [21]. The antibiotic cocktail, consisting of metronidazole (MedChemExpress, HY-B0318), neomycin sulfate (MedChemExpress, HY-B0470), ampicillin (MedChemExpress, HY-B0522), and vancomycin (MedChemExpress, HY-B0671), was added to sterile drinking water at final concentrations of 1, 1, 1, and 0.5 g/L, respectively. Mice in the ABX+FMT-Model and ABX+FMT-PD groups had free access to this antibiotic-containing drinking water for 7 consecutive days. Following antibiotic treatment, a 3-day recovery period was allowed before initiating fecal microbiota transplantation.

The preparation and administration of the fecal microbiota suspension were performed according to a previously published method [22]. On the day of transplantation, fresh fecal samples were collected from donor mice in the Model and PD-H groups. Briefly, 0.1 g of feces was

suspended in 1 mL of sterile PBS, thoroughly vortexed, and filtered through a sterile 100-µm cell strainer. Recipient mice in the ABX+FMT-Model and ABX+FMT-PD groups were administered 200 µL of the fecal suspension by oral gavage once daily. The suspension administered to the ABX+FMT-Model group was derived from Model donor mice, and the suspension administered to the ABX+FMT-PD group was derived from PD-H donor mice. Fecal microbiota transplantation continued for 2 weeks.

Sample collection, euthanasia, and tissue processing

At the scheduled endpoint, mice were deeply anesthetized with 3% isoflurane in oxygen. Adequate anesthetic depth was confirmed by the loss of the righting reflex and the absence of a pedal withdrawal response. While mice remained under deep anesthesia, whole blood was collected by terminal cardiac puncture. Immediately after blood collection, cervical dislocation was performed as a secondary physical method to ensure euthanasia. Death was confirmed by the absence of spontaneous respiration, heartbeat, corneal reflex, and response to toe pinch before tissue collection. One portion of the blood was collected into EDTA-coated tubes, while the remaining blood was allowed to clot for serum preparation. Tumors, spleens, intestinal tissues, and fecal samples were then collected. Tumors were excised, photographed, weighed, and divided for subsequent analyses. Samples intended for histological analysis were fixed in 4% paraformaldehyde, whereas the remaining samples were stored at -80°C until further analysis.

16S rRNA gene sequencing and analysis

Total genomic DNA was extracted from fecal samples using a commercial DNA extraction kit (Thermo Fisher, 1159U64). The V3-V4 region of the bacterial 16S rRNA gene was amplified using universal primers, and the amplicons were sequenced on an Illumina NovaSeq platform using a 2 × 250-bp paired-end strategy. Given its superior effects on tumor growth, immune responses, and inflammatory markers, the PD-H group was selected for gut microbiota sequencing. The Control, Model, and PD-H groups were included, with three fecal samples analyzed per group.

Raw paired-end reads were processed using the QIIME 2 pipeline (version 2022.2). Reads were quality-filtered, denoised, merged, and screened for chimeric sequences using DADA2. An amplicon sequence variant (ASV) table was generated, and taxonomic classification was performed using the SILVA 138 reference database. Alpha diversity was evaluated using the ACE and Shannon indices. Differences in alpha diversity and taxon abundance among the three groups were assessed using the Kruskal-Wallis test, followed by false discovery rate (FDR) correction for multiple comparisons. Differences in beta diversity were evaluated using permutational multivariate analysis of variance (PERMANOVA). Linear discriminant analysis effect size (LEfSe) was used to identify differentially abundant taxa, with an LDA score threshold of 4.0.

Metagenomic sequencing and functional annotation analysis

Total DNA extracted from fecal samples was subjected to shotgun metagenomic sequencing. Taxonomic assignments and relative abundances were determined using the lowest common ancestor algorithm. KEGG pathway abundances were calculated for each sample. Principal coordinate analysis was performed to evaluate differences in microbial taxonomic composition and functional profiles. Differential KEGG pathways were analyzed using STAMP with Welch's t-test, and *P* values were adjusted for multiple comparisons. An adjusted *P* value < 0.05 was considered statistically significant. Three fecal samples from each of the Control, Model, and PD-H groups were analyzed.

RT-qPCR

RNA was extracted from xenograft tumor tissues or isolated splenic human CD4⁺ T cells using TRIzol® (Invitrogen). After quantification, 1 µg of total RNA was converted to cDNA with the PrimeScript™ RT Kit (Takara) per manufacturer guidelines. qPCR was conducted on a QuantStudio™ 5 (Applied Biosystems) using SYBR® Premix Ex Taq™ II (Takara) to assess *TNF-α*, *IL-6*, *IL-17A*, and *IL-10* in isolated splenic CD4⁺ T cells and the expression of *NF-κB*, *JAK2*, *STAT3*, *IL-17A*, and *TNF-α* in tumor tissues. RT-qPCR was performed in a total reaction volume of 20 µL. The amplification conditions were as follows: initial denaturation at 95°C for

Table 1. Primer sequences

<i>GAPDH-F</i>	5'-GTCTCCTCTGACTTCAACAGCG-3'
<i>GAPDH-R</i>	5'-ACCACCCTGTTGCTGTAGCCAA-3'
<i>IL-17-F</i>	5'-CGGACTGTGATGGTCAACCTGA-3'
<i>IL-17-R</i>	5'-GCACTTTGCCTCCCAGATCACA-3'
<i>JAK2-F</i>	5'-CCAGATGGAACTGTTGCTCAG-3'
<i>JAK2-R</i>	5'-GAGGTTGGTACATCAGAAACACC-3'
<i>STAT3-F</i>	5'-CTTTGAGACCGAGGTGTATCACC-3'
<i>STAT3-R</i>	5'-GGTCAGCATGTTGTACCACAGG-3'
<i>NF-κB-F</i>	5'-TGAACCGAACTCTGGCAGCTG-3'
<i>NF-κB-R</i>	5'-CATCAGCTTGCAGAAAGGAGCC-3'
<i>TNF-α-F</i>	5'-CTCTTCTGCTGCTGCACTTTG-3'
<i>TNF-α-R</i>	5'-ATGGGCTACAGGCTTGCTACTC-3'
<i>IL-6-F</i>	5'-CCACCGGGAACGAAAGAGAA-3'
<i>IL-6-R</i>	5'-GAGAAGGCAACTGGACCGAA-3'
<i>IL-10-F</i>	5'-CGAGATGCCTTCAGCAGAGT-3'
<i>IL-10-R</i>	5'-GGCAACCCAGGTAACCCCTTAG-3'

30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 30 s. A melting-curve analysis was subsequently performed to verify the specificity of the amplified products. GAPDH was used as the internal reference gene, and relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in **Table 1**.

Western blot analysis

Tumor tissues were lysed, and the supernatants were collected after centrifugation. Proteins were separated by 8% or 10% SDS-PAGE and transferred onto PVDF membranes. After blocking, the membranes were incubated with the following primary antibodies: anti-β-actin (Proteintech, 20536-1-AP, 1:10,000), anti-IL-17 (Proteintech, 87785-2-RR, 1:1,000), anti-TNF-α (Proteintech, 17590-1-AP, 1:1,000), anti-NF-κB p65 (Abcam, ab76311, 1:1,000), anti-phospho-NF-κB p65 (phospho S536) (Abcam, ab76302, 1:1,000), anti-JAK2 (Abclonal, A28566, 1:2,000), anti-phospho-JAK2 (Abclonal, AP1575, 1:4,000), anti-STAT3 (Abcam, ab32500, 1:1,000), anti-phospho-STAT3 (phospho Y705) (Abcam, ab76315, 1:2,000), anti-CD8A (Abclonal, A23305PM, 1:500), anti-Granzyme B (Proteintech, 13588-1-AP, 1:500), and anti-Foxp3 (Proteintech, 85128-6-RR, 1:1,000). The membranes were incubated with primary antibodies overnight at 4°C. The following day, the PVDF membranes were washed three times with TBST and incubated with an

HRP-conjugated secondary antibody (Proteintech, RGAR001, 1:10,000) for 2 hours at room temperature. After incubation, protein bands were visualized using an ECL chemiluminescent substrate and imaged with a chemiluminescence imaging system (Tanon-5200, Tanon Science & Technology Co., Ltd., Shanghai, China). Band intensities were quantified using ImageJ software. β-Actin served as the loading control to calculate the relative expression levels of the target proteins.

Hematoxylin and eosin (H&E) staining

Tumor tissues were fixed in 10% neutral-buffered formalin for 24 h, embedded in paraffin, and sectioned at a thickness of 4 μm. Sections were deparaffinized, rehydrated, stained with hematoxylin and eosin, dehydrated, cleared, and mounted with coverslips. Histopathological features were evaluated and imaged using an Olympus BX53 light microscope.

