Aquaculture Research

Aquaculture Research, 2013, **44**, 1613–1624 doi: 10.1111/are.12055

Effects of continuous and alternate administration of β-glucan and mannan-oligosaccharide on the growth, immunity and resistance against *Vibrio splendidus* of sea cucumber *Apostichopus japonicus* (Selenka)

Nan Bai, Wenbing Zhang, Kangsen Mai, Min Gu & Wei Xu

Key Laboratory of Aquaculture Nutrition and Feeds (Ministry of Agriculture), Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, Qingdao 266003, China

Correspondence: W Zhang, Key Laboratory of Aquaculture Nutrition and Feeds (Ministry of Agriculture), Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, Qingdao 266003, China. E-mail: wzhang@ouc.edu.cn

Abstract

A 4-week growth trial was conducted to compare the effects of different feeding strategies of dietary immunostimulants on the growth, immunity and resistance against *Vibrio splendidus* of sea cucumbers *Apostichopus japonicus* (Selenka). Six feeding strategies were set, including feeding immunostimulants-free diet continuously (control), feeding dietary β-glucan (1.25 g kg^{-1} diet) continuously, feeding dietary mannan-oligosaccharides (MOS; 2.00 g kg^{-1} diet) continuously, feeding β-glucan 2 days followed by MOS 5 days alternately, feeding β-glucan 5 days followed by MOS 2 days alternately and feeding β-glucan 7 days followed by MOS 7 days alternately. The sea cucumbers fed immunostimulants showed higher specific growth rate (SGR) and lower cumulative mortality than control ($P < 0.05$). When sea cucumbers were fed with β-glucan continuously, total coelomocytes counts and superoxide anion were significantly higher than control on the 4th day ($P < 0.05$). However, these two immune parameters were not significantly higher than those in control after the 18th day ($P > 0.05$). While sea cucumbers continuously fed MOS, these two immune parameters were not significantly higher than control until the 15th day. All immune parameters of the sea cucumbers fed with β-glucan and MOS alternately were significantly higher than those in control during the experiment ($P < 0.05$). The sea cucumbers fed with β-glucan 7 days followed MOS 7 days alternately showed the highest SGR and second lowest cumulative mortality. It was suggested that this feeding strategy is most suitable for sea cucumbers.

Keywords: *Apostichopus japonicus*, β-glucan, mannan-oligosaccharide, immunity, *Vibrio splendidus*

Introduction

The sea cucumber *Apostichopus japonicus* (Selenka) is a traditional food and invigorant in China. Because of the increasing demand for sea cucumber products in recent years, farming and sea ranching of *A. japonicus* have developed rapidly and grown into a prosperous sector in northern China (Chen 2003). The rapid expansion and high intensity of sea cucumber farming resulted in serious diseases, such as skin ulceration and peristome tumescence (Zheng, Liu, Sun, Qu, Dong & Liu 2010). These diseases caused serious economic loss and limited the sustainable development of this industry (Deng, He, Zhou, Liu, Tan, Wang, Jing, Gao & Liu 2009). Therefore, it is very urgent to find ways to control the diseases.

Immunostimulant could boost the immune system of animals and enhance their resistance against infections. It has been widely used in fish and shrimp industries to prevent diseases (Sakai 1999; Smith, Brown & Hauton 2003; Ringø, Olsen, Gonzalez Vecino, Wadsworth & Song 2012). However, information on immunostimulants of sea cucumber is less available (Li, Sun, Zheng & Hao 2009; Wang, Sun, Jin, Xu, Wang, Ren & Wang 2009). In a previous work, some immunostimulants

(β-glucan, mannan-oligosaccharides (MOS), CpG oligodeoxynucleotide, lactoferrin and vitamin C) were screened *in vitro* based on their effects on immune responses of *A. japonicus* coelomocytes, and β-glucan was confirmed to be an immunostimulant for sea cucumber *in vitro* (Gu, Ma, Mai, Zhang, Wang & Bai 2010). Furthermore, Zhao, Ma, Zhang, Ai, Mai, Xu, Wang and Liufu (2011) suggested that the optimal dietary level of β-glucan for cultured *A. japonicus* is 1.25 g kg^{-1}. Moreover, to make good use of the synergistic effects of different immunostimulants, combinations of dietary β-glucan and MOS (Gu, Ma, Mai, Zhang, Bai & Wang 2011), *Bacillus subtilis* and fructooligosaccharide (Zhang, Ma, Mai, Zhang, Liufu & Xu 2010) were also investigated in *A. japonicus*.

