

## Research Article

# Protease Hydrolysates Ameliorates Inflammation and Intestinal Flora Imbalance in DSS-Induced Colitis Mice

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Wheat germ and fish skin usually has not been completely utilized and sometimes may be discarded, thus causing a lot of waste. Here, we aim at exploring the therapeutic anti-inflammatory effects of protease hydrolysates of wheat germ and fish skin on the ulcerative colitis (UC) mice. In the current study, wheat germ protein hydrolysates (WGPH) and fish skin gelatin hydrolysates (FSGH) treated mice had a longer colon than the DSS-induced mice. Moreover, protease hydrolysates reversed DSS-induced gut dysbiosis. Protease hydrolysates were likely to shift the balance of the intestinal flora on inflammation. In summary, these findings suggested that protease hydrolysates might serve as a latent therapy for UC treatment.

## 1. Introduction

Ulcerative colitis (UC) is recognized as a type of chronic and recurrent inflammatory bowel disease (IBD) [1]. Unfortunately, UC patients often experienced abdominal pain and bloody diarrhea, and the course of the disease is quite long [2]. Additionally, the recurrence of UC is always along with grave complications and disappointing prognosis [3, 4]. Moreover, the incidence of UC has been rising year by year, particularly in the developed countries [5]. However, the etiology of UC is still unclear. Thus, it is emerging to find effective therapy for UC.

The continuous improvement in the understanding of UC has evidenced that heredity, environmental factors, dietary habits, immune factors, and gut microbiota are intently related to the occurrence of UC [6, 7]. Among them, the interaction between gut microbiota and the host immune system is considered as the major origin of colonic inflammation [8, 9]. Moreover, the amount of some ill bacteria (including *Bacteroides fragilis*, *Escherichia coli*, and *Helicobacter*) is flourishing in the UC patients' intestinal tract, which is markedly higher than that in healthy ones and is probably connected to the incidence of UC [10, 11]. On this basis, researchers have observed the correlation between UC and gut microbiota [12]. The ordinary gut microbiota

composes the intestinal mucosal barrier of the human body and shields the intestines [13]. Once the intestinal flora is disturbed, it may cause inflammation of the colon [14].

The incidence of inflammation is increasing year by year, and the drugs for treating inflammation are always expensive along with side effects [15, 16]. Given the concerns about the side effects of long-term use of these drugs, food-based functional food as alternatives to anti-inflammatory drugs has become a research hotspot [17]. A number of studies have reported that some nutrients or food components exert beneficial effects on UC well beyond their conventional nutritional value [18]. Previous studies have shown that the wheat oligopeptides have a protective effect on DSS-induced colitis in mice [19, 20]. Vitexin and anapigenin-8-C-glucoside have been shown that they could possess the positive effects on human health as well as gut microbiota [21]. Corn proteolysis product has been proved to exert a good anti-inflammatory effect on trinitrobenzene sulfonic acid-induced colitis in rats [22]. Fish protein hydrolysates have multiple biological activities, containing antioxidative, anticancer, lipid homeostasis modulation, antihypertensive activities, anti-inflammatory, and neuroprotective effects which make them promising nutraceutical ingredients for application in food [23]. Moreover, oral