Peripheral blood leukocyte preparation and flow cytometric analysis

Peripheral blood was collected into EDTA-coated tubes, and erythrocytes were lysed to obtain leukocytes. The cells were washed with PBS and incubated with APC-conjugated anti-human CD8 and FITC-conjugated anti-human CD4 antibodies for 20 min at room temperature in the dark. After washing, the cells were analyzed by flow cytometry. The proportions of human CD4⁺ and CD8⁺ T cells were quantified using FlowJo software. The antibodies used in this study to detect CD4⁺ and CD8⁺ T cells in peripheral blood included: APC anti-human CD8 antibody (BioLegend, 344722) and FITC anti-human CD4 antibody (BioLegend, 357405).

Preparation of splenic single-cell suspensions and isolation of CD4⁺ T cells

After euthanasia, spleens were aseptically removed and transferred into ice-cold PBS. Single-cell suspensions were prepared, and erythrocytes were lysed. The cells were then washed with PBS, counted, and used for subsequent experiments. CD4⁺ T cells were isolated using an EasySort™ Human CD4⁺ T Cell Isolation Kit (Elabscience, MIH002N) according to the manufacturer's instructions. The purified cells were resuspended in RPMI-1640 medium and used for flow cytometry, cell culture, ELISA, and RT-qPCR.

Flow cytometric analysis

Purified splenic CD4⁺ T cells were stained to detect Treg and Th17 subsets. For Treg detection, antibody staining against CD4, CD25 and Foxp3 was performed on cells at 4°C in the dark. For Th17 detection, the cells were stained with anti-CD4 and anti-IL-17A antibodies. The following antibodies were used: PE anti-human CD4 antibody (BioLegend, 300550), FITC anti-human IL-17A antibody (BioLegend, 512304), APC anti-FOXP3 monoclonal antibody (PCH101; Thermo Fisher, 17-4776-42), and FITC anti-human CD25 antibody (BioLegend, 302604).

Immunofluorescence (IF) staining

Fresh tumor tissues were embedded and then cut into 5-µm frozen sections. The sections were fixed in cold acetone, blocked with 5% normal goat serum. After blocking, sections were incubated with primary antibodies, followed by DAPI nuclear counterstaining. Images were captured using a Leica TCS SP8 confocal microscope. For tumor immunofluorescence, the antibodies utilized were: FITC anti-human CD8 antibody (BioLegend, 344703) or FITC anti-human CD4 antibody (BioLegend, 357405).

ELISA

Tumor tissues were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors on ice. The lysates were centrifuged at 12,000×g for 15 minutes at 4°C, and the supernatants were collected. The concentrations of TNF-α (mlbio, ml077385), IL-17A (mlbio, ml105734), NF-κB p65 (mlbio, ml105589), JAK2 (mlbio, ml105594), and STAT3 (mlbio, ml037546) in tumor lysates were measured using commercial ELISA kits. After culturing, the supernatants of isolated CD4⁺ T cells were collected by centrifugation. TNF-α (mlbio, ml077385), IL-6 (TW-reagent, TW-E8937), IL-17A (mlbio, ml105734), and IL-10 (mlbio, ml064299) concentrations were measured using commercial ELISA kits.

Statistical analysis

All data are presented as the mean ± standard deviation (SD). Longitudinal data, such as body weight and tumor volume, obtained from the same animals at multiple time points, were analyzed using two-way repeated-measures

analysis of variance (ANOVA), with treatment group as the between-subject factor and time as the within-subject factor. Bonferroni's multiple-comparisons test was subsequently performed for comparisons between groups at individual time points. Data involving multiple groups measured at a single time point were analyzed using one-way ANOVA followed by Tukey's post hoc test. A *P* value of less than 0.05 was considered statistically significant. Statistical analyses and graph generation were performed using GraphPad Prism software (Version 5.0, USA).

Results

High-dose Pulsatilla decoction remodels the gut microbiota composition in colorectal cancer mice

To evaluate the effect of PD on the gut microbiota, we analyzed the composition and diversity of microbial communities in the Control, Model, and PD-H groups. A total of 226 ASVs were shared among the three groups. The Model group harbored 622 unique ASVs, compared with 465 in the PD-H group, indicating that CRC modeling markedly altered the microbial community, a shift partially reversed by high-dose PD (**Figure 1A**).

Alpha diversity was evaluated using the ACE and Shannon indices. Compared with the Control group, the Model group showed lower ACE and Shannon indices, indicating reductions in microbial richness and overall community diversity. Compared with the Model group, both indices were increased in the PD-H group and tended to approach the levels observed in the Control group. These results suggest that high-dose PD treatment was associated with partial restoration of CRC-related alterations in gut microbial richness and diversity (**Figure 1B, 1C**). Also, Principal Coordinate Analysis (PCoA) was used to analyze beta diversity. PCoA revealed clear separation between the Model and Control groups, whereas the PD-H samples were positioned closer to the Control samples, suggesting partial normalization of the overall microbial community structure after PD treatment (**Figure 1D**).

Taxonomic analysis revealed significant changes in microbial compositions at the phylum and family levels. The phyla of *Bacteroidota*,