Sea cucumbers need more than 1 year to grow before harvest (Chen 2003). Therefore, long-term using of immunostimulants is needed. However, some researchers have proved that long-term oral administration of a single immunostimulant may cause the immunity fatigue (Chang, Chen, Su & Liao 2000; Bricknell & Dalmo 2005; Sajeevan, Philip & Singh 2008). For example, our previous work on white shrimp *Litopenaeus vannamei* showed that shrimps fed with dietary β-glucan could not maintain the immune parameters significantly higher than those fed without β-glucan for 42 days (Bai, Zhang, Mai, Wang, Xu & Ma 2010). Immunity fatigue limited the application of immunostimulants.

Research on the immune system of drosophila revealed that different non-self particles would react with different receptors (peptidoglycan recognition protein or β-glucan recognition protein) and stimulate the generation of different immune factors, such as drosomycin, cecropin and diptericin (Hultmark 2003). Because of the conservation of immune system, it is suggested that alternate administration of different immunostimulants may activate different parts of immune system of *A. japonicus* and take advantage of different immunostimulants to solve the immunity fatigue.

Yeast β-glucan, products known as β-1.3/1.6-glucans, is suggested to be the most potent immune system enhancer of all the different β-glucans (Ringø *et al.* 2012). MOS are obtained from the cell walls of yeast *Saccharomyces cerevisiae* and widely used in animal feed to improve gastrointestinal health and performance (Ringø, Olsen, Gifstad, Dalmo, Amlund, Hemre & Bakke 2010). In this study, a 4-week growth trial was conducted to compare the effects of continuous administration of β-glucan or MOS, and alternate administration of these two immunostimulants on the growth, immunity and resistance against *V. splendidus* infection of *A. japonicus*. The aim of this study was to investigate if alternate administration of two immunostimulants could eliminate the potential immunity fatigue in *A. japonicus* and detect the better strategy to use immunostimulants for *A. japonicus*.

Materials and methods

Experimental design and diets

Based on nutritional requirements of *A. japonicus* (Zhu, Mai, Zhang, Wang & Xu 2005), a basal diet was formulated (Table 1). β-glucan (Angel Company, Hubei, China; extracted from yeast *Saccharomyces cerevisiae*) and MOS (Bio-Mos®, Alltech, USA) were added into basal diet, respectively, to prepare two experimental diets. There were six treatments with different feeding strategies using the basal diet and the two experimental diets respectively. The different feeding strategies are presented in Table 2. The diets were prepared by thoroughly mixing the dry ingredients with fish oil and then adding cold water until a stiff dough resulted. This stiff dough was then passed through an extruder with a diameter 1.0 mm and dried in an oven at 40°C. After drying, the diets were broken up and sieved into the length between 0.30 and 0.45 mm. Diets were stored in plastic bags in −20°C until use.

Table 1 Composition of the basal diets (% dry weight)

Ingredients	Percentage
Silkworm tail meal[a]	50.0
Soybean meal[a]	15.0
Fish meal[b]	8.0
Fish oil[c]	1.0
Soy lecithin[d]	1.0
$Ca(H_2PO_4)_2$	1.0
Wheat flour	22.5
Vitamin premix[e]	0.5
Mineral premix[e]	1.0

[a]Purchased from Shangdong Liuhe Group, Qingdao, China.
[b]Crude protein 67.5% (dry weight basis), crude lipid 7.8% (dry weight basis).
[c]Purchased from Cisan Fisheries company, Weihai, China.
[d]Purchased from Jiakangy Beijing Company, Beijing, China.
[e]Kindly provided by Qingdao Master Biotechnology, Qingdao, China.

