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Effects of *Nannochloropsis* Concentration in Diet on Growth, Survival and Anti-inflammatory Cytokine (Interleukin-10) Production of the Sea Cucumber *Apostichopus japonicus*.

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Abstract

Two experiments were conducted to investigate the effects on growth, survival and anti-inflammatory cytokine (Interleukin-10) using with Nannochloropsis concentration in diets of sea cucumber *Apostichopus japonicus*. In the first experiment, 60 days feeding trail was conducted to evaluate the growth performance and survival of the sea cucumber fed on six experimental diets containing different inclusion level of *Nannochloropsis oculata* (0%, 2%, 4%, 6%, 8%, 10%) in recirculating aquaculture system. Specific growth rate (SGR) and food conversion efficiency (FCE) of sea cucumber fed diet containing 8% *Nannochloropsis oculata* algae was significantly higher than that of other diets ($P<0.05$). Results of the experiment suggest that dietary inclusion with 8% *Nannochloropsis oculata* may improve growth of sea cucumber. The second experiment was conducted to determine IL-10 gene expression where mice splenocytes were stimulated with 10 µg/ml of each diet fed sea cucumber extracts for 2 hours. The result showed that IL-10 gene expression levels were significantly increased in 6% and 8% *Nannochloropsis oculata* algae containing diets fed sea cucumber extracts. This result suggests that proper combination of *Nannochloropsis oculata* could up-regulate of IL-10 gene expression. Such detailed information could be helpful in further development of more appropriate diets for sea cucumber culture.

Keywords: Sea cucumber (*Apostichopus japonicus*), *Nannochloropsis oculata*, growth, interleukin (IL)-10.

Introduction

Sea cucumber, *Apostichopus japonicus* is temperate species, which has long been exploited as an important fishery resource in Russia, China, Japan and South Korea (Sloan, 1984). Market demand for this species has increased because of its aphrodisiac and curative properties. However, wild production of sea cucumber has declined because of over-exploitation (Conand, 2004). As a commercially important species for mariculture, sea cucumber *Apostichopus japonicus* has been widely cultured in Asia because of its nutritional and economic value in recent years (Chen, 2004; Sun *et al*., 2004; Yuan *et al*., 2006).

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Apostichopus japonicus is deposit-feeders that ingest sediment with organic matter (Moriarty, 1982; Michio *et al*., 2003), and their gut contents have lots of macroalgae and seaweed, shell fragments from mollusks, crustaceans and barnacles, echinoderm ossicles, many pelagic and benthic foraminifera and diatoms (Hauksson, 1979; Zhang *et al*., 1995).

Sea cucumbers have the ability to synthesize long-chain polyunsaturated fatty acids in diets (Hai-Bo Yu *et al*., 2015). Lipids of sea cucumber play essential roles in the metabolic activities of organisms (Sargent *et al*., 2002; Tocher, 2003). For instance, most of the animals cannot synthesize longer chain polyunsaturated fatty acids such as eicosapentaenoic acid, arachidonic acid and docosahexaenoic acid. Phytoplankton and some bacteria are responsible to form these polyunsaturated fatty acids and are transferred through the food web (Volkman *et al*., 1989).

Traditionally, sea cucumbers are cultured without supplement feeds. But recently more and more farmers have started to feed the sea cucumbers with macroalgae to increase production (Shi *et al*., 2013). Formulated diets for sea cucumbers are commonly made of macroalgal powder. Among macroalgae, brown algal *Sargassum thunbergii* is widely distributed over shallow coastal area in Korea, Japan and China and commonly used as a feed in sea cucumber culture (Battaglene *et al*. 1999). However, it is difficult to satisfy demand for sea cucumber culture because this algal species is not produced commercially and its use as feed ingredients is also expensive. Moreover, in recent years, more and more *S. thunbergii* has been harvested with the rapid expansion of sea cucumber farming scale, which results in severe damage to *S. thunbergii* resource (Yuan 2005). Meanwhile, sea cucumber contained high n-6 fatty acids, low n-3 fatty acids and lower ratio of n-3/n-6 fatty acids when fed with commercial diets (Feng Jin *et al*., 2016). But for many allergic and inflammatory diseases like asthma, n-3 fatty acids and good balance of n-3/n-6 ratio is very important. So, reducing the *S. thunbergii* content of sea cucumber feed will be one strategy to increase the sustainability of the sea cucumber culture.