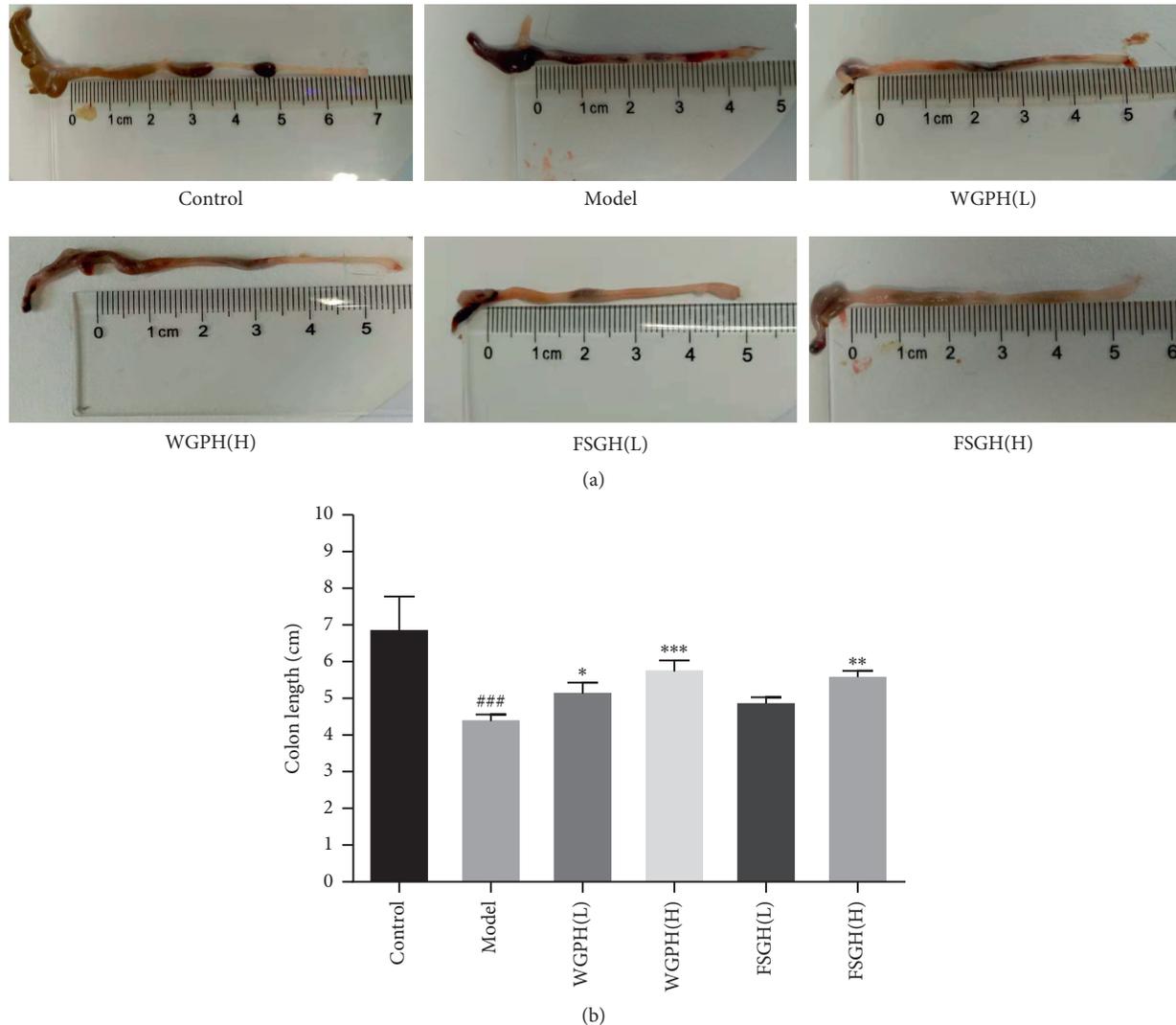


FIGURE 1: Protease hydrolysates could alleviate the symptoms of DSS-induced UC mice. (a) Protease hydrolysates validly improved the shorten of the colon tissue. (b) Quantitative results of colon length in different groups. ### $P < 0.001$  vs. control; \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. model.

bovine collagen peptides have also been proved to possess the analgesic and anti-inflammatory effects on patients with arthritis [24].

However, the specific mechanism of the effects of WGP and FSG on colitis is still unclear. Therefore, the DSS-induced UC mouse model was utilized to detect the intervention of foodborne peptides on UC and observe the effects of peptides on the intestinal tract, which would provide new idea for the treatment of UC.

## 2. Materials and Methods

**2.1. Protein Isolation Preparation.** Wheat gluten protein peptide was extracted from our laboratory (Jiangsu Jianda Wukesong Biotechnology Co., Ltd). Male c57 bl/6 mice, 6–8-week-old, weight 18~24 g, were selected and purchased from Jiangsu University Experimental Animal Center, license SCXK (Su) 2018–0012. Dextran

sulfate sodium salt (DSS) salt buy in MP Biomedicals Company.

The raw wheat germ was obtained and cleaned to remove contaminants. N-hexane (1 : 8, w/w) was used to defat the wheat germ flour [25, 26], and the defatted wheat germ flour (DWGF) was scattered into the 1M NaCl solution (1 : 8, w/v) and stirred for 30 min at ambient temperature [27, 28]. Then, the pH was adjusted to 9.5. After stirring for 30 min, the suspension was centrifuged at 8,000 g for 20 min at 4°C. Then, the pH of the supernatant was adjusted to 4.0 and centrifuged again at 8,000 g for 20 min at 4–8°C. Finally, the precipitate was obtained [29, 30].

The skin of tilapia was homogenized with distilled water (1 : 20, w/v). Alcalase (7674 U/g protein) was added to the suspension, and the pH was adjusted to 9.0. Then, it was hydrolyzed for 4.92 h at 55°C. Afterwards, the reaction was terminated by heating the solution in a waterbath at 95°C for 20 min. The supernatant was