Table 2 Groups and feeding strategies

Treatment Abbreviations	Feeding Strategy
Control	Sea cucumbers were fed with basal diet continuously
Treatment 1	Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) continuously
Treatment 2	Sea cucumbers were fed with diet containing MOS (2000 mg kg^{-1}) continuously
Treatment 3	Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 2 days and then with diet containing MOS (2000 mg kg^{-1}) for 5 days alternately
Treatment 4	Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 5 days and then with diet containing MOS (2000 mg kg^{-1}) for 2 days alternately
Treatment 5	Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 7 days and then with diet containing MOS (2000 mg kg^{-1}) for 7 days alternately

Experimental animals and culture condition

A. japonicus juveniles were bought from a commercial hatchery (Qingdao, China) and acclimated in a re-circulated seawater system for 2 weeks prior to feeding experiment.

One thousand four hundred and forty sea cucumbers (initial mean weight 6.80 ± 0.30 g) with similar size were randomly distributed to six treatments and each treatment had six replicates. Each 150-L cylindrical fibreglass tank with 40 sea cucumbers was used as a replicate. The sea cucumbers were fed to apparent satiation once a day at 18:00 hours. Before feeding, the tanks were cleaned to remove the waste matter. During the acclimation and 4-week feeding trial, the air pump was opened continuously and the water temperature was maintained at 14–16°C; pH, 7.8–8.2; salinity, 31–32‰.

Experimental procedure

During the 4-week feeding experiment, sea cucumbers were sampled for nine times for analysis of immune parameters. The nine sampling time points were on the 1st, 4th, 7th, 11th, 15th, 18th, 22nd, 25th and 29th day respectively. For each sampling, three tanks in one treatment were randomly chosen, and four sea cucumbers from each tank were randomly sampled. The sea cucumbers were washed five times in sterile ice cold sea water, then dipped in 70% alcohol and rinsed in sterile ice cold sea water. After that, the sterile knife was used to make a small incision on the side of the body and 1-mL sterile syringe was used to collect the coelomic fluid. The coelomic fluid was quickly mixed with the sterile anticoagulant solution (0.02 mol L^{-1} EGTA, 0.48 mol L^{-1} NaCl, 0.019 mol L^{-1} KCl and 0.068 mol L^{-1} Tris-HCl, adjust pH to 7.6 and osmotic pressure to 780 mOsm Kg^{-1}) modified from Xing, Leung and Chia (1998). Compared with the anticoagulant solution formula published by Xing *et al.* (1998), the density of NaCl used in this study was increased to adjust osmotic pressure to 780 mOsm kg^{-1}. Based on our previous work on *A. japonicus* coelomocytes primary culture (Gu *et al.* 2010), this osmotic pressure is optimal for *A. japonicus* coelomocytes. The coelomic fluid from four sea cucumbers within a tank was pooled and used for immune parameters assay. At the end of the 4-week growth experiment, the remaining sea cucumbers were weighted to monitor growth and injected with pathogen bacteria *V. splendidus* suspension for a 14-day challenge test.

Immune parameters assay

Total coelomocytes counts (TCC)

One drop of the anticoagulant coelomic fluid was placed on a Buker haemocytometer to measure the total coelomocytes counts (TCC) under optical microscope (XPS-BM-2GA; Shanghai BM optical institution manufacture Co., Ltd., Shanghai, China). The coelomocytes were counted manually in all 25 squares (=0.1 mm^3).

Phagocytosis

One thousand μL of the anticoagulant coelomic fluid was used to measure phagocytosis. Coelomocytes were collected using centrifugation at 1000 × *g* for 10 min at 4°C (Sorvall Legend RT, Germany), and washed twice with isotonic buffer (0.001 mol L^{-1} EGTA, 0.53 mol L^{-1} NaCl, 0.01 mol L^{-1} Tris–HCl, pH 7.6). The present composition of the isotonic buffer was modified from Xing *et al.* (1998). Compared with the isotonic buffer composition published by Xing *et al.* (1998),

the density of NaCl used in this study was increased to adjust osmotic pressure to 780 mOsm kg^{-1}. Based on our previous work on *A. japonicus* coelomocytes primary culture (Gu *et al.* 2010), this osmotic pressure is optimal for *A. japonicus* coelomocytes. After being washed with isotonic buffer, coelomocytes were re-suspended in L-15 cell culture medium (Invitrogen Corporation, CA, USA) supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin sulphate and 0.39 mol L^{-1} NaCl. Cell viability was determined to be greater than 95% using the trypan blue exclusion test. Then, the cell suspension was adjusted to 10^7 cells mL^{-1}. Aliquots of 500 μL cell suspension were dispensed into wells of 48-well culture microplates for phagocytosis assay.

