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Inhibitory effects of β-type glycosidic polysaccharide from *Pleurotus eryngii* on dextran sodium sulfate-induced colitis in mice

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Abstract: The aim of the present study was to determine the inhibiting effects and the potential underlying mechanisms of a novel *Pleurotus eryngii* β-type glycosidic polysaccharide (WPEP) on colitis. To achieve this, sixty CD-1 (ICR) mice were divided into six groups including heathy and colitic mice treated with or without WPEP at two different doses (n=10). Results showed that WPEP displayed significant inhibitory effect on colitis as indicated by a lowered disease activity index in treated colitic mice compared to the untreated colitic mice (2.78 ± 0.50 to 1.80 ± 0.17). Decrease in pro-inflammatory cytokines concentrations and pro-inflammatory protein expressions and increase in colon length (9.31 ± 0.59 cm to 10.89 ± 1.20 cm) along with histological improvements were also observed in the treated colitic mice compared to the untreated colitic mice. the present study. Flow cytometry and western blotting analysis revealed that these anti-colitis effects were associated with decreased accumulation of CD 45+ Immune Cells, CD45+F480+ Macrophages and CD45+Gr1+ Neutrophils. Moreover, 16s rRNA sequencing analysis of gut microbiota revealed that WPEP partially reversed gut microbiota dysbiosis in the colitis mice including the decreased abundance of *Akkermansia muciniphila* (35.80 ± 9.10% to 18.24 ± 6.23%) and *Clostridium cocleatum* (2.34 ± 1.78% to 0.011 ± 0.003%) and increased abundance of *Bifidobacterium pseudolongum* (3.48 ± 2.72% to 9.65 ± 3.74%), *Lactobacillus reuteri* (0.007 ± 0.002% to 0.21 ± 0.12%), *Lactobacillus salivarius* (1.23 ± 0.87% to 2.22 ± 1.53%) and *Ruminococcus bromii* (0.009 ± 0.001% to 3.83 ± 1.98%). In summary, our results demonstrated that WPEP could be utilized as a functional food component in colitis management as well as a potential prebiotic agent to improve inflammation-related disorders.

Keywords: β-type glycosidic polysaccharide**;** Gut microbiota; Inflammatory bowel disease; *Pleurotus eryngii*

1. Introduction

Inflammatory bowel disease (IBD), a term for ulcerative colitis (UC) and Crohn's disease (CD), is associated with chronic and long-term inflammation reaction [1]. Specifically, as the most prevalent IBD, UC has been linked with increasing morbidity in the world, including the western and the developing countries. It commonly occurs in the colon and results in diarrhoea, bloody mucus is stool and colon shortening [2]. Presently, drug intervention is the major strategy utilized for UC mitigation. However, this approach is not only expensive but also is exerts significant side effects, which severely impose restrictions on its application as a therapeutic option. Hence, exploiting novel, preventive and inexpensive therapeutic strategies with low side effects and high efficiency is needed in the present UC inhibition and management. With deepening research related to UC inhibition, the application of functional components including flavonoids, lutein, and other antioxidants obtained from specific natural foods (berries, fruits and grains), have widely been studied with significant improvement effects on colitis symptoms being revealed [3-4].

In recent years, edible mushrooms, with high active polysaccharide fractions, have been proved to be an effective way of preventing and inhibiting UC development with efficient activities and negligible side effects [5-6]. Numerous studies have been carried out with results revealing impressive protective effects of edible mushroom polysaccharides (EMPs) against IBD [7-9]. As a large class of natural biopolymers, EMPs have been reported mainly as novel glucans possessing significant anti-inflammatory effects in the colon. Latest studies have documented the feasibility of EMPs as a beneficial therapy for IBD management based on their key role attributed to the crosstalk between immune functions and intestinal flora, which has been

considered the main functioning mechanisms of EMPs during IBD ameliorating process [10-11]. Based on their non-digestible ability, EMPs could be considered as novel prebiotics due to their increasing significant effect on the body's resistance against invading pathogens in addition to their strong association the crosstalk in the colon. Therefore, the exploitation of intestinal flora modulation effects induced by EMPs supplementation could be considered as a novel therapeutic strategy in UC inhibition.

Pleurotus eryngii, which exhibits various unique biological activities due to its abundant non-digestible polysaccharide fractions content (PEPs), has attracted numerous researchers' attention in recent years [12]. Latest findings have demonstrated antitumor [13], anti-obesity [14], hepatoprotective and hypolipidemic activities of PEPs [15]. Moreover, its immuno-stimulating activity has been studied and shown to be the primary function in body health [16]. However, its gut microbiome modulation effect, as well as the correlation with the above biological activities has not been exhaustively revealed. In our previous studies, the intestinal flora regulation effects and alteration impacts on intestinal immune proteins expression of a novel crude *P. eryngii* polysaccharide (0.2, 0.4 and 0.8 g/kg body weight) with immune modulatory activity on mice were investigated [17-18]. Additionally, the anti-inflammation activity of its purified β-type glycosidic polysaccharide fraction on lipopolysaccharide stimulated RAW264.7 cells along with its toxicity on normal RAW264.7 cells were also evaluated. From these findings we hypothesized that this β-type glycosidic polysaccharide could be considered as a potential ingredient for UC treatment through its gut microbiome modulation ability. Hence, the major goal of the present study was to investigate the inflammatory attenuation response efficacy of WPEP and related mechanisms under the chronic colitis mice model. The findings would

demonstrate the significance of this novel water-soluble *P. eryngii* β-type glycosidic polysaccharide as a food-based IBD treatment approach as well as providing a basis for the promotion of edible mushrooms as functional foods with efficient biological effects.

2. Materials and Methods

2.1. Materials and Chemicals

Fresh *P. eryngii* (second batch of the autumn cultivation of 2017, Nanjing, China) was purchased from the local market. The antibodies for western blot were purchased from Cell Signaling Technology (Danvers, MA). All other chemicals were supplied by Fisher chemicals (Hampton, NH).