Therefore, it is critical to find good substitutes for *S. thunbergii* to relieve the pressure on natural *S. thunbergii* resource and produce more n-3 fatty acids containing sea cucumber. *Nannochloropsis oculata* algae might be an important choice.

Nannochloropsis oculata is one of the main groups of seawater phytoplankton but also occur in fresh and brackish water. *Nannochloropsis oculata* have a diameter of about 1-2 μm and a very simple ultrastructure with reduced structural elements that easily digest by aquatic animals. *Nannochloropsis oculata* is considered a promising alga for aquaculture because of its ability to accumulate high levels of polyunsaturated fatty acids. *Nannochloropsis oculata* offers high levels of polyunsaturated fatty acids (PUFAs), especially ecosapentaenoic acid (EPA, 20:5n-3) (Kandilian *et al*., 2013).

Sea cucumbers have many therapeutic effects against various diseases (Bordbar *et al*., 2011; Guo *et al*., 2015). Moreover, sea cucumber extracts have potent biological effects and have antiviral, anti-cancer, antibacterial, anti-oxidant, anti-inflammation effects (Esmat *et al*., 2013; Kiani *et al*., 2014; Wijesinghe *et al*., 2013). In China and Malaysia, sea cucumbers have been traditionally used for the remedy of different inflammatory diseases like asthma. IL-10 is a potent anti-inflammatory cytokine that down regulates the synthesis of Th1 (T helper 1) and Th2 (T helper 2)-associated cytokines, chemokines, and inflammatory enzymes. It plays a vital role for the mitigation of allergic responses. But till now, there are no reports demonstrating the effect of sea cucumber on IL-10 production.

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In the present study, the effects of different concentration of *Nannochloropsis oculata* algae in prepared feeds on growth, survival and anti-inflammatory cytokine, IL-10 production of the sea cucumber *Apostichopus japonicus* were examined.

Materials and Methods

Experiment 1

Sea Cumber Collection and Acclimatization

The experiment was carried out for sixty days in the laboratory of Marine Biology and Aquaculture, Gyeongsang National University, Republic of Korea. Sea cucumbers used in this experiment were collected from the Goseong Sea cucumber farm. Prior to the experiment, sea cucumbers were transferred to the laboratory in fiberglass aquaria and acclimated for 14 days at 19°C. Temperature of water bath was regulated by a thermostat, which controlled the on / off switch of a 2000-W electric heater. During acclimation period, sea cucumbers were fed with algal powder (*S. thunbergii*) and sea mud.

Experimental Diets

Six experimental diets designed as Diet 1 (control), Diet 2, Diet 3, Diet 4, Diet 5 and Diet 6 were prepared. Ingredients and proximate compositions of experimental diets were presented in Table 1. Diet 1 was used as the control diet where *Nannochloropsis oculata* powder was not used. For diets 2, 3, 4, 5 and 6 *Nannochloropsis oculata* powder were used with the percentages of 2%, 4%, 6%, 8% and 10%, respectively. All ingredients were ground into fine powder through a 200 μm mesh, thoroughly mixed and stored at −20 °C.

Experiment Design

After 2 days' starvation, 240 sea cucumbers with initial wet body weights of 2.92±0.06g were randomly selected from acclimatized sea cucumbers and placed in equal number into 24 fiberglass aquaria (45×60×50 cm^3) to form 6 groups in tetraplicates. The initial body weights of sea cucumber were measured individually as described in Battaglene *et al.* (1999). The 6 groups were fed once daily (17:00 h) with different experimental diets such as Diet 1, Diet 2, Diet 3, Diet 4, Diet 5 and Diet 6 respectively at a feeding rate of 5% body weight per day for 60 days.