Phagocytosis of sea cucumber coelomocytes against *Staphyloccocus aureus* (S2014; purchased from Sigma-Aldrich Co., LLC, St Louis, MO, USA) was studied using flow cytometry according to Esteban, Rodríguez, Cuesta and Meseguer (2005) with slight modifications. In brief, the bacteria was grown in trypticase soy broth (TSB) containing 50 μg mL^{-1} fluorescein isothiocyanate (FITC; F7250; purchased from Sigma-Aldrich Co.) at 28°C for 12 h in dark with constant shaking to label the bacteria. The FITC-labelled bacteria were adjusted to 10^9 cells mL^{-1} in PBS and heat-killed at 60°C for 15 min. An aliquot of 50 μL FITC-labelled bacteria was added to each 500 μL coelomocytes (10^7 cells mL^{-1}) and incubated in dark at 18°C for 1 h. Then, the cells were placed on ice to stop phagocytosis and 500 μL ice-cold L-15 medium was added. The fluorescence of the extracellular bacteria was quenched by adding 80 μL ice-cold trypan blue (0.4% in PBS). The samples were analysed in a flow cytometry (FC-500, Beck-Man Coulter, USA) with an argon-ion laser at 488 nm. Each analysis is performed on 10 000 cells, which were acquired at the rate of 300 cells s^{-1}. Phagocytosis was defined as the percentage of cells with one or more ingested bacteria within the total cell population (10 000 cells).

Superoxide anion (O_2^-) production

Production of intracellular O_2^- by coelomocytes was evaluated using the nitroblue tetrazolium (NBT, Sigma) method according to Song and Hsieh (1994) with modifications. In brief, 50 μL of the anticoagulant coelomic fluid was dispensed into wells of 96-well plates. Coelomocytes were centrifuged at 1000 × *g* for 10 min at 18°C (Sorvall Legend RT, Germany) and the supernatant was removed. Aliquots of 200 μL 0.2% NBT dissolved in sterile Hank's balanced salt solution were added to coelomocytes and incubated for 30 min at 18°C. Supernatant was removed from each well and the cells were then fixed by adding 200 μL 100% methanol and incubating for 10 min. Subsequently, the cells were washed twice with 70% methanol to remove unreduced NBT, and air-dried. Reduced NBT was dissolved by adding 120 μL 2 mol L^{-1} KOH, followed by 140 μL DMSO. The production of superoxide anion was expressed as the absorption value at 630 nm.

Superoxide dismutase activity and total soluble protein

Three hundred μL of the anticoagulant coelomic fluid was used to measure intracellular superoxide dismutase activity (SOD). Coelomocytes were collected using centrifugation at 1000 × *g* for 10 min at 4°C (Sorvall Legend RT, Germany) and were re-suspended with 300 μL of ice-cold isotonic buffer (0.001 mol L^{-1} EGTA, 0.53 mol L^{-1} NaCl, 0.01 mol L^{-1} Tris–HCl, pH 7.6) modified from Xing *et al.* (1998). The coelomocytes were then homogenized on ice with a sonicator (Sonic, Vibra Cell, USA) for 10 s at 20% amplitude and centrifuged at 12 000 × *g* for 5 min (Heraeus Biofuge Stratos, Germany). Supernatants were collected for SOD activity and protein content assay.

Total soluble protein of coelomocyte lysate was measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute, China), based on the method described by Bradford (1976). Bovine serum albumin (P0834; purchased from sigma) was used as standard.

SOD activity was measured by its ability to inhibit superoxide anion generated by xanthine and xanthine oxidase reaction system according to Wang and Chen (2005) using an SOD detection kit (Nanjing Jiancheng Bioengineering Institute, China). The optical density was measured at 550 nm. One unit of SOD was defined as the amount required for inhibiting the rate of xanthine reduction by 50% in 1-mL reaction system. Specific activity was expressed as SOD units mg^{-1} protein.

Challenge test

A virulent strain of *Vibrio splendidus* was provided by Yellow-sea Fishery Research Institute, Chinese Academy of Fishery Sciences (Qingdao, China). The strain was originally isolated from sea

cucumbers diagnosed of skin ulceration disease (Zhang, Wang & Rong 2006).