2.2. Animals and Dosage Information

The water-soluble *P. eryngii* β-type glycosidic polysaccharide (WPEP, 167 kDa), occurring as a heteropolysaccharide with β-type glycosidic linkages and mainly composed of xylose, mannose, glucose and galactose with a molecular ratio of 21.35: 3.28: 73.22: 1.63 was prepared based on our previous study (Supplemental Methods and Supplemental Table. 1). A dextran sodium sulfate (DSS)-induced colitis mouse model was utilized and followed the protocol in Figure. 1. Specifically, sixty 8-week-old male CD-1 (ICR) mice were purchased from the Charles River laboratories (Wilmington, MA). The animals were maintained under the ambient temperature of 25 ± 2 °C, relative humidity (50 ± 5%), and a 12/12 h of light-dark cycle, and were fed on a diet and drink ad libitum with weights monitored during each cycle to detect the potential stress of different treatments utilized in the present study on animals. After 1 week of acclimation, all mice were randomly divided into six groups (ten mice per group) and fed on a standard AIN-93G diet (Supplemental Table. 2). Mice of groups named DSS, DSP and DSHP

were treated with 1.5% w/v DSS in drinking water for four cycles (5 days of DSS treatment per cycle followed with a 7 days recovery for each of the first 3 DSS cycles) to induce colitis, and supplemented with WPEP under a dose of 0, 0.2 and 0.8 g/kg mice body weight through intragastric administration during the whole process. In the meantime, the healthy mice in the other three groups, named CT, CTP and CTHP, were treated with regular drinking and supplemented with WPEP under a dose of 0, 0.2 and 0.8 g/kg mice body weight, respectively. All the protocols were performed on the basis of National Institutes of Health Guide for the care and use of laboratory animals and approved by the Ethical Committee of Experimental Animal Center of University of Massachusetts, Amherst (#2014-0079). At the end of the protocol, mice feces were collected individually and used for the stool DNA extraction. Afterwards, the mice were sacrificed and subjected to gross necropsy. Colons were collected, opened longitudinally and macroscopically inspected. Then all the colon tissues were cut into halve longitudinally and frozen at -80 °C for further study. Liver, spleen and kidney were collected, weighed and used to calculate the tissue indices.

2.3. Disease Activity Index (DAI)

Mice stools were collected in the beginning and ending of each cycle to observe the stool consistency and stool blood presence which were considered as the two major colitis symptoms. The DAI of mice in different groups was calculated utilizing three-point scale as follows: i) stool consistency: 0, normal; 1, soft but still formed; 2, very soft; 3, diarrhoea. ii) blood in stool: 0, negative hemoccult; 1, positive hemoccult; 2, visible blood traces in stool; 3, rectal bleeding.

2.4. Histopathological Analysis of Colon Tissues

The histopathological analysis of mice harvested colon tissues in CT, DSS, DSP and DSHP

groups were conducted as follows. Briefly, colon tissues were fixed by formalin as Swiss-rolls, and then processed for paraffin embedding, sectioning, and at last staining with hematoxylin and eosin (H&E) to observe and evaluate the histological improvements, including colon dysplasia, immune cells infiltration and mucosal ulceration under a microscope (×150, ×300, ×600).

2.5. Determination of Immune Cells in Colonic Mucosa

Colonic mucosa protein fractions of mice colonic mucosa in CT, DSS, DSHP groups were split with an EDTA (5 mmol/L) containing HBSS solution. Then a fluorescence-activated cell sorting (BD Biosciences, San Jose, CA) was used to obtain the single cell suspension followed with immunofluorescence histochemical staining of FITC labelled CD45 antibody, PerCP/Cy5.5 labelled F4/80 antibody or isotype control antibody (Bio Legend, San Diego, CA). Lastly, a BD LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA) combined with a FlowJo 13.0 software were utilized to analyze the proportion of total immune cells, neutrophils and macrophages in colonic mucosa.

2.6. Determination of Pro-Inflammatory Cytokine in Colonic Mucosa

Frozen colon tissues of mice in CT, DSS and DSHP groups were melted under an ice water environment and then cleaned with PBS solution buffer (4 ℃), followed by gentle dissection from the colon muscularis propria to obtain the colonic mucosa. RIPA buffer (Boston Bioproducts, Ashland, MA) containing protease inhibitors (proteinase, phosphatase inhibitor I, phosphatase inhibitor II) was used to extract the mucosa protein fractions. After the protein concentration was determined through Bicinchoninic Acid Assay (BCA), cytokines (IL-1β, IL-2, IL-6, TNF-α, INF-γ, IL-10) concentrations in colonic mucosa protein fractions were

analyzed utilizing a multiplex cytokine immunoassay-MSD 96-Well MULTI-SPOT kit (Meso Scale Discovery, MD, USA) in accordance with the manufacturer's instructions.

2.7. Western Blotting Analysis

Colonic mucosa protein fractions of mice colonic mucosa in CT, DSS, DSHP groups were harvested utilizing a RIPA buffer containing protease inhibitors (Boston Bioproducts, Ashland, MA). Then, the protein contents in the collected mucosa protein lysate was determined by a BCA Protein assay kit. Afterwards, lysate containing 30 μg mucosa protein per well were loaded and separated on a 12% sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE), followed by electroblotting onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Then the membranes were blocked (2 h, room temperature) with 5% (g/100mL) skim milk, incubated (4 ℃) with different primary antibodies (Abcam, Cambridge, MA, USA) overnight. After washing three times through TBST buffer, membranes were incubated with HRP-conjugated secondary antibodies (2 h, room temperature). Lastly, the protein bands were visualized and quantified with the enhanced chemiluminescence (ECL) detection system (Bio-rad, California, USA).

2.8. Fecal DNA Extraction and 16S rRNA High Throughput Analysis

Frozen stools of mice in CT, DSS, DSP and DSHP groups were melted under an ice water environment and utilized for the extraction of DNA contents through a QIAamp DNA stool kit (Qiagen, Inc., Shanghai, China) according to the manufacturer's instructions. A UV-vis spectrophotometer (NanoDrop 2000, Waltham, MA) was used for the measurement of extracted DNA concentrations. All the DNA samples were stored at -20 °C for the future 16S rRNA sequencing study. The 16S rRNA high throughput analyses of fecal DNA samples were

carried out as follows. Briefly, universal primers tailed with Illumina barcoded adapters were used to amplify the bacterial V3-V4 region of the 16S ribosomal gene during the PCR process. Afterwards, the products of PCR were purified using the Agencourt XP Ampure XP Beads (Beckman Coulter, Danvers, MA). After the qualification of the amplicon through ScreenTape Assay on Tape Station 2200 (Agilent Technologies, Santa Clara, CA), samples were pooled in equal proportions and sequenced the paired end in an Illumina MiSeq at the IGA Technology Services. Particularly, 30% PhiX genome was spiked to avoid the focusing and phasing issues resulting from the sequencing of "low diversity" libraries.