Rearing Conditions

During the experiment, aeration was provided continuously and to ensure water quality 2/3 volume of the water in each aquarium was exchanged every day. Seawater temperature was controlled at 19 ± 2.0 °C. Temperature of water reservoir was regulated by a thermostat, which controlled the on / off switch of a 2000-W electric heater. Dissolved oxygen was maintained above 5.0-7.0 mg/ L, the levels of ammonia in the water of aquaria were less than 0.25 mg/ L. Other conditions were salinity 32 ± 1 psu; pH 7.7–8.3; photoperiod 24 h dark. The aquaria were wrapped in black carbon paper to maintain continuous dark period. The longer and darker light conditions are better for a population of *A. japonicus* to induce sea cucumbers to feed continuously.

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Procedure and Sample Collection

Twenty-four sea cucumbers were sampled from the acclimated sea cucumbers simultaneously while experimental sea cucumbers were selected to determine the initial body weight of the experimental sea cucumbers. During the experiment, sea cucumbers were fed once per day (at about 17:00 h). Uneaten feed was collected from aquaria by siphon at 24h later and dried at 65°C to constant weight for calculation use. Sea cucumbers faeces were also collected by siphon once per day (16:00h). The faeces were dried at 65°C to constant weight and those from each aquarium were pooled for further analysis. At the end of sixty days experiment, all the experimental sea cucumbers were deprived of food to clear their guts for 2 days, weighed and then dried at 65°C until constant weight was achieved.

Data Calculation

Survival rate (SR), specific growth rate (SGR), ingestion rate (IR), faeces production rate (FPR) and food conversion efficiency (FCE) were calculated as follows:

$$SR\ (\%) = 100 \times (N_2/N_1)$$

$$SGR\ (\%\ d^{-1}) = 100\ (\ln W_2 - \ln W_1)/T$$

$$IR\ (g\ g^{-1}\ d^{-1}) = I/[T\ (W_2 + W_1)/2]$$

$$FPR\ (g\ g^{-1}\ d^{-1}) = F/[T\ (W_2 + W_1)/2]$$

$$FCE\ (\%) = 100\ (W_2 - W_1)/I$$

where N_1 is the number of individuals alive at start of experiment and N_2 is the number of individuals alive at end of experiment; W_1 and W_2 are initial and final combined dry weights of all 10 sea cucumbers in each aquarium; T is the experimental period; I is the dry weight of the total feed ingested and F is the dry weight of faeces.

Experiment 2

Preparation of Sea Cucumber Extract

At first, sea cucumbers were cleaned and removed the visceral organs. After that, sea cucumbers were cut into small pieces and homogenized. 200g of samples were boiled in 400 ml distilled water for 20 min. After removing solid materials from the water, the boiled water was vaporized using a microwave until the mixture was reduced by 50%. After centrifugation of the extracts at 500 × g for 10 min, a 5-fold volume of 100% ethyl alcohol was added to the supernatant and incubated at 20°C for 24 h. After that, the supernatant was discarded. The extract pellet was washed with 70% EtOH and centrifuged under the same conditions. The supernatant was discarded and the pellet was evaporated under a vacuum. The final extracts were prepared by re-suspending the pellet in 20mL distilled water (Lee *et al.*, 2016).

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IL-10 Gene Expression

In order to analysis IL-10 gene expression, mice splenocytes were stimulated with 10 µg/ml of each experimental diet fed sea cucumber extracts for 2 hours. The total RNAs were isolated by Qiazol reagent (Qiagen Scienece, USA) according to the manufacturer's protocols. 2 µg of total RNAs were transcribed using M-MLV reverse transcriptase (Promega, USA), according to the manufacturer's protocols. IL-10 mRNA expression levels were synthesis by real-time PCR using the iCyclerTM (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used for reference gene. IL-10 and GAPDH primer sequence are shown in **Table** 2.

