Am J Transl Res 2021;13(1):38-56
www.ajtr.org /ISSN:1943-8141/AJTR0114144

Original Article

The effect of menthol supplement diet on colitis-induced colon tumorigenesis and intestinal microbiota

Lei Luo[1,2], Jing Yan[1], Bingyu Chen[1,2], Yi Luo[3], Lina Liu[1,4], Zhiguang Sun[1], Yin Lu[5,6]

[1]Institute of First Clinical Medicine, Nanjing University of Chinese Medicine, Nanjing 210023, Jiangsu, China; [2]Department of Gastroenterology, Jiangsu Provincial Second Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Chinese Medicine, Nanjing 210017, Jiangsu, China; [3]Department of Oncology, Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing 210028, Jiangsu, China; [4]Department of Liver Disease, Jiangsu Provincial Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Chinese Medicine, Nanjing 210029, Jiangsu, China; [5]Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210029, Jiangsu, China; [6]Jiangsu Collaborative Innovation Center of Traditional Chinese Medicine (TCM) Prevention and Treatment of Tumor, Nanjing University of Chinese Medicine, Nanjing 210029, Jiangsu, China

Received May 11, 2020; Accepted December 1, 2020; Epub January 15, 2021; Published January 30, 2021

Abstract: Natural phytochemicals are extensively considered to potentially ameliorate or reverse the pathological progression of colitis-associated colon cancer (CAC). The present study aimed to evaluate the therapeutic effect of menthol on CAC and the promoting effect on the gut microbiome and metabolites. In this study, azoxymethane (AOM) combined with dextran sulfate sodium (DSS) was adopted to build CAC mouse models. H&E staining was performed to identify the pathological damage of colon tissue. By immunohistochemistry and immunofluorescence, the expression levels of β-catenin and Ki67 were measured. The mRNA expression of inflammatory cytokines and myeloperoxidase (MPO) was evaluated through RT-PCR. The infiltration of immune cells was measured by flow cytometry analysis. With 16SrDNA sequencing technology, the composition of gut microbiome were detected. To determine the concentration of short-chain fatty acids (SCFAs) in the feces, gas chromatography coupled to mass spectrometry (GC-MS) was performed. A significant inhibiting effect of menthol on AOM/DSS-induced tumorigenesis was observed, as indicated by the significantly fewer small adenomas, lower disease activity index (DAI) scores and histopathological scores, lower expression of proliferation biomarkers (β-catenin and Ki67) and pro-inflammatory cytokines (IL-6, TNF-α and MPO), and decreased immune cells infiltration. As suggested from the results of 16SrDNA sequencing, compared with AOM/DSS (AD) group, MSD exhibited higher α-diversity and shared more similar β-diversity with the control (Ctrl). Moreover, a higher abundance of butyrate-producing bacteria (Allobaculum, Roseburia and Intestinimonas) and the higher fecal butyrate concentrations were measured in the MSD compared with the AD group. MSD effectively ameliorated AOM/DSS-induced tumorigenesis and facilitated the predominant growth of butyrate-producing bacteria.

Keywords: Menthol supplement diet, colitis-associated colon cancer, gut microbiome, butyrate

Introduction

Accumulated evidences indicate that CAC is an important lethal complication of inflammatory bowel disease (IBD), accounting for approximately 15% of IBD deaths [1]. Severity of intestinal inflammation, extent of lesions, and duration of symptoms show positive correlations with the pathogenesis of CAC [2, 3]. Moreover, anti-inflammatory therapies have been suggested to effectively relieve or prevent the conversion of IBD to CAC [4, 5]. Medicinal plants, especially plant components and phytochemicals exhibiting anti-inflammatory biological activity, have been extensively considered novel therapeutic agents to restrict the pathogenesis of IBD and reduce the incidence of adverse events. Furthermore, over the past few years, intestinal microbiota has aroused broad attention to reveal the crucial role and mechanism of

intestinal inflammation in the initiation and progression of colon adenoma. Various probiotics or natural medicinal plants and chemical components with prebiotic properties, (e.g., *Bacteroides fragilis* [6], American Ginseng [7] and Apple Polysaccharide [8]), have suggested a significant inhibiting effect on AOM/DSS-induced colon tumorigenesis by modifying the gut microbiome.

Menthol, one of the major ingredients that leading to the cooling effect or sensation of mint plants, has extensively used as an additive in a range of pharmaceuticals, cosmetics, confectionery and toothpastes [9]. Moreover, anti-inflammatory, antibacterial, antifungal, antiviral, antiallergic, antipruritic, analgesic properties and other biological properties of menthol have been supported by various clinical and experimental evidences [9, 10]. Over the past few years, menthol has been reported to effectively improve the macroscopic and microscopic lesions of experimental colitis rat model [11, 12]. Besides, it has been generally evidenced that diets rich in menthol significantly have an effect on the gastrointestinal and ruminal microbiota of piglets and sheep [13, 14]. However, after searching the literature, rare convincing evidence has been found regarding the therapeutic effect of menthol on AOM/DSS-induced CAC and its effect on intestinal microbes and metabolites.

In this study, the effect of menthol against AOM/DSS-induced CAC was assessed. The results of this study revealed that MSD significantly reduced the number of small adenoma, DAI scores, expression of proliferation biomarkers (β-catenin and Ki67), pro-inflammatory cytokine (IL-6, TNF-α and MPO) and infiltration of immune cells (leukocytes, neutrophils and MDSCs) in spleens. Furthermore, menthol was observed to significantly facilitate the expansion of butyrate-producing bacteria and increase the concentration of butyrate in the feces, which might be one of mechanisms of menthol inhibiting AOM/DSS-induced tumorigenesis.

Materials and methods

Animal and experimental design

Overall, 60 male C57BL/6 mice aged 4 weeks and weighing about 20 g were provided by Nanjing Institute of Biomedicine of Nanjing University. All animals were maintained in specific pathogen free facility with 12:12 h light-dark cycle, 23°C ± 2°C and 55% ± 5% humidity. The mentioned mice were fed adaptively for a week before being intervened to ensure the comparability of baseline characteristics of intestinal microbiota in each group. Besides, these mice were fed and administered by specialized technicians to eliminate the effect of other factors on intestinal microbes. The mice were allowed to access food and water *ad libitum* and randomly split into 5 groups, i.e., Control (Ctrl) group, AOM/DSS (AD) group, MSD low dose (AD/Men1%) group, MSD medium dose (AD/Men1.5%) group and MSD high dose (AD/Men2%) group. The variations in body weight and food intake were weighed and recorded twice a week, while the diet, drinking water and litter were changed. The methods abided by the Institutional Animal Care and Usage Committee of the National Institutes of Health approved by the Research Ethical Committee of Nanjing University of Chinese Medicine.

Preparation of normal diet and MSD

Both the normal diet and the MSD were formulated and offered by Jiangsu Synergy Pharmaceutical Bioengineering Company. To be specific, the normal diet fat supply reached 12%, the carbohydrate supply reached 67.4%, and the protein supply reached 20.6%. Low (1%), medium (1.5%) and high (2%) doses MSD respectively represented 1 g, 1.5 g and 2 g of menthol (Sigma-Aldrich) per 100 g of normal diet. All diets should be rigorously mixed in the preparation to ensure that the various ingredients were evenly distributed and then undergo the ultraviolet sterilization to avoid the impact of exogenous bacteria on the intestinal flora of mice.

CAC mouse model

As indicated from one existing study, a mouse model of CAC was constructed by combining AOM and DSS, an extensively used mouse model in the field of colon "inflammatory cancer transformation", for its excellent repeatability and simplicity [15]. **Figure 1** suggests that mice were injected with AOM (10 mg/kg, Sigma-Aldrich) intraperitoneally diluted in isotonic saline solution, then a normal diet or MSD

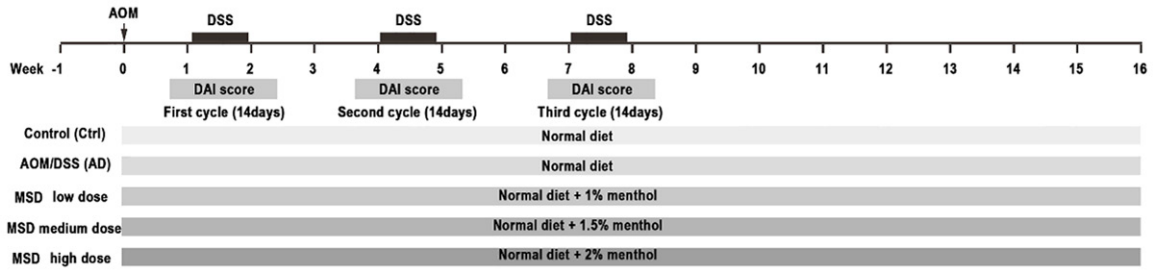


Figure 1. Experimental scheme for construction of the mouse model of CAC. First, AOM (10 mg/kg) was injected intraperitoneally at week 0, followed by 3 cycles of 2% DSS drinking water (1 week/cycle) in the 2, 5, and 8 weeks. All mice randomly fell to 5 groups, i.e., Control (Ctrl) group, AOM/DSS (AD) group, MSD low dose (AD/Men1%) group, MSD medium dose (AD/Men1.5%) group and MSD high dose (AD/Men2%) group.

was maintained for 7 days. Afterwards, these mice received immediate 3 cycles (7 days per cycle) of drinking water supplemented by 2% DSS (Mp Biomedicals, USA), each cycle was separated by 2 weeks of normal drinking water (without DSS). Throughout the experiment, all mice maintained a normal diet or MSD. After 16 weeks, the mice were euthanized, and then organs and tissues were collected and stored as described below for subsequent analyses.

Tissue collection and storage

After 16 weeks, the mice in each group were sacrificed by neck removal. Next, the liver, spleen, colon and intestinal contents were collected, weighed and then photographed. In brief, the colon tissue received a careful longitudinal dissection and the intestinal contents were collected in 1.5 ml sterile centrifuge tubes and immediately snap-frozen in liquid nitrogen, then they were stored in -80°C for intestinal flora analyses. Moreover, the colon tissue was washed with PBS buffer and then flattened. Next, under a dissecting microscope, the number and size of colon polyps were observed. Finally, colon tissue was fixed in 10% formalin buffer for 24 hours and then embedded in paraffin for H&E staining, immunohistochemistry and immunofluorescence.

Detection of DAI

The previous scoring scheme [16, 17] used to detect intestinal DAI was adopted and revised in the present study. Briefly, the DAI scores complied with the body weight loss, stool consistency and bleeding in the feces. Since existing studies suggested that menthol exerts a significant weight-loss effect, which may adversely affect the inhibiting effect of menthol on AOM/DSS-induced tumorigenesis. Therefore, in the scoring scheme of this study, the weight loss entry in the DAI scoring rules was canceled. To be specific, the scoring method of stool consistency was score 0 (normally formed stool), score 1 (stool loose with some stool particles), score 2 (watery stool). Rectal bleeding was detected with the fecal occult blood kit (o-toluidine method), and the specific scoring rules were score 0 (negative, no significant bleeding), score 1 (positive occult blood), score 2 (positive occult blood with a small amount of visible bleeding) and score 3 (positive occult blood with a large amount of visible bleeding).