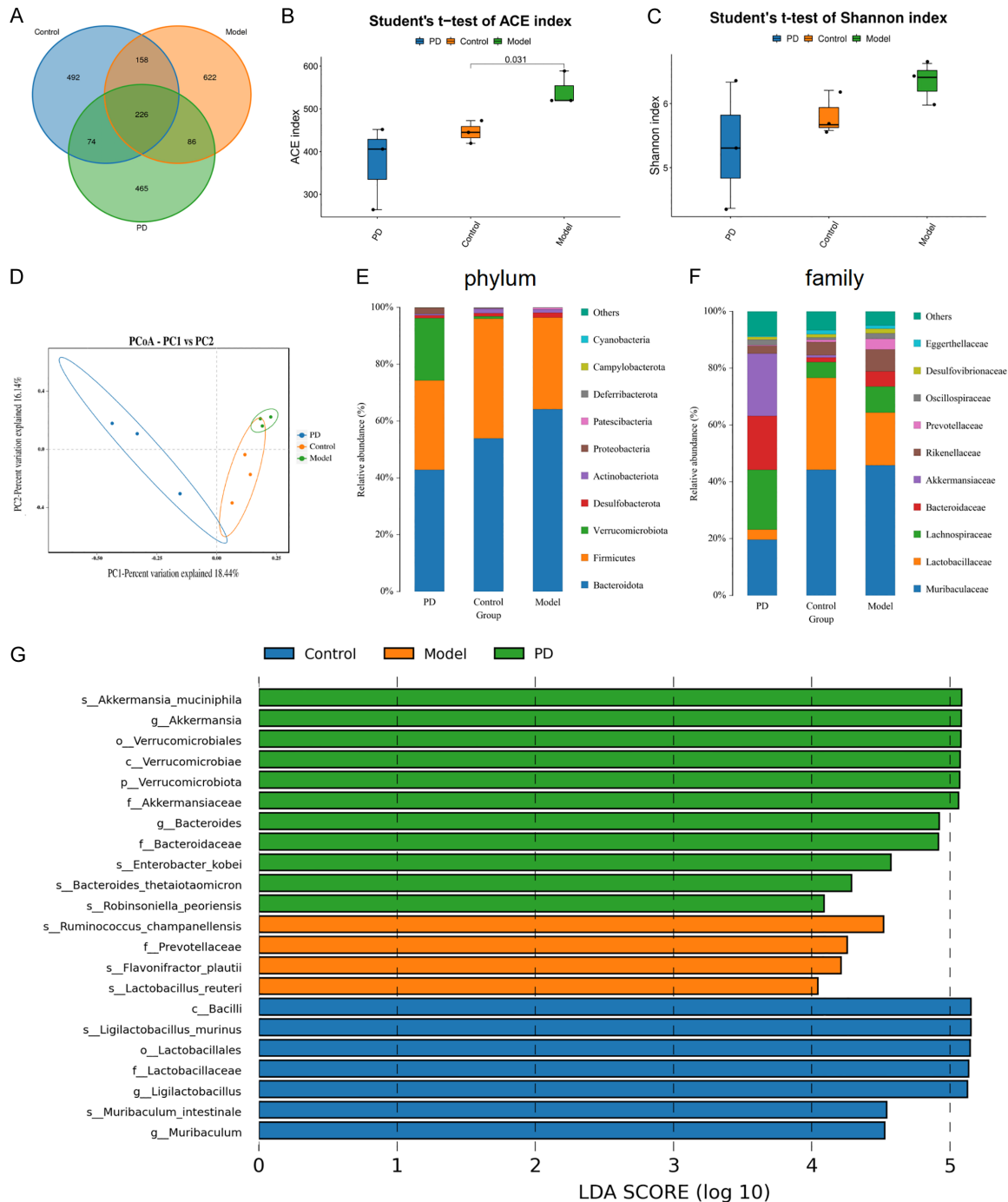


Figure 1. High-dose Pulsatilla decoction alleviates gut microbiota dysbiosis in colorectal cancer mice. (A) Venn diagram illustrating the number of shared and unique ASVs among the Control, Model, and PD groups. (B, C) Alpha diversity of the gut microbiota, as assessed using the ACE index (B) and Shannon index (C). Data are presented as mean $\pm$ SD. (D) PCoA plot based on Bray-Curtis distances, visualizing the dissimilarities in microbial community structure (beta diversity) across the three experimental groups. Each point represents an individual sample. (E) Relative abundance of the dominant microbial taxa at the phylum level. (F) Relative abundance of the dominant microbial taxa at the family level. (G) LefSe analysis identifying the key taxa that were differentially abundant among the groups (LDA score > 4.0). PD represents the PD-H group treated with Pulsatilla Decoction at 16 g/kg/day. The microbiome analysis included the Control, Model, and PD-H groups and was not designed to assess dose-dependent microbiota alterations.

Firmicutes, and *Verrucomicrobiota* were the major compositions of the murine gut microbiota. Significant changes were also observed in response to PD treatment that caused a significant reduction in the proportional abundance of *Bacteroidota* in comparison to the Model group. Conversely, the distribution of *Verrucomicrobiota*, which were nearly undetectable in the Model mice, was significantly elevated after the administration of PD. PD treatment also induced marked changes at the family level. The latter in particular reversed the CRC-related depletion of *Akkermansiaceae* and enhanced the abundance of *Bacteroidaceae*, and simultaneously lowered the percentage of *Lachnospiraceae* (Figure 1E, 1F).

LEfSe identified taxa that were differentially enriched among the three groups using an LDA score threshold of 4.0. The Control group was enriched in *Bacilli*, *Ligilactobacillus reuteri*, and *Muribaculum intestinale*. The Model group was enriched in *Robinsoniella peoriensis*, *Flavonifractor plautii*, and *Prevotellaceae*. The PD-H group was enriched in the *Akkermansia muciniphila* lineage, from the phylum *Verrucomicrobiota* to the species *A. muciniphila*, as well as *Bacteroidaceae* and *Bacteroides thetaiotaomicrons* (Figure 1G).

High-dose Pulsatilla decoction improves gut microbiota structural and functional dysbiosis in colorectal cancer mice

To further investigate the effects of PD on the gut microbiota's structure and function, we performed metagenomic sequencing. PCoA based on genus-level relative abundance showed a clear separation between the Control and Model groups in the ordination space, whereas the PD-treated samples tended to shift toward the Control group (Figure 2A). Genus-level taxonomic composition analysis revealed that the relative abundances of *Akkermansia*, *Bacteroides*, and *Parabacteroides* were decreased in the Model group, while *Prevotella* was increased. After high-dose PD treatment, the relative abundances of these potentially beneficial genera were partly recovered, and the abnormal enrichment of *Prevotella* was reduced (Figure 2B). Functional PCoA analysis showed a clear separation between the Model and Control groups. Compared with the Model group, the PD group shifted closer to the Control group (Figure 2C). These results suggest that

high-dose PD treatment is associated with partial restoration of CRC-related gut microbiota dysbiosis.

To examine the effect of PD on gut microbial functions, we analyzed metagenomic KEGG pathway abundance among the groups. Gut microbiota functional profiles differed across the treatment groups (Figure 2D). Compared with the Control group, the Model group showed enrichment of pathways related to pathogen infection and host inflammatory responses, including shigellosis, lipopolysaccharide biosynthesis, and bacterial chemotaxis. These results indicate that CRC modeling was associated with a pro-inflammatory shift in microbial function (Figure 2E). After high-dose PD treatment, several metabolism-related pathways were increased, including fructose and mannose metabolism, glycolysis/gluconeogenesis, and histidine metabolism. This pattern suggests that PD treatment may help improve microbial carbohydrate utilization and amino acid metabolic functions (Figure 2F).

Pulsatilla decoction inhibits tumor growth in colorectal cancer mice

To evaluate the in vivo effects of PD, tumor formation was monitored during the whole experiment. Tumor volume was measured throughout the study, and representative images of excised tumors were taken on day 20 (Figure 3A). PD treatment did not cause significant body weight loss (Figure 3B), indicating no overt systemic toxicity at the tested doses. Tumor growth curves showed that tumor volume in the Model group increased over time. Compared with the Model group, the treatment groups showed significant inhibition in tumor growth (Figure 3C). On day 20, tumor volume was reduced in the 5-FU, PD-M, and PD-H groups, whereas the PD-L group did not show a clear reduction (Figure 3D). Tumor weight was also lower after 5-FU or PD treatment (Figure 3E). These results suggest that PD suppressed tumor growth in mice bearing transplanted colorectal tumors.

H&E staining showed that tumor tissues in the Model group contained densely arranged tumor cells with marked atypia and obvious inflammatory cell infiltration. After PD treatment, tumor cell atypia and inflammatory infiltration were reduced (Figure 3F). These pathological changes suggest that PD treatment altered tumor tis-

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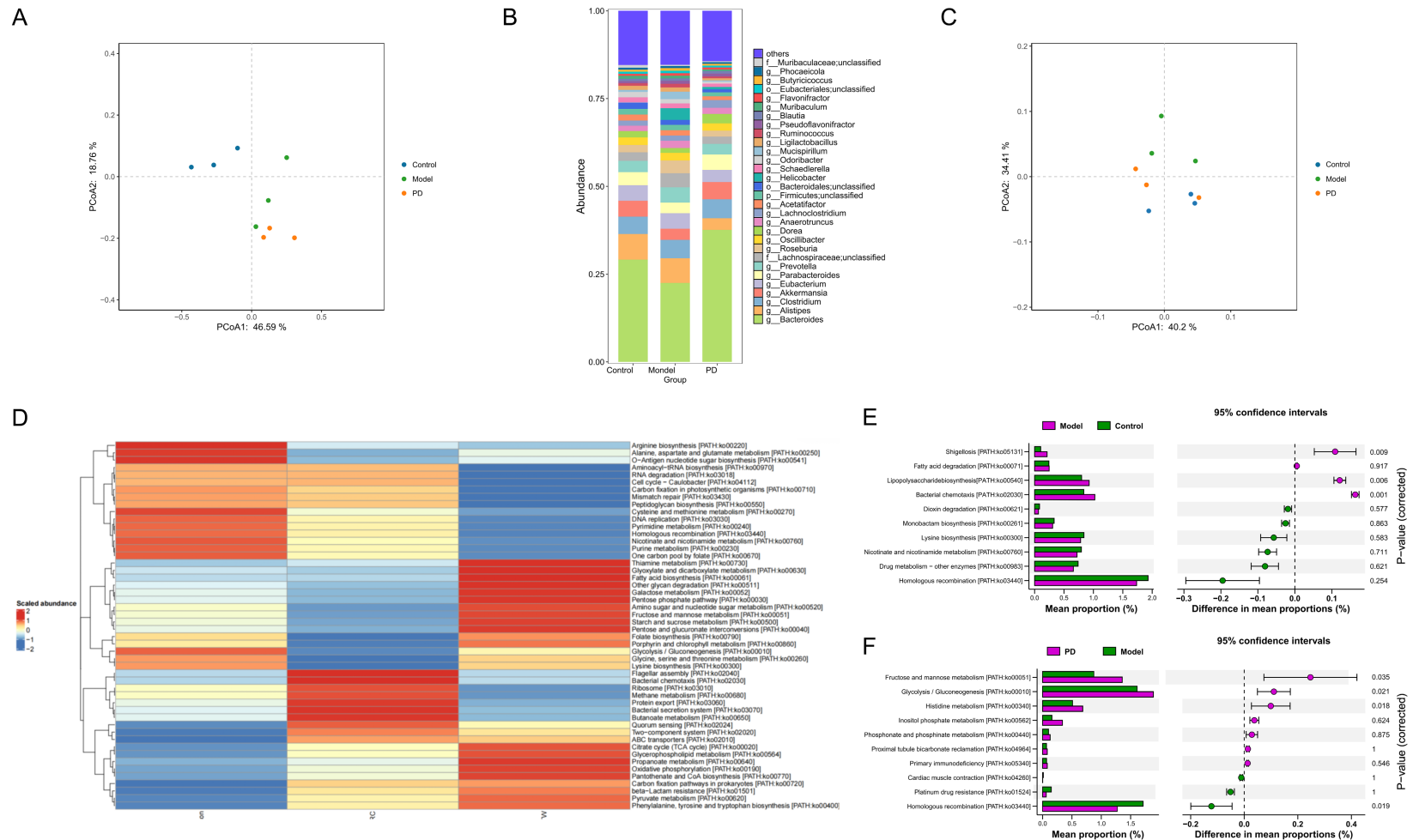