2.9. Bioinformatic analysis

The high-quality sequences were assigned to samples according to barcodes. Mothur software was used for the alpha-diversity analysis to identify the species diversity, including the indices of Chao, Simpson, Shannon and Coverage. Overall, the validated sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity using UPARSE embedded in Quantitative Insights into Microbial Ecology software package (QIIME, version 1.8.0, http://bio.cug.edu.cn/qiime/). Community structure comparisons, such as Jaccard tree clustering, PCoA, Unifrac and heatmap analyses were performed using Mothur and R software packages (http://www.R-project.org).

2.10. Statistical analysis

Statistical analysis was performed with one-way analysis of variance (ANOVA) (more than two groups) or Student's t-test (two groups) using SPSS 24.0 software (Statistical Package for Social Sciences, SPSS Inc., Chicago). Means ± standard deviation (SD) was used for the data presentation of independently performed experiments. A p-value of <0.05 was considered

192 statistically significant.

193 **3. Results**

194 **3.1. Inhibition of WPEP on DSS-Induced Colitis Symptoms in Mice**

195 No accidental deaths were reported the 60 experimental mice, and no significant differences of
196 the food and water intake (data not shown), body weights (Table. 1), tissue indices (Table. 2)
197 in different treatments were observed. There was no observable toxicity impact of WPEP
198 gavage on mice's body health. Clinical DAI values of mice in DSS group (Table. 3) displayed
199 an extremely significant difference ($p < 0.01$) compared to DSHP group, suggesting a
200 beneficial improvement of WPEP supplementation on colitis. Colonic length was significantly
201 shortened in the DSS-induced mice colitis (9.31 ± 0.59 cm) compared to the healthy mice
202 (10.99 ± 0.90 cm) ($p < 0.01$) while WPEP treatment reversed this symptom significantly (10.81
203 ± 0.67 cm of DSP group and 10.89 ± 1.20 cm of DSHP group) (Figure. 2 A and Table. 2).
204 Based on the health situation of mice with different treatment, H&E-stained colorectal sections
205 were utilized to evaluate the goblet cells loss, crypts distortion, inflammatory cell infiltration
206 and epithelial injury of mucosa and submucosa in DSS, DSP, and DSHP groups (Figure. 2 B).
207 From this, we observed that the colonic architecture of mice in DSP and DSHP groups
208 exhibited less inflammation reaction including epithelial wall damage and crypt distortion
209 compared to the DSS group.

210 **3.2. Regulation Effects of WPEP on Immune Cells in Colonic Mucosa**

211 The total immune cells and their macrophages and neutrophils proportion in colonic mucosa in
212 different treatment mice groups were analyzed using flow cytometry and double staining
213 technique to reveal the inhibitory effects of WPEP gavage on immune cell enrichment in colon

induced by DSS stimulation. As shown in Table. 4, the total immune cells, macrophages and neutrophils proportion in colonic mucosa of mice in DSS group were extremely increased (1.38-, 2.12- and 2.33-fold, respectively) compared to the CT group ($p < 0.01$) while a high dose WPEP intake significantly reduced this increase. Specifically, the proportions of total immune cells and their macrophages and neutrophils were 3.12 ± 1.58%, 6.35 ± 3.43% and 8.48 ± 2.85%, exerting 45.55%, 75.00% and 69.85% reduction, respectively compared to the mice with DSS treatment.

3.3. Reduction of WPEP on Pro-Inflammatory Cytokines Secretion in Colon Mucosa

The production of pro-inflammatory cytokines in the mice colonic mucosal layer was significantly increased due to the damage of the colon tissues and enrichment of immune cells during the chronic inflammation period. The main goal of this section was to further evaluate the inhibition effects of WPEP supplementation on DSS-induced colitis symptoms. From the preliminary experimental results, no significant inhibition effects on pro-inflammatory cytokines secretion were observed (data not shown) in low dose group (DSP) and high dose group (DSHP) mice. Common pro-inflammatory cytokines, viz, IL-1β, IL-2, IL-6, TNF-α, INF-γ, and IL-10 were determined. As shown in Table. 5, concentrations of the detected colonic mucosa pro-inflammatory cytokines were all elevated in DSS treatment compared to the CT group ($p < 0.05$) and a beneficial reverse impact was found with the WPEP intake. IL-1β concentration in DSS group increased 8.7-fold compared to the CT group (11.00 ± 2.03 pg/mg), and only 40.74 ± 9.18 pg/mg was determined in colitis mice supplemented with WPEP. The concentration of IL-2 in DSS group was 0.43 ± 0.29 pg /mg, suggesting a significant difference compared with CT group (0.03 ± 0.01 pg/mg) which was restored to 0.13 ± 0.06

pg/mg with WPEP gavage. Since the pro-inflammatory factor changed in different groups, the concentrations of IL-6 in CT, DSS and DSP groups were 1.15 ± 0.60 pg/mg, 100.87 ± 33.87 pg/mg, and 6.10 ± 2.18 pg/mg, respectively. In addition, a WPEP supplementation WPEP diet exhibited potent inhibitory effects on DSS-induced overproduction of TNF-α, INF-γ, and IL-10 in colonic mucosa.

3.4. Impacts of WPEP on the Expression of Colonic Mucosa Pro-Inflammatory Proteins

Western blotting analysis was utilized to reveal the anti-inflammation mechanisms of WPEP on DSS-induced mice colitis. This section looked at the expression levels of functional proteins related to inflammation, oxidative stress, and intestinal tight junction function, which are considered as the three primary pathogenesis signal pathways of IBD. As shown in Figure. 2 C and D, the p65 and p50 proteins involved in NF-κB signal pathway, the phosphorylation level of p38 protein in MAPK signal pathway and the expression level of key proteins of TLR-4 signal pathway were investigated. Expression levels of selected key inflammatory proteins in colonic mucosa of DSS group, especially p65, COX-2, iNOS and TLR-4, exhibited significant increase compared with CT group ($p < 0.01$). However, the expression levels of these functional proteins in colitis mice with WPEP gavage significantly decreased by 97.12%, 74.80%, 94.04% and 85.48%, respectively compared with DSS group, which reflected a potential functional signal pathway related mechanism of WPEP against IBD symptoms.