H&E staining

According to the previous perfect pathological tissue staining protocol [18], the colon tissue were sliced into 5 μm thickness with a paraffin slicer, stained with H&E and then observed under a light microscopy. Subsequently, the pathological and morphological variations of the mentioned tissues were evaluated by professionals (not related to the experiment) and scored by complying with the following criteria: ① Destruction of the crypt structure: labeled 0-3 score according to the damage degree of the crypt; ② Infiltration of inflammatory cells: labeled 0-3 score according to the degree of infiltration of inflammatory cells in colon tissue; ③ Dysplasia of intestinal wall: labeled 0-3 score in accordance with the degree of dysplasia.

Immunohistochemistry

The tissue slices with a thickness of 5 μm were first dried in a 60°C incubator, and then dewaxed and rehydrated in serial xylene solu-

Table 1. The sequences of primers used in this experiment

Gene names	sequences of primers
GAPDH	5'-GGTTGTCTCCTGCGACTTCA-3'
	5'-TGGTCCAGGGTTTCTTACTCC-3'
IL6	5'-CCGGAGAGGAGACTTCAC-3'
	5'-TCCACGATTTCCCAGAGA-3'
IL10	5'-GCTCTTACTGACTGGCATGAG-3'
	5'-CGCAGCTCTAGGAGCATGT-3'
TNF-α	5'-TAGCCAGGAGGGAGAACAGA-3'
	5'-TTTTCTGGAGGGAGATGTGG-3'
MPO	5'-AGCTGCAGTACGTCAAGCAGGA-3'
	5'-TTTGCAAACTGTATTTCGCTCTGG-3'

tions and gradient ethanol solution. Subsequently, the slices were immersed in 0.1 mol/l sodium citrate antigen retrieval solution (PH6.0) and placed in a microwave oven and heated for 10-15 minutes for antigen retrieval. 3% H_2O_2 was added dropwise to the slices for 5-10 minutes to eliminate endogenous peroxidase activity. 5% BSA was added dropwise to the tissue, then the slices were incubated in the humidity box at 37°C for 60-120 minutes. Anti-β-catenin antibody (BD Biosciences) was added to the sliced tissue in the humidity box at 4°C overnight. Next, the slices were incubated with biotin-labeled secondary antibody in a humidity box at 37°C for 30-60 minutes. According to the instructions of DAB horseradish peroxidase color kit, HRP-labeled avidin was then applied to the slices for 15-30 minutes at 37°C. Afterwards, the developer developed the color and counterstained with a drop of hematoxylin about 10 seconds. Finally, the sections were observed under a light microscopy, and the positive expression range was semi-quantitatively analyzed with Image-Pro Plus6 software.

Immunofluorescence

Tissue section, dewaxing, rehydration and antigen repair followed the same operation procedures as immunohistochemistry. Next, tissue sections were incubated with primary antibody (Ki67, Cell Signaling) in a humidified box at 4°C overnight. Subsequently, the sections were incubated with the 1:500 diluted fluorescent secondary antibody Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) at ambient temperature in the dark for 2-3 hours. Then, DAPI was added to the sections at ambient temperature for 10 minutes. Next, the sections were covered with anti-quenching agent and glycerin. Finally, the sections were observed and photographed under a fluorescence microscopy within 1 week.

Real-time quantitative polymerase chain reaction (RT-PCR)

SYBR green-based RT-PCR was adopted to detect mRNA expression in the colon tissue. Briefly, following the operating instructions, total RNA was extracted with TRIzol agent (Thermo Fisher Scientific, USA). Besides, SYBR Green Master kit (Bio-Rad, USA) was applied for RT-PCR analysis. **Table 1** lists the primer sequences employed in this study. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression acted as the internal control.

Flow cytometry analysis of immune cells infiltration in spleens

After the animals were euthanized, the spleens were isolated on the ultra-clean platform. The spleens were grinded and filtered via a 200-mesh screen and preparation of a single cell suspension. The red blood cells were lysed with red blood cell lysate and then centrifuged at 1000 rpm for 5 minutes. The collected spleen cell suspensions underwent the staining with FITC-conjugated anti-mouse CD45 antibody, PE-conjugated anti-mouse CD11b antibody and PerCP-conjugated anti-mouse Ly-6G/Ly-6C (Gr-1) antibody (BioLegend), respectively. Afterwards, the samples were analyzed with BD Accuri C6 and the data were processed with FlowJo Version 7.6 software.

Fecal genomic DNA extraction and microbial analysis

The fecal genomic DNA was extracted from the frozen solid feces according to the operating instructions of the fecal genomic DNA extraction kit (DP328). The Illumina Hiseq 2500 platform was adopted to sequence and analyze the v4-v5 region of the 16SrRNA gene of microorganisms in feces. In brief, the valid sequence of all samples was first obtained according to barcode, and then the quality control filtering was performed on the quality of the reads and pairs of reads were merged into a sequence based on the overlapping relationship between PEreads. Finally, the high-quality

sequence of each sample was obtained by complying with the barcode and primer sequence, the sequence direction was corrected, and the chimera was removed according to the positive and negative barcode and primer directions in the process. Usearch software was adopted to de-chimerize and cluster the data. The 97% similarity criterion was used to cluster to obtain OTU (Operational Taxonomic Units), considered to represent a species. The dilution curve of the alpha diversity index was plotted by Qiime software, and the reasonable leveling parameters were selected according to the dilution curve. To analyze the obtained flattened OUT, the qiime software was employed. First, a read was extracted from the OTU as a representative sequence, and the sortmerna method was used to compare the representative sequence with the 16S database for the classification of each OTU. LDA EffectSize (LEfSe) analysis was conducted to calculate the magnitude of the difference between groups by linear discriminant analysis (LDA) (Kruskal-Wallis test was performed to evaluate the statistical significance and the screening criteria were *p*-value < 0.05 and LDA score > 3).

SCFAs detection

GC-MS was used to measure the concentration of SCFAs in feces. Briefly, nearly 100 mg feces were dissolved in 1 ml of NaOH solution (0.005 mol/l) and then centrifuged at 13000 rpm for 10 min. Subsequently, 500 μl supernatant were transferred to a glass tube, and 10 μl of internal standard (d3-hexanoic acid, 500 μg/ml) was added to the supernatant. Next, a mixed solution of each standard was prepared and diluted in a gradient, followed by the addition of 10 μl of internal standard (d3-hexanoic acid, 500 μg/ml) to each tube. Afterwards, 500 μl 1-propanol/pyridine (3:2) reagent and 100 μl n-propyl chloroformate were introduced for derivatization. Finally, the concentration of SCFAs was measured by GC-MS (Thermo).

Statistical analysis

All data are presented in the form of mean ± standard deviation. Log-rank (Mantel-Cox) was adopted to conduct the survival analysis. The statistical comparison between three or more groups was analyzed by the one-way analysis of variance (ANOVA). Besides, all statistical analysis and graph drawing were conducted with GraphPad Prism 8 or R statistical software. $P < 0.05$ was considered a statistically significant difference between groups (*P < 0.05, **P < 0.01, ***P < 0.001).

Results

MSD increases the weight loss but has no significant effect on the survival rate of AD mice

In this study, the effect of MSD on the survival rate, body weight and food consumption of AD mice was continuously observed in 8 weeks after AOM injection. As schematized in **Figure 2A**, no statistically significant difference in survival analysis was identified between MSD and AD groups (P = 0.4795). Similar to existing studies, AD mice exhibited a significantly lower body weight compared with the Ctrl group. Moreover, MSD further increased the weight loss of AD mice (**Figure 2B**). Furthermore, no significant correlation was suggested between weight loss and food consumption since no significant difference was observed in food consumption between groups (**Figure 2C**).

MSD significantly increases colon length and reduce liver and spleen weights of AD mice

After 16 weeks, all mice were euthanized, and their colon, liver and spleen were weighed, collected and stored. Compared with the AD group, MSD significantly increased the colon length (**Figure 3A**, **3B**), decreased the liver and spleen weights (**Figure 3C**, **3D**). It has been evidenced that the colon length shows a negative correlation with the intensity of inflammation in the intestine [19], while the liver and spleen weights are positively correlated with systemic inflammation [20]. Accordingly, this study reasonably concluded that the significantly prolonged colon length and decreased liver and spleen weights in the MSD group may be achieved due to the excellent anti-inflammatory effect of menthol.

MSD significantly inhibits the intestinal inflammatory activity and reduces the number of small adenoma of AD mice

The stool consistency and fecal bleeding of mice were continuously observed during the three DSS cycles. Since menthol could significantly induce weight loss [21], which is slightly

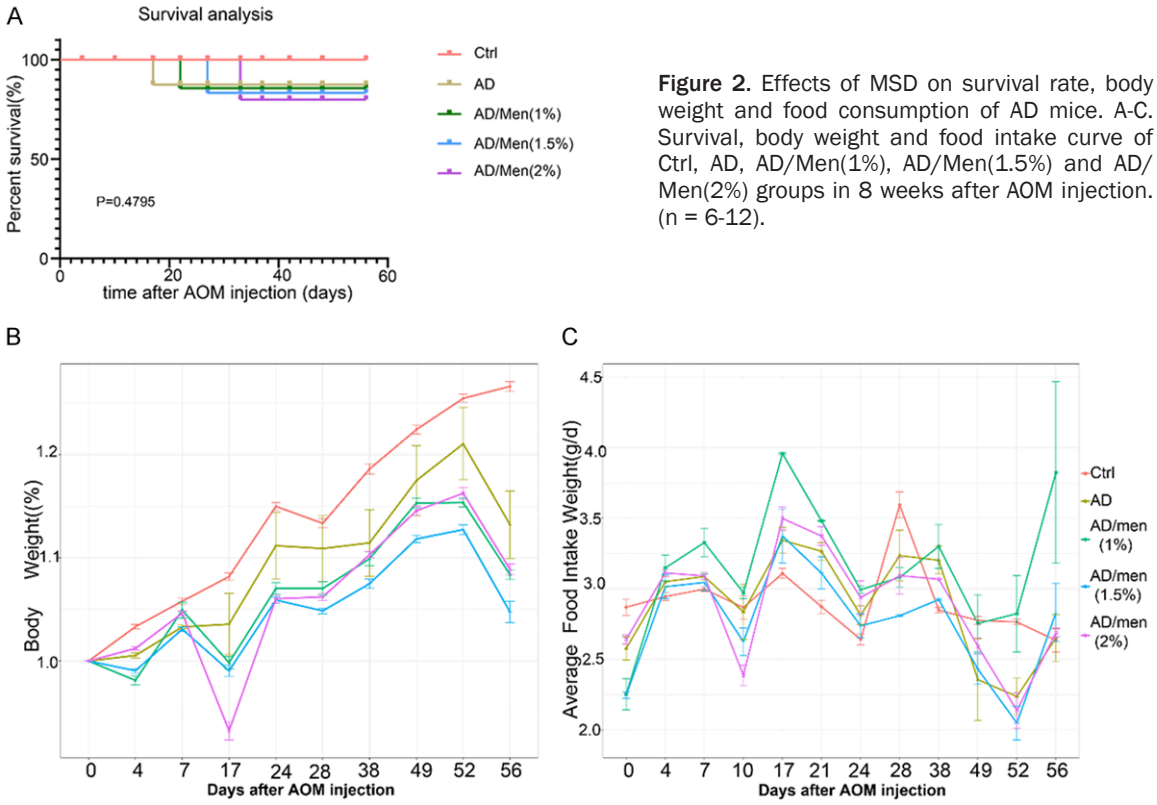


Figure 2. Effects of MSD on survival rate, body weight and food consumption of AD mice. A-C. Survival, body weight and food intake curve of Ctrl, AD, AD/Men(1%), AD/Men(1.5%) and AD/Men(2%) groups in 8 weeks after AOM injection. (n = 6-12).

