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The Preventative Effect of Dietary *Apostichopus japonicus* on Intestinal Microflora Dysregulation in Immunosuppressive Mice Induced by Cyclophosphamide

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Abstract

Sea cucumbers are recognized as food and drug resources with many nutritional benefits, and *Apostichopus japonicus* is a kind of sea cucumber with good quality. Processing methods have some effect on its quality. This study aimed to explore the effects of *Apostichopus japonicus* with three different processing methods (dried, instant, and enzymatic sea cucummbers) on intestinal microflora dysregulation using a cyclophosphamide (cy) induced immunosuppressive mouse model. The expression of lysozyme, immunoglobulin A (IgA), and polymeric immunoglobulin receptor (pIgR) in the intestine and gut microbiota were investigated. The results showed that three types of *A. japonicus* could improve mucosal immunity and regulate gut microbiota. Dietary *A. japonicus* could reverse microbial imbalance, including increasing the bacterial diversity, enhancing the number of *Bifidobacterium,* and changing the bacterial composition. The most effect was observed with dried *A. japonicus*. Expression of lysozyme and IgA in the intestine was significantly increased. This study identified positive effects of dietary *A. japonicus* on mucosal immunity, particularly on gut microbiota, suggesting that dietary *A. japonicus* may aid in improving mucosal immunity and preventing exogenous infection. Additionally, the processing method has some effect on immunomodulatory function.

Keywords

Gut Microbiota, Mucosal Immunity, *Apostichopus japonicus*, Cyclophosphamide

*Hongjie Shi and Rong Zheng contributed equally to this paper.

1. Introduction

The mammalian intestine is covered by a large area (400 m^2) of mucosal tissue. It is one site in the body, which communicates with the external environment [1]. The mucosa is constantly in contact with a lot of material, including foods, probiotics and other harmless substances, and viruses, parasites, and other harmful organisms or substances which can invade the body through the mucosa [2]. In order to protect the body, the mucosal tissue forms part of the immune system with a tight defense function. It plays an important role in preventing the invasion of pathogens and also incorporates humoral and cellular immune functions [3]. Lysozyme and secretory IgA (SIgA), which is composed of two IgA molecules and is transported by pIgR, are two important mucosal immune system effectors. They help prevent infection and remove antigens crossing the mucosal barrier [4] [5]. Our previous study had indicated that cyclophosphamide could induce the expression of lysozyme and sIgA [6]. There are a large number of bacteria inhabiting the mammalian gut, constituting the body's normal microbes [7]. The gut microbiota, as part of the intestinal mucosal barrier, plays an important role in maintaining intestinal homeostasis [8] [9]. Disturbances in gut microbiota homeostasis are thought to contribute to severe gastrointestinal disorders [10] [11]. It has been reported that cyclophosphamide (cy) causes intestinal microflora imbalance, resulting in bacterial transfer to mesenteric lymph nodes and spleen [12]. Infection is common during chemotherapy, resulting in diarrhea, stomach ache, emesis, etc [13]. It is painful for patients, making a search for suitable treatments imperative.

Apostichopus japonicus is a species of sea cucumber with good quality, which is popular in Asia as a foodstuff. It contains many bioactive materials such as polysaccharide, collagen, and saponin, which contribute to its numerous physiological effects. It was reported that different processing method could change the ingredient of sea cucummber [14]. Some believe that Sea cucumbers are not suitable for the cancer patients treated with chemotherapeutic drugs and this set a limitation to the application aspect of *A. japonicus.* However, this study aimed to investigate the effects of *A. japonicus,* prepared using different processing methods, on mucosal immunity and gut microbiota to clarify whether *A. japonicus* could reduce tumor-related pain as a dietotherapy method. Additionally, we explored the effects of processing methods on nutritional benefits of *A. japonicus*. Our results might provide a basic knowledge for cancer patients to choose right sea cucumber products for the sake of their health.