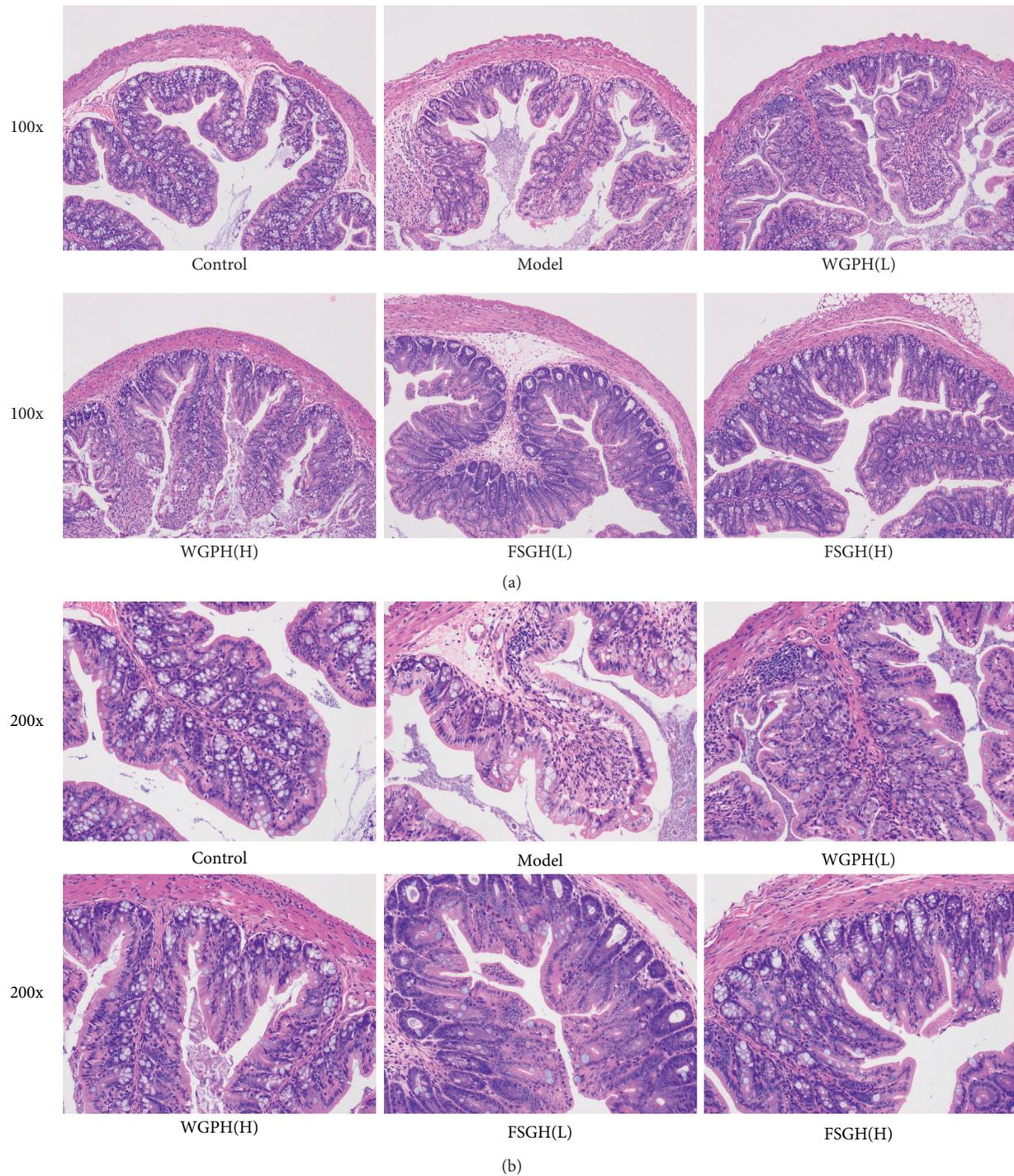


FIGURE 2: Histological observation and evaluation. (a) Representative photos of colonic segments ( $\times 100$ ). (b) Representative photos of colonic segments ( $\times 200$ ).

collected by centrifugation at 10,000 rpm for 15 min and then freeze-dried [31].

**2.2. The Design of Experiments.** Sixty male C57BL/6 mice (6–8-week-old) were obtained from Jiangsu University and housed at a constant temperature and humidity room. All mice were divided into 6 groups randomly with 10 mice each; they

were the control group, DSS group, DSS + WGPH (300 mg/kg-d), DSS + WGPH (600 mg/kg-d), DSS + FSGH (300 mg/kg-d), and DSS + FSGH (600 mg/kg-d). We treated the mice in the control group with pure water, and 3% DSS solution was supplied to the other five groups to induce UC disease. The WGPH and FSGH were orally administered once a day at the same time according to the abovementioned volume for 7 days. The control and DSS groups were given normal saline, and all