3.5. Improvement of Gut Microbiota Diversity in Mice with DSS-Induced Colitis

Next, we sought to determine whether WPEP could ameliorate IBD symptoms through its modulation on the structure and composition of intestinal flora in DSS-treated mice by analyzing 16S rRNA sequence. Firstly, the number of total and specific OTUs in feces of each

mice treatment group were analyzed under a 97% similarity screening criterion. As portrayed in Figure. 3 A, the number of OTUs shared by all mice was shown using central number while the outer circle ellipse number represents the unique OTUs' number of single mouse. From this, which we found that the number of mutual OTUs in feces of all mice was 44 while the number of unique OTUs in each mouse was not significantly different ($p > 0.05$). In addition, the OTUs' differences of mice feces in different groups, the specific number of mutual or unique OTUs were analyzed using Venn diagram (Figure. 3 B). As shown, the number of mutual OTUs in mice feces between CT group and DSS group was 256 which changed to 285 between the CT group and DSP group, indicating that WPEP innervation could promote the disorder of intestinal flora structure in IBD mice to a healthy situation. Moreover, an Alpha diversity analysis of intestinal flora among different treatment groups was carried out, including the sequencing depth indices (Observed species and Good's coverage), flora diversity indices (Shannon's diversity and Simpson's diversity), and flora abundance indices (Chao and ace). indices in All the intestinal flora diversity of mice in the five groups (CT, CTP, CTHP, DSP, DSHP) displayed a significant increase compared to the mice in DSS group (Figure. 3 C-H), further illustrating an intestinal flora disorder recovery effect of WPEP on IBD mice.

Besides, the differences of mice intestinal flora in CT, DSS, DSP and DSHP groups were further revealed through principal component analysis (PCA) and PLS-DA analysis. As shown in Figure. 4 A and B, an extremely significant difference was observed between CT group and DSS group. Beneficial reverse effects were observed under the intervention of WPEP, suggesting a positive role of WPEP supplementation as novel component against IBD through gut microbiota modulation. Bacterial taxonomic profiling in different levels of fecal microbiota

from different treatments was done and shown in Supplemental Figure. 1. Heatmaps of bacterial taxonomic profiling in different levels, including phylum, class, order, family, Genus level (Supplemental Figure. 2), and species level (Figure. 4 C) of mice fecal microbiota from different treatments was also done. From these, we identified the specific species of gut microbiota induced by WPEP gavage during its activity on DSS-induced colitis and illustrated its variation trends. Six species of mice intestinal flora in WPEP-treated group namely *Akkermansia muciniphila*, *Clostridium cocleatum*, *Bifidobacterium pseudolongum*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, and *Ruminococcus bromii* changed significantly compared with DSS group. In addition, the specific relative abundance of the selected six gut microbiota species were further shown in Table. 6 from which two of the species viz *Akkermansia muciniphila* and *Clostridium cocleatum* significantly increased with DSS stimulation. The other four species displayed a significantly decreasing trend ($p < 0.05$). However, the relative abundances of *Akkermansia muciniphila* and *Clostridium cocleatum* in WPEP-treated colitis mice decreased from 35.80 ± 9.10% to 18.24 ± 6.23% and 2.34 ± 1.78% to 0.01 ± 0.003%, respectively. Relative abundance of the other four species manifested a reversed trend with WPEP supplementation, suggesting a deep correlation between the IBD symptoms inhibition and gut microbiota structure disorder improvement in DSS-induced colitis mice.

Lastly, the correlation of the six identified species with pro-inflammatory cytokine secretion was analyzed using Pearson's correlation estimation (Figure. 4 D) based on the key role of gut microbiota during the IBD pathogenesis process. The results indicated that the relative abundance of *Akkermansia muciniphila*, *Clostridium cocleatum*, *Bifidobacterium*

pseudolongum, *Lactobacillus reuteri* and *Lactobacillus salivarius* was correlated to the secretion level of the determined pro-inflammatory cytokines. From this we confirmed that *Akkermansia muciniphila* and *Clostridium cocleatum* had a significant positive correlation with the cytokines' secretion, while *Bifidobacterium pseudolongum*, *Lactobacillus reuteri* and *Lactobacillus salivarius* had a negative correlation with the cytokines' secretion.

4. Discussion

Previous studies have proven that the proportion of neutrophils in colonic tissues was negatively correlated with the integrity of the colonic epithelial tissue. This means that the accumulation of neutrophils in colonic mucosa prompts the rupture of colonic epithelial cells and the disappearance of the colon goblet cells, eventually destroying the intestinal mucosal barrier [19-20]. Thus, the anti-inflammation activity mechanisms of WPEP could be considered primarily through its protection effects on colonic tissues consequently leading to a decrease in colonic mucosa immune cells. Moreover, the present study revealed that WPEP could modulate key functional proteins of specific signal pathways which were related to colonic inflammation happening from microscopic level. Specifically, there existed a deep correlation between the production of IL-6 and TNF-α, as well as the damage of colon tissues. As one of the earliest and most important pro-inflammatory cytokines, the excessive production of TNF-α would stimulate further IL-6 secretion and was suggested by the similar decreasing trends of TNF-α and IL-6 concentration in DSP group [21-22]. similar inhibition effects of IL-2, IL-10 and INF-γ concentrations of mice in DSP group compared to DSS group were also been proved to be closely related with an increase in colitis symptoms [23]. Elevation of the expression levels of p65 and p50 have also been revealed to be related to the activation NF-κB signal pathway along

with the intestinal inflammation development [24]. On the other hand, the phosphorylation increase of p38 protein in MAPK pathway, which is also considered to be related to NF-κB signal pathway, has been investigated. The expression levels of other three key proteins namely COX-2, iNOS and TLR-4, which have been proven to be highly relevant to the NF-κB signal pathway activation, were also determined [25]. Positive correlation has been proved between the expression level of COX-2 and NF-κB pathway activation while activation of iNOS could result to NO secretion thus promoting the pro-inflammatory concentration through NF-κB pathway. TLR-4, known as the key protein of TLRs signal pathway, has also been shown to display a significant key role in the NF-κB pathway activation process [26]. In summary, the increasing expression levels of these proteins in colitis mice were reversed upon WPEP gavage, suggesting that WPEP could exert its anti-inflammation activities mainly through inhibition on NF-κB pathway activation.