2. Materials and Methods

2.1. Preparation of Dietary *A. japonicus*

Fresh *A. japonicus* were purchased from Nanshan markets in Qingdao. The sea cucumbers were boiled for 10 min and processed in three different ways. To produce dried *A. japonicus,* the boiled sea cucumbers were put in a cold-air oven at 20˚C for 6 days. They were soaked in distilled water for 2 days and boiled for 30 - 40 minutes. After cooling, the sea cucumbers were soaked in distilled water for 2 - 3 days at 4˚C. To

prepare instant *A. japonicus,* the boiled sea cucumbers were kept in the condiments for 1 hour and sterilized at 121˚C for 15 minutes). Finally, *A. japonicus* was treated with papain at 50˚C for 12 hours and the papain inactivated at 100˚C for 10 min. The resulting mixture was centrifuged and the supernatant retained. The processed *A. japonicus* were stored at −40˚C until use. The sea cucumbers were crushed and dissolved in saline before being administered to the mice.

All reagents used in the animal experiments were of analytical purity.

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. The mice had free access to standard laboratory pellet chow (Kangda, Jinan, China) and fresh water.

The mice were randomly assigned to 5 groups (10 mice/group): normal control group, model group, and *A. japonicus* groups with three different processing methods. The *A. japonicus* groups were given one of three sea cucumber regimens followed by 512 $mg \cdot kg^{-1}$ by gavage for 28 days, while the normal and model groups were given normal saline as control. Except for the normal group, the others were treated with cyclophosphamide (50 $mg \cdot kg^{-1}$) by intraperitoneal injection on the 26^{th} and 27^{th} feeding day. The normal mice were injected with saline as control. The mice body weights were recorded every two days. The feces were collected on the 28^{th} day. Mice were sacrificed by cervical dislocation on the 29^{th} day 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) and the standards for laboratory animals of China (GB 14922-94, GB 14923-94 and GB/T 14925-94).

2.3. DNA Extraction

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

2.4. PCR-DGGE Analysis

Bacterial universal primers GC-338F (5'-CGCCCGGGGCGCGCCCCGGGGCGGGGC GGGGGCGC-GGGGGGCCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGC GGCTGCTGG-3') were used to amplify the 16S rDNA gene V3 region by PCR. 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 × 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 at 94˚C for 5 min, 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% (7 M 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 PCR

Bifidobacteria and *Lactobacillus* were assayed by quantitative real-time PCR. 25 μL of the reaction volume was used for the quantitative real-time PCR assay that consisted of 12.5 μL of Maxima SYBR Green qPCR Master Mix (Roche, Switzerland), 10 μM of primers (0.75 μL each of forward and reverse primers), 8.5 μL ddH_2O, and 2.5 μL 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 supplementary **Table S**.

2.6. Western Blot Analysis

The expression levels of lysozyme, immunoglobulin A (IgA) and polymeric immunoglobulin receptor (pIgR) in the small intestine were investigated by western blot analysis. Briefly, the tissues were homogenized in RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.4) and put on ice for 20 minutes and then centrifuged at 12,000 × g for 5 min at 4˚C. The protein concentration of the supernatant was measured using the BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) with bovine serum albumin as the standard sample. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyl difluoride (PVDF) membranes (GE, Fairfield, CT, USA). The membrane was blocked with TBST (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.6) containing 5% skim milk (BD, Franklin Lakes, NJ, USA) and incubated with the indicated primary antibody in TBST overnight at 4˚C. Subsequently, the membrane was incubated with the secondary antibody and visualized by enhanced chemiluminescence (ECL) kit (GE, Fairfield, CT, USA). Equal protein loading was assessed by blotting for GAPDH.

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

Specific groups	Primers name	Primer sequence
Bifidobacterium	Bif-F	CCTACGGGAGGCAGCAG
	Bif-R	ATTACCGCGGCTGCTGG
Lactobacillus	Lac-F	AGCAGTAGGGAATCTTCCA
	Lac-R	ATTTCACCGCTACACATG

Bacteria-specific primers were designed by *Primer Premier* 5.0 software.

2.7. Statistical Analysis

A one-way analysis of variance between different groups was carried out using *SPSS V* 18.0 software. Data were presented as mean ± standard error and statistical significance was defined to be $p < 0.05$.