Before the challenge test, *V. splendidus* was grown in Difco™ marine broth 2216 (REF279110; purchased from Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 28°C for 24 h with constant shaking as the stock culture for the challenge test. After centrifuged at 5000 × *g* for 10 min at 4°C (Sorvall Legend RT, Germany), the bacterial pellets were re-suspended in PBS to form a concentration of 10^9 colony forming unit (CFU) mL^{-1}.

After the 4-week feeding experiment, sea cucumbers in the same treatment were pooled. Then, 18 sea cucumbers in each treatment were randomly chosen and injected with 0.1-mL PBS. These 18 sea cucumbers were used as the blank injection. Another pooled 90 sea cucumbers in the same treatment were randomly selected and injected with 0.1-mL bacterial suspension (10^9 CFU mL^{-1} in PBS). After injection, these 90 sea cucumbers were randomly divided into three replicates. All sea cucumbers were observed once daily for 14 days and the cumulative mortality was recorded.

Calculations and statistical analysis

The growth data were expressed as the specific growth rate (SGR). It was calculated as follows: Specific growth rate (SGR)% = [Ln (final weight)–Ln (initial weight)]/time (day) × 100

Results are presented as mean ± S.E. (standard error of means). SPSS (Version 15.0) programs were used for the statistical analysis. One-way analysis of variance (One-way ANOVA) and Tukey's multiple comparison were used to determine whether significant difference existed between the treatments. All tests used a significance level of $P < 0.05$.

Results

Growth

As can be seen from Figure 1, sea cucumbers fed with immunostimulants, regardless of continuous or alternate feeding, showed significantly higher SGR than those in the control group ($P < 0.05$). There was no significant difference in the SGR between sea cucumbers fed with β-glucan continuously and those fed with MOS continuously ($P > 0.05$). The SGR of sea cucumbers with the feeding strategy of 2-day β-glucan/5-day MOS alternately was significantly higher than those with 5-day β-glucan/2-day MOS ($P < 0.05$). However, SGRs in these two treatments were not significantly different from those of sea cucumbers fed with β-glucan or MOS continuously ($P > 0.05$). The highest SGR (3.6941) was observed in the sea cucumbers fed with β-glucan 7 days followed by MOS 7 days alternately (Treatment 5).

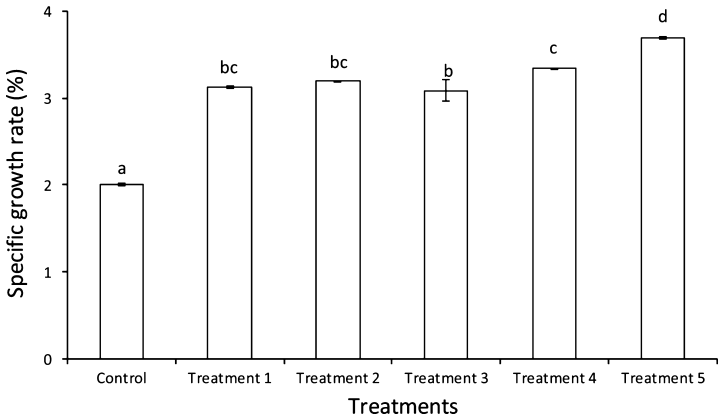

Figure 1 Specific growth rate (SGR) of *A. japonicus* fed with basal diet (control) and diets containing β-glucan (1250 mg kg^{-1}) or MOS (2000 mg kg^{-1}) under different feeding strategies for 29 days. Treatment 1, sea cucumbers were fed with diet containing β-glucan continuously; Treatment 2, sea cucumbers were fed with diet containing MOS continuously; Treatment 3, sea cucumbers were fed with diet containing β-glucan 2 days and then with diet containing MOS 5 days alternately; Treatment 4, sea cucumbers were fed with diet containing β-glucan 5 days and then with diet containing MOS 2 days alternately; Treatment 5, sea cucumbers were fed with diet containing β-glucan 7 days and then with diet containing MOS 7 days alternately. Each bar represents mean value from six replicates with standard error of means. Data with different letters are significantly different ($P < 0.05$).