related to the deterioration of the disease, a revised DAI scoring strategy without considering weight loss was adopted. As expected, AD mice achieved a higher DAI scores than the Ctrl group, demonstrating a large scale inflammatory response in the intestine of AD mice. More importantly, during the three DSS cycles, significantly lower DAI scores were identified in MSD mice, compared with AD mice (**Figure 4A-C**). After 16 weeks, according to the size of adenoma (1-3 mm, 3-5 mm and > 5 mm), the number of adenomas in each group was counted. It is noteworthy that in the adenomas category of 1-3 mm, MSD was observed to significantly reduce the number of small adenomas compared with AD mice. However, in the adenomas category of 3-5 mm and > 5 mm, no significant difference was identified in the incidence of adenomas between the MSD and AD (**Figure 4D**, **4E**).

MSD significantly alleviates the pathological damage and reduces the expression of proliferative biomarkers of AD mice

To further confirm the inhibiting effect of MSD on AOM/DSS-induced microscopic pathological destruction of colon tissue, the pathological H&E staining was performed, and the expressions of proliferative proteins (β-catenin and Ki67) were detected. Compared with AD mice, MSD significantly alleviated the intestinal pathological damage, as embodied by a relatively more complete crypt structure, longer villi and fewer inflammatory cells infiltration and lower pathology scores (**Figure 5A**, **5B**). Furthermore, a significantly lower expression of β-catenin and Ki67 protein in the colon tissue of MSD was observed, compared with AD (**Figure 5C-E**). Overall, the mentioned results suggested that menthol had the potential to inhibit AOM/DSS-induced colon tumorigenesis for its significant anti-proliferative effect.

MSD significantly regulates the gene expression of inflammatory cytokines and myeloperoxidase (MPO) in colon tissue

The gene expression levels of inflammatory cytokines (IL-6, TNF-α, IL-10) and neutrophil biomarkers (MPO) in the terminal colon tissue were determined to verify the inhibiting effect of MSD on intestinal inflammation and oxidative stress at the micro-molecular level. Complying with the pathological staining results, significantly lower expression of pro-inflammatory cytokines (IL-6 and TNF-α) and relatively higher expression of anti-inflammato-

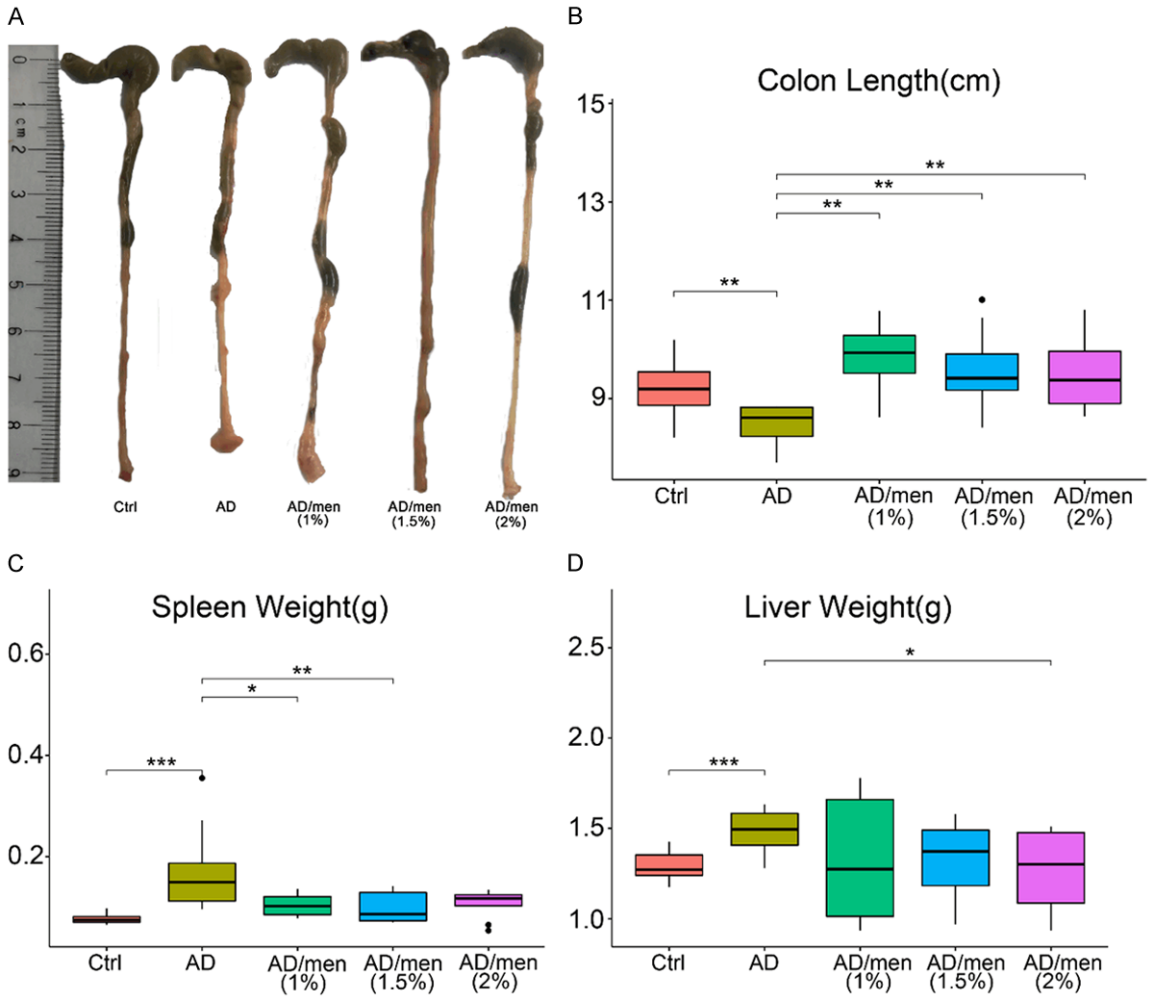


Figure 3. Effects of MSD on the colon length, the liver and spleen weights of AD mice. A. Representative photograph of colons. B-D. Colon length, liver and spleen weights of the Ctrl, AD, AD/Men(1%), AD/Men(1.5%) and AD/Men(2%) groups. (n = 6-12).

ry cytokines (IL-10) were detected in MSD mice, compared with AD mice (**Figure 6A-C**). Higher MPO levels mean more severe tissue destruction and inflammation, as well as neutrophil activation and infiltration [22]. Fortunately, MSD was observed to significantly suppress the expression of MPO in colon tissue in a dose-dependent manner (**Figure 6D**). In summary, the mentioned findings demonstrated that the significant intestinal protective effect of MSD may be associated with its regulation of inflammatory cytokines and MPO gene expression.

MSD significantly reduces the infiltration of immune cells in the spleens of AD mice

The infiltration of immune cells in the spleens was examined to demonstrate the effect of MSD on the alleviation of AOM/DSS-induced colon tumorigenesis. As revealed from the results of the flow cytometry analysis, MSD dose-dependently reduced the infiltration of leukocytes ($CD45^+$), neutrophils ($CD45^+Gr1^+$), and MDSCs ($CD11b^+Gr1^+$) in the spleens of AD mice (**Figure 7A-F**). These results suggested that menthol could exert its biological effects in the process of anti-inflammatory and anti-tumor by regulating the relevant components of the immune system.

MSD significantly improves the structural characteristics of intestinal flora in AD mice

It has been increasingly reported that there is a significant intestinal microflora disorder (reduction of probiotics and proliferation of pathogenic bacteria) in patients with intestinal

