

The Modulatory Effect of Dietary *Apostichopus japonicus* on Mice with Ulcerative Colitis Induced by Trinitrobenzene Sulfonic Acid

Hongjie Shi¹, Hong Sun², Rong Zheng¹, Shangyun Lu¹, Fang Liu¹, Na Zhang¹, Changhu Xue¹, Qingjuan Tang^{1*}

¹College of Food Science and Engineering, Ocean University of China, Qingdao, China

²Emergency Medicine, Shandong Jining No. 2 Peoples Hospital, Jining, China

Email: *tangqingjuan@ouc.edu.cn

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Abstract

Sea cucumber is a food with nutritional benefits distributing mainly in Asia, and *Apostichopus japonicus* (*A. japonicus*) is a kind of sea cucumber whose quality is better than any others. However, different processing methods make various effect on its quality. In this study, we evaluated the protection effect of *A. japonicus* with different processing methods on mice with ulcerative colitis induced by trinitrobenzene sulfonic acid (TNBS), especially on the intestinal microflora. The expression of IFN- γ /IL-4 and IL-1 β in gut, and intestinal microbiota were discussed. The results revealed that three different processing methods of *A. japonicus* could decrease the expression of inflammatory cytokines, except for the expression of IFN- γ /IL-4 treated with enzymatic, and dried *A. japonicus* was the most efficient. *A. japonicus* could change the microbiotic imbalance relatively back to normal in terms of bacterial diversity and composition, meanwhile increase the abundance of *Bifidobacteria*, *Lactobacillus* and *Clostridium leptum*. The elements of protein, polysaccharide in dried, instant, enzymatic *A. japonicus* are 73.09%, 65.06%, 57.42% and 6.72%, 5.46%, 5.45% respectively. This study indicated that *A. japonicus* have a good improving effect on ulcerative colitis, especially on the microbiome, and processing methods had an effect on alleviation of ulcerative colitis, which might be associated with content of protein and polysaccharide.

Keywords

Apostichopus japonicus, Ulcerative Colitis, Trinitrobenzene Sulfonic Acid, Intestinal Microbiota

1. Introduction

Ulcerative colitis (UC) and Crohn's disease are two major forms of inflammatory bowel disease. UC can lead to disorder of the colonic mucosa in some area and finally extends to the entire colon [1]. As a common drug, trinitrobenzene sulfonic acid (TNBS) is widely used to induce colitis in mice model [2]. Studies have suggested that UC results from growth production of cytokines, such as TNF, IL, and interferon family members [3]. IL-1 β and IFN- γ are pro-inflammatory cytokines, while IL-4 is an anti-inflammatory cytokine, which are secreted by Th1 and Th2 respectively [4]. A proper balance of the cytokines is necessary to make intestine healthy [5]. TNBS could change the cytokines, such as TNF- α , IFN- γ , IL-4 and IL-1 β , and even some study take these cytokines as UC markers [6]. As residents with a large population living in the gastrointestinal tract, gut microbiota have much relationship with UC [7]. Studies revealed that some special gut microflora could lead to UC or make it worse. *Bacteroidetes* and *Firmicutes* are dominant in small intestine, whose total population account for more than 90% in all phylotypes [8]. The different ratio of *Bacteroidetes* and *Firmicutes* usually serves as the change of microbial structure [9]. Being the probiotics, *Bifidobacteria* and *Lactobacillus* could alleviate some disease by supplying with some nutrition, such as vitamin C and so on [10]. *Clostridium leptum* is a producer of butyrate, which contains large quantity of enzymes to make use of fiber and polysaccharide [11].

It is reported that Chinese herbal formula Yupingfeng powder have anti-colitis activity, which is consisted of Radix Astragali (Huang-Qi), Rhizoma Atractylodis Macrocephalae (Bai-Zhu), and Radix Saposhnikoviae (Fang-Feng) [12]. As a chinese herb, sea cucumber is also a kind of traditional food. Sea cucumbers belong to phylum Echinodermata and have many bioactive materials such as phospholipids, and *A. japonicus* is a very precious sea cucumber which is popular in Asia as luxury food. It revealed that *A. japonicus* could restore mice intestinal mucosal immunity after being immunosuppressive [13]. Some believe that Sea cucumbers are not suitable for the UC patients, and patients are not at a good digestive absorption condition, which will make the situation even worse. Xue *et al.* reported that the different processing method might change the ingredient of sea cucummbur [14]. This study tried to explore and evaluated by gut microbiota and cytokines whether different processing methods of *A. japonicus* have a good effect on UC.