Figure 2. High-dose Pulsatilla decoction improves the structural and functional dysbiosis of the gut microbiota in colorectal cancer mice. A. Principal coordinate analysis (PCoA) based on the relative abundance of microorganisms at the genus level. B. Relative abundance of dominant microbial taxa at the genus level. C. Principal coordinate analysis (PCoA) based on microbial functional abundance. D. Changes in the relative abundance of KEGG functional pathways of gut microbiota based on metagenomic sequencing results. Colors represent normalized pathway abundance levels, with red indicating higher relative abundance and blue indicating lower relative abundance. E, F. Comparison of relative abundance differences in KEGG functional pathways among different groups based on metagenomic sequencing results. P values were adjusted for multiple comparisons. PD represents the PD-H group treated with Pulsatilla Decoction at 16 g/kg/day. The microbiome analysis included the Control, Model, and PD-H groups and was not designed to assess dose-dependent microbiota alterations.

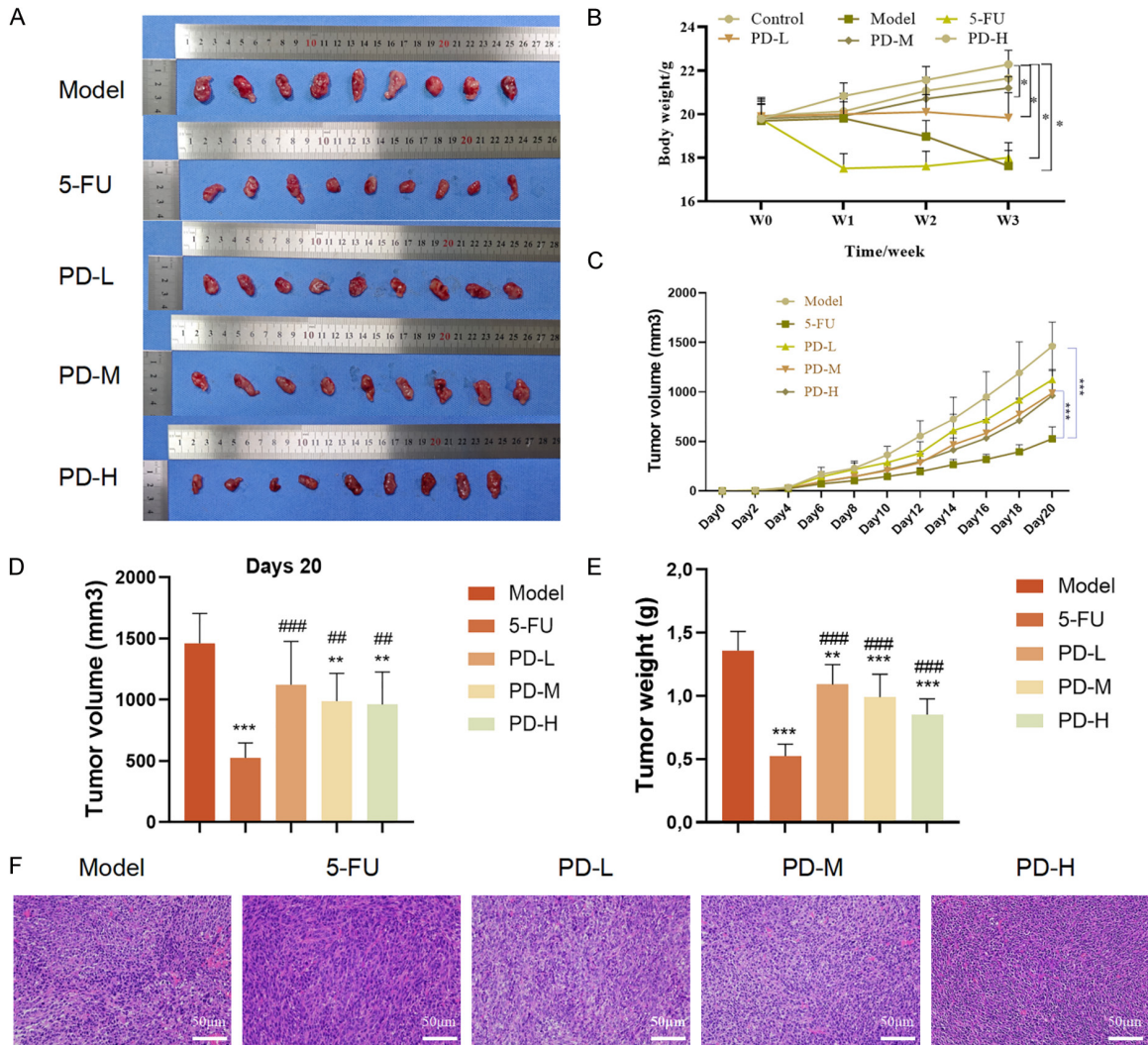


Figure 3. Pulsatilla decoction inhibits tumor growth in colorectal cancer mice. A. Representative images of excised tumors from each group of mice (n=9). B. Changes in body weight during the experimental period (n=9). C. Tumor volume growth curves for each group of mice during the experimental period (n=9). D. Statistical analysis of tumor volume in each group on day 20 (n=9). E. Statistical analysis of terminal tumor weight for each group (n=9). F. H&E staining was performed on tumor tissues from the Model, 5-FU, and PD-treated tumor-bearing groups (n=3), magnification: $\times 200$. $**P < 0.01$, $***P < 0.001$ compared with the Model group. $##P < 0.01$, $###P < 0.001$ compared with the 5-FU group.

sue morphology and reduced inflammatory cell infiltration.

Pulsatilla decoction suppresses colorectal cancer by modulating CD4⁺/CD8⁺ T-Cell-mediated immunity

In CRC, CD4⁺ and CD8⁺ T cells are both involved in anti-tumor immune responses. CD8⁺ T cells can directly kill tumor cells, whereas CD4⁺ helper T cells support CD8⁺ T-cell activity via interactions with antigen-presenting cells. To evaluate the immunomodulatory effects of PD, we

analyzed these T-cell populations. Flow cytometry showed that peripheral CD4⁺ and CD8⁺ T cells were reduced in the Model group compared with the Control group. PD treatment partly reversed this reduction and increased the circulating levels of both T-cell subsets in a dose-related manner (Figure 4A, 4B, 4E, 4F). Immunofluorescence analysis further showed that PD treatment was associated with increased CD4⁺ and CD8⁺ T-cell infiltration in tumor tissues, also with a dose-related trend (Figure 4C, 4D, 4G, 4H).