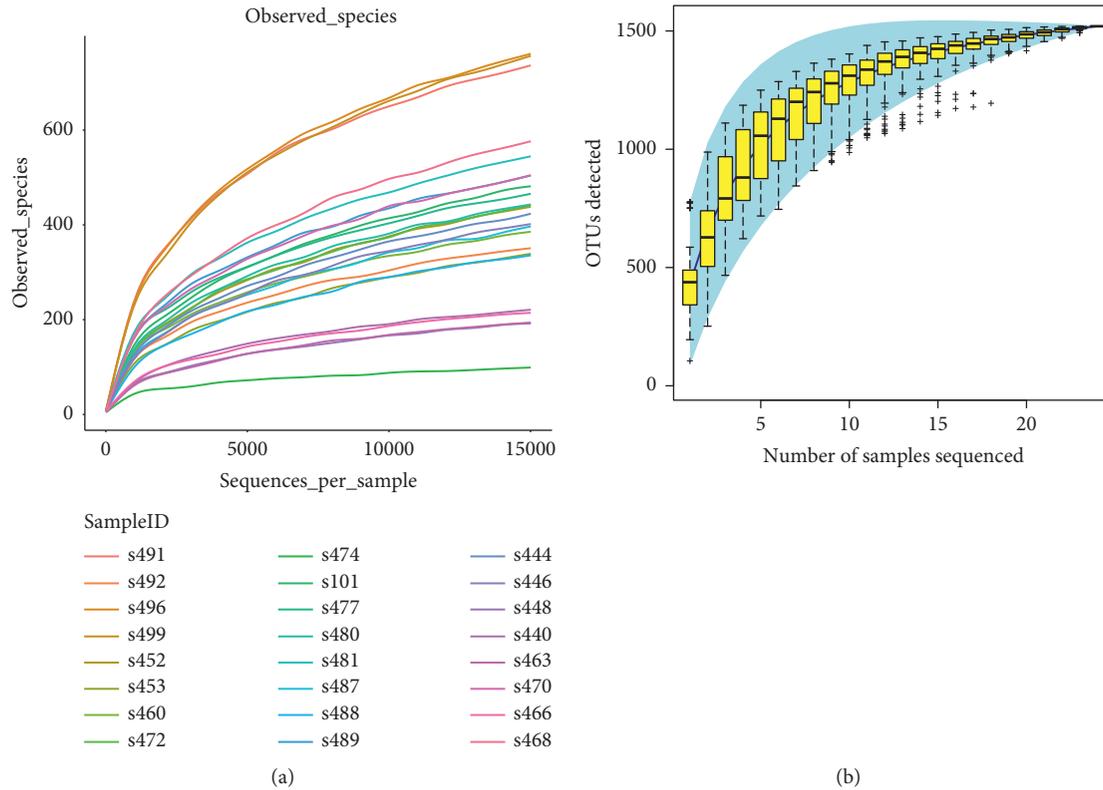


FIGURE 3: Rarefaction curves species (a) and accumulation curves (b) OTUs clustered at 97% sequence identity of all the samples.

the mice were endowed for cervical dislocation on the 15th day for anatomical sampling. All experiments were approved by the Institution Animal Care and Use Committee of Jiangsu University (UJS-IACUC-201920960) and met the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**2.3. HE Assay.** The mice' colon tissues were cut into 5  $\mu\text{m}$  thick slices. After dewaxing and hydration, the slices were incubated in hematoxylin solution for about 3–5 min and later hatched in ethanol hydrochloric acid for 15 sec [32]. Subsequently, the sections were washed by PBS solution, and then, the sections were placed in eosin solution and incubated for about 2–3 min. Ultimately, the slices were dehydrated and transparent, and then, the staining results could be observed under a light microscope (Olympus, Tokyo, Japan) [33].

**2.4. Gut Microbiota Analysis.** For gut microbiota analysis, first, we collected the mice fecal samples and obtained the DNA of different groups through the Stool DNA Kit (Omega Bio-Tek, Inc., USA) according to the manufacturer protocols. Then, the spectrophotometer, as well as 1% agarose gel electrophoresis, was separately used for detecting the concentration and purity of microbial DNA. Next, the V3-V4 region of the bacteria 16S rRNA was enlarged, and two general primers 343F (TACGGRAGGCAGCAG) and 798R (AGGGTATCTAATCCT) were used through PCR assay.

The conditions of PCR assay were listed as follows: primary denaturation at 94°C for 5 min; 32 cycles of 94°C for 30 sec, annealing at 55°C for 30 sec, and extension 72°C for 60 sec; and the final step was an extension at 72°C for 7 min. PCR amplification was carried out in a total volume of 25  $\mu\text{L}$  reaction mixture including 3  $\mu\text{L}$  BSA, 12.5  $\mu\text{L}$  2xTaq Plus Master Mix, 30 ng of template DNA and ddH<sub>2</sub>O, and 1  $\mu\text{L}$  of each primer. We collected and purified the PCR products via QIAquick Gel Extraction Kit (QIAGEN, Germany). After that, we prepared the amplicon pools for sequencing, size, and quantity of the amplicon library through the Agilent 2100 Bioanalyzer (Agilent, USA) as well as the Library Quantification Kit for Illumina, separately [12].

Next, the samples were then sequenced through the Illumina HiSeq platform following the manufacturer's instructions (LC-Bio). Flash was used to assign, truncate, and merge the paired-end reads. We assigned the sequences with  $\geq 97\%$  similarity to the same operational taxonomic units (OTUs) via VSEARCH (v2.3.4). After choosing the representative sequences for each OTU, the RDP (Ribosomal Database Project) classifier was then used to assign the taxonomic data. Then, we used the map software (V 7.310) to investigate the differences of the dominant species and utilized the QIIME (Version 1.8.0) to calculate all of the indices in our samples [34].

**2.5. Statistical Analysis.** SPSS (18.0 version, SPSS, Inc., USA) and GraphPad (6.0 version) were used to determine each group's statistic data. All data were presented as means  $\pm$  SD.