2. Materials and Methods

2.1. Materials

The fresh sea cucumbers *A. japonicus* were purchased from Nanshan markets in Qingdao. All the sea cucumbers were boiled for 10 min, then three different processes were taken. To produce dried *A. japonicus*, the boiled sea cucumbers were placed into the cold air oven at 20°C for 6 days, then soaked in distilled water for 2 days and boiled for 30 minutes, and these were soaked again for 3 days at 4°C after cooled. Like instant *A. japonicus*, the sea cucumber were put into the condiments for 1hour and sterilized at

121°C for 15 minutes. As enzymatic *A. japonicus*, the sea cucumber were dealt with papain at 50°C for 12 hours after treated like the instant one, then inactivated the papain at 100°C for 10 mins and centrifuged for supernatant. After those, they were stored in the refrigerator at -40°C. The sea cucumbers were crushed and dissolved in saline before given to the mice.

All the reagents used in the animal experiments were analytically pure.

2.2. Animal Maintenance and Treatment

Male balb/c mice (19 ± 1 g, 4 weeks old) were obtained from Vital River (Beijing, China). During the experimental period, the mice were housed in a room maintained under a 12 h light/dark cycle at 24°C and a relative humidity of $65\% \pm 15\%$. Mice had free access to standard laboratory pellet chow (Kangda, Jinan, China) and fresh water.

After one week adaption time, the mice were randomly assigned to five groups with 10 in each group following by normal, TNBS model and *A. japonicus* groups treated with three different processing methods. Except for the normal group, the others were treated with 0.2 mL 1.5% TNBS through enteroclysis from the beginning for once. The normal mice were taken saline instead as control. The *A. japonicus* groups were given $512 \text{ mg}\cdot\text{kg}^{-1}$ [13] sea cucumbers by gavage for 10 days respectively, while the normal and model groups were given saline as control. The body weights of mice were recorded every two days. The feces were collected on the 10th day. Following overnight fasting, the mice were sacrificed by cervical dislocation on the 11th day after anaesthetized with diethyl ether and the small intestine was collected for analysis. All experimental procedures were conducted according to the guidelines provided by the ethical committee of experimental animal care at Ocean University of China (OUC, Qingdao, China).

2.3. DNA Extraction

Bacterial genomic DNA was extracted from the fecal samples with the QIAamp DNA stool kit (Qiagen, Germantown, MD), according to the manufacturer's instruction.

2.4. Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis Analysis

Bacterial universal primers GC-338F (5'-CGCCCGGGCGCGCCCCGGGGCGGGG-CGGGGGCGC-GGGGGCCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGC-GGCTGCTGG-3') were used to amplify the 16S rDNA gene V3 region by PCR. The PCR amplification was carried out in a MJ Mini Personal Thermal Cycler (BIO-RAD, America). Each reaction was performed in a total volume of 50 μL containing DNA template 100 ng, $10 \times$ PCR buffer 5 μL , dNTP mix (10 mM) 3.2 μL , MgCl_2 (25 mM) 3 μL , each primer (20 mM) 0.5 μL , and 5 U/ μL of Taq DNA polymerase (Takara, Japan) 1 μL . The thermal cycler profile consisted of an initial denaturation step of 5 min at 94°C followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. DGGE was carried out with a Dcode System apparatus (Bio-Rad,

Herts, UK) and a gradient from 35% - 55% (7M urea and 40% deionized formamide were considered to be the 100% denaturant). The gel images were converted into digital data using Canoco. Principal component analysis (PCA) and cluster analysis were employed to compare the gut microbiota composition among groups. Important bands were retrieved, sequenced (Sangon Biotech Co. Ltd., Shanghai, China) and identified in Genbank, as described [15].