Statistical Analysis

Statistics was performed using software SPSS 18.0 with possible differences among diet treatments being tested by one-way ANOVA. Duncan's multiple range tests were used to analyze the differences among treatments. Differences were considered significant at a probability level of 0.05.

Results

Experiment 1

Survival and Growth

The sea cucumbers in the different treatments showed high survival rates (100%). For all treatments during this period, no sea cucumbers were died. At the starting of the experiment, there were no significant differences in wet and dry body weights of sea cucumbers among diet treatments (P>0.05) (Table 3). At the end of the experiment, final wet and dry body weights of sea cucumbers fed with diet 5 was significantly higher than those fed with other diets (P<0.05) (Table 3).

SGR of the sea cucumbers varied in different diet treatments and showed a descending order of diet 5>diet 4>diet 3>diet 6>diet 2>diet 1. The highest values of SGR (1.54% d^{-1}) was observed in treatments fed with diet 5 (Fig. 1) ($P < 0.05$). The sea cucumbers fed diet 1 showed significantly lower SGR (0.57% d^{-1}) than those fed other diets ($P < 0.05$). No difference in SGR was found among groups fed diet 3 and diet 6(P<0.05) (Figure 1).

Ingestion Rate and Faeces Production Rate

Ingestion rates (IR, see Figure 2) and faeces production rates (FPR, see Figure 3) of the sea cucumbers varied in different diet treatments and showed the same ascending order of diet 6>diet 5>diet 4>diet 3>diet 2>diet 1. Sea cucumber fed with diet 6 showed the lowest IR (0.32 g g^{-1} d^{-1}) ($P < 0.05$) and FPR (0.26 g g^{-1} d^{-1}) ($P < 0.05$) among all treatments.

Food Conversion Efficiency

Food conversion efficiency (FCE) is presented in Figure 4. FCE of the sea cucumbers fed with diet 5 was 3.48%, which was significantly higher than those fed with other diets except diet 4 and diet 6 ($P < 0.05$). Sea cucumbers fed diet 1 showed the lowest FCE (0.79%).

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Experiment 2

Anti-Inflammatory Cytokine, Interleukin (IL)-10 Expression Level

In order to establish optimal combination percentage of *Nannochloropsis oculata*, we synthesize IL-10 gene expression levels. Splenocytes were stimulated with each experimental diet fed sea cucumber extracts for 2 hours. Result showed that IL-10 gene expression levels were significantly increased in diet 4 and diet 5 compared to other experimental diet (Figure 5). However, IL-10 gene expression levels were decreased by diet 2 and 6 contrast to diet 1. These results suggest that combination of *Nannochloropsis oculata* could up-regulate of IL-10 gene expression and need optimum concentration.

Discussion

Experiment 1

In all treatments survival rates of sea cucumbers were excellent (100%) and were higher than the rates reported in previous similar studies (Hai-Bo Yu *et al*., 2015; Slater and Carton, 2007; Zhou *et al*., 2006). The results showed that *A. japonicus* might have the ability to tolerate the different proportion of *Nannochloropsis oculata* algae in diet.

S. thunbergii is considered to be the most commonly used for sea cucumber *A. japonicus* food, but a substitution of it is desiderated due to severe exhaustion of natural *S. thunbergii* resource (Yuan, 2005; Wang *et al*. 2006). There are lots of different microalgae in gut contents of sea cucumber in nature (Hauksson 1979). Eicosapentaenoic acid, the fatty acids biomarker of diatom, *nannochloropsis*, accounts for the higher mass fraction among polyunsaturated fatty acids in the body wall of *A. japonicus* (Kharlamenko, Zhukova, Khotimchenko, Svetashev & Kamenev 1995; Budge, Parrish & Mckenzie 2001; Gao, Yang & Xu 2009; Ce Shi *et al*. 2015). This indicate that diatom, *nannochloropsis* may have a great contribution as alternative source of *A. japonicus* food. This study showed that the SGR of sea cucumber *A. japonicus* fed with 8% *Nannochloropsis oculata* containing prepared diet was as high as those fed with control diet (Figure 1). These result suggested that the prepared diet containing *Nannochloropsis oculata* algae may perform better than the traditional feed where only used *S. thunbergii* algae.