On the other hand, gut microbiota alteration effect of WPEP on colitis mice was revealed and the microflora structure in CT, DSS and DSP group showed significant differences in the levels of phylum, class, order, family, genus and species. The observed gut microbiota regulation effects of WPEP could be considered mainly due to its β-type glycosidic linkages reported in our previous study. As a newly identified β-type glycosidic polysaccharide, WPEP exerted non-digestible characteristics enabling it to reach the colon and interact with the gut microbiota to perform its specific functional activities. Six species of gut microbiota were identified and their correlation with colonic pro-inflammatory cytokines secretion proved. Specifically, *Akkermansia muciniphila* and *Clostridium cocleatum*, considered as strains with intestinal mucin biodegradable function, have been closely related to the occurrence of intestinal

346 inflammation [27-29]. Increase of these two intestinal flora species in DSS group was mainly due
347 to the colonic tissues damage induced by DSS stimulation leading to the release of abundant
348 gut mucin in the colon which would act as a substrate for their proliferation. However, intestinal
349 tissue damage was inhibited after WPEP treatment which in turn inhibited the increase of these
350 two species' abundance. The other four species with abundance reduction in the DSS group
351 have been illustrated to display positive effects against IBD and were shown to increase with
352 WPEP treatment. Of these, *Bifidobacterium pseudolongum*, *Lactobacillus reuteri* and
353 *Lactobacillus salivariushave* been shown to be probiotic for the treatment of colonic cancer
354 associated with enteritis [29-31]. The last identified species was *Ruminococcus bromii*, which is
355 considered to be related to the butyric acid production. *Ruminococcus bromii* has shown
356 multiple biological activities, especially its improvement on intestinal inflammation. Previous
357 studies have demonstrated the relationship between the six identified species and colitis
358 symptoms, including the intestinal tissue repair, colonic epithelial barrier protection, and
359 intestinal inflammation improvement [32-34], which were in congruent with the results of the
360 Pearson's correlation estimation analysis between the six and the pro-inflammatory cytokines
361 secretion resulting from WPEP supplementation of IBD mice.
362 In summary, the present study revealed the inhibition effects of WPEP on colitis were related
363 to its modulation of gut microbiota. However, considering the differences between
364 experimental animals and humans, future studies were warranted to validate the underlying
365 mechanisms of actions of WPEP. This is important to lay a solid scientific foundation for the
366 utilization of WPEP as novel functional food ingredients with anti-inflammatory activities
367 through gut microbiota regulation.

5. Conclusion

The present study demonstrated that WPEP supplementation significantly reversed the development DSS-induced colitis in mice through multiple factors including the enrichment inhibition of immune cells proportion and pro-inflammatory cytokines overproduction which were revealed to correlate with the key functional protein expression related to NF-κB signal pathway in mice colonic mucosa. For the first time, we revealed colitis inhibition effects of WPEP in colitis mice where the reversed impacts were found to correlate strongly with gut microbiota dysbiosis. These results demonstrated the significant role and underlying mechanisms of a newly identified water-soluble *P. eryngii* polysaccharide as a potential prebiotic to alleviate colitis. This provides a strong scientific basis for the possible application of this functional component as a novel approach for colitis symptoms improvement. However, further studies with more sample size, as well as clinical model are needed in the future to determine and reveal the specific impacts of intestinal flora species on gut inflammation.

Abbreviations List

WPEP: *Pleurotus eryngii* β-type glycosidic polysaccharide; UC: Ulcerative colitis; CD: Crohn's disease; EMPs: Edible mushroom polysaccharides; PEPs: *Pleurotus eryngii* polysaccharide fractions; DSS: Dextran sodium sulfate; DAI: Disease activity index; OUT: Operational taxonomic unit; PCA: Principal component analysis; PLS-DA: Partial least squares discrimination analysis.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

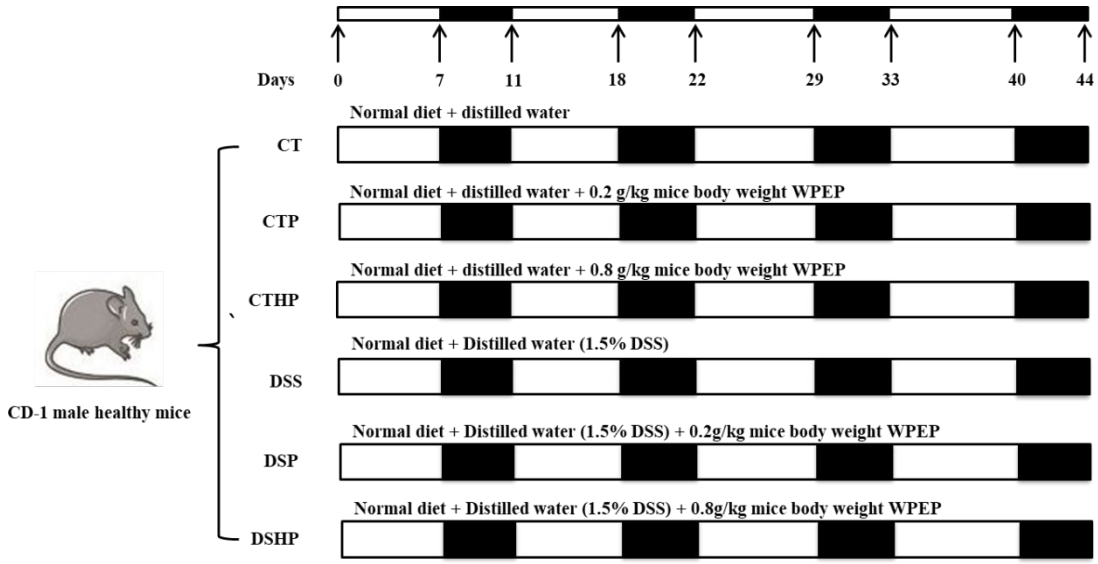
Figure. 1. Sketch map of the animal experiment.

Figure. 2. Amelioration effect of WPEP on DSS-induced colitis symptoms in mice. A, Colon length of mice with different treatments; B, Colon stain image from mice with different treatments; C, Pro-inflammatory proteins expression level in colonic mucosa of mice with different treatments.

Figure. 3. The Core-Pan OTU, Venn and Alpha differences analysis of gut microbiota in mice with different treatments. A, Core-Pan OTU; B, Venn; C, Observed species; D, Chao; E, Ace; F, Shannon's diversity; G, Simpson diversity; H, Good's coverage.