2.5. Real-Time Quantitative Polymerase Chain Reaction

Quantifications of *Bacteroidetes*, *Firmicutes*, *Clostridium leptum*, *Bifidobacteria* and *Lactobacillus* were assayed by quantitative real-time PCR. 25 μ L of the reaction volume was used for the quantitative real-time PCR assay that consisted of 12.5 μ L of Maxima SYBR Green qPCR Master Mix (Roche, Switzerland), 0.75 μ L 10 μ M of primers (each of forward and reverse primers), 8.5 μ L ddH₂O, and 2.5 μ L of template. The thermal conditions consisted of an initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s and extension at 72°C for 30 s. The sequences of the primers used in this study are described in **Table 1**.

2.6. Statistical Analysis

A one-way analysis of variance between different groups were operated by *SPSS V18.0* software. Data were presented as mean \pm standard error and statistical significance was defined to be $p < 0.05$.

3. Results

3.1. Changes of Structure in the Gut Microbiota by Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

The principal components analysis (PCA) of the fingerprints revealed that the mice groups were made into three clusters, with first principal component (PC1) accounting

Table 1. The 16S rRNA gene-targeted group-specific primers of bacteria.

Specific groups	Primers name	Primer sequence
<i>Bacteroidetes</i>	Bfr-F	CTGAACCAGCCAAGTAGCG
	Bfr-R	CCGCAAACCTTCACAACACTGACTTA
<i>Firmicutes</i>	Fir-F	GGAGYATGTGGTTTAATTCGAAGCA
	Fir-R	AGCTGACGACAACCATGCAC
<i>Bifidobacteria</i>	Bif-F	CCTACGGGAGGCAGCAG
	Bif-R	ATTACCGCGGCTGCTGG
<i>Lactobacillus</i>	Lac-F	AGCAGTAGGGAATCTTCCA
	Lac-R	ATTCACCGCTACACATG
<i>Clostridium leptum</i>	Clept-F	GCACAAGCAGTGGAGT
	Clept-R	CTTCCTCCGTTTTGTCAA

for 49.7% of the variation (**Figure 1(a)**). The model and dried *A. japonicus* groups were one of clusters, the normal group was another cluster, while the instant and enzymatic *A. japonicus* groups belonged to the last one. It showed that TNBS could change the composition of gut microbiota, and the cluster dendrogram indicated the dried *A. japonicus* group was closer to normal group compared with model one (**Figure 1(b)**). These data suggested that three kinds of *A. japonicus* had different effects on gut microbial imbalance induced by TNBS, and dried *A. japonicus* could keep the balance.

According to the 16S rRNA V3 region PCR-DGGE patterns, the overall composition after the TNBS treatment revealed significance different from that of normal group (**Figure 2(a)**). The bands in the model group changed significantly and comparing with normal group, the intensity of some bands decreased. This result indicated that TNBS had changed the structure and diversity of gut microbiota. The difference of gut flora between *A. japonicus* groups had no significance and the diversity index of microbiota showed that the diversity of model group increased (**Table 2**), but in the virtual figure (**Figure 2(b)**) of PCR-DGGE fingerprint dried *A. japonicus* was more similar to normal group than others.

3.2. The Significant Different Bands in Denaturing Gradient Gel Electrophoresis (DGGE) Fingerprints

In model group, intensities of band A1, bandA2, bandA4, bandA6, bandA8, bandA9, bandA10 were significantly reduced. By sequence analysis, they were belonging to *Clostridiales bacterium CIEAF 019* (99% homology), *Akkermansia muciniphila* (99%), *Barnesiella* sp. *EBA4-14* (99%), *Barnesiella intestinihominis* (99%), *Anaerostipes hadrus* (99%), *Gram-negative bacterium cL10-2b-4* (90%), *Porphyromonadaceae bacterium*