3. Results

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

According to the 16S rRNA V3 region PCR-DGGE patterns, the overall composition after the Cy treatment showed a significant difference from that in normal mice (Figure 1(A)). After treated with Cy, the number of bands in model group reduced sharply, and the intensities of bands in some migration locations decreased compared with the normal group. The results indicated that the diversity and structure of the gut microbiota in the model group changed after Cy treatment. The gut microbiota differences in mice treated with *A. japonicus* were reduced notably. The structures of the gut microbiota in the *A. japonicus* groups were similar with that in the normal group. The results were also shown clearly in the virtual figure (Figure 2(B)) of PCR-DGGE fingerprints. The diversity indexes of the microbiota in every group showed that the diversity of intestinal flora in the model group decreased, and *A. japonicus* helped to reverse the changes (Table 1), especially in the dried *A. japonicus* group.

The principal components analysis (PCA) of the fingerprints showed that mice were classified into three clusters, with the first principal component (PC1) accounting for

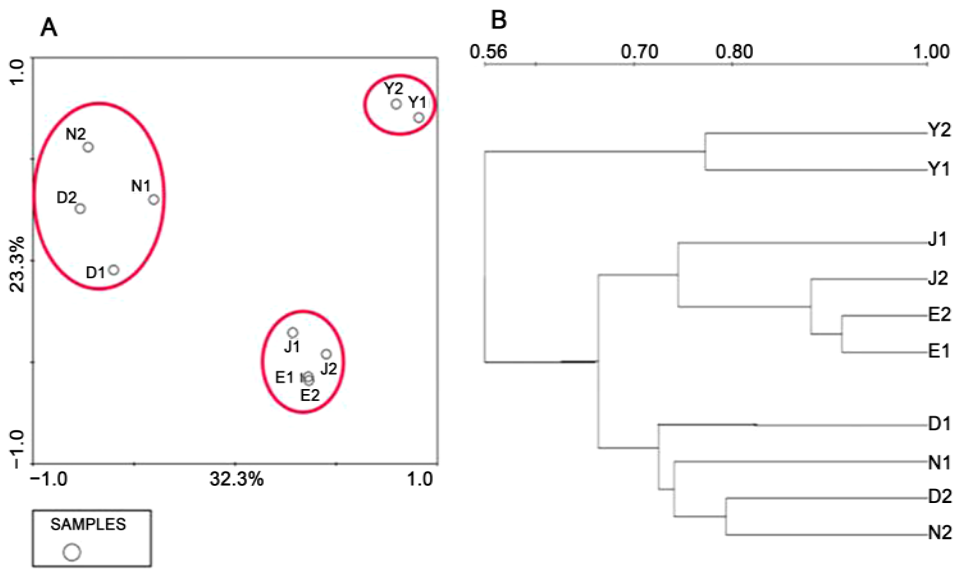

Figure 1. PCA and cluster analysis of DGGE fingerprint. (A) The principal components analysis (PCA) scores plot according to the DGGE fingerprint. (B) Clustering of gut microbiota based on distances between different groups. N serves as normal group and Y, D, J, E represent model, dried, instant and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