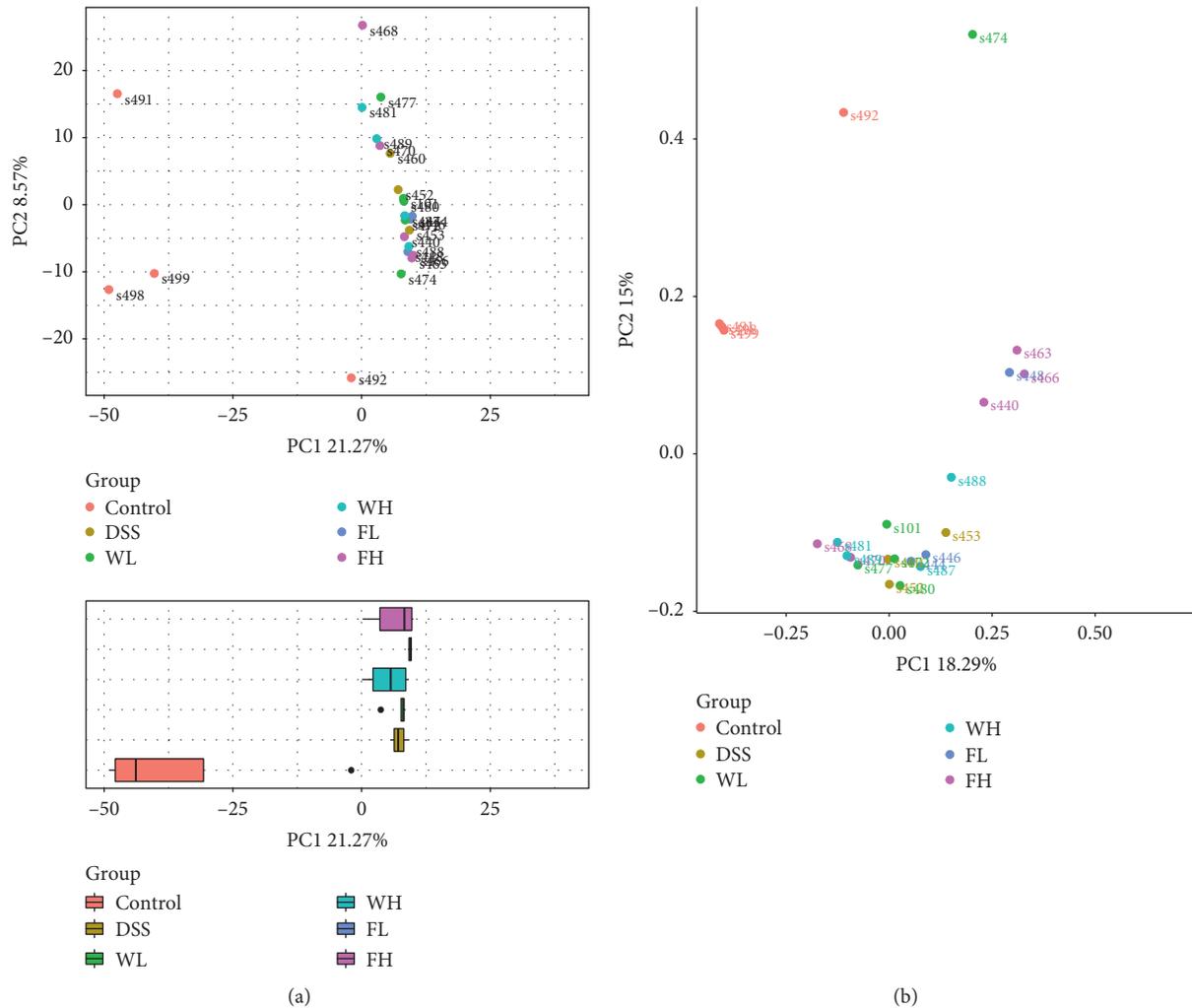


FIGURE 4: The variation of fecal microbiota based on 16S rRNA gene sequences among different treatment group samples including principal component analysis (PCA) (a) and principal coordinates analysis (PCoA) (b).

All experiments were independently implemented at least three times. Moreover, Student' *t*-test and one-way ANOVA were used to compare two groups or more than two groups. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

### 3. Results

**3.1. Protease Hydrolysates Could Alleviate the Symptoms of DSS-Induced UC Mice.** As shown in Figures 1(a) and 1(b), it could be seen that the colon length was markedly shortened in the model group, when comparing with control group. The colon length in the DSS + WGPH and DSS + FSGH groups was significantly longer than that in the DSS group. The colon length of the high-concentration group of DSS + WGPH was longer than that of the low-concentration group. Compared with the model group, the colon longer changes induced by different concentrations of WGPH in mice were alleviated to a certain extent, and the improvement effect of high-concentration (600 mg/kg.d) was better than that of low-concentration WGPH (300 mg/kg.d).

**3.2. Histological Observation and Evaluation.** As shown in Figures 2(a) and 2(b), the control group expressed normal colonic mucosal epithelium and H&E staining of the blank group showed normal colonic mucosal epithelium. On the contrary, the model group exhibited broad-scale glandular destruction and notably appeared inflammatory cell infiltration in the submucosa with the induce of DSS. What is more, compared with the model group, DSS + WGPH and DSS + FSGH groups both expressed a milder degree of congestion and edema.

### 3.3. Microbial Diversity Analysis

**3.3.1.  $\alpha$ -Diversity Analysis.** As shown in Figure 3, the rarefaction curve (Figure 3(a)) and species accumulation curves (Figure 3(b)) evidenced that the curve of each treatment group tends to be flat, which indicated that the amount of sequencing data was reasonable and the sample depth was reliable. It has been showed that the sample size and the library volume we constructed were large enough to represent the vast majority of bacteria in the human intestinal