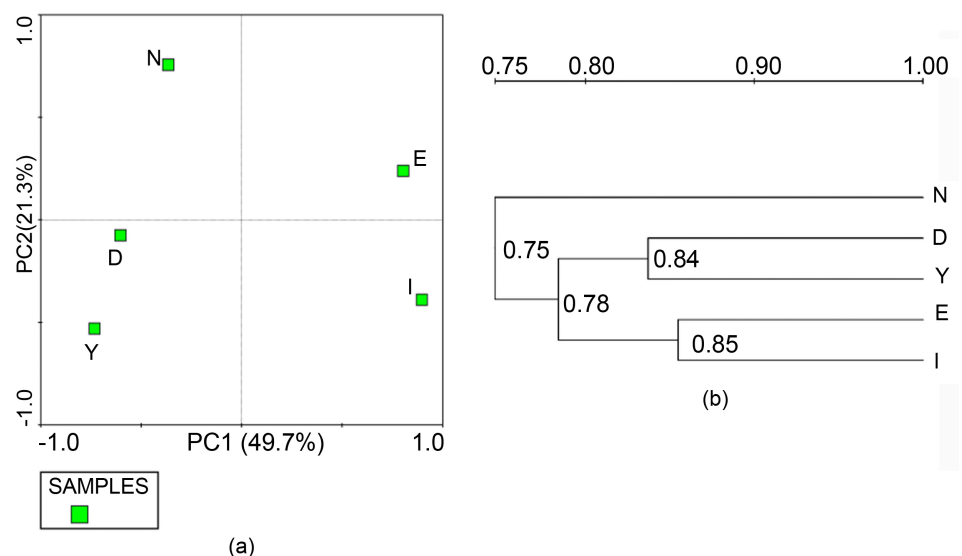


Figure 1. PCA and cluster analysis of DGGE fingerprint. (a) The principal components analysis (PCA) scores plot according to the DGGE fingerprint; (b) Clustering of gut microbiota based on distances between different groups. N serves as normal group and Y, D, J, E represent model, dried, instant and enzymatic groups, respectively.