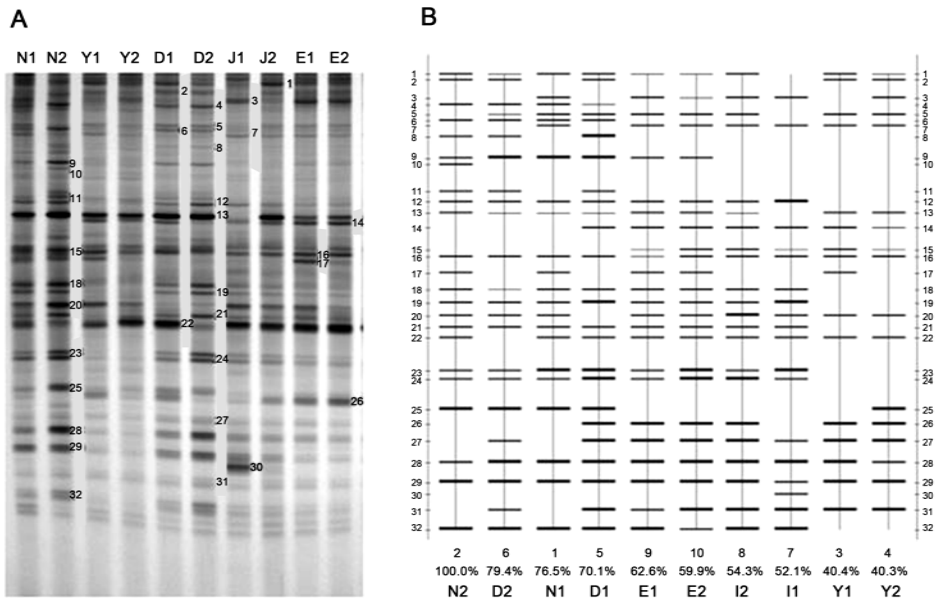

Figure 2. Comparison of gut microbiota composition between the mouse groups. (A) Denaturing gradient gel electrophoresis (DGGE) fingerprinting of V3 region of 16S rRNA genes from faecal bacterial communities. (B) The virtual map of DGGE electrophoresis. Every sample included feces of five mice and feces in 1 and 2 were different five ones. N is for normal group and Y, D, J, E represent model, dried, instant and enzymatic groups, respectively.

Table 1. Analysis of the microbiota diversity of mice intestine.

Groups	Shannon-Wiener Index (*H*)	Evenness (*E*)	Richness (*S*)
Normal	3.094	0.994	23
Model	2.766	0.987	17
Dried	3.161	0.995	24
Instant	3.005	0.996	21
Enzymatic	3.155	0.993	24

Each group includes 10 mice. We selected 5 fecal samples randomly into one detecting sample and the other five ones was another sample. Every data was mean of two detecting samples.

32.3% of the variation (**Figure 1(A)**). The normal and dried *A. japonicus* group belonged to one cluster, the instant and enzymatic *A. japonicus* group belonged to another cluster, and the model group was in a third cluster. It confirmed that Cy caused changes in gut microbiota, and *A. japonicus* helped reverse the changes. The cluster dendrogram showed similar results (**Figure 1(B)**). These results suggest that *A. japonicus* could ameliorate the gut microbiota imbalance induced by Cy in mice.

3.2. Identification of the Significantly Different Bands in Denaturing Gradient Gel Electrophoresis (DGGE) Fingerprints

In model mice, the intensities of band 6 (b6), b9, b12, b24, b25 and b28 were significantly reduced. By sequence analysis, these bands were related to Prevotella dentalis DSM 3688 (96% homology), *Moryella indoligenes* (99%), Barnesiella intestinihominis

(99%), Ruminococcaceae bacterium LM158 (98%), Clostridiales bacterium CIEAF 020 (97%), Johnsonella sp. oral taxon 166 (97%) (**Figure 2(A)** and **Table 2**). These bacteria belong to Bacteroidetes and Clostridia groups. Bacteroidetes is an intestinally adapted bacterium involved in carbohydrate fermentation, polysaccharide and steroid metabolism, and maintaining intestinal physiological functions, amongst other functions [16]. Most Clostridial bacteria can hydrolyze polysaccharide and protein. The reduction of *Bacteroidetes* and *Clostridia* may have an effect on digestion and absorption of polysaccharide and protein. The intensities of bands in *A. japonicus* groups increased to near normal levels. We speculate that administration of *A. japonicus* helped to restore normal intestinal physiological function.