Figure. 4. Improvement of gut microbiota diversity in mice with DSS-induced colitis. A and B, Principle Components Analysis (PCA) and PLS-DA analysis of mice gut microbiota based on OTU abundances; C, Heatmaps of the bacterial taxonomic profiles in species level of fecal microbiota in mice with different treatments; D, Heatmap of the abundance of the identified six kinds of specific bacteria species correlated with the pro-inflammatory cytokine secretion in colonic mucosa. * means significant correlations under the Pearson's correlation estimation, $p < 0.05$; ** means significant correlations under the Pearson's correlation estimation, $p < 0.01$.

Figure. 1



Note: Black shade represents 1.5% DSS water treatment, 10 mice/group.

Figure. 2

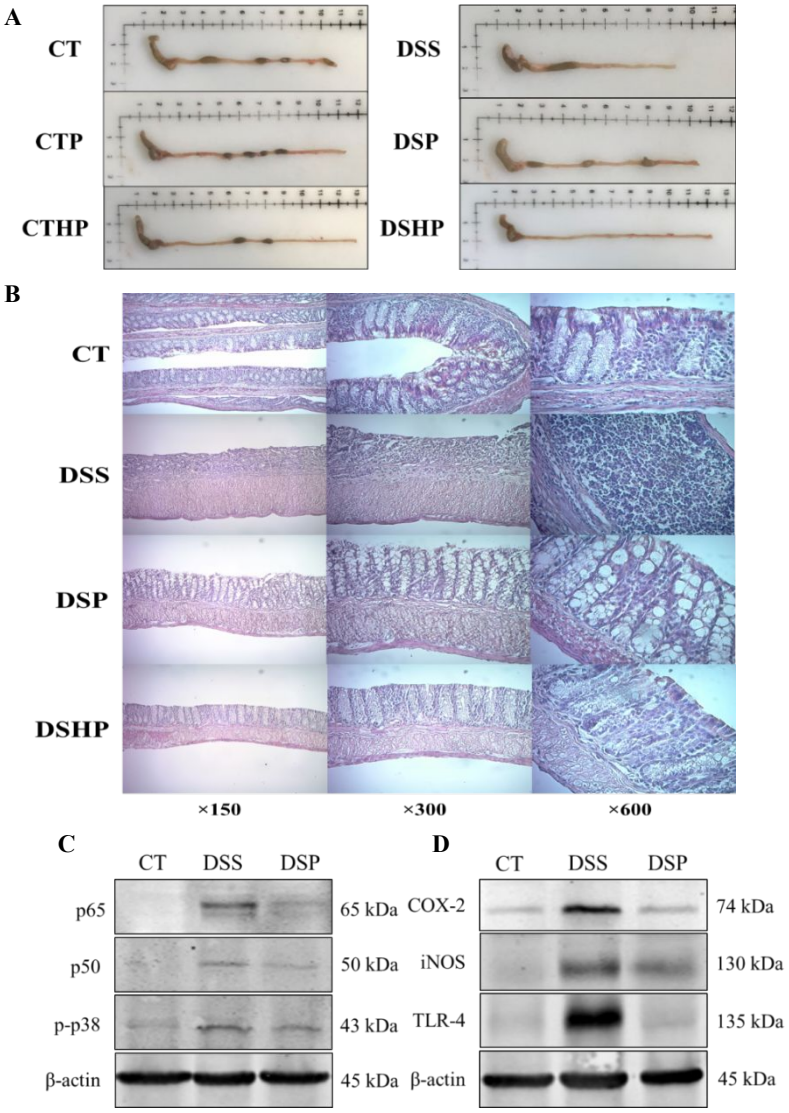
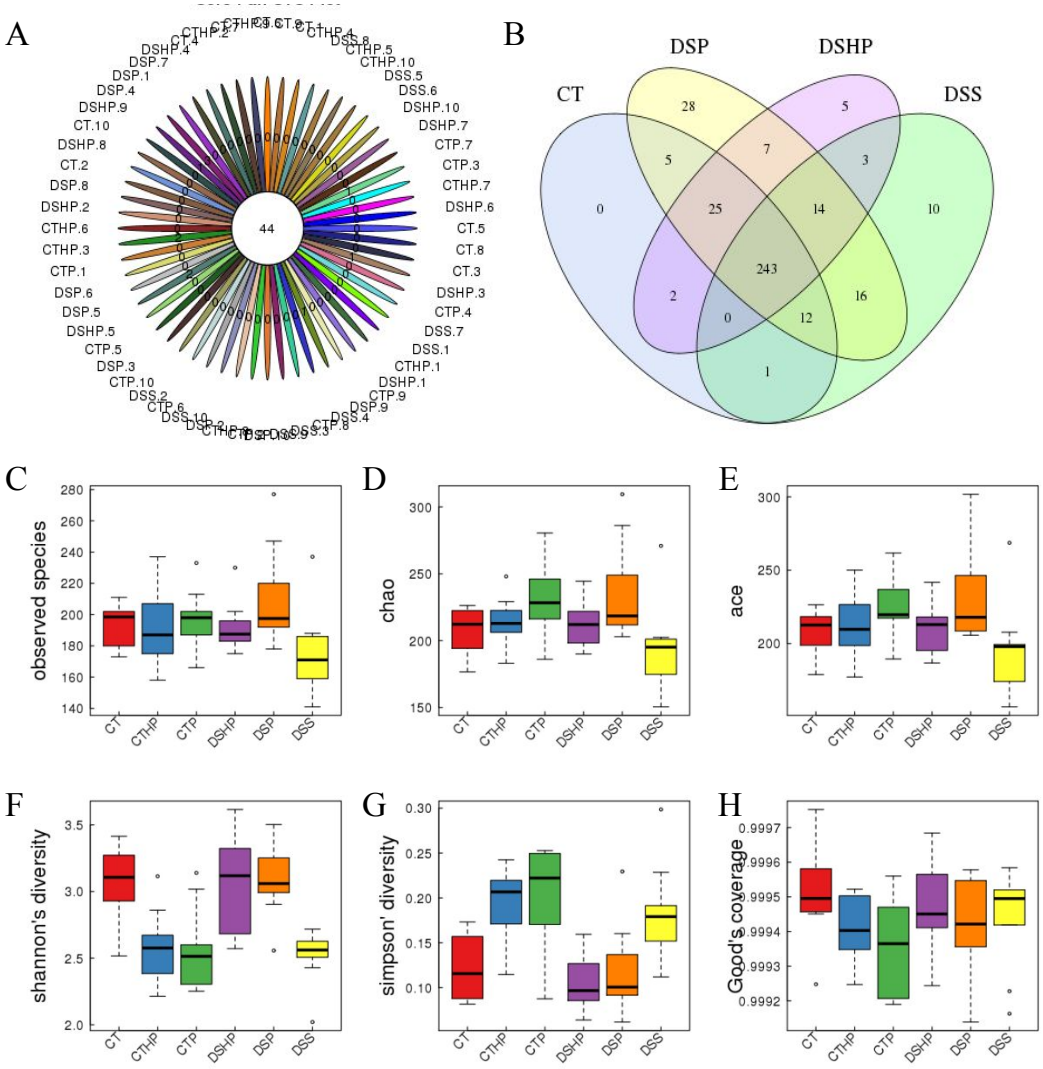