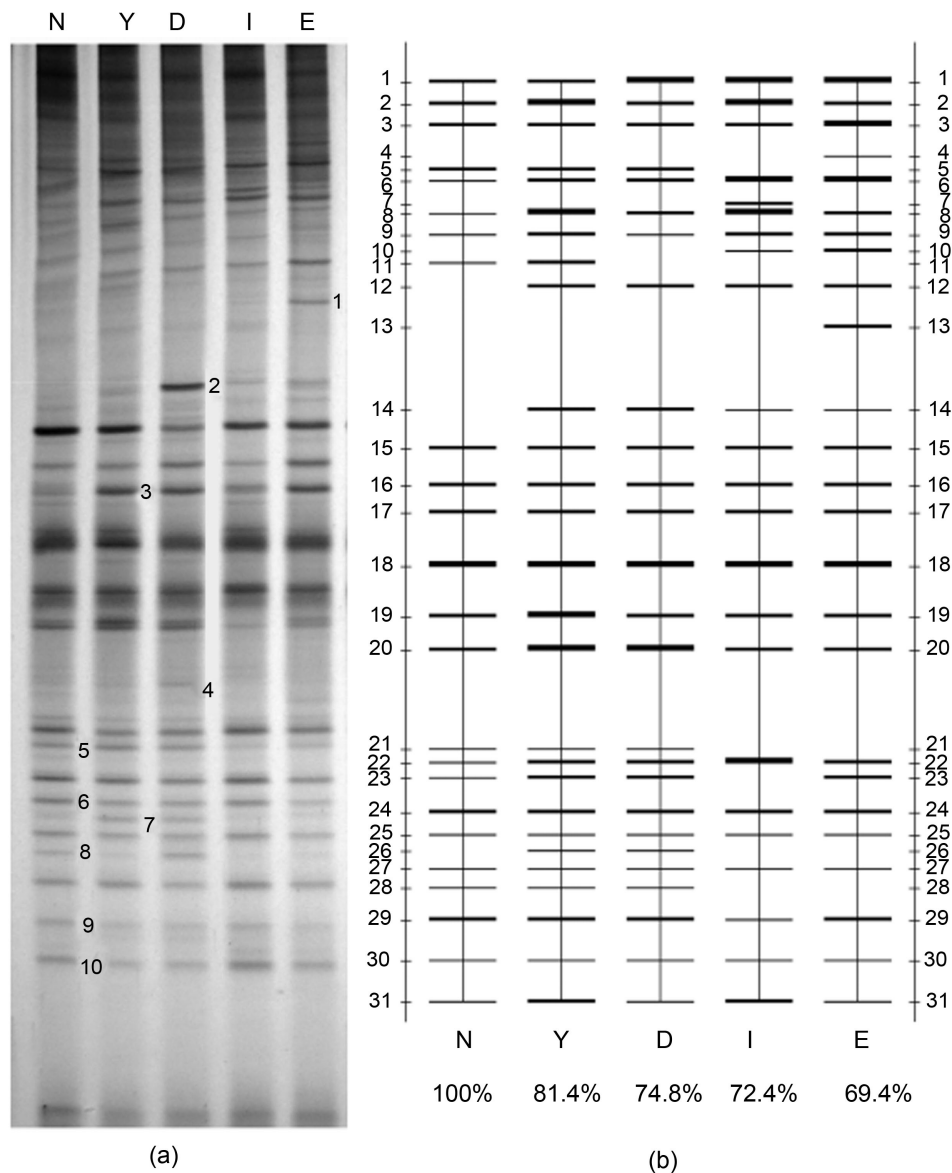


Figure 2. The difference of microbiota composition in the mouse groups. (a) Denaturing gradient gel electrophoresis (DGGE) fingerprinting of V3 region of 16S rRNA genes from faecal bacterial communities; (b) The virtual map of DGGE electrophoresis. Each sample included feces of ten mice. N is for normal group and Y, D, J, E represent model, dried, instant and enzymatic groups, respectively.

Table 2. Analysis of the microbiota diversity of mice intestine.

Groups	Shannon-Wiener Index (<i>H'</i>)	Evenness (<i>E</i>)	Richness (<i>S</i>)
N	2.979	0.925	24
Y	3.052	0.916	27
D	3.044	0.924	26
I	2.977	0.937	23
E	3.057	0.938	25

C941 (92%) (Figure 4(a) and Table 3). BandA4, A6, A10 were involved in *Porphyromonadaceae* as well as other two bands A5 and A7. Studies revealed that *Porphyromonadaceae* were related to some diseases. Band A1, A2, A8, A9 were in *Clostridia*, *Verrucomicrobiaceae*, *Lachnospiraceae* and unknown bacteria respectively. Most of *Clostridia* could digest polysaccharide and protein, which is important for the absorption of nutrition. *Verrucomicrobiaceae* increased significantly in the dried group and *Verrucomicrobiaceae* was involved in producing butyrate which had a good effect on colon.

3.3. Quantity Changes of Normal Bacteria Revealed by Real-Time Quantitative Polymerase Chain Reaction (RT-PCR)

The quantity of *Bacteroidetes*, *Firmicutes*, *Clostridium leptum*, *Bifidobacteria* and *Lactobacillus* were detected by real time-PCR. Data revealed that the ratio of *Bacteroidetes* and *Firmicutes* decreased after treatment of TNBS in model group (Figure 3(a)). *A. japonicus* could help increase the ratio significantly in the instant group. The quantity of *Clostridium leptum* decreased significantly ($p < 0.01$) in model group, while it increased in dried group (Figure 3(b)). Both *Bifidobacteria* and *Lactobacillus* decreased significantly in the model group caused by TNBS (Figure 3(c) and Figure 3(d)). *A. japonicus* could improve the quantity of *Bifidobacteria* and *Lactobacillus* but with no

Table 3. The analysis results of DGGE gel bands recovery sequence.

Band Number	Similar strain	Accession number	Similarity	Classification
BandA1	<i>Clostridiales bacterium CIEAF019</i>	AB702936	99	<i>Clostridia</i> ; <i>Clostridiales</i>
BandA2	<i>Akkermansia muciniphila</i>	NR_074436	99	<i>Verrucomicrobiaceae</i> ; <i>Akkermansia</i>
BandA3	<i>Lactobacillus reuteri</i>	EF412975	99	<i>Lactobacillaceae</i> ; <i>Lactobacillus</i>
BandA4	<i>Barnesiella sp. EBA4-14</i>	EF608208	99	<i>Porphyromonadaceae</i> ; <i>Barnesiella</i>
BandA5	<i>Porphyromonadaceae bacterium S9 HS-8</i>	KF007168	91	<i>Porphyromonadaceae</i> ; unclassified <i>Porphyromonadaceae</i>
BandA6	<i>Barnesiella intestinihominis</i>	NR_113073	99	<i>Porphyromonadaceae</i> ; <i>Barnesiella</i>
BandA7	<i>Barnesiella sp. 177</i>	KJ572412	90	<i>Porphyromonadaceae</i> ; <i>Barnesiella</i>
BandA8	<i>Anaerostipes hadrus</i>	NR_104799	99	<i>Lachnospiraceae</i> ; <i>Anaerostipes</i> .
BandA9	<i>Gram-negative bacterium cL10-2b-4</i>	AY239469	90	<i>Bacteria</i>
BandA10	<i>Porphyromonadaceae bacterium C941</i>	JF803519	92	<i>Porphyromonadaceae</i> ; unclassified <i>Porphyromonadaceae</i>

Ten important bands were cut from the gel, sequenced and then identified in Genbank.