Pulsatilla decoction attenuates colorectal cancer

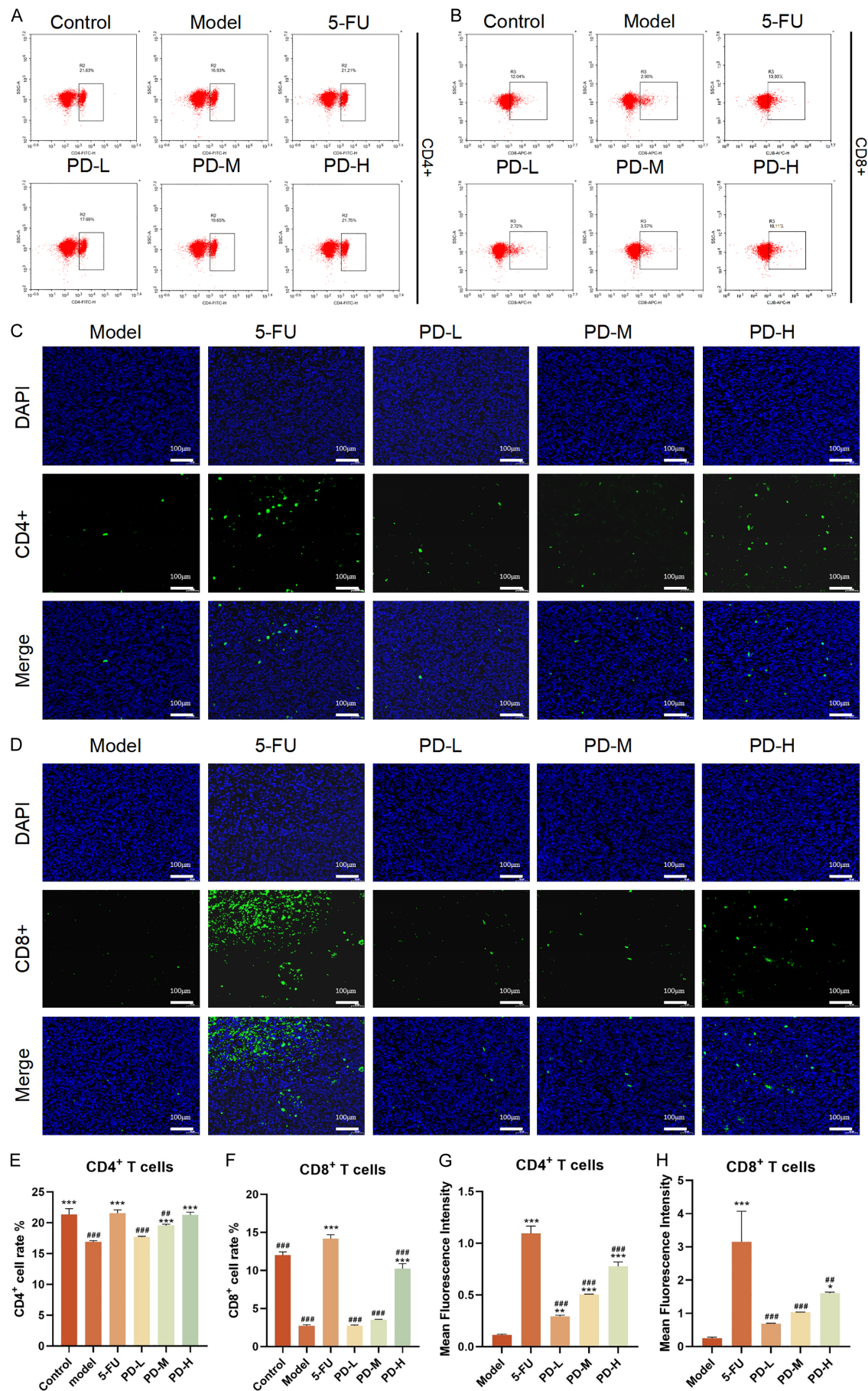


Figure 4. Pulsatilla decoction suppresses colorectal cancer by modulating CD4⁺/CD8⁺ T-cell-mediated immunity. A, B. Representative flow cytometry plots showing the populations of CD4⁺ and CD8⁺ T cells in peripheral blood from each experimental group (n=3). C, D. Representative immunofluorescence images displaying the infiltration of CD4⁺ and CD8⁺ T cells within the tumor microenvironment (n=3), magnification: ×200. E, F. Quantification of the percentage of circulating CD4⁺ and CD8⁺ T cells (n=3). G, H. Quantification of the mean fluorescence intensity for CD4⁺ and CD8⁺ T cells in tumor tissues (n=3). ***P* < 0.01, ****P* < 0.001 compared with the Model group. ##*P* < 0.01, ###*P* < 0.001 compared with the 5-FU group.

Pulsatilla decoction regulates splenic CD4⁺ T-cell differentiation imbalance and inflammatory levels in mice

To assess the effect of PD on splenic CD4⁺ T-cell differentiation, we isolated CD4⁺ T cells from spleen tissues for flow cytometric analysis. Compared with the Control group, the Model group showed a lower proportion of CD4⁺CD25⁺FOXP3⁺ Treg cells, suggesting that CRC modeling was associated with impaired splenic immune balance. 5-FU treatment increased the proportion of Treg cells. PD treatment also increased Treg cells in a dose-related manner among the PD-L, PD-M, and PD-H groups (**Figure 5A, 5C**).

Further analysis showed that the proportion of CD4⁺IL-17A⁺ Th17 cells was higher in the Model group than in the Control group, indicating enhanced Th17-related inflammation in the spleen. Both 5-FU and PD treatment reduced the proportion of CD4⁺IL-17A⁺ cells, and the effect of PD showed a dose-related trend (**Figure 5B, 5D**). Together, these findings suggest that PD treatment may help restore the splenic Treg/Th17 balance and reduce CRC-associated peripheral immune dysregulation.

To evaluate whether PD was associated with changes in inflammatory responses, we measured cytokine levels in cell culture supernatants by ELISA and detected related mRNA expression by qPCR. Compared with the Control group, TNF-α, IL-6, and IL-17A levels were increased in the Model group, whereas IL-10 levels were decreased. This pattern suggests enhanced inflammatory activation in the Model group (**Figure 6A-D**). After PD treatment, TNF-α, IL-6, and IL-17A secretion decreased, while IL-10 levels increased, in a dose-dependent manner (**Figure 6A-D**). The mRNA expression results were broadly consistent with the ELISA findings (**Figure 6E-H**). Collectively, these results indicate that PD treatment reduced pro-inflammatory cytokine expression and increased IL-10 levels, suggesting alleviation of inflammatory responses.

Pulsatilla decoction reduces inflammation in colorectal cancer by inhibiting the NF-κB/JAK2/STAT3 pathway

To elucidate the molecular mechanisms of PD, we assessed the activity of the NF-κB and JAK2/STAT3 pathways within tumor tissues. Immunoblotting revealed that PD treatment resulted in a dose-dependent decline in the phosphorylation of NF-κB, JAK2, and STAT3 relative to the Model group (**Figure 7A-G**). In line with the changes in pathway activation, PD treatment was associated with lower protein levels of IL-17 and TNF-α, with a dose-related trend (**Figure 7H, 7I**). Protein and gene expression analyses further supported this pattern. ELISA showed reduced total protein levels of NF-κB, JAK2, and STAT3 in the PD-treated groups (**Figure 7J-L**). RT-qPCR also showed lower mRNA expression of pathway-related genes compared with the Model group, and the reduction was more evident at higher PD doses (**Figure 7M-Q**). Because tumors were not formed in the Control mice, pathway analyses were conducted only in tumor-bearing groups.

Overall, PD treatment was associated with reduced tumor burden, decreased inflammatory cytokine expression, and lower NF-κB/JAK2/STAT3 pathway activation. Whether these changes are connected with the observed gut microbiota remodeling requires further investigation.

Fecal microbiota from PD-H donors can partially transfer their antitumor effects, local tumor immune regulation, and changes in NF-κB/JAK2/STAT3-related proteins

To evaluate whether gut microbiota modulated by PD contributes to its antitumor effects, recipient mice were pretreated with antibiotics (ABX) and then received FMT from donor mice in either the Model or PD-H group. Compared to the ABX+FMT-Model group, the ABX+FMT-PD group exhibited smaller tumors and lower tumor weights at the experimental endpoint (**Figure 8A, 8B**). Endpoint tumor