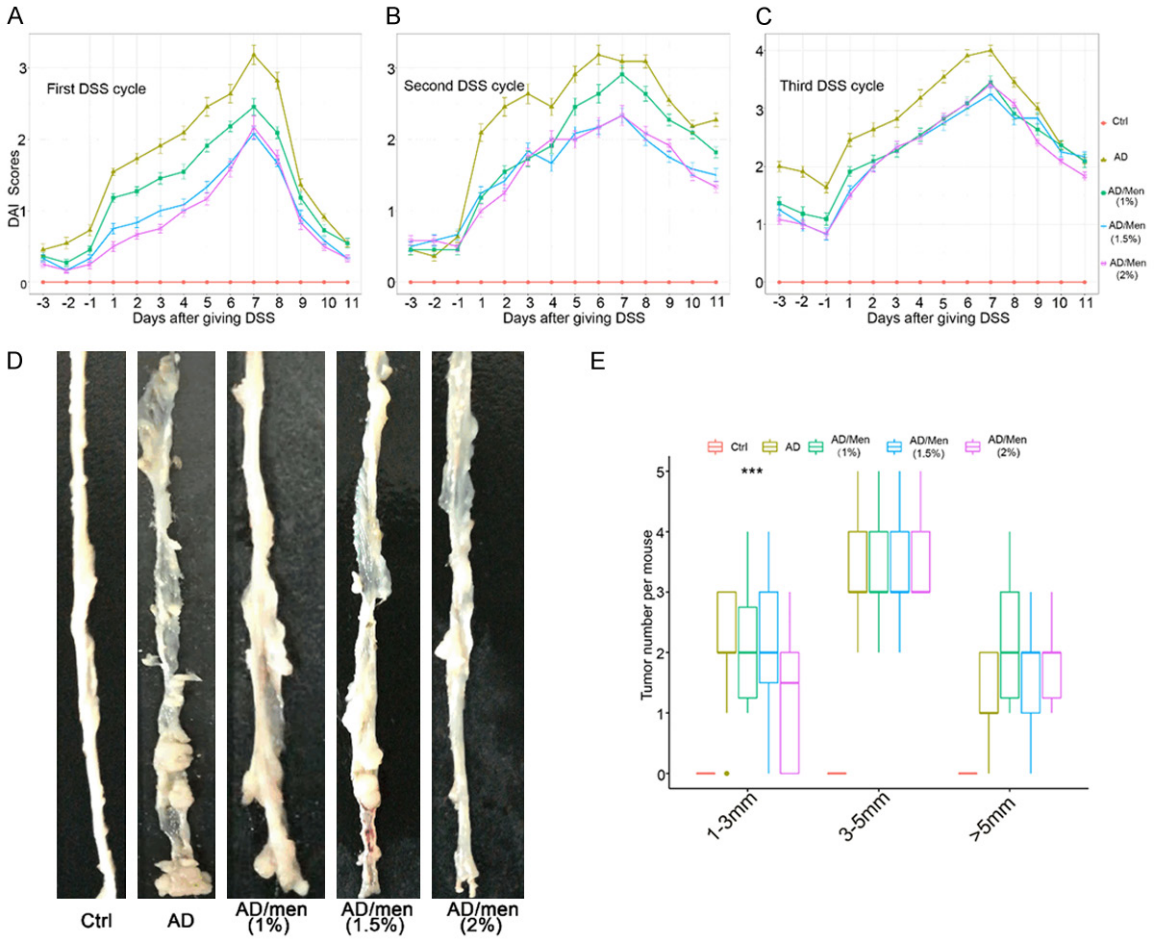


Figure 4. Effects of MSD on the DAI scores and the number of adenomas with different diameters. A-C. DAI scores of mice in Ctrl, AD, AD/Men(1%), AD/Men(1.5%) and AD/Men(2%) groups during three DSS cycles. D, E. Representative photograph and the adenomas number with a diameter of 1-3 mm, 3-5 mm and > 5 mm. (n = 6-12).

inflammatory diseases [23]. At present, regulation of intestinal microbes has become a significantly promising intervention in the treatment of intestinal inflammation-related diseases [24]. Thus, the effect of MSD on the intestinal flora of AD mice was investigated in depth in this study to elucidate the mechanism of menthol inhibiting AOM/DSS-induced colon tumorigenesis. Consistent with existing findings [25], AD mice were observed to exhibit significantly lower species α-diversity (chao1, shannon and simpson index) compared with the Ctrl group. It is noteworthy that MSD (men group) achieved a significantly improved microbial community α-diversity (shannon and simpson index) compared with the AD (**Figure 8A-C**). In addition, the differences of species communities between samples through β-diversity analysis were further compared. **Figure 8D**, **8E** show a clear separation of the microbial community between the groups as observed by the principal coordinates analysis. The distance distribution of the samples in MSD and Ctrl group was closer according to the Weight UniFrac analysis (**Figure 8E**), demonstrating that they have a more similar species community. Moreover, compared with AD, the intestinal flora of MSD was observed to exhibit significantly higher abundance of Allobaculum, which is generally considered an important strain for the synthesis and secretion of SCFAs [26, 27] (**Figure 8F**, **8G**). To find out the significantly different communities or species, LEfSe analysis was conducted. This analysis showed that the abundance of Deferribacteres (phylum level) and Prevotellaceae UCG_001, Mucispirillum, Roseburia, Intestinimonas, Thauera (genus level) were significantly higher in the MSD (**Figure 8H**, **8I**). To be specific, Roseburia and Intestinimonas

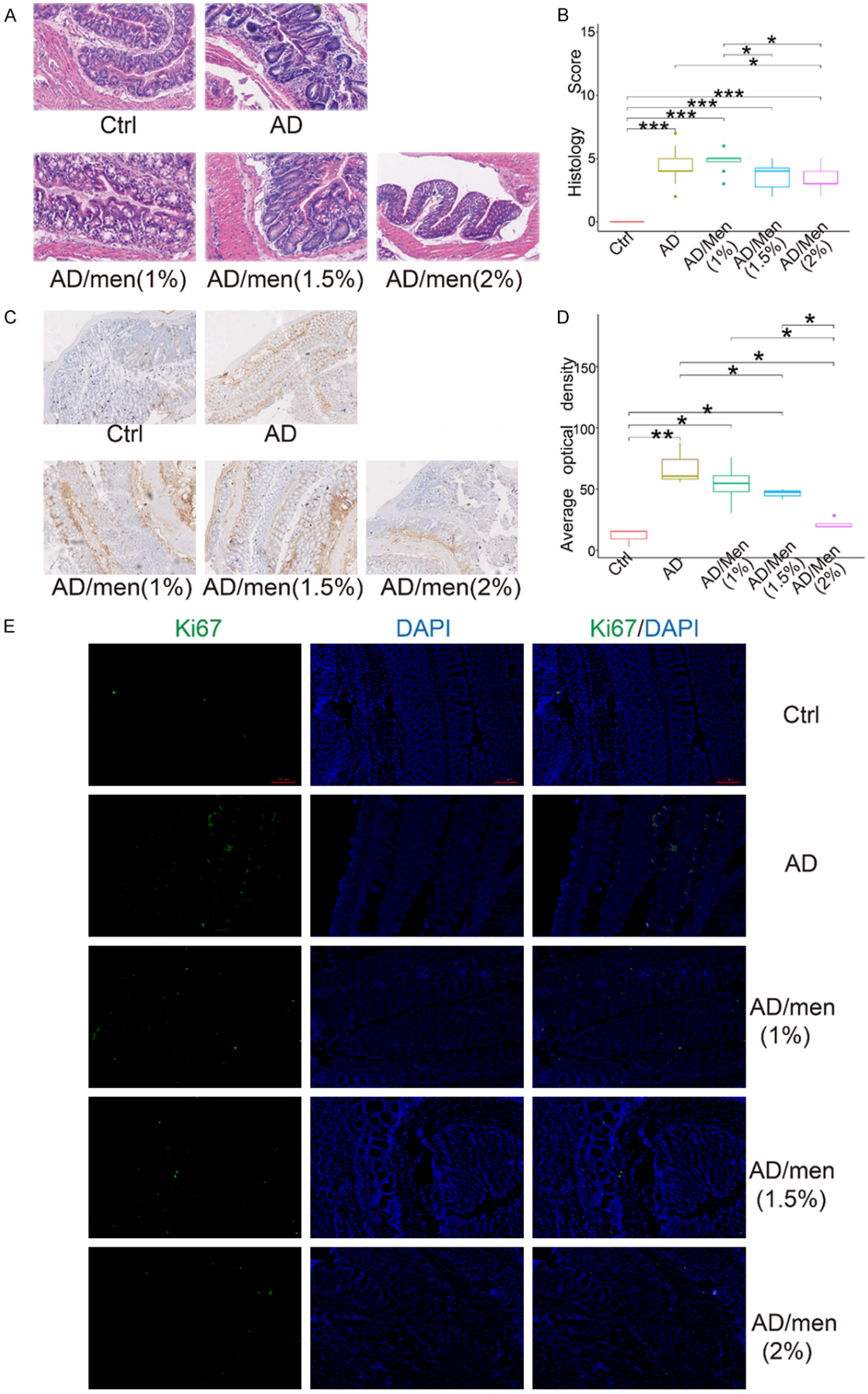

A
Ctrl
AD
AD/men(1%)
AD/men(1.5%)
AD/men(2%)
B
Score
Histology
Ctrl
AD
AD/Men (1%)
AD/Men (1.5%)
AD/Men (2%)
C
Ctrl
AD
AD/men(1%)
AD/men(1.5%)
AD/men(2%)
D
Average optical density
Ctrl
AD
AD/Men (1%)
AD/Men (1.5%)
AD/Men (2%)
E
Ki67
DAPI
Ki67/DAPI
Ctrl
AD
AD/men (1%)
AD/men (1.5%)
AD/men (2%)

Figure 5. Effects of MSD on the pathological damage and the expression of β-catenin and Ki67 of colon tissue. A, B. H&E staining (original magnification ×40) and pathological scores. C, D. Epithelial cell proliferation was analyzed by immunohistochemical staining of β-catenin and quantification of immunohistochemical images. E. Representative immunofluorescence images. (n = 4-6).