A
CT
CTP
CTHP
DSS
DSP
DSHP
B
CT
DSS
DSP
DSHP
×150
×300
×600
C
CT DSS DSP
p65
65 kDa
p50
50 kDa
p-p38
43 kDa
β-actin
45 kDa
D
CT DSS DSP
COX-2
74 kDa
iNOS
130 kDa
TLR-4
135 kDa
β-actin
45 kDa

Figure. 3


A
B
CT
DSP
DSHP
DSS
28
5
7
5
3
0
25
14
10
243
2
16
0
12
1
C
observed species
D
chao
E
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G
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H
Good's coverage
CT
CTHP
CTP
DSHP
DSP
DSS

Figure. 4

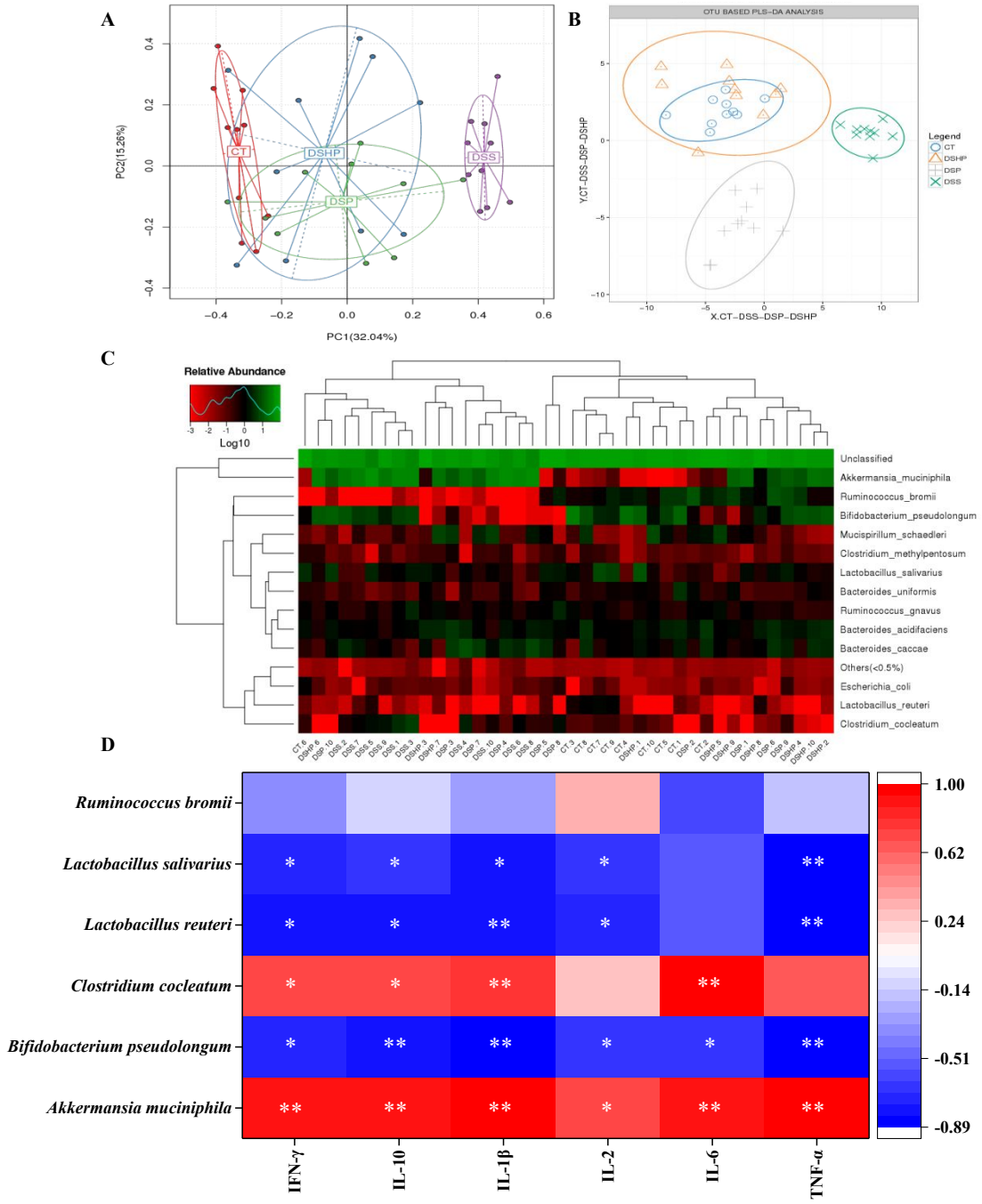
A
PC2(15.26%)
PC1(32.04%)
CT
DSHP
DSP
DSS
B
OTU BASED PLS-DA ANALYSIS
Y:CT-DSS-DSP-DSHP
X:CT-DSS-DSP-DSHP
Legend
CT
DSHP
DSP
DSS
C
Relative Abundance
Log10
Unclassified
Akkermansia_muciniphila
Ruminococcus_bromii
Bifidobacterium_pseudolongum
Mucispirillum_schaedleri
Clostridium_methylpentosum
Lactobacillus_salivarius
Bacteroides_uniformis
Ruminococcus_gnavus
Bacteroides_acidifaciens
Bacteroides_caccae
Others(<0.5%)
Escherichia_coli
Lactobacillus_reuteri
Clostridium_cocleatum
D
Ruminococcus bromii
Lactobacillus salivarius
Lactobacillus reuteri
Clostridium cocleatum
Bifidobacterium pseudolongum
Akkermansia muciniphila
IFN-γ
IL-10
IL-1β
IL-2
IL-6
TNF-α
1.00
0.62
0.24
-0.14
-0.51
-0.89

Table. 1 Effects of different treatments on mice body weight (n=10).