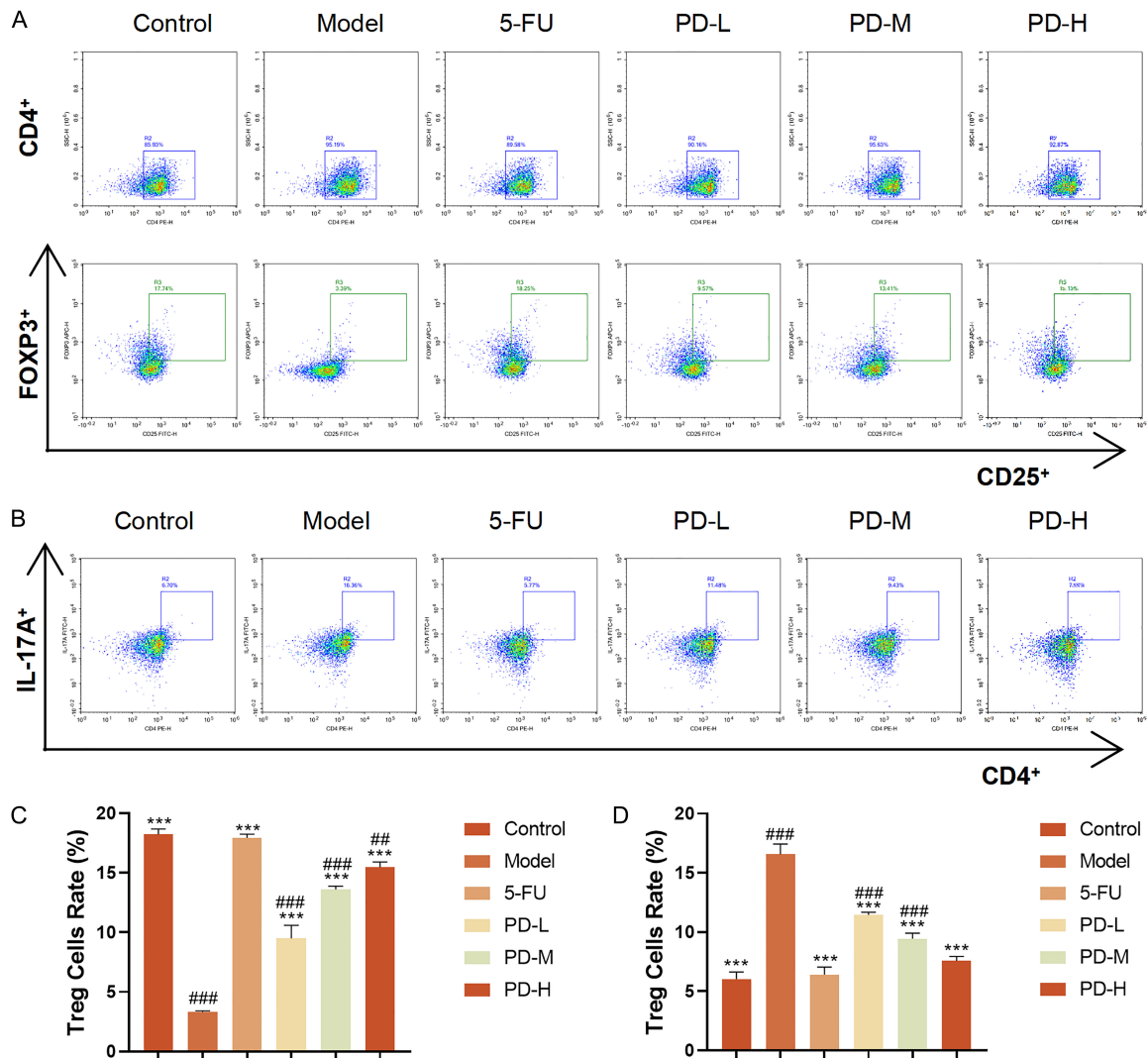


Figure 5. Pulsatilla decoction restores the balance of splenic Treg/Th17 differentiation in colorectal cancer mice. A. Representative flow cytometry dot plots illustrating the proportion of CD4⁺CD25⁺FOXP3⁺ Treg cells among splenic CD4⁺ T cells in each group (n=3). B. Representative flow cytometry dot plots illustrating the proportion of CD4⁺IL-17A⁺ Th17 cells among splenic CD4⁺ T cells in each group (n=3). C. Statistical analysis of the proportion of Treg cells in each group (n=3). D. Statistical analysis of the proportion of CD4⁺IL-17A⁺ Th17 cells in each group (n=3). ****P* < 0.001 compared with the Model group. ##*P* < 0.01, ###*P* < 0.001 compared with the 5-FU group.

weight in the ABX+FMT-Model group was similar to that in the Model group. The antitumor effect observed in the PD-H group was stronger than that in the ABX+FMT-PD group (Figure 8A, 8B).

Body weights of mice in all groups remained generally stable throughout the experiment. At the experimental endpoint, mice in the PD-H group had significantly higher body weights than those in the Model group, while no significant difference was observed between the ABX+FMT-Model and ABX+FMT-PD groups (Figure 8C).

ELISA results showed that levels of JAK2, STAT3, and NF-κB in tumor tissues were lower in the ABX+FMT-PD group than in the ABX+FMT-Model group (Figure 8D-F). Western blot analysis revealed relatively low CD8A and granzyme B expression in the Model and ABX+FMT-Model groups, indicating weak local cytotoxic immune responses within the tumors (Figure 8G-J). In contrast, the ABX+FMT-PD group showed increased CD8A and granzyme B expression in tumor tissues compared to the ABX+FMT-Model group, suggesting that FMT from PD-H-treated donors may enhance local cytotoxic antitumor immunity (Figure 8G-J). Additionally, Foxp3

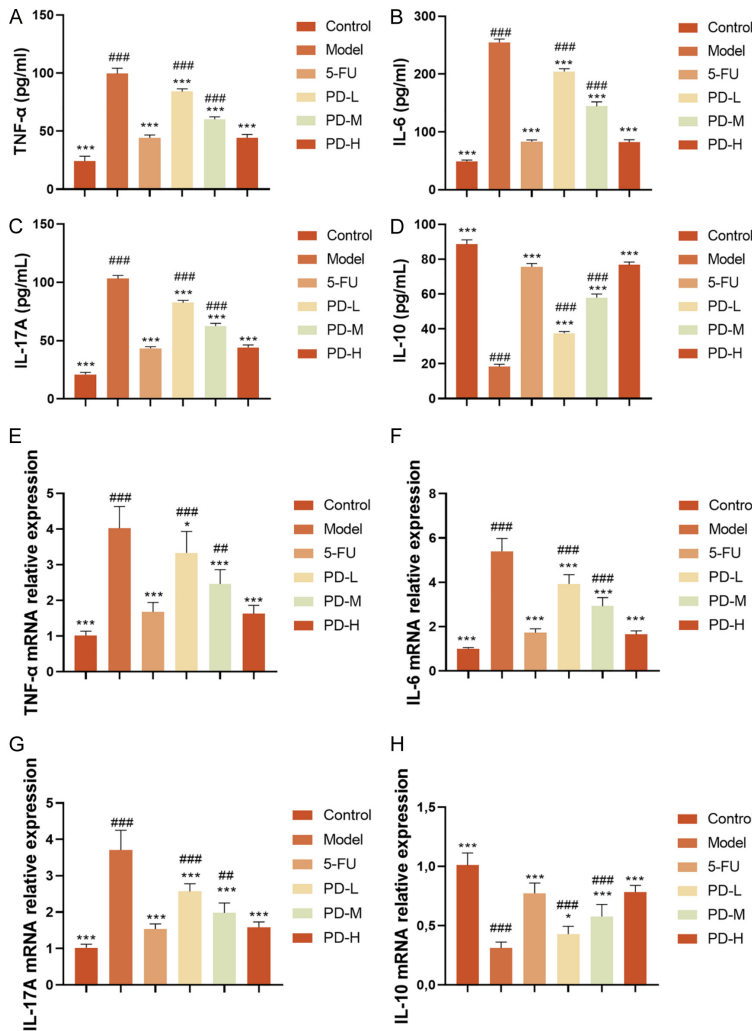


Figure 6. Pulsatilla decoction regulates inflammatory cytokine secretion and gene expression in splenic CD4⁺ T cells. A-D. ELISA analysis of inflammatory cytokine levels in the culture supernatants of CD4⁺ T cells from each group (n=3). E-H. qPCR analysis of the mRNA expression levels of inflammatory cytokines in CD4⁺ T cells from each group (n=3). ****P* < 0.001 compared with the Model group. ##*P* < 0.01, ###*P* < 0.001 compared with the 5-FU group.

expression was reduced in tumor tissues from the ABX+FMT-PD group, suggesting attenuation of regulatory T-cell-associated immunosuppression within the tumor microenvironment.

Collectively, these results indicate that transplantation of fecal microbiota from PD-H-treated donor mice inhibited tumor growth, reduced NF- κ B, JAK2, and STAT3 levels, and improved the local immune status of the tumor. These findings suggest that the antitumor effects of PD may be partially mediated through modulation of the gut microbiota. However, a direct effect of PD on the host or tumor tissues cannot be excluded.

Discussion

In this study, PD treatment reduced tumor burden in mice bearing transplanted CRC tumors. This effect was accompanied by changes in gut microbiota composition, microbial functional profiles, T-cell immune responses, inflammatory cytokine expression, and NF- κ B/JAK2/STAT3 signaling.