3.3. Quantitative Changes in Probiotic Bacteria Revealed by Real-Time Quantitative Polymerase Chain Reaction (RT-PCR)

A number of bacteria are friendly and helpful to hosts, but in low abundance, such as probiotic *Bifidobacterium* and *Lactobacillus*. *Bifidobacterium* can reduce intestinal pH to inhibit the growth of harmful bacteria, produce vitamins and amino acids, stimulate immune responses, protect the intestinal barrier, etc. [17] [18] [19]. *Lactobacillus* can ferment carbohydrates to produce lactic acid and help digestion and absorption, stimulate immunoglobulin production to enhance the host immune function, etc [20] [21]. Hence, the amount of probiotic *Bifidobacterium* and *Lactobacillus* was quantified by real-time PCR in this study. The results show that Cy caused a significant reduction of *Bifidobacterium* (**Figure 3(A)**). *A. japonicus* administration increased the quantity of *Bifidobacterium* and dried *A. japonicus* showed a significant effect ($p < 0.05$). However, the amount of *Lactobacillus* showed no obvious change in this model (**Figure 3(B)**). These data imply that *A. japonicus* intake protects the intestine by increasing the quantity of *Bifidobacterium*.

Table 2. The analysis results of DGGE gel bands recovery sequence.

Band Number	Similar Strain	Accession Number	Similarity	Classification
Band 4	Gram-negative bacterium cTPY-13	AY239461	99	Bacteria
Band 6	Prevotella dentalis DSM 3688	NR_102481	96	Bacteroidia; Prevotella
Band 9	*Moryella indoligenes*	DQ377946	99	Clostridia; Moryella
Band 12	*Barnesiella intestinihominis*	NR_113073	99	Bacteroidetes; Barnesiella
Band 18	bacterium NLAE-zl-P919	JQ607737	97	Bacteria
Band 19	bacterium 26-4b2	AF524856	90	Bacteria
Band 23	Gram-negative bacterium cTPY-13	AY239461	93	Bacteria
Band 24	Ruminococcaceae bacterium LM158	KJ875867	98	Clostridia; Ruminococcaceae
Band 25	Clostridiales bacterium CIEAF 020	AB702928	97	Clostridia Clostridiales
Band 28	*Johnsonella* sp. oral taxon 166	GU407052	97	Clostridia; Johnsonella

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

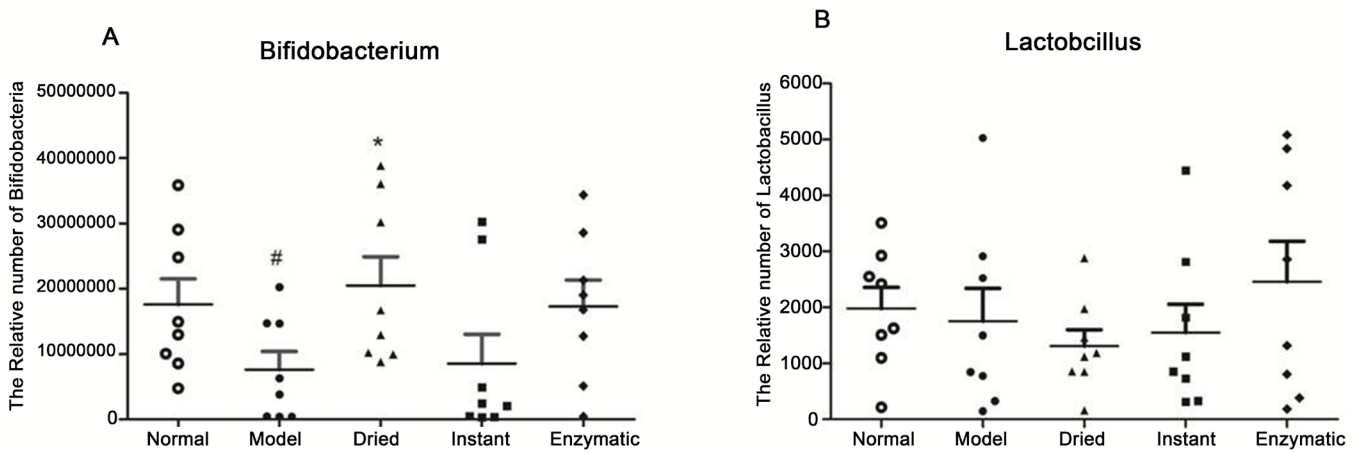

Figure 3. Intestinal microflora compositions in different groups of mice were checked by quantitative real-time PCR analysis of (A) Bifidobacterium, and (B) lactobacillus. Values are expressed as mean ± S.E.M (n = 8 per group). $^{\#}p < 0.05$, different from the normal group; $^{*}p < 0.05$ different from the model group.