Treatments	Body Weight							
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
CT	34.1 ± 2.28	34.9 ± 2.88	36.6 ± 2.67	37.7 ± 2.67	39.3 ± 3.23	40.0 ± 3.50	40.7 ± 4.11	40.8 ± 3.71
CTP	33.9 ± 2.18	34.6 ± 2.37	35.7 ± 2.95	37.6 ± 3.13	38.5 ± 3.72	38.7 ± 3.92	39.7 ± 4.08	39.8 ± 3.85
CTHP	34.1 ± 1.97	34.7 ± 2.00	35.6 ± 2.17	36.9 ± 2.92	38.5 ± 2.88	38.6 ± 2.27	39.2 ± 2.70	39.6 ± 3.10
DSS	34.0 ± 1.63	34.6 ± 1.78	36.1 ± 2.47	37.0 ± 2.45	38.8 ± 2.82	39.0 ± 3.35	39.1 ± 3.92	39.7 ± 3.91
DSP	34.2 ±1.23	34.7 ± 1.83	35.3 ± 2.58	36.9 ± 2.84	37.9 ± 3.41	38.6 ± 3.37	38.7 ± 3.30	39.5 ± 2.80
DSHP	34.2 ± 1.23	34.3 ± 1.83	36.1 ± 2.47	37.9 ± 1.91	39.1 ± 2.13	40.0 ± 2.71	40.3 ± 2.26	41.0 ± 2.16

The present data were expressed the mean ± SD.

Table. 2 The tissue indexes and colon length of mice with different treatments (n=10).

Treatments	Tissue indexes			Colon length (cm)
	Liver	Spleen	Kidney	
CT	0.053 ± 0.003	0.003 ± 0.001	0.014 ± 0.002	10.99 ± 0.90
CTP	0.052 ± 0.005	0.002 ± 0.001	0.015 ± 0.002	11.93 ± 0.94
CTHP	0.052 ± 0.006	0.003 ± 0.002	0.015 ± 0.003	11.41 ± 0.69
DSS	0.056 ± 0.005	0.004 ± 0.002	0.017 ± 0.003	9.31 ± 0.59*
DSP	0.055 ±0.004	0.003 ± 0.001	0.017 ± 0.002	10.81 ± 0.67
DSHP	0.053 ± 0.005	0.003 ± 0.001	0.016 ± 0.001	10.89 ± 1.20

The present data were expressed the mean ± SD.

* means significant difference between DSS group and CT group, $p < 0.05$

Table. 3 Effects of WPEP gavage on disease score indexes of colitis mice (n=10).

Treatments	Disease score indexes								
	Day 0	Day 7	Day 11	Day 18	Day 22	Day 29	Day 33	Day 40	Day 44
DSS	0	0	0.70 ± 0.27	0	1.60 ± 1.00	0.78 ± 0.24	2.67 ± 0.38	1.29 ± 0.52	2.78 ± 0.50
DSP	0	0	0.30 ± 0.05	0	0.50 ± 0.29	0.40 ± 0.17	1.60 ± 0.44	0.20 ± 0.04	1.60 ± 0.57
DSHP	0	0	0.30 ± 0.15	0	1.10 ± 1.16	0.30 ± 0.13	1.50 ± 0.44	0.40 ± 0.13	1.80 ± 0.17**

The present data were expressed the mean ± SD.

** means extremely significant difference between DSS group and DSHP group, $p < 0.01$.

Table. 4 Regulation of WPEP on immune cells proportion in colonic mucosa of mice with different treatments (n=10).

Treatments	Immune cells proportion (%)		
	CD 45+ Immune Cells	CD45+F480+ Macrophage	CD45+Gr1+ Neutrophil
CT	2.42 ± 1.16	8.14 ± 5.48	8.44 ± 5.78
DSS	5.73 ± 1.82**	25.40 ± 10.70**	28.13 ± 12.29**
DSP	2.90 ± 1.59	11.70 ± 4.76	12.28 ± 4.14
DSHP	3.12 ± 1.58	6.35 ± 3.43	8.48 ± 2.85

The present data were expressed the mean ± SD.

** means extremely significant difference between DSS group and DSHP group, $p < 0.01$.

Table. 5 Regulation of WPEP on pro-inflammatory cytokines secretion in colonic mucosa of mice with different treatments (n=10).

Treatments	Pro-inflammatory cytokines concentrations (pg/mg)					
	IL-1β	IL-2	IL-6	TNF-α	INF-γ	IL-10
CT	11.00 ± 2.03	0.03 ± 0.01	1.15 ± 0.60	2.46 ± 0.70	0.08 ± 0.06	0.09 ± 0.05
DSS	95.76 ± 25.67**	0.43 ± 0.29*	100.87 ± 33.87**	15.32 ± 3.44**	1.75 ± 0.82**	1.26 ± 0.43**
DSP	40.74 ± 9.18**	0.13 ± 0.06*	6.10 ± 2.18**	6.18 ± 1.25**	0.45 ± 0.27*	0.49 ± 0.40*

The present data were expressed the mean ± SD.

* means significant difference compared to CT group, $p < 0.05$; ** means extremely significant difference compared to CT group, $p < 0.01$.

Table. 6 Relative abundances of specific gut microbiota species displayed with significant differences in mice with different treatments (n=10).

Treatments	Relative abundances of specific gut microbiota species (%)					
	Akkermansia muciniphila	*Clostridium cocleatum*	*Bifidobacterium pseudolongum*	*Lactobacillus reuteri*	*Lactobacillus salivarius*	*Ruminococcus bromii*
CT	0.008 ± 0.004	0.100 ± 0.066	15.39 ± 8.89	0.63 ± 0.48	3.66 ± 2.40	1.95 ± 1.54
DSS	35.80 ± 9.10**	2.34 ± 1.78*	3.48 ± 2.72*	0.007 ± 0.002**	1.23 ± 0.87*	0.009 ± 0.001**
DSP	18.03 ± 12.70*	0.043 ± 0.015	3.35 ± 1.14*	0.075 ± 0.059**	1.83 ± 1.32	4.39 ± 2.44
DSHP	18.24 ± 6.23*	0.011 ± 0.003	9.65 ± 3.74	0.21 ± 0.12*	2.22 ± 1.53	3.83 ± 1.98

The present data were expressed the mean ± SD.

* means significant difference compared to CT group, $p < 0.05$; ** means extremely significant difference compared to CT group, $p < 0.01$.