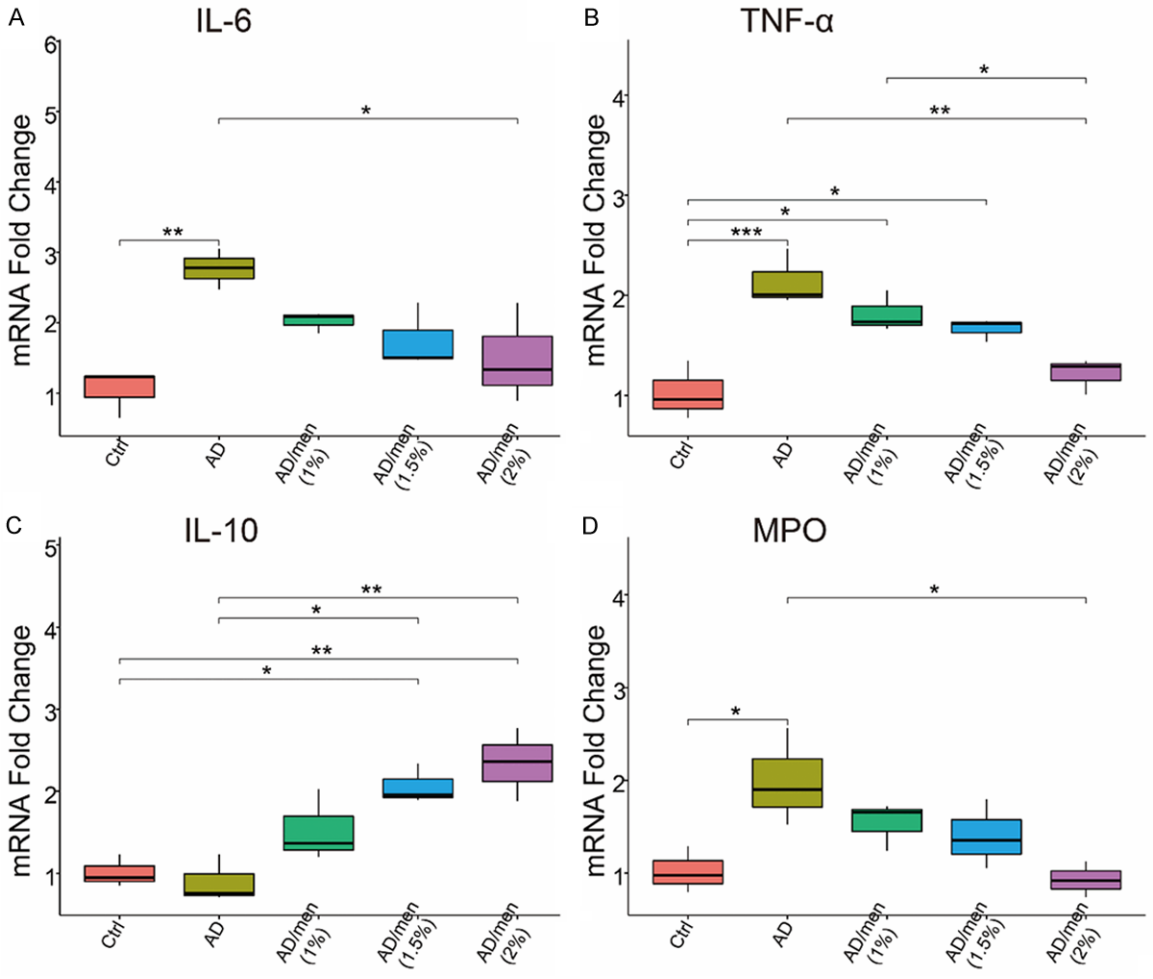
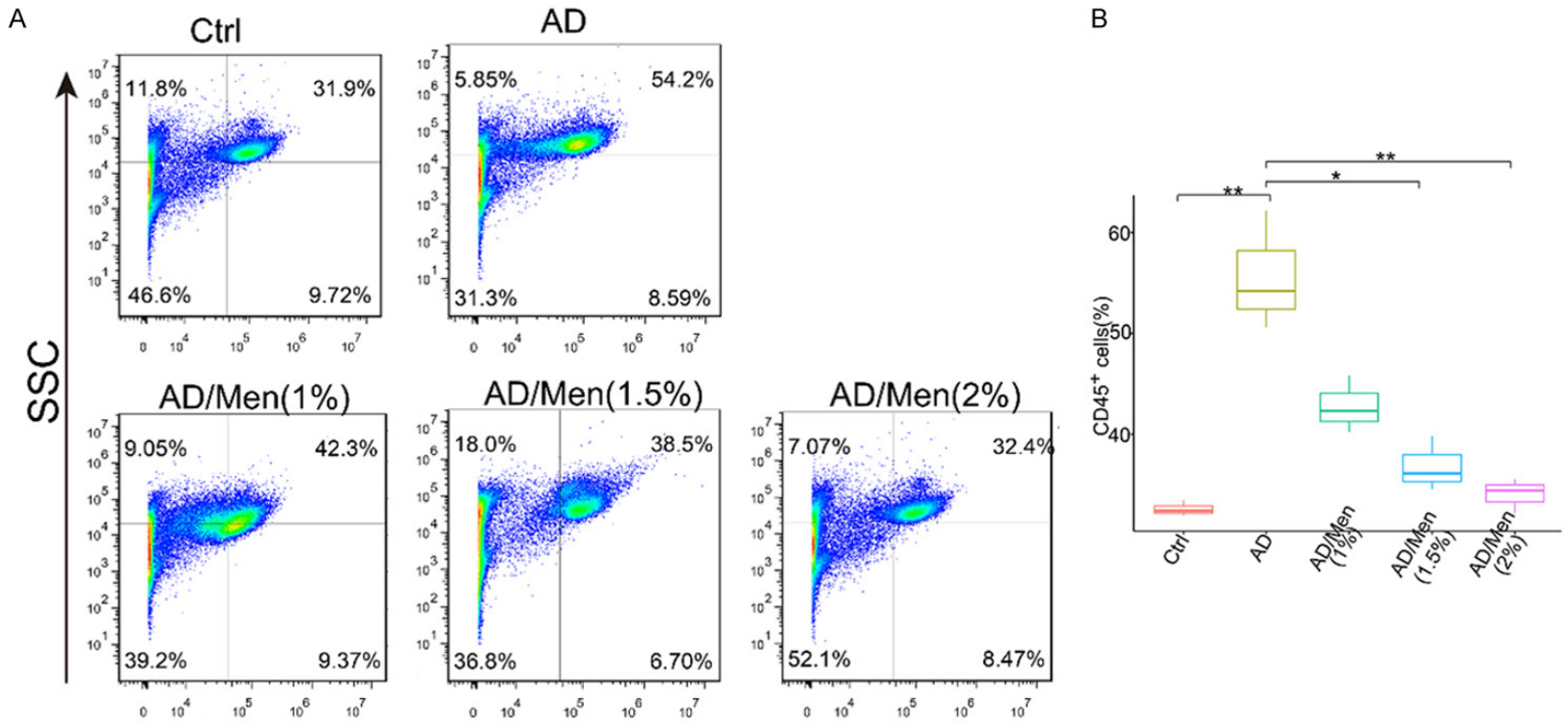


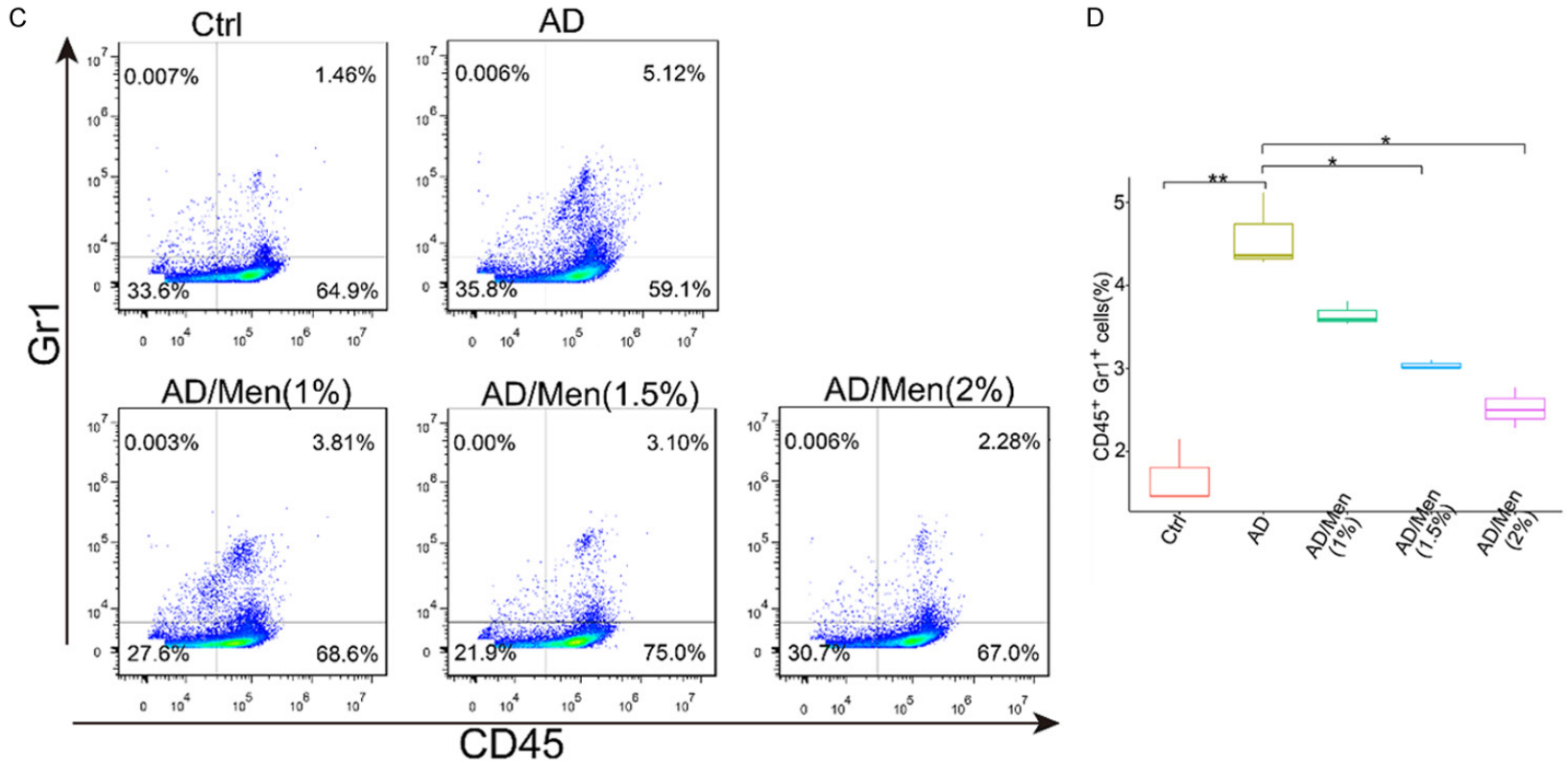
Figure 6. Effects of MSD on the gene expression of inflammatory cytokines and MPO. A-D. The mRNA expression of IL-6, TNF-α, IL-10 and MPO in distal colon tissue determined by real-time qPCR. (n = 3-5).

were considered by numerous studies to significantly promote the production of butyrate by metabolism and fermentation of indigestible carbohydrates (dietary fiber) in the intestine [28, 29]. Based on the mentioned results, this study reasonably speculates that the mechanism of MSD alleviating AOM/DSS-induced colon tumorigenesis may be associated with expansion of butyrate-producing bacteria.

MSD significantly increases the concentration of butyrate in the feces of AD mice

As a key derivative of intestinal microbes, SCFAs are gradually considered to be critical to maintain the integrity of intestinal epithelial structure and intestinal immune homeostasis [30, 31]. To confirm the effect of MSD on the SCFAs in the feces of AD mice, GC-MS was conducted. Consistent with the above mentioned, we found that compared with the AD, the concentrations of various SCFAs (e.g., butyrate, isobutyrate, isovalerate, propionate, valeric acid and acetate) in feces of MSD mice increased, of which butyrate, propionate and acetate were relatively more significant (**Figure 9A-F**). These results further confirmed the hypothesis here that menthol inhibited AOM/DSS-induced colon tumorigenesis is in


A
Ctrl
AD
AD/Men(1%)
AD/Men(1.5%)
AD/Men(2%)
SSC
11.8%
31.9%
46.6%
9.72%
5.85%
54.2%
31.3%
8.59%
9.05%
42.3%
39.2%
9.37%
18.0%
38.5%
36.8%
6.70%
7.07%
32.4%
52.1%
8.47%
B
CD45+ cells(%)
60
50
40
**
*
**
Ctrl
AD
AD/Men (1%)
AD/Men (1.5%)
AD/Men (2%)


C
Ctrl
AD
AD/Men(1%)
AD/Men(1.5%)
AD/Men(2%)
0.007%
1.46%
33.6%
64.9%
0.006%
5.12%
35.8%
59.1%
0.003%
3.81%
27.6%
68.6%
0.00%
3.10%
21.9%
75.0%
0.006%
2.28%
30.7%
67.0%
Gr1
CD45
D
CD45+ Gr1+ cells(%)
Ctrl
AD
AD/Men (1%)
AD/Men (1.5%)
AD/Men (2%)