3.4. *A. japonicus* Ameliorated Intestinal Mucosal Immunity in Immunosuppressive Mice

Lysozyme is an important bactericidal substance in body fluids, which works as a mucosal chemical barrier and represents the innate mucosal immunity effector. SIgA is composed of two immunoglobulin A (IgA) molecules, joined by a J-chain and a secretory component. pIgR transporting dimeric IgA (dIgA) across the epithelial cell function is a necessary condition for the secretion of sIgA to exocrine fluid [22]. Hence, the expression of lysozyme, IgA and pIgR was detected to reflect the mucosal immune function.

The results showed that expression of lysozyme increased with *A. japonicus* intake, while that in the model group reduced significantly ($p < 0.05$) (**Figure 4(A)**). Compared with the normal group, the IgA and pIgR expression of mice in model group decreased significantly (**Figure 4(B)** and **Figure 4(C)**). Administration of dried and instant *A. japonicas* significantly improved the expression of IgA ($p < 0.01$). The three types of *A. japonicus* could all increase pIgR expression; however, they showed no statistical significance. All these data indicated that *A. japonicus* intake could ameliorate intestinal mucosal injury induced by Cy.

4. Discussion

Cy is one of the most common agents in the treatment of cancer [23] [24]. It has higher lethality towards rapidly proliferating cells, and this is not limited to tumor tissue. The intestinal epithelium is composed of rapidly proliferating cells, and is therefore susceptible to Cy. This results in destruction of mucosal barrier and translocation of intestinal microbiota [25] [26]. In this study, the RT-PCR and PCR-DGGE results showed that Cy caused an imbalance in gut microbiota, including decreased abundance of *Bifidobacterium*, reduced diversity, and altered microflora composition. *A. japonicus* could reverse these changes. Additionally, the modulation effect of dried *A. japonicus* on gut