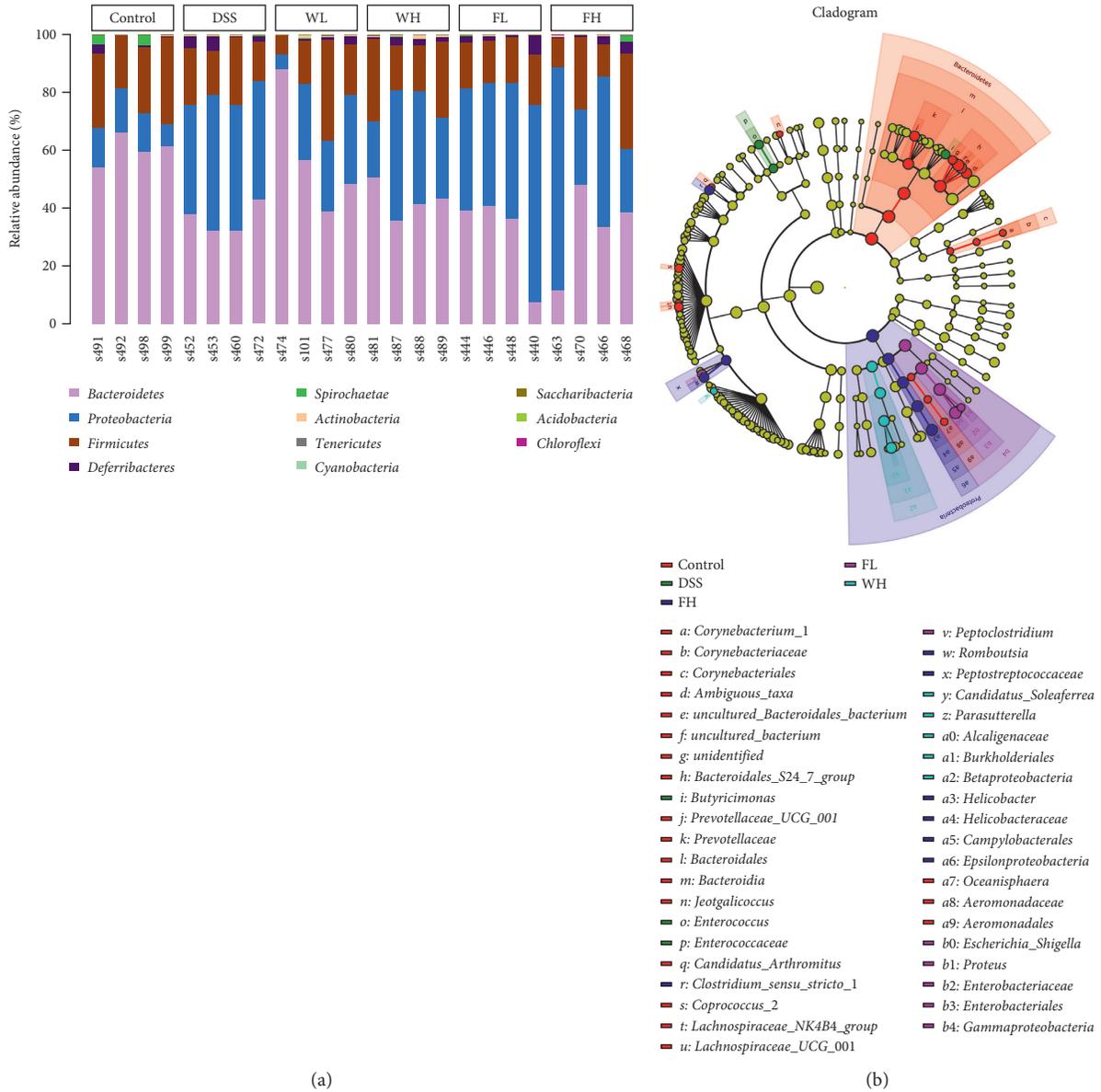


FIGURE 5: Continued.

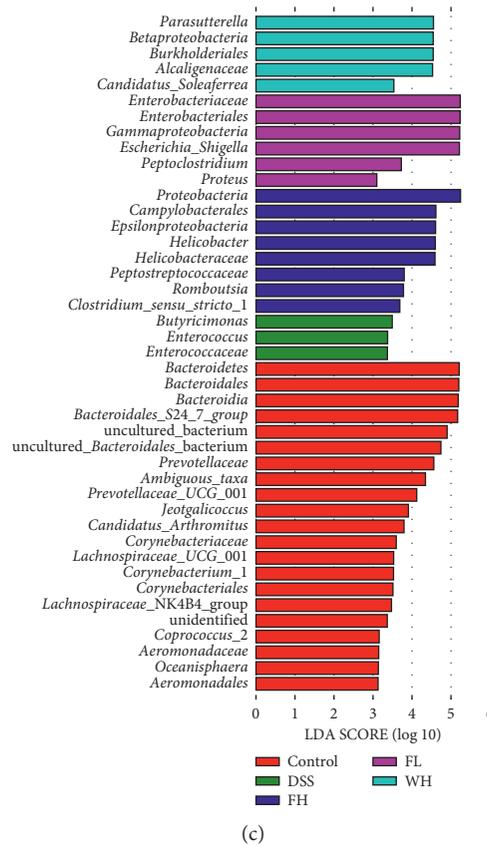


FIGURE 5: Structural comparison of fecal flora among different groups. (a) Bacterial taxonomic profiling at the phylum level. (b) LEfSe analysis of fecal flora among different groups. (c) LEfSe analysis showing the distribution histogram of different groups in gut microbiota (LDA score > 4).

flora, and the species richness and library diversity were saturated.

**3.3.2.  $\beta$ -Diversity Analysis.** The results of principal component analysis (PCA) (Figure 4(a)) and principal coordinate analysis (PCoA) of weighted (Figure 4(b)) suggested no overlap between the control group and DSS model group, demonstrating that the number of differential OTUs was high. The samples of the WGPH high-dose administration group and FSGH high and low-dose administration groups were similar to those of the normal group.