The present results showed that among the six prepared experimental diet treatments, SGR of the sea cucumbers fed with Diet 5 (1.54 % d^{-1}) was significantly higher than the other diet($P<0.05$) (Figure 1). Ce Shi *et al*. (2015) reported that SGR of the sea cucumbers *A. japonicus* was 1.36% d^{-1} when fed 70% *S. thunbergii* algae, 20% sea mud and 10% white fish meal containing diet. Ying Liu *et al*. (2009) reported that when sea cucumber fed 60% *S. thunbergii* algae and 40% sea mud, SGR of the sea cucumbers *A. japonicus* was 1.40% d^{-1}. Thus it is clear that the certain proportion of *Nannochloropsis oculata* with *S. thunbergii* algae was good for sea cucumber culture.

SGR of the sea cucumbers were significantly affected by different experimental diets. In this study, SGR of the sea cucumbers were increased with increasing level of *Nannochloropsis oculata* until 8% after that decreased (Figure 1). Holothurians like sea cucumber have no specialized organ for grinding or gland for chemical digestion (Massin, 1982), digestive enzyme activities are very low and have very little cellulose activity (Wang *et al*., 2007). Therefore, sea cucumber *A. japonicus* are able to assimilate a specific amount of cellulose content.

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Ingestion rates (IR) of sea cucumber were significantly affected by different experimental diets. There was a negative relationship between IR and the protein level. In natural ecosystem, low nutritional value of sediment consumed by deposit feeders means those animals need to consume large amounts of sediment in order to obtain a net input of energy (Santos *et al*., 1994; Hudson *et al*., 2004). Vice-versa, when food quality becomes better, internal appetite regulation would work actively to decrease food ingestion. In this study, ingestion rate of sea cucumbers decreased when protein content of the diets increased. The ingestion rate showed a descending order (Figure 2), whereas the food conversion efficiencies showed an opposite trend (Figure 4). The same phenomenon was also found in other echinoderms. McBride et al. (1998) reported that sea urchin (*Strongylocentrotus franciscanus*), prepared diets of different protein levels resulted in different ingestion rate. Otero-Villanueva *et al*. (2004) also found in *Psammechinus miliaris* that lowest ingestion rate was related to high energetic diet.

Experiment 2

Regulatory T cells (Treg cells), known as suppressor T cells, are subpopulation of T cells and modulate the immune systems (Kikodze *et al*., 2016). IL-10 is one of the Treg cells and known as a key regulator of immunity to many infection or inflammatory disease (Gutierrez-Murgas *et al*., 2016). For instance, high levels of IL-10 gene expressions have protective effect against malaria parasites infection (Niikura *et al*., 2011). Conversely, lack of IL-10 promote cell apoptosis during virus infection in small intestine (Pan *et al*., 2014). In previous study, we already investigated that administration of sea cumber total extract can upregulate of IL-10 and ameliorate asthma disease (Lee *et al*., 2016). Here, we suggested optimal combination of *Nannochloropsis oculata* algae to increase Interleukin (IL)-10 gene expression.

In conclusion, the results of experiment 1 suggest that dietary inclusion with 8% *Nannochloropsis oculata* algae may improve growth of juvenile sea cucumber and experiment 2 suggest that proper combination of *Nannochloropsis oculata* could up-regulate of IL-10 gene expression. Such detailed information could be helpful in further development of more appropriate diets for culture of sea cucumber.