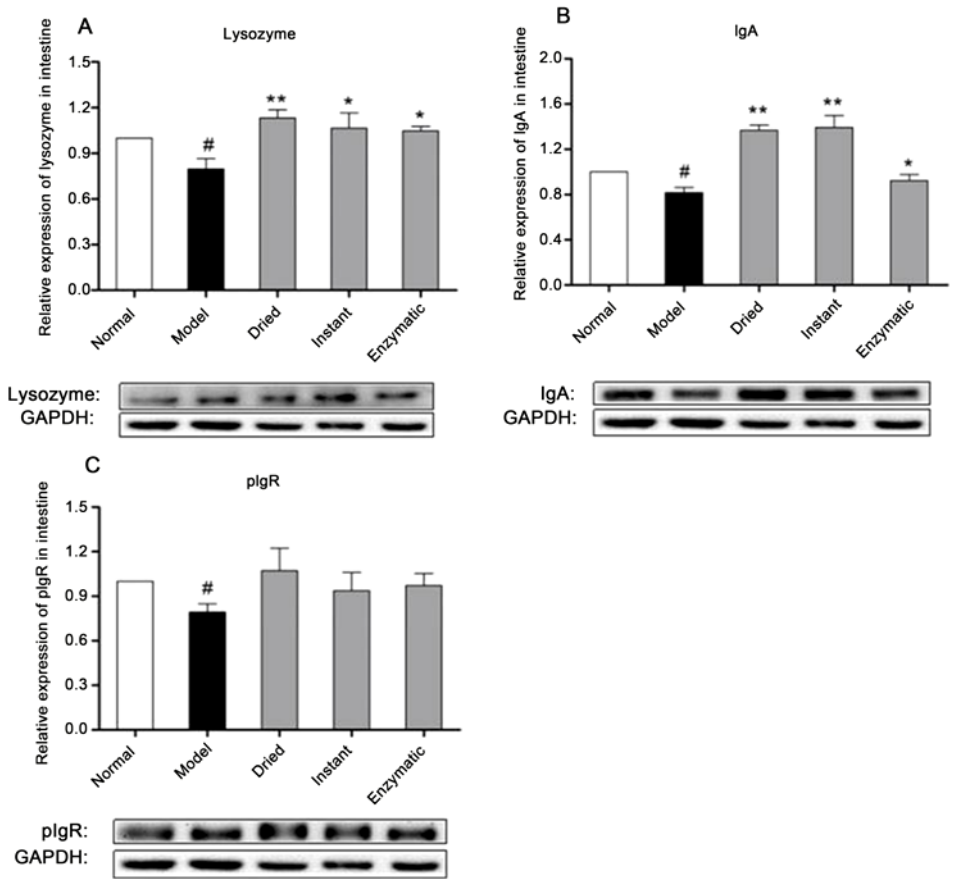

Figure 4. Relative expression of lysozyme, IgA and pIgR in intestine. Lysozyme, IgA and pIgR expression was analyzed by western blotting and GAPDH was the loading control. Each value represents the mean ± S.E.M. of 8 mice in each group. #Significantly different from normal group, $p < 0.05$; *Significantly different from model group, $p < 0.05$; **Significantly different from model group, $p < 0.01$.

microbiota was better. Otherwise, the expression of IgA, pIgR and lysozyme decreased significantly in the model group, which was consistent with previous reports [27] [28] [29]. Dietary *A. japonicus* could enhance the expression of IgA, pIgR, and lysozyme, and three preparations of *A. japonicas* prepared using different processing methods did not show significant differences.

The effect of dietary *A. japonicus* on mucosal immunity was discussed in our previous study [6]. Here, we focused on its effect on intestinal microflora. The human intestinal microflora includes 300 - 500 different species of bacteria [30]. They affect the health of the body, including digestion and absorption, energy metabolism, and immune regulation [31] [32]. Gut microbiota are involved in the digestion of complex macromolecules, particularly fermented polysaccharides, and also provide short-chain fatty acids by enzymatic reaction. They can produce bacteriocins and other antagonists to prevent adhesion and colonization of pathogenic bacteria. Microflora may enhance immune cell reactivity to antigen stimulation, activate lymphoid tissue, and increase sIgA biosynthesis [33] [34]. The RT-PCR results showed that *Bifidobacterium* de-

creased in the model group and the alteration of *Bifidobacterium* in the dried *A. japonicus* group compared to controls was more significant than the other two groups. Hence, we analyzed changes in gut microbiota using the PCE-DGGE method. DGGE electrophoresis showed that the number and composition of bands changed in the model group, indicating an imbalance of gut microbiota. The electrophoresis patterns of *A. japonicus* groups were similar to those of the normal group, while the effect of dried *A. japonicus* was better. We detected polysaccharide and protein in three kinds of *A. japonicas,* as processing method affects proportions of sea cucumber nutrients. The results showed that the polysaccharide content of dried, instant, and enzymatic *A. japonicus* was 6.72%, 5.46%, and 5.45%, and the protein content was 73.09%, 65.06%, and 57.42%, respectively. We speculate that the regulatory effect of dietary *A. japonicus* on gut microbiota was related to amounts of a specific active ingredient. Our study have revealed that dried *A. japonicus* have better effect on intestinal mucosa protection, but we still didn't know which component in *A. japonicus* was related with protection of mucosa damage induced by cy. 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 effcet on mucosa barrier. These conclusions need further validation.

5. Conclusion

In conclusion, the present study suggests that dietary *A. japonicus* may regulate gut microbiota and increase the expression of IgA, pIgR and lysozyme to ameliorate small intestinal mucosal disorders caused by Cy. Processing methods affect the nutritional benefits of sea cucumbers, especially its microbiota regulating function. These observations might provide a new mechansm for dietary *A. japonicus* to reduce possible infectious complications resulting from chemotherapy.

Acknowledgements

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