**3.3.3. Cluster Analysis.** Metastatistical analysis illustrated that, at the phylum level, there were three phyla, containing *Bacteroidetes*, *Proteobacteria*, and *Firmicutes*, that displayed significant differences in the relative abundance between the control and model groups. Furthermore, these three phyla in the model group also displayed apparent differences in the relative abundance with the hydrolysates group (Figure 5(a)). In an in-deep step, to identify the fecal microbiota meaningful changes among the different groups, the relative abundance of 44 genera was presented by branch diagram via LEfSe analysis (Figures 5(b) and 5(c)). Clearly, different groups showed different genera levels. As shown in Figures 5(b) and 5(c), *Bacteroidales*, *Prevotellaceae*,

*Ambiguous\_taxa*, *Prevotellaceae\_UCG\_001*, *Corynebacteriaceae*, *Lachnospiraceae*, and *Aeromonadaceae* are the major components of the intestinal community of rats in the normal group. *Enterococcaceae*, *Butyricimonas*, and *Enterococcus* are the components of the intestinal community of rats in the model group. *Alcaligenaceae*, *Burkholderiales*, *Betaproteobacteria*, and *Parasutterella* were shown as the important parts of the high dose of the WGPH group and *Enterobacteriaceae*, *Gammaproteobacteria*, *Escherichia\_Shigella*, *Proteobacteria*, *Campylobacteriales*, and *Epsilonproteobacteria* are the principal parts of different dose of the FSGH group. To further identify the top 10 characteristic bacterial genera among the different groups, as shown in Figure 6, DSS increased the relative abundance of *Parasutterella*, *Allobaculum*, *Desulfovibrio*, *Lachnospiraceae*, and *Mucispirillum*. Meanwhile, DSS decreased the relative abundance of *Escherichia\_Shigella*, *Bacteroides*, *Helicobacter*, *Parabacteroides*, and *Alloprevotella*. However, the abundances of the main bacterial genus could significantly be changed by WGPH and FSGH (Figure 6).

## 4. Discussion

Colitis, a type of inflammatory bowel disease, is recognized as one of the world's refractory diseases due to its unknown cause, difficulty in healing, and recurring attacks [1]. Its

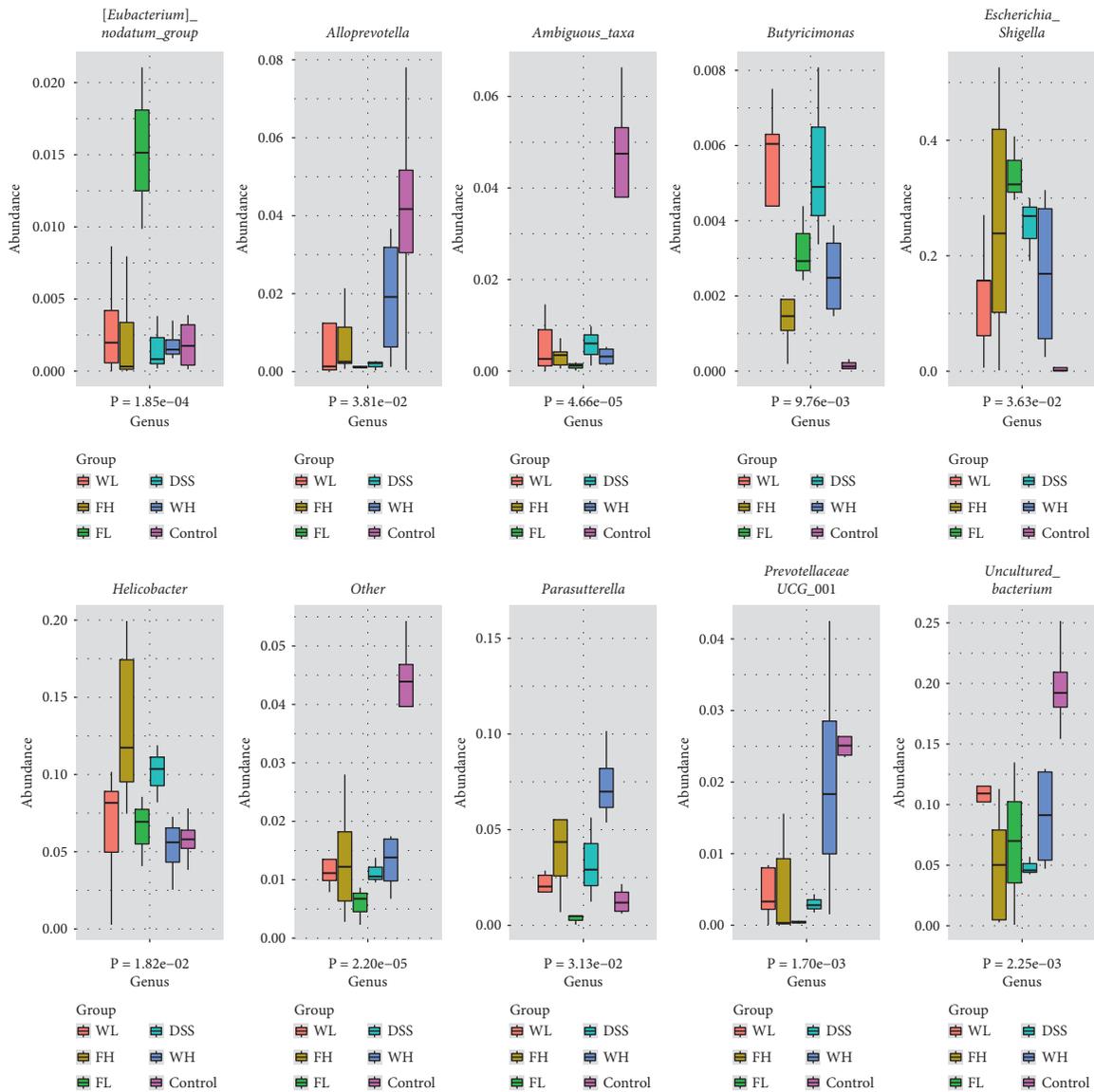


FIGURE 6: Boxplot analysis of relative abundance of the top 10 species among different groups at the genus level.