The gut microbiota changes observed in this study were complex and closely associated with PD treatment. The combined analysis of taxonomic abundance and LEfSe showed that PD increased the relative abundance of the *Verrucomicrobiota* lineage, extending from the phylum level to the species *Akkermansia muciniphila*. At the same time, PD treatment reduced the abundance of *Bacteroidota* and *Lachnospiraceae*. Recent studies have shown that enterotoxigenic *Bacteroides fragilis* and its toxin BFT can impair intestinal barrier integrity and promote CRC-related inflammation and tumor progression through STAT3-, IL-17A-, and NF- κ B-associated signaling [23-25]. However, the role of *Lachnospiraceae* in CRC is heterogeneous across genera and species. Therefore, the reduced family-level abundance observed

after PD treatment should be interpreted as an association rather than evidence of a uniformly beneficial microbial shift [26]. Metagenomic analysis further showed that PD changed microbial functions. The Model group was enriched in shigellosis, lipopolysaccharide biosynthesis, and bacterial chemotaxis, indicating a pro-inflammatory microbial state. PD increased fructose and mannose metabolism, glycolysis/gluconeogenesis, and histidine metabolism, suggesting improved microbial metabolism and reduced inflammatory functions [27]. This study selected only the PD-H group for 16S rRNA and metagenomic sequenc-

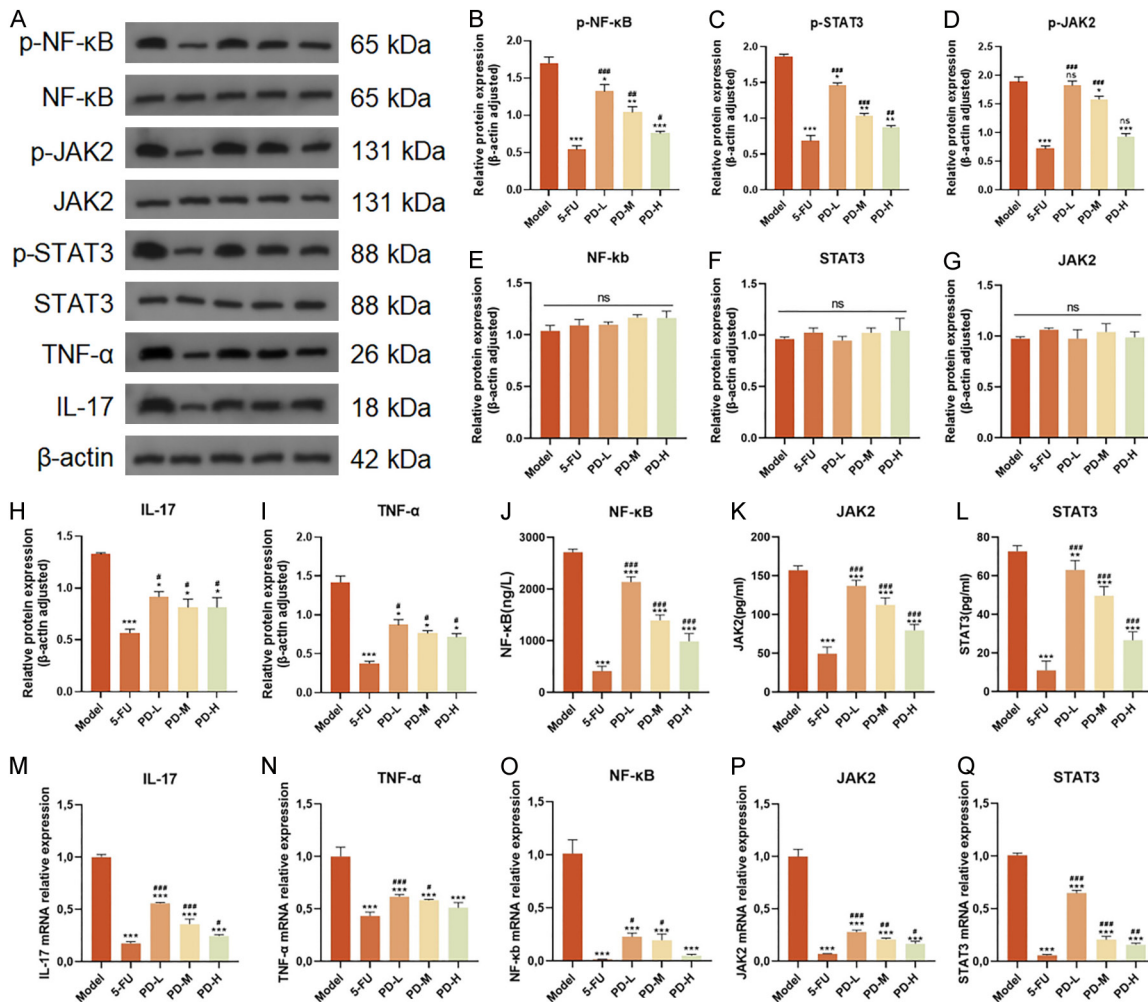


Figure 7. Pulsatilla decoction inhibits the NF- κ B/JAK2/STAT3 pathway in colorectal cancer. A. Representative Western blot bands for the indicated proteins (n=3). B-I. The quantification of Western blot bands for p-NF- κ B, p-STAT3, p-JAK2, NF- κ B, STAT3, JAK2, IL-17, and TNF- α (n=3). J-L. ELISA analysis of protein concentrations for NF- κ B, JAK2, and STAT3 in tumor tissues (n=3). M-Q. qRT-PCR analysis of relative mRNA expression for NF- κ B, JAK2, STAT3, IL-17, and TNF- α in tumor tissues (n=3). *** P < 0.001 compared with the Model group. * P < 0.05, ** P < 0.01, ### P < 0.001 compared with the 5-FU group.

ing because this dose exhibited the most pronounced antitumor, immunomodulatory, and anti-inflammatory effects in vivo. Therefore, the microbiome analysis was designed to describe changes in the microbiome at the optimal effective dose, rather than to evaluate dose-dependency. Based on these results, we cannot determine whether PD exerts dose-dependent effects on the gut microbiome. Given that gut dysbiosis disrupts immune homeostasis and facilitates tumor immune evasion in CRC, we investigated the abundance of CD4⁺ and CD8⁺ T cells in both peripheral blood and tumor tissues. Our results demonstrate that the populations of these critical T cells were significantly

expanded by direct PD treatment. Restoration of these T-cell compartments suggests that PD may improve systemic and local anti-tumor immune surveillance. This immune-restorative effect is consistent with findings that gut microbiota can regulate anti-cancer immunity and the tumor immune microenvironment [28, 29].

In addition to CD4⁺/CD8⁺ T-cell responses, PD corrected the imbalance of splenic CD4⁺ T-cell differentiation. PD increased Treg cells and IL-10 while decreasing Th17 cells and pro-inflammatory cytokines. These results indicate that PD alleviates CRC-associated peripheral immune dysregulation and restrains Th17-type