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Table 1. Ingredients and composition of experimental diets for *Apostichopus japonicus* (% dry matter basis)

Ingredients	Diet 1 (Control)	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Nannochloropsis oculata powder	0	2	4	6	8	10
Wheat flour	10	8	6	4	2	0
Seaweed powder	20	20	20	20	20	20
Soybean meal	8	8	8	8	8	8
Shell fish powder	8	8	8	8	8	8
Shell powder	2	2	2	2	2	2
Calcium phosphate	2	2	2	2	2	2
Yeast protein	5	5	5	5	5	5
Soyabean lecithin	4	4	4	4	4	4
Mineral	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin	0.5	0.5	0.5	0.5	0.5	0.5
Sea mud	40	40	40	40	40	40
Proximate composition (%)						
Crude protein	17.50	18.51	19.11	19.71	20.31	20.91
Crude lipid	3.34	3.70	4.06	4.42	4.78	5.14
Ash	43.50	43.65	43.78	43.93	44.06	44.20

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Table 2. Primers Used for Real-Time PCR

Primer	Sequence
GAPDH- for*	5/-TAC CCC CAA TGT GTC CGT C-3/
GAPDH-rev†	5/-AAG AGT GGG AGT TGC TGT TGA AG-3/
IL-10-for	5/-GCT ATG CTG CCT GGT CTT ACT G-3/
IL-10-rev	5/-TCC AGC TGG TCC TTT GTT TG-3/

for*; forward; rev†; reverse.

Table 3. Initial and Final wet weight (WW), dry weight (DW) of *Apostichopus japonicus* fed different test diets (mean±SE)

Experimental Diets	Initial WW (g)	Initial DW (g)	Final WW (g)	Final DW (g)
Diet 1	2.97±0.15	0.27	4.20±0.12	0.38
Diet 2	2.86±0.08	0.26	4.78±1.03	0.44
Diet 3	2.85±0.16	0.26	5.49±0.94	0.50
Diet 4	2.96±0.08	0.27	6.73±1.18	0.61
Diet 5	2.86±0.14	0.26	7.20±1.78	0.66
Diet 6	2.96±0.19	0.27	5.74±1.04	0.52

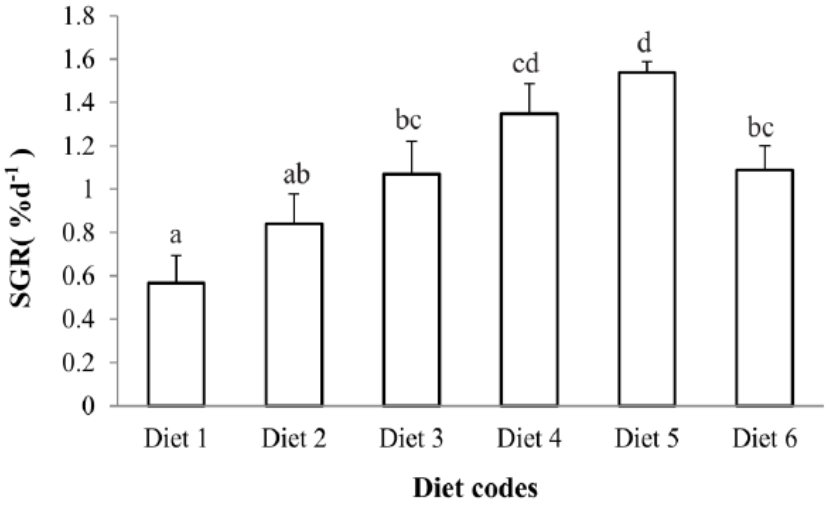

Figure 1. Specific growth rate of *Apostichopus japonicus* fed different test diets. Different letters indicate significant differences ($P < 0.05$) between treatments within the same group, and bars represent standard errors.