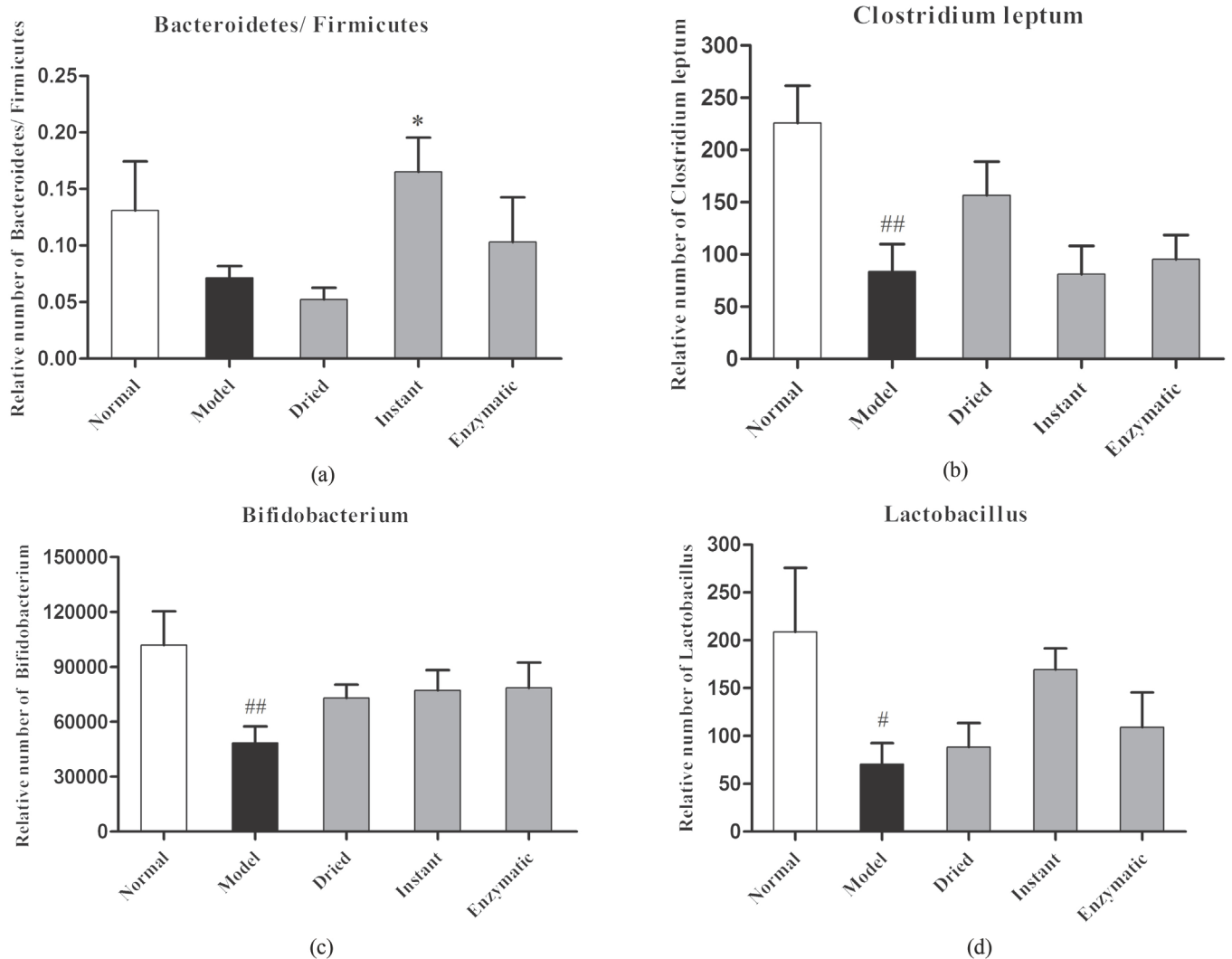


Figure 3. The quantity of gut microflora analysed by real time-PCR in different groups. (a) *Bacteroidetes* and *Firmicutes*; (b) *Clostridium leptum*; (c) *Bifidobacteria*; and (d) *Lactobacillus*. Values are expressed as mean \pm S.E.M. #Significantly different from normal group, $p < 0.05$; *Significantly different from model group, $p < 0.05$.

significance. The data indicated that *A. japonicus* administration could help against the damage of TNBS by increasing the quantity of *Clostridium leptum*, *Bifidobacteria* and *Lactobacillus*.