Immune parameters assay

Values of total coelomocytes counts (TCC, $12.58 \pm 0.12 \times 10^6$ cell mL^{-1}, mean ± S.E.), superoxide anion production (OD630 0.385 ± 0.014), SOD activity (58.34 ± 0.42 U mg^{-1} protein) and phagocytosis (14.55 ± 0.08%) of the sea cucumbers fed with β-glucan continuously were significantly higher ($P < 0.05$) than those in the control group ($11.42 \pm 0.15 \times 10^6$ cell mL^{-1}, OD630 0.215 ± 0.022, 55.67 ± 0.19 U mg^{-1} protein, 12.42 ± 0.21%) on the 4th day (Tables 3–6). After the 18th day, TCC and superoxide anion production of the sea cucumbers continuously fed with β-glucan were not significantly higher than those in the control ($P > 0.05$; Table 3 and Table 4). However, SOD activity and phagocytosis of the sea cucumbers fed with β-glucan continuously were significantly higher than those in the control during the 4-week feeding trial ($P < 0.05$; Table 5 and Table 6).

Values of TCC ($15.12 \pm 0.87 \times 10^6$ cell mL^{-1}) and superoxide anion production (OD630 0.430 ± 0.031) of the sea cucumbers fed with MOS continuously were significantly higher ($P < 0.05$) than those in the control group ($13.18 \pm 0.06 \times 10^6$ cell mL^{-1} and OD630 0.295 ± 0.011 respectively) on the 15th day (Tables 3 and 4). SOD activity and phagocytosis of the sea cucumbers fed with MOS continuously were significantly higher than those in the control group all along the experiment (Tables 5 and 6).

Overall, compared with those in the control, the analysed immune parameters of sea cucumbers fed with β-glucan and MOS alternately, or fed with β-glucan continuously, were significantly higher on the 4th day ($P < 0.05$). After the 18th day, the TCC in the sea cucumbers fed with β-glucan continuously was no longer significantly higher ($P > 0.05$) than that in the control group (Table 3). However, TCC of sea cucumbers fed with β-glucan and MOS alternately was still significantly higher ($P < 0.05$) than that in the control group. The same thing occurred in superoxide anion production (Table 4). SOD activity and phagocytosis were significantly higher than those in the control group all along the experiment (Tables 5 and 6).

Susceptibility to the V. splendidus

During the 14 days, all the sea cucumbers injected with PBS survived. By contrast, mortality occurred on the 4th day for the sea cucumbers in the control

Table 3 Total coelomocytes counts (10^6 cells per mL coelomic fluid) of *A. japonicus* fed with basal diet and diets containing β-glucan or MOS under different feeding strategies for 29 days*

	Sampling time (Day)								
Treatment	**1**	**4**	**7**	**11**	**15**	**18**	**22**	**25**	**29**
Control[1]	11.33 ± 0.08	11.42 ± 0.15^a	11.17 ± 0.38^a	12.03 ± 0.04^b	13.18 ± 0.06^a	11.37 ± 0.22^a	11.20 ± 0.24^a	13.87 ± 0.09^a	13.42 ± 0.22^a
Treatment 1[2]	11.42 ± 0.31	12.58 ± 0.12^b	12.97 ± 0.03^b	12.82 ± 0.28^c	15.17 ± 0.16^c	11.82 ± 0.06^b	11.78 ± 0.11^{ab}	13.10 ± 0.06^a	13.90 ± 0.08^a
Treatment 2[3]	11.35 ± 0.18	11.88 ± 0.09^a	11.29 ± 0.45^a	11.37 ± 0.07^a	15.12 ± 0.87^c	15.83 ± 0.29^d	14.50 ± 0.09^c	14.67 ± 0.09^b	14.70 ± 0.12^b
Treatment 3[4]	11.37 ± 0.23	14.55 ± 0.45^c	13.01 ± 0.43^b	13.00 ± 0.31^c	13.88 ± 0.17^b	12.57 ± 0.29^c	13.22 ± 0.11^b	14.47 ± 0.52^b	15.52 ± 0.20^c
Treatment 4[5]	11.41 ± 0.33	12.38 ± 0.15^b	13.47 ± 0.57^c	13.58 ± 0.42^d	15.37 ± 0.06^d	15.62 ± 0.27^d	15.40 ± 0.23^d	14.07 ± 0.20^b	15.45 ± 0.26^c
Treatment 5[6]	11.41 ± 0.17	12.48 ± 0.14^b	13.40 ± 0.49^c	13.80 ± 0.23^d	14.28 ± 0.22^b	15.45 ± 0.05^d	13.47 ± 0.20^b	14.95 ± 0.15^b	14.05 ± 0.38^b