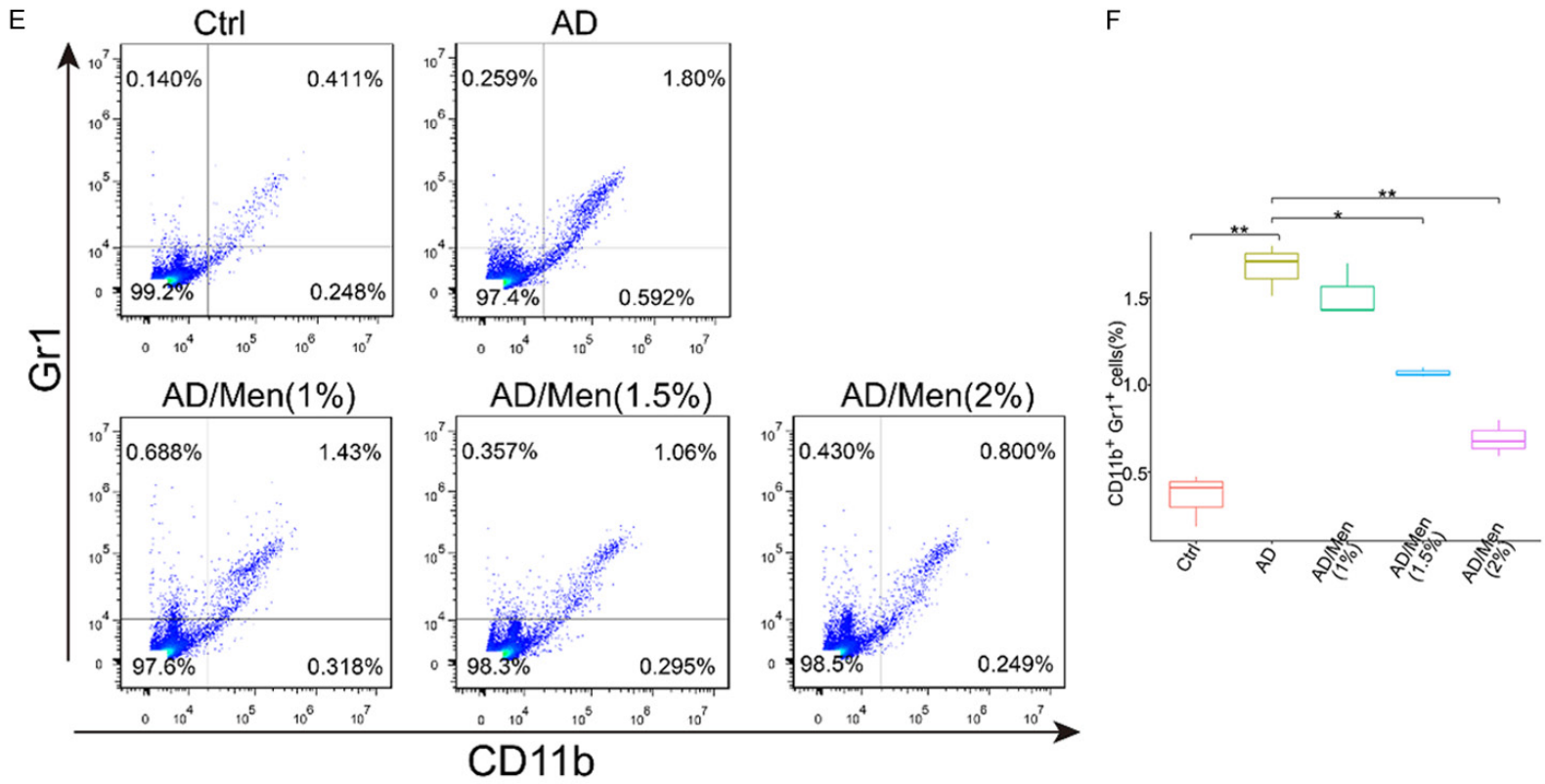
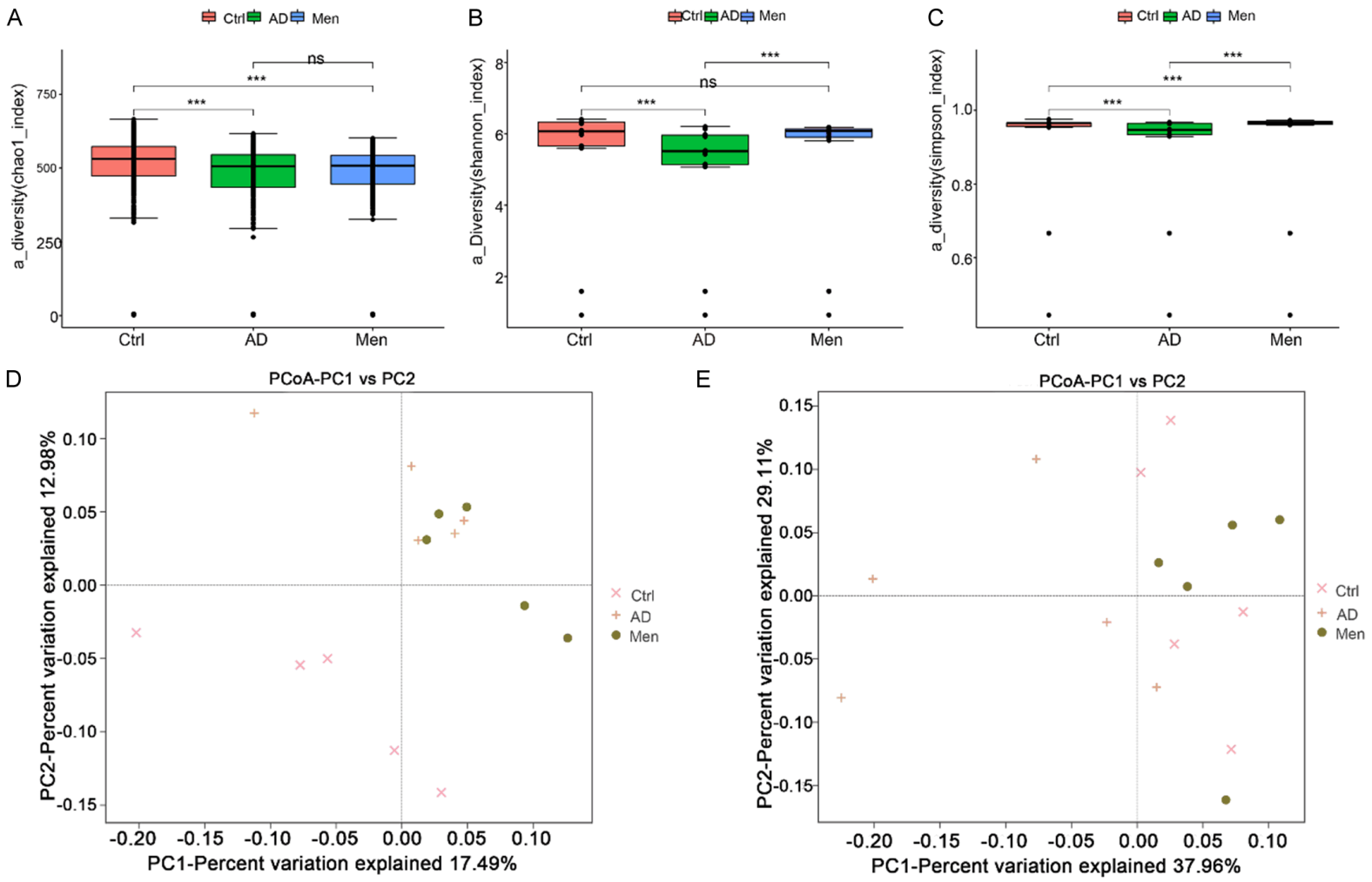


Figure 7. Effect of MSD on immune cells infiltration in the spleen of AD mice. Flow cytometry analysis of immune cells: (A, B) leukocytes ($CD45^+$), (C, D) neutrophils ($CD45^+Gr1^+$) and (E, F) MDSCs ($CD11b^+Gr1^+$). (n = 4-6).


A
Ctrl AD Men
a_diversity(chao1_index)
ns
***
***
Ctrl
AD
Men
B
Ctrl AD Men
a_Diversity(shannon_index)
***
ns
***
Ctrl
AD
Men
C
Ctrl AD Men
a_diversity(simpson_index)
***
***
***
Ctrl
AD
Men
D
PCoA-PC1 vs PC2
PC2-Percent variation explained 12.98%
PC1-Percent variation explained 17.49%
Ctrl
AD
Men
E
PCoA-PC1 vs PC2
PC2-Percent variation explained 29.11%
PC1-Percent variation explained 37.96%
Ctrl
AD
Men