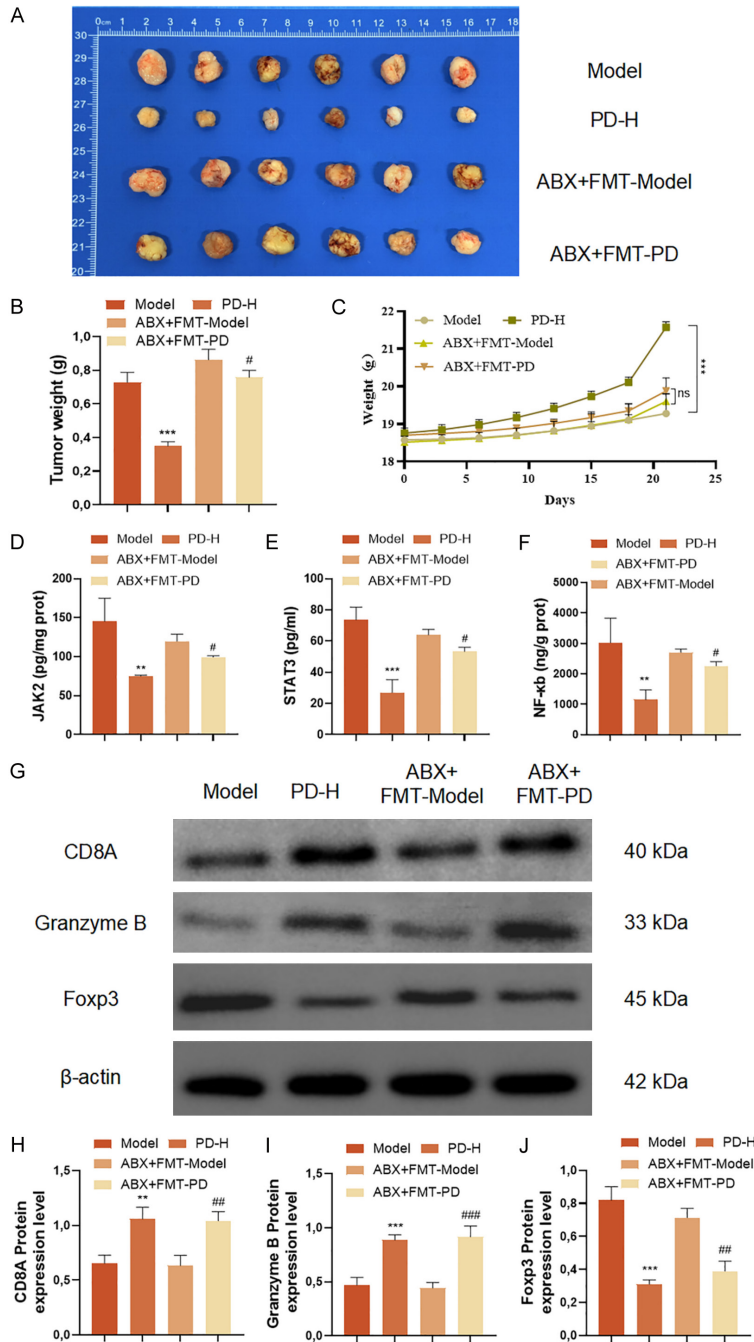


Figure 8. Fecal microbiota from PD-H-treated donors partially transfers tumor-inhibitory effects, modulates local antitumor immunity, and alters NF-κB/JAK2/STAT3-related protein levels. **A.** Representative images of tumors collected from each group at the experimental endpoint (n=6). **B.** Tumor weight at the experimental endpoint (n=6). **C.** Changes in mouse body weight during the experiment (n=6). **D-F.** ELISA measurements of JAK2, STAT3, and NF-κB levels in tumor tissues (n=6). **G.** Western blot analysis of CD8A, granzyme B, and Foxp3 expression in tumor tissues (n=3). **H-J.** Densitometric analysis of the relative protein expression levels of CD8A, granzyme B, and Foxp3 (n=3). ** $P < 0.01$ and *** $P < 0.001$ versus the Model group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ versus the ABX+FMT-Model group.

inflammation. Recent research suggests that the Treg/Th17 axis in CRC is highly plastic and context-dependent; nevertheless, excessive IL-17A and Th17-skewed inflammation can promote inflammatory tumor progression, whereas Treg-associated IL-10 can restrict uncontrolled intestinal inflammation [13, 14]. Thus, the observed restoration of the Treg/Th17 balance may be one of the key mechanisms through which PD reduces inflammation-associated CRC progression.

Integrative analysis of microbiota, immune, and inflammatory data showed that PD treatment increased the abundance of *Akkermansia muciniphila* and reduced the abnormal enrichment of *Prevotella*. These changes were accompanied by lower relative abundances of microbial pathways related to lipopolysaccharide biosynthesis and pathogen-associated inflammatory responses. Such microbial shifts may reduce microbiota-derived inflammatory stimuli [30]. This may help reduce Th17-skewed inflammation, as shown by decreased IL-17A, TNF-α, and IL-6 levels and increased IL-10 production. At the same time, PD increased CD4⁺ and CD8⁺ T-cell responses in peripheral blood and tumor tissues and restored the splenic Treg/Th17 balance. These findings suggest that the microbiota changes after PD treatment were closely related to improved anti-tumor immune and reduced inflammatory activation.

The inhibition of NF-κB/JAK2/STAT3 signaling in tumor tissues may further explain the

connection between microbiota-related inflammatory regulation and tumor growth suppression. Gut dysbiosis and microbial products can increase inflammatory cytokine production. These cytokines may then activate NF- κ B and IL-6/JAK/STAT3 signaling. In our study, PD reduced TNF- α and IL-17A expression and lowered the phosphorylation levels of NF- κ B p65, JAK2, and STAT3. These results suggest that PD may weaken the inflammatory loop linking dysbiotic microbiota, Th17-related cytokines, and tumor-promoting inflammatory signaling.

To further evaluate the gut microbiota's role in PD's antitumor effects, antibiotic pretreatment combined with fecal microbiota transplantation (FMT) was performed. FMT from PD-H-treated donors inhibited tumor growth and reduced NF- κ B, JAK2, and STAT3 levels in tumor tissues, even in recipient mice not directly treated with PD. These findings suggest that the gut microbiota modulated by PD may contribute to transferable antitumor and inflammation-regulating effects. FMT from PD-H-treated donors also increased CD8A and granzyme B expression while decreasing Foxp3 expression in tumor tissues. CD8A indicates the infiltration of CD8⁺ T cells into tumors, and granzyme B is a key effector molecule in cytotoxic lymphocyte-mediated killing of target cells [31]. Thus, increased CD8A and granzyme B expression may reflect enhanced local cytotoxic immune activity. Conversely, reduced Foxp3 expression may signify an attenuation of Foxp3⁺ regulatory T-cell-associated immunosuppression in the tumor microenvironment [12]. These immune changes, coupled with reduced NF- κ B, JAK2, and STAT3 levels and inhibited tumor growth, support the hypothesis that the gut microbiota, reshaped by PD, mitigates pro-tumor inflammatory signaling by modulating the tumor's local immune status. Notably, direct PD treatment increased the proportion of splenic CD4⁺CD25⁺FOXP3⁺ cells, whereas FMT from PD-H-treated donors reduced the overall Foxp3 protein level in tumor tissues. This difference may reflect distinct regulatory patterns between peripheral immune homeostasis and local immunosuppression within the tumor microenvironment. The increased proportion of splenic Treg cells might be associated with the alleviation of systemic inflammation, while reduced Foxp3 expression in tumor tissues could indicate weakened local Treg-related immuno-

suppressive signals. Additionally, the overall improvement in tumor growth observed with ABX+FMT-PD was weaker than that produced by direct PD-H treatment. This suggests that the gut microbiota may mediate only a portion of PD's antitumor effects and cannot fully account for all its actions. It remains possible that active components of PD are absorbed through the intestine and directly act on tumor tissues, immune cells, or other host tissues. Therefore, PD's antitumor effects likely involve both partial mediation by the gut microbiota and direct pharmacological actions, rather than a single mechanism.

This study has several limitations. First, the FMT experiment demonstrated that gut microbiota from PD-treated mice could partially transfer the tumor-inhibitory and immunomodulatory effects of PD. However, without an ABX+PD-H group, this study cannot directly determine whether PD's effects are significantly weakened after gut microbiota depletion or the extent to which its antitumor activity depends on the gut microbiota. Second, although *Akkermansia muciniphila* was enriched in the PD-H group, specific bacterial strains and microbiota-derived metabolites were not further validated. Therefore, a causal relationship linking *A. muciniphila*, the Treg/Th17 balance, and NF- κ B/JAK2/STAT3 signaling could not be established in the present study. Third, gut microbiota sequencing was performed only in the Control, Model, and PD-H groups. Thus, the current findings reflect only microbiota changes induced by high-dose PD and cannot determine whether the effects of low-, medium-, and high-dose PD on the gut microbiota are dose-dependent. Finally, PD is a compound prescription consisting of multiple medicinal herbs. Because active fractions were not separated and individual components were not validated, the specific active constituents and their relative contributions remain unclear. In addition, the concentrations of PD-derived constituents in plasma and tumor tissues were not measured, and pharmacokinetic or tissue-distribution analyses were not performed. Therefore, the direct systemic actions of absorbed PD components cannot be distinguished from microbiota-mediated effects. Future studies will combine microbiota depletion, validation of specific bacterial strains and metabolites, microbiota analysis across differ-

ent PD doses, and screening for active components, to further clarify the mechanisms underlying PD's effects.

In conclusion, PD inhibited tumor growth in mice with colorectal cancer, which was accompanied by improvements in gut microbiota composition and function, modulation of T-cell immune responses, and suppression of NF- κ B/JAK2/STAT3 signaling. The FMT experiment further demonstrated that the gut microbiota from PD-treated mice could partially transfer the tumor-inhibitory and immunomodulatory effects to recipient mice that did not directly receive PD, suggesting that the gut microbiota contributes to the effects of PD. *Akkermansia muciniphila* may be a candidate bacterial species associated with PD treatment. However, its specific role and the causal relationships among *A. muciniphila*, the Treg/Th17 balance, and inflammatory signaling pathways require further validation. Overall, these findings support the involvement of the gut microbiota in PD's anti-colorectal cancer effects, although direct pharmacological actions of PD cannot be excluded.

Acknowledgements

This study was supported by Tianshan Talents Program: High-Level Talent Development Project in Medicine and Healthcare (No. TSYC-202301B080) and Annual Special Project of the In-Hospital Research Program, Xinjiang Uygur Autonomous Region Hospital of Traditional Chinese Medicine (No. ZYYNDZX202502).

Disclosure of conflict of interest

None.

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