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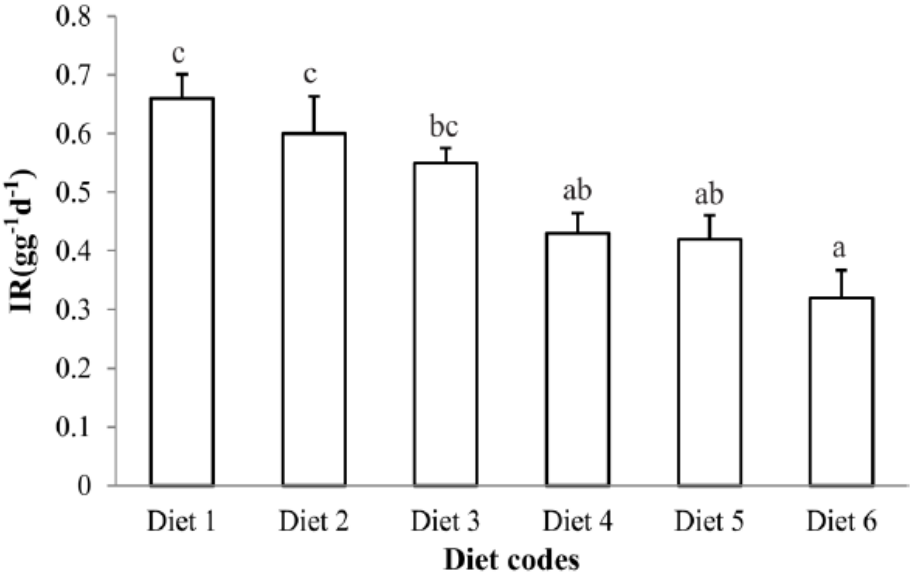

Figure 2. Ingestion rate (IR) of *Apostichopus japonicus* fed different test diets. Different letters indicate significant differences ($P < 0.05$) between treatments within the same group, and bars represent standard errors

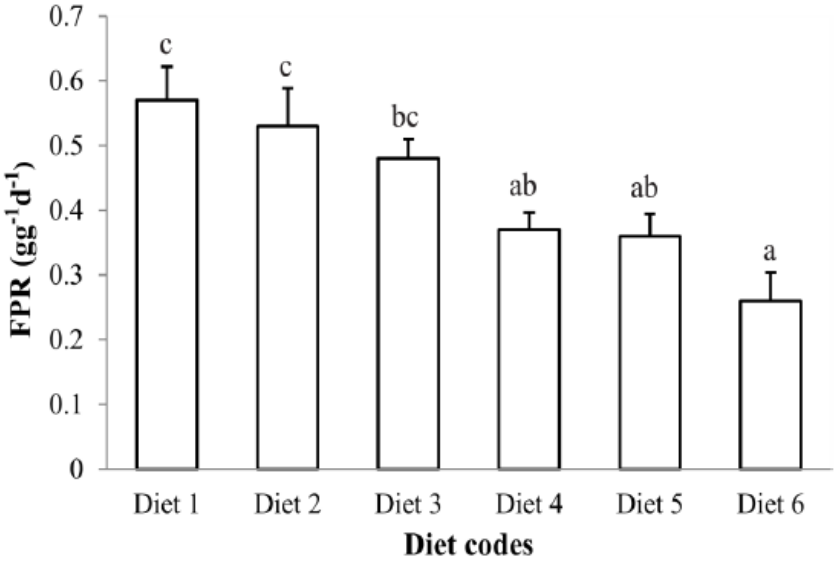

Figure 3. Faeces production rate (FPR) of *Apostichopus japonicus* fed different test diets. Different letters indicate significant differences ($P < 0.05$) between treatments within the same group, and bars represent standard errors.