3.4. *A. japonicus* Inhibit the Upregulated Pro-Inflammatory Cytokines of Ulcerative Colitis Mice

The results indicated that *A. japonicus* reduced the mRNA expression of IL-1 β , while it increased significantly ($p < 0.05$) in model group (Figure 4(a)). Compared with normal group, the expression of IFN- γ /IL-4 also upregulated notably (Figure 4(b)). Dried and instant *A. japonicus* improved the expression of IFN- γ /IL-4, and the dried group had significant effects ($p < 0.01$). Three types of *A. japonicus* could decrease the expression of IL-1 β , but only dried and enzymatic groups showed statistical significance ($p < 0.05$).

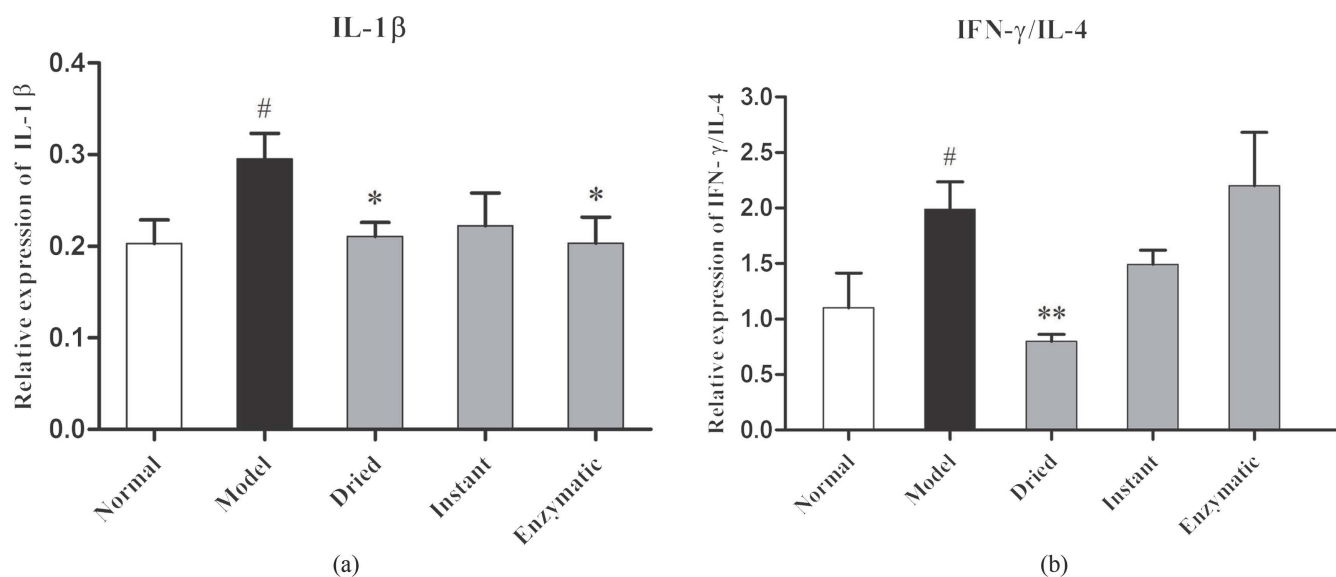


Figure 4. Relative expression of IL-4, IFN- γ and IL-1 β in small intestine. IL-4, IFN- γ and IL-1 β relative expression were analyzed by RT-PCR. Each value represents Mean \pm S.E.M of 10 mice in each group. [#]Significantly different from normal group, $p < 0.05$; ^{*}Significantly different from model group, $p < 0.05$; ^{**}Significantly different from model group, $p < 0.01$.

These data implied that *A. japonicus* might alleviate the ulcerative colitis induced by TNBS in mice.