[1]Sea cucumbers were fed with basal diet continuously.
[2]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) continuously.
[3]Sea cucumbers were fed with diet containing MOS (2000 mg kg^{-1}) continuously.
[4]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 2 days and then with diet containing MOS (2000 mg kg^{-1}) for 5 days alternately.
[5]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 5 days and then with diet containing MOS (2000 mg kg^{-1}) for 2 days alternately.
[6]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 7 days and then with diet containing MOS (2000 mg kg^{-1}) for 7 days alternately.
*Data were expressed as mean ± S.E. Data in the same column with different letters are significantly different ($P < 0.05$).

Table 4 Superoxide anion (OD630) in coelomocytes of *A. japonicus* fed with basal diet and diets containing β-glucan or MOS under different feeding strategies for 29 days*

	Sampling time (Day)								
Treatment	**1**	**4**	**7**	**11**	**15**	**18**	**22**	**25**	**29**
Control[1]	0.275 ± 0.011	0.215 ± 0.022^{a}	0.290 ± 0.003^{ab}	0.270 ± 0.046^{a}	0.295 ± 0.011^{a}	0.290 ± 0.011^{b}	0.285 ± 0.011^{a}	0.225 ± 0.016^{a}	0.295 ± 0.04^{a}
Treatment 1[2]	0.265 ± 0.015	0.385 ± 0.014^{b}	0.390 ± 0.031^{d}	0.415 ± 0.007^{b}	0.445 ± 0.021^{c}	0.205 ± 0.025^{a}	0.310 ± 0.025^{abc}	0.235 ± 0.003^{a}	0.29 ± 0.022^{a}
Treatment 2[3]	0.255 ± 0.022	0.200 ± 0.018^{a}	0.260 ± 0.042^{a}	0.275 ± 0.025^{a}	0.430 ± 0.031^{c}	0.330 ± 0.016^{c}	0.315 ± 0.016^{bc}	0.270 ± 0.018^{b}	0.365 ± 0.05^{b}
Treatment 3[4]	0.245 ± 0.023	0.370 ± 0.007^{b}	0.330 ± 0.013^{c}	0.410 ± 0.010^{b}	0.410 ± 0.020^{c}	0.320 ± 0.028^{c}	0.350 ± 0.028^{d}	0.275 ± 0.020^{b}	0.370 ± 0.049^{b}
Treatment 4[5]	0.260 ± 0.014	0.375 ± 0.009^{b}	0.310 ± 0.008^{bc}	0.430 ± 0.051^{b}	0.435 ± 0.036^{c}	0.315 ± 0.012^{c}	0.335 ± 0.012^{cd}	0.290 ± 0.016^{b}	0.340 ± 0.023^{b}
Treatment 5[6]	0.265 ± 0.015	0.380 ± 0.017^{b}	0.340 ± 0.009^{c}	0.410 ± 0.003^{b}	0.370 ± 0.021^{b}	0.340 ± 0.007^{c}	0.355 ± 0.007^{d}	0.280 ± 0.007^{b}	0.370 ± 0.015^{b}

[1]Sea cucumbers were fed with basal diet continuously.
[2]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) continuously.
[3]Sea cucumbers were fed with diet containing MOS (2000 mg kg^{-1}) continuously.
[4]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 2 days and then with diet containing MOS (2000 mg kg^{-1}) for 5 days alternately.
[5]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 5 days and then with diet containing MOS (2000 mg kg^{-1}) for 2 days alternately.
[6]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 7 days and then with diet containing MOS (2000 mg kg^{-1}) for 7 days alternately.
*Data were expressed as mean ± S.E. Data in the same column with different letters are significantly different ($P < 0.05$).