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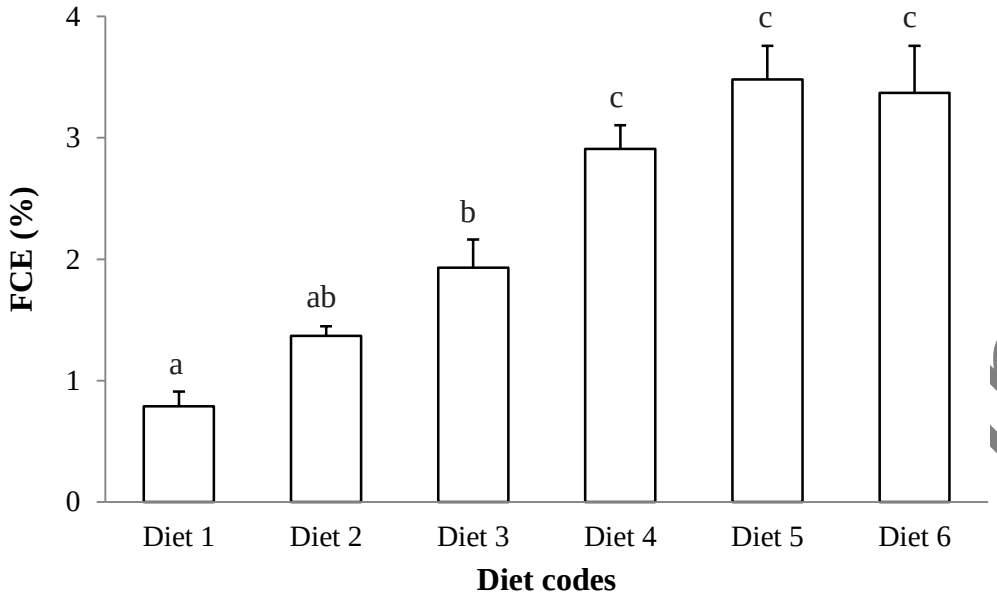

Figure 4. Food conversion efficiency (FCE) of *Apostichopus japonicus* fed different test diets. Different letters indicate significant differences ($P < 0.05$) between treatments within the same group, and bars represent standard errors.

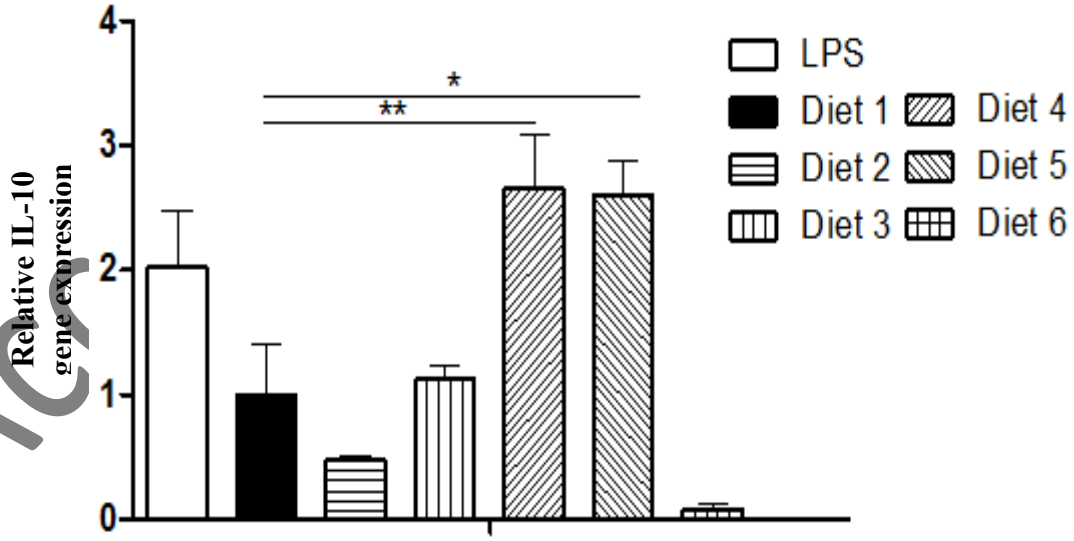

Figure 5. IL-10 gene expression. IL-10 gene expression were significantly increased by administration of diet 4 and 5 (* ; P=0.0173, ** ; p=0.0080).