etiology may involve genetics, environment, diet, immunity, psychology, and many other aspects [6, 7]. The current treatments are mostly aminosalicic acid drugs (such as sulfasalazine), adrenal glycosides, and immunosuppressants [35]. However, these treatments generally have large side effects and limited efficacy. Preventing and reducing the side effects of drugs for the treatment of inflammatory bowel disease [15, 16] and at the same time, developing new, safe, and effective drugs that interfere with inflammatory bowel disease has become one of the research hotspots in the global medical field [17]. Foodborne protease hydrolysates, as natural polymer compounds, have a wide range of sources [36]. They are safe and nontoxic and also have a wide range of functional activities such as antiangiogenesis, anti-oxidation, immune regulation, three high (high sugar, high fat, and high blood pressure), antiaging, anti-inflammatory, and so on [37, 38]. According to research reports at home and abroad, protease hydrolysates have received extensive

attention in the treatment and improvement of inflammatory bowel disease [39].

DSS-induced UC in mice is one of the most mature methods at present [40]. The influencing factors of DSS modeling include molecular weight and concentration of DSS, research environment, mouse species, and administration time [41]. In our study, after 7 days of DSS intervention, the mice in the DSS group lost weight and shortened the length of colon. The colon length and histopathological score in the polypeptides group were much improved than that in the DSS-induced model group.

Gut microbiota is the most complex and the highest population of symbiotic microbial ecosystems in the human body. They are recognized as an essential factor in the body's health and homeostasis [13]. They have profound effects on human physiology and nutrition [42]. Besides, the changes in gut microbiota are likely to be related to intestinal diseases [14]. Studies based on 16S rRNA gene sequencing showed

that the two major categories of *Bacteroides* and *Firmicutes* are the main groups of intestinal microbes. The typical symptoms of UC are inflammation and ulcers in the rectum, colonic mucosa, and submucosa [43]. Gut microbiota is a key protective factor of the intestinal mucosa [44]. Previous studies have reported colonizing the fecal bacteria from healthy donors into patients with inflammatory bowel disease, which could significantly improve the symptoms of patients [45]. Previous studies expressed that microbial diversity research could reflect the abundance and diversity of microbial communities through a single-sample diversity analysis (alpha diversity), which included a series of statistical analysis indexes to estimate the species abundance and diversity of environmental communities [46, 47]. In our study, the results showed that the intestinal flora balance system of DSS-induced colitis mice was severely broken, and the intake of wheat germ protein hydrolysates (WGPH) from wheat as well as the fish skin gelatin hydrolysates (FSGH) from tilapia skin could alleviate the imbalance of flora and reduce the symptoms of inflammatory diseases. Based on 16S rRNA gene sequencing, the content samples of mice in the normal group, DSS-induced colitis mice group, and different dose of hydrolysates treatment groups were analyzed. After analyzing the  $\alpha$ -diversity between different groups, the results evidenced that the sample size and the library volume we constructed were large enough to represent the vast majority of bacteria in the human intestinal flora, and the species richness and library diversity were saturated. PCoA analysis expressed that the distance between the control group and the DSS group was far from each other, indicating that there was a great difference in the composition of the flora between the two groups [48]. After giving different doses of hydrolysates from wheat and fish protein, each administration group deviated from the model group to a certain extent, indicating that each dose could improve the intestinal flora diversity of colitis rats to a certain extent, which was the same as the previous conclusion. The study also suggests that high-dose WGPH, as well as low and high-dose FSGH, could improve the structure of the intestinal flora of rats in the model group, which indicated better colitis improvement and relief effects.

According to the results of the phylum level, there were three phyla, containing *Bacteroidetes*, *Proteobacteria*, and *Firmicutes*, that displayed significant differences in the relative abundance between the control and model groups. Furthermore, these three phyla in the model group also displayed apparent differences in the relative abundance with the polypeptides group. Moreover, considering the results of the family level, after DSS modeling, in comparison to the control group, the bacteria in the intestines of DSS-induced mice had undergone tremendous. However, the intestinal flora of the polypeptides group was closer to that of the control group. Compared with the DSS-induced model group, *Parasutterella*, *Allobaculum*, *Desulfovibrio*, *Lachnospiraceae*, and *Mucispirillum* were significantly reduced in the blank and polypeptides groups, while the relative abundance of *Escherichia Shigella*, *Bacteroides*, *Helicobacter*, *Parabacteroides*, and *Alloprevotella* increased ( $P < 0.05$ ).

In summary, the oral administration of WGPH and FSGH exhibited therapeutic effects on DSS-induced UC in mice. The wheat germ protein hydrolysates and fish skin gelatin hydrolysates could be considered as a novel attractive intestinal microecological improver for UC treatment.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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