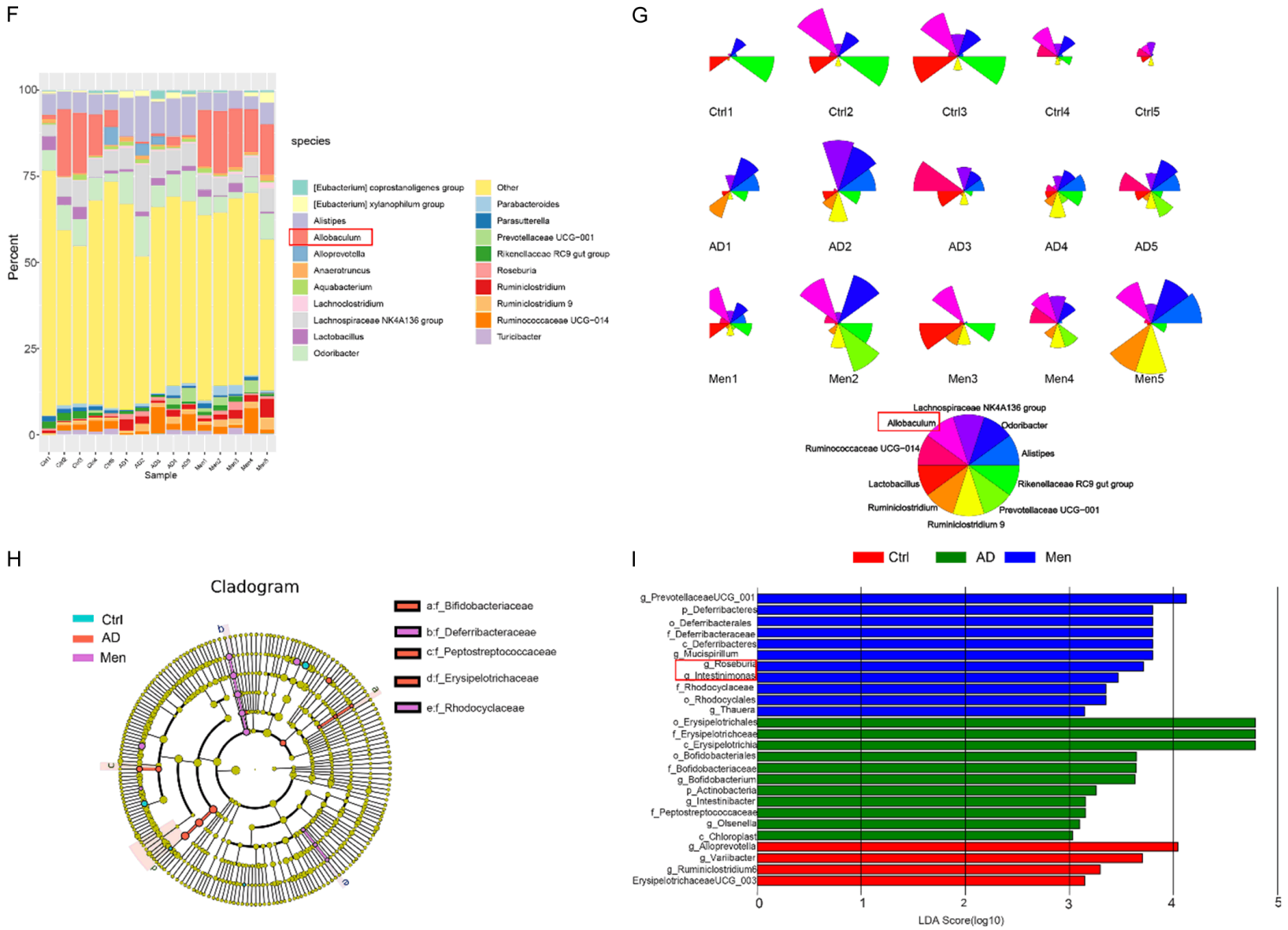


Figure 8. Effect of MSD on the structure and composition of intestinal flora. Intestinal microbial analysis of Ctrl, AD and Men (AD/Men2%) group was conducted using 16SrDNA sequencing technology. (A-C) Effect of MSD on α-diversity of fecal microbiota, assessed by chao1, shannon and simpson index, respectively. (D, E) Effect of MSD on β-diversity of fecal microbiota (unweighted UniFrac) (D) and (weighted UniFrac) (E), evaluated by principal coordinate analysis (PCoA). (F, G) Effect of MSD on relative abundance of the microbiota at genus level. (H, I) LEfSe cladogram and LDA diagram (LDA score > 3) of each group.

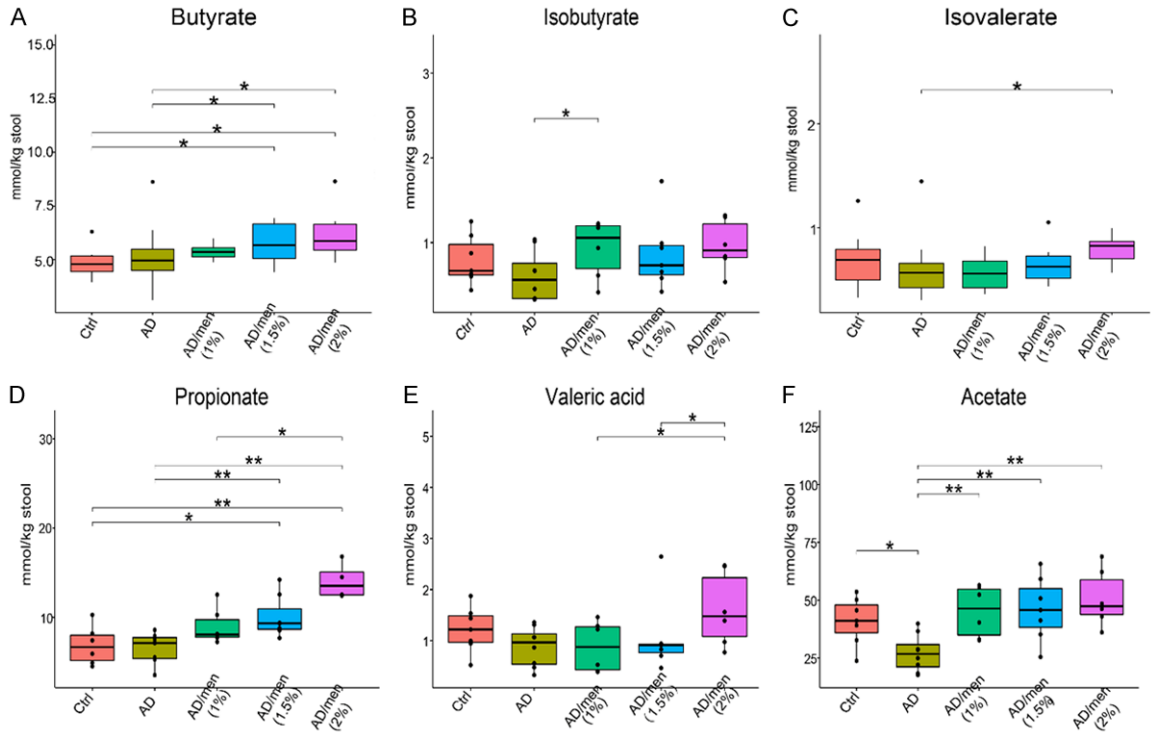


Figure 9. Effect of MSD on the concentration of SCFAs in the feces of AD mice. A-F. Concentrations of butyric acid, isobutyric acid, isovaleric acid, propionic acid, valeric acid and acetic acid in feces of the Ctrl, AD, AD/Men(1%), AD/Men(1.5%) and AD/Men(2%) groups. (n = 6-12).

part attributed to the increased concentration of butyrate in the feces of AD mice.

Discussion

According to 2018 global cancer statistics, colorectal cancer remains a malignant tumor that jeopardizes human health with a sharp increase in morbidity and mortality [32]. Existing studies confirmed that the risk of IBD with a 10, 20, and 30 years eventually progressing to CRC was 2%, 8%, and 18% higher than healthy people, respectively [3]. Anti-inflammatory therapies (e.g., 5-aminosalicylic acid) could reduce the risk of CAC in patients with IBD by 49% [33], whereas extensively reported clinical adverse effects have limited their clinical application [34]. A variety of natural plant ingredients (e.g., anthocyanin [35], HMPL-004 [36] and Wormwood [37]) have been reported to significantly mitigate the clinical symptoms and improve the prognosis of IBD, as well as reducing or avoiding the corresponding adverse effects. Thus, exploring more effective natural plant ingredients as dietary supplements is critical to prevent and care CRC developed from IBD.

Menthol, a monoterpene natural plant ingredient derived from the peppermint plant *Mentha x piperita* (Lamiaceae), has been extensively applied to skin diseases, digestive system diseases for years because of its excellent anti-inflammatory, antibacterial and analgesic pharmacological effects [38-40]. Some recent reports claimed that menthol significantly restricted the pathological progress of acetic acid-induced acute colitis for its significant anti-inflammatory and antioxidant effects [11, 12]. However, to the best of the knowledge of the authors, there has been no relevant report regarding the therapeutic effect of menthol on AOM/DSS-induced CAC, which is closer to the pathological characteristics of human CAC characterized by distant location tumor and aggressive adenocarcinoma [41]. In this study, one of the significant findings is that MSD was observed to significantly ameliorate the AOM/DSS-induced colon tumorigenesis. It is macroscopically indicated as significantly reduced number of 1-3 mm adenomas in a dose-dependent manner, extended colon length, reduced liver and spleen weights and substantially lower DAI score, as well as microscopically manifested as a significant decrease

in pathological score, expression of proliferation protein (β-catenin and Ki67), pro-inflammatory cytokine (IL-6, TNF-α and MPO) and immune cells infiltration in spleens, while the up-regulated gene expression of anti-inflammatory cytokine (IL10) in distal colon tissue. Though the survival analysis identified no significant effect of menthol on the survival rate of AD mice, the high-dose MSD mice achieved a relatively longer survival time compared with medium and low doses. In addition, this study speculates that the weight loss caused by MSD may be attributed to the weight loss effect of menthol [21], instead of the nutritional consumption caused by intestinal inflammation and tumors.

Intestinal microbiota and metabolites have aroused considerable researchers in recent years to clarify the chemopreventive and therapeutic effects of healthy diets or natural medicine ingredients on colon cancer. Fortunately, significantly higher microbial butyrate-producers (Allobaculum, Roseburia and Intestinimonas) abundance and fecal butyrate concentration were observed in MSD, complying with existing studies reporting that resistant starch restricted AOM/DSS-induced tumorigenesis by manipulating intestinal microbiota [42]. It is worth noting that a clinical study reported that compared with healthy controls, patients with advanced colon cancer were observed to have significantly diminished butyrate-producing bacteria (Clostridium, Roseburia and Eubacterium spp.) and lower fecal butyrate concentrations, complying with the findings of this study [43]. Furthermore, a randomized controlled crossover study revealed that increasing fecal butyrate concentration by dietary fiber supplementation could significantly reduce the increasing risk of colon cancer caused by a diet high in red meat [44]. Increasing experimental evidences supported that butyrate exerts its anti-tumor effect via various mechanisms (e.g., anti-inflammatory and immunomodulatory effects) [45, 46], thereby inhibiting proliferation and promoting apoptosis of neoplastic colonocytes [47]. On the whole, the results of the mentioned studies corroborate the hypothesis here that the inhibiting effect of MSD on AOM/DSS-induced tumorigenesis may be associated with the increased concentration of butyrate in feces caused by the expansion of butyrate-producing bacteria.

Conclusions

In summary, this study demonstrated that MSD significantly increases the abundance of butyrate-producing bacteria and the concentration of butyrate in feces of AD mice, thereby reducing the incidence of small adenoma, mitigating intestinal inflammation and suppressing colon tissue proliferation. This study suggested that menthol may be a potential therapeutic agent for prevention and treatment of CAC.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant nos. 81673795, 81603578, 81503536 and 81-773947).

Disclosure of conflict of interest

None.

Abbreviations

CAC, colitis-associated colon cancer; MSD, menthol supplement diet; MDSC, myeloid-derived suppressor cell; DAI, disease activity index; H&E, haematoxylin and eosin; RT-PCR, real-time quantitative polymerase chain reaction analysis; GC-MS, Gas chromatography coupled to mass spectrometry detection; SCFAs, short-chain fatty acids; MPO, myeloperoxidase; AOM, azoxymethane; DSS, dextran sulfate sodium; ns, no significance; IBD, inflammatory bowel disease; Ctrl, Control; AD, AOM/DSS; AD/Men1%, MSD low dose; AD/Men1.5%, MSD medium dose; AD/Men2%, MSD high dose.

Address correspondence to: Zhiguang Sun, Institute of First Clinical Medicine, Nanjing University of Chinese Medicine, 138 Xianlin Avenue, Nanjing 210023, Jiangsu, China. E-mail: pr_zhiguangsun@163.com

References

[1] Vagefi PA and Longo WE. Colorectal cancer in patients with inflammatory bowel disease. Clin Colorectal Cancer 2005; 4: 313-319.

[2] Manninen P, Karvonen AL, Huhtala H, Aitola P, Hyöty M, Nieminen I, Hemminki H and Collin P. The risk of colorectal cancer in patients with inflammatory bowel diseases in Finland: a follow-up of 20 years. J Crohns Colitis 2013; 7: e551-557.