4. Discussion

TNBS is a common drug to induce ulcerative colitis [16]. It could destroy mucosa of small intestine, which is an important barrier for organism. IFN- γ , IL-4 and IL-1 β are cytokines which play great role in the health of the intestine, while IL-4 is an anti-inflammatory cytokine and IFN- γ and IL-1 β are pro-inflammatory cytokines. However they all play important roles in maintaining intestinal mucosa. Seriously, that change leads to produce more pro-inflammation cytokines making much more suffering and be much easier for the bacteria to translocate into the inner gut [17] [18]. In this study, the expression of IFN- γ /IL-4 and IL-1 β increased significantly in the model group, and that was consistent with the reports [19] [20]. *A. japonicus* could decrease the expression of IFN- γ /IL-4 and IL-1 β , and only dried showed significance in both IFN- γ /IL-4 and IL-1 β among the three different processing methods. *Bacteroidetes* and *Firmicutes* are predominant in gut microflora. The change of ratio between *Bacteroidetes* and *Firmicutes* reflects different structures of intestinal microbiota. The majority species of *Clostridium leptum* are the producers of butyrate which is the major and important energy of intestinal epithelial cells of colon. Some bacteria are good for the body, such as *Bifidobacteria* and *Lactobacillus*, and they can provide some nutritions by fermentating fiber or hydrlyzing protein and fat acid. The results of PCR-DGGE and RT-PCR indicated that cyclophosphamide broke the balance of gut microbiota, including reducing microbial diversity and composition, and abundance of the ratio between *Bacteroidetes* and *Firmicutes*, *Clostridium leptum*, *Bifidobacterium* and *Lactobacillus*.

A. japonicus could reverse all of these changes. The instant and enzymatic could increase the ratio of *Bacteroidetes* and *Firmicutes*, while the three methods all could improve the abundance of *Clostridium leptu*, *Bifidobacteria* and *Lactobacillus*. The instant had the best effect on regulating the *Bacteroidetes/Firmicutes* and *Lactobacillus*, with the dried regulating *Clostridium leptum*, but they all got no difference on the *Bifidobacteria*.

As a big family living in the gut, intestinal flora have much effect on the health [21]-[23]. So we drew attention to the intestinal microflora. The gastrointestinal tract in an adult human being has been measured to contain approximately 500 to 1000 distinct bacterial species [24]. Their DNA is more than 10 times of an adult, and this give them an ability to synthesize many kinds of enzymes, some of which even don't exist in mammals. That made them more capable to help hydrolyze and absorb some complex component, especially for fermentation polysaccharide to acquire short chain fat acid (SCFA) [25]-[28]. Meanwhile microbiota or butyrate produced by commensal microorganisms was associated with the balance between pro- and anti-inflammatory mechanisms [29]-[31]. The DGGE electrophoresis revealed that there was no remarkable difference between the normal and model group, and it might predict that we didn't give enough amount of TNBS. However, in analysis of microbiota diversity, PCA and cluster, we got the difference among the group and the dried had more similarity with normal group compared with other groups. The variation of *Clostridium leptu* in dried was much better than other two groups. Therefore, dried *A. japonicus* had the best effect on modulating the intestinal microbiota. This study might help UC patients to choose the right products of sea cucumbers.

Xue *et al.* reported that the different processing method might change the ingredient of sea cucumber [14]. Therefore, we checked the protein and polysaccharide in the three types of *A. japonicus*. The result indicated that the protein of dried, instant and enzymatic *A. japonicus* was 73.09%, 65.06%, 57.42%, and polysaccharide was 6.72%, 5.46%, 5.45% respectively. Although the mechanism needs to be explored deeply, our present study confirmed that the different component of three *A. japonicus* might have influence on gut microflora, which could contributed to different effects on UC. Collectively, Our study have revealed that dried *A. Japonicus* have better effect on UC, but we still didn't know which component in *A. Japonicus* works on UC. In the future study, we need to figure out the effective component and how the component interact with intestinal microbial species, if the production of SCFA deprived by microbiota have an effect on UC.

5. Conclusion

A. japonicus could decrease the expression of IFN- γ /IL-4 and IL-1 β , and regulate intestinal microbiota to alleviate mice ulcerative colitis caused by TNBS. The processing methods indeed have an effect on the content of sea cucumber nutrients, especially for modulation of gut microflora. This study might provide a new method for *A. japonicus* to reduce the pain of UC patients.

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