[3] Eaden JA, Abrams KR and Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 2001; 48: 526-535.
[4] Nieminen U, Jussila A, Nordling S, Mustonen H and Färkkilä MA. Inflammation and disease duration have a cumulative effect on the risk of dysplasia and carcinoma in IBD: a case-control observational study based on registry data. Int J Cancer 2014; 134: 189-196.
[5] Bonovas S, Fiorino G, Lytras T, Nikolopoulos G, Peyrin-Biroulet L and Danese S. Systematic review with meta-analysis: use of 5-aminosalicylates and risk of colorectal neoplasia in patients with inflammatory bowel disease. Aliment Pharmacol Ther 2017; 45: 1179-1192.
[6] Lee YK, Mehrabian P, Boyajian S, Wu WL, Selicha J, Vonderfecht S and Mazmanian SK. The protective role of bacteroides fragilis in a murine model of colitis-associated colorectal cancer. mSphere 2018; 3: e00587-18.
[7] Wang CZ, Yu C, Wen XD, Chen L, Zhang CF, Calway T, Qiu Y, Wang Y, Zhang Z, Anderson S, Wang Y, Jia W and Yuan CS. American ginseng attenuates colitis-associated colon carcinogenesis in mice: impact on gut microbiota and metabolomics. Cancer Prev Res (Phila) 2016; 9: 803-811.
[8] Li Y, Wang S, Sun Y, Xu W, Zheng H, Wang Y, Tang Y, Gao X, Song C, Long Y, Liu J, Liu L and Mei Q. Apple polysaccharide protects ICR mice against colitis associated colorectal cancer through the regulation of microbial dysbiosis. Carbohydr Polym 2020; 230: 115726.
[9] Kamatou GP, Vermaak I, Viljoen AM and Lawrence BM. Menthol: a simple monoterpene with remarkable biological properties. Phytochemistry 2013; 96: 15-25.
[10] Trombetta D, Castelli F, Sarpietro MG, Venuti V, Cristani M, Daniele C, Saija A, Mazzanti G and Bisignano G. Mechanisms of antibacterial action of three monoterpenes. Antimicrob Agents Chemother 2005; 49: 2474-2478.
[11] Ghasemi-Pirbaluti M, Motaghi E and Bozorgi H. The effect of menthol on acute experimental colitis in rats. Eur J Pharmacol 2017; 805: 101-107.
[12] Bastaki SM, Adeghate E, Amir N, Ojha S and Oz M. Menthol inhibits oxidative stress and inflammation in acetic acid-induced colitis in rat colonic mucosa. Am J Transl Res 2018; 10: 4210-4222.
[13] Patra AK, Park T, Braun HS, Geiger S, Pieper R, Yu Z and Aschenbach JR. Dietary bioactive lipid compounds rich in menthol alter interactions among members of ruminal microbiota in sheep. Front Microbiol 2019; 10: 2038.
[14] Maenner K, Vahjen W and Simon O. Studies on the effects of essential-oil-based feed additives on performance, ileal nutrient digestibility, and selected bacterial groups in the gastrointestinal tract of piglets. J Anim Sci 2011; 89: 2106-2112.
[15] Thaker AI, Shaker A, Rao MS and Ciorba MA. Modeling colitis-associated cancer with azoxymethane (AOM) and dextran sulfate sodium (DSS). J Vis Exp 2012; 4100.
[16] Sun H, Lou Y, Porturas T, Morrissey S, Luo G, Qi J, Ruan Q, Shi S and Chen YH. Exacerbated experimental colitis in TNFAIP8-deficient mice. J Immunol 2015; 194: 5736-5742.
[17] Rangan P, Choi I, Wei M, Navarrete G, Guen E, Brandhorst S, Enyati N, Pasia G, Maesincee D, Ocon V, Abdulridha M and Longo VD. Fasting-mimicking diet modulates microbiota and promotes intestinal regeneration to reduce inflammatory bowel disease pathology. Cell Rep 2019; 26: 2704-2719, e2706.
[18] Fischer AH, Jacobson KA, Rose J and Zeller R. Hematoxylin and eosin staining of tissue and cell sections. CSH Protoc 2008; 2008: pdb.prot4986.
[19] Borrelli F, Fasolino I, Romano B, Capasso R, Maiello F, Coppola D, Orlando P, Battista G, Pagano E, Di Marzo V and Izzo AA. Beneficial effect of the non-psychotropic plant cannabinoid cannabigerol on experimental inflammatory bowel disease. Biochem Pharmacol 2013; 85: 1306-1316.
[20] Ferreira PS, Spolidorio LC, Manthey JA and Cesar TB. Citrus flavanones prevent systemic inflammation and ameliorate oxidative stress in C57BL/6J mice fed high-fat diet. Food Funct 2016; 7: 2675-2681.
[21] Sakellariou P, Valente A, Carrillo AE, Metsios GS, Nadolnik L, Jamurtas AZ, Koutedakis Y, Boguszewski C, Andrade CM, Svensson PA, Kawashita NH and Flouris AD. Chronic l-menthol-induced browning of white adipose tissue hypothesis: a putative therapeutic regime for combating obesity and improving metabolic health. Med Hypotheses 2016; 93: 21-26.
[22] Aratani Y. Myeloperoxidase: its role for host defense, inflammation, and neutrophil function. Arch Biochem Biophys 2018; 640: 47-52.
[23] Wong SH and Yu J. Gut microbiota in colorectal cancer: mechanisms of action and clinical applications. Nat Rev Gastroenterol Hepatol 2019; 16: 690-704.
[24] Sun J and Kato I. Gut microbiota, inflammation and colorectal cancer. Genes Dis 2016; 3: 130-143.
[25] Ibrahim A, Hugerth LW, Hases L, Saxena A, Seifert M, Thomas Q, Gustafsson J, Engstrand L and Williams C. Colitis-induced colorectal cancer and intestinal epithelial estrogen receptor beta impact gut microbiota diversity. Int J Cancer 2019; 144: 3086-3098.

[26] Wang G, Li X, Zhao J, Zhang H and Chen W. Lactobacillus casei CCFM419 attenuates type 2 diabetes via a gut microbiota dependent mechanism. Food Funct 2017; 8: 3155-3164.
[27] Zhang X, Zhao Y, Xu J, Xue Z, Zhang M, Pang X, Zhang X and Zhao L. Modulation of gut microbiota by berberine and metformin during the treatment of high-fat diet-induced obesity in rats. Sci Rep 2015; 5: 14405.
[28] Bui TP, Shetty SA, Lagkouvardos I, Ritari J, Chamlagain B, Douillard FP, Paulin L, Piironen V, Clavel T, Plugge CM and de Vos WM. Comparative genomics and physiology of the butyrate-producing bacterium Intestinimonas butyriciproducens. Environ Microbiol Rep 2016; 8: 1024-1037.
[29] Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M, de Los Reyes-Gavilán CG and Salazar N. Intestinal short chain fatty acids and their link with diet and human health. Front Microbiol 2016; 7: 185.
[30] Kim CH, Park J and Kim M. Gut microbiota-derived short-chain fatty acids, T cells, and inflammation. Immune Netw 2014; 14: 277-288.
[31] Parada Venegas D, De la Fuente MK, Landskron G, González MJ, Quera R, Dijkstra G, Harmsen HJM, Faber KN and Hermoso MA. Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. Front Immunol 2019; 10: 277.
[32] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.
[33] Velayos FS, Terdiman JP and Walsh JM. Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies. Am J Gastroenterol 2005; 100: 1345-1353.
[34] Di Paolo MC, Paoluzi OA, Pica R, Iacopini F, Crispino P, Rivera M, Spera G and Paoluzi P. Sulphasalazine and 5-aminosalicylic acid in long-term treatment of ulcerative colitis: report on tolerance and side-effects. Dig Liver Dis 2001; 33: 563-569.
[35] Sodagari HR, Farzaei MH, Bahramsoltani R, Abdolghaffari AH, Mahmoudi M and Rezaei N. Dietary anthocyanins as a complementary medicinal approach for management of inflammatory bowel disease. Expert Rev Gastroenterol Hepatol 2015; 9: 807-820.
[36] Tang T, Targan SR, Li ZS, Xu C, Byers VS and Sandborn WJ. Randomised clinical trial: herbal extract HMPL-004 in active ulcerative colitis - a double-blind comparison with sustained release mesalazine. Aliment Pharmacol Ther 2011; 33: 194-202.
[37] Krebs S, Omer TN and Omer B. Wormwood (artemisia absinthium) suppresses tumour necrosis factor alpha and accelerates healing in patients with Crohn's disease - a controlled clinical trial. Phytomedicine 2010; 17: 305-309.
[38] Farco JA and Grundmann O. Menthol--pharmacology of an important naturally medicinal "cool". Mini Rev Med Chem 2013; 13: 124-131.
[39] Merat S, Khalili S, Mostajabi P, Ghorbani A, Ansari R and Malekzadeh R. The effect of enteric-coated, delayed-release peppermint oil on irritable bowel syndrome. Dig Dis Sci 2010; 55: 1385-1390.
[40] Sabzghabaee AM, Nili F, Ghannadi A, Eizadi-Mood N and Anvari M. Role of menthol in treatment of candidial napkin dermatitis. World J Pediatr 2011; 7: 167-170.
[41] Parang B, Barrett CW and Williams CS. AOM/DSS model of colitis-associated cancer. Methods Mol Biol 2016; 1422: 297-307.
[42] Hu Y, Le Leu RK, Christophersen CT, Somashekar R, Conlon MA, Meng XQ, Winter JM, Woodman RJ, McKinnon R and Young GP. Manipulation of the gut microbiota using resistant starch is associated with protection against colitis-associated colorectal cancer in rats. Carcinogenesis 2016; 37: 366-375.
[43] Hibberd AA, Lyra A, Ouwehand AC, Rolny P, Lindegren H, Cedgård L and Wettergren Y. Intestinal microbiota is altered in patients with colon cancer and modified by probiotic intervention. BMJ Open Gastroenterol 2017; 4: e000145.
[44] Humphreys KJ, Conlon MA, Young GP, Topping DL, Hu Y, Winter JM, Bird AR, Cobiac L, Kennedy NA, Michael MZ and Le Leu RK. Dietary manipulation of oncogenic microRNA expression in human rectal mucosa: a randomized trial. Cancer Prev Res (Phila) 2014; 7: 786-795.
[45] Meijer K, de Vos P and Priebe MG. Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health? Curr Opin Clin Nutr Metab Care 2010; 13: 715-721.
[46] Chen J and Vitetta L. Inflammation-modulating effect of butyrate in the prevention of colon cancer by dietary fiber. Clin Colorectal Cancer 2018; 17: e541-e544.
[47] Zeng H, Safratowich BD, Wang TTY, Hamlin SK and Johnson LK. Butyrate inhibits deoxycholic-acid-resistant colonic cell proliferation via cell cycle arrest and apoptosis: a potential pathway linking dietary fiber to cancer prevention. Mol Nutr Food Res 2020; 64: e1901014.