Table 5 Superoxide dismutase (SOD) activity (units per mg protein) of *A. japonicus* fed with basal diet and diets containing β-glucan or MOS under different feeding strategies for 29 days*

	Sampling time (Day)								
Treatment	**1**	**4**	**7**	**11**	**15**	**18**	**22**	**25**	**29**
Control[1]	55.34 ± 1.35	55.67 ± 0.19^{a}	52.49 ± 0.64^{a}	55.74 ± 1.41^{a}	55.80 ± 0.57^{a}	51.55 ± 0.26^{a}	54.86 ± 0.82^{a}	53.44 ± 2.55^{a}	52.12 ± 0.49^{a}
Treatment 1[2]	55.31 ± 0.69	58.34 ± 0.42^{b}	56.36 ± 0.47^{b}	58.24 ± 1.13^{b}	58.15 ± 0.29^{b}	56.29 ± 0.30^{b}	55.21 ± 0.54^{b}	55.31 ± 2.18^{b}	53.67 ± 1.62^{b}
Treatment 2[3]	54.62 ± 1.21	61.39 ± 1.81^{b}	57.84 ± 0.26^{b}	61.16 ± 1.73^{b}	62.31 ± 1.61^{c}	60.56 ± 1.66^{b}	58.78 ± 1.12^{c}	62.87 ± 1.98^{d}	61.36 ± 1.56^{d}
Treatment 3[4]	55.12 ± 1.99	58.85 ± 0.16^{b}	55.28 ± 2.01^{b}	61.49 ± 1.14^{c}	67.79 ± 0.54^{d}	63.35 ± 0.39^{c}	62.05 ± 1.07^{d}	61.06 ± 1.23^{c}	56.52 ± 1.88^{c}
Treatment 4[5]	55.03 ± 0.85	58.45 ± 0.93^{b}	56.10 ± 1.53^{b}	62.87 ± 1.99^{c}	61.76 ± 1.55^{c}	63.59 ± 0.93^{c}	56.89 ± 1.04^{bc}	54.93 ± 2.09^{b}	53.64 ± 1.87^{b}
Treatment 5[6]	55.19 ± 1.66	57.51 ± 0.62^{b}	56.28 ± 0.46^{b}	63.55 ± 2.47^{c}	58.03 ± 2.10^{b}	62.90 ± 0.72^{c}	61.66 ± 0.85^{d}	54.66 ± 0.78^{b}	53.98 ± 0.78^{b}

[1]Sea cucumbers were fed with basal diet continuously.
[2]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) continuously.
[3]Sea cucumbers were fed with diet containing MOS (2000 mg kg^{-1}) continuously.
[4]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 2 days and then with diet containing MOS (2000 mg kg^{-1}) for 5 days alternately.
[5]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 5 days and then with diet containing MOS (2000 mg kg^{-1}) for 2 days alternately.
[6]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 7 days and then with diet containing MOS (2000 mg kg^{-1}) for 7 days alternately.
*Data were expressed as mean ± S.E. Data in the same column with different letters are significantly different ($P < 0.05$).

Table 6 Phagocytosis (%) of *A. japonicus* fed with basal diet and diets containing β-glucan or MOS under different feeding strategies for 29 days*

Treatment	Sampling time (Day)							
	1	4	7	11	15	18	22	25	29
Control[1]	12.69 ± 0.11	12.42 ± 0.21^{a}	13.80 ± 0.10^{a}	12.99 ± 0.23^{a}	12.21 ± 0.17^{a}	13.50 ± 0.17^{a}	12.81 ± 0.14^{a}	14.43 ± 0.26^{a}	12.00 ± 0.13^{a}
Treatment 1[2]	13.53 ± 0.15	14.55 ± 0.08^{b}	15.69 ± 0.18^{b}	14.70 ± 0.17^{b}	14.19 ± 0.09^{b}	15.81 ± 0.09^{b}	17.40 ± 0.43^{b}	16.32 ± 0.19^{b}	14.70 ± 0.21^{b}
Treatment 2[3]	12.78 ± 0.13	14.82 ± 0.17^{b}	15.70 ± 0.15^{b}	15.39 ± 0.12^{b}	14.01 ± 0.24^{b}	16.11 ± 0.24^{b}	17.55 ± 0.18^{b}	17.10 ± 0.13^{b}	16.26 ± 0.22^{b}
Treatment 3[4]	12.93 ± 0.14	16.74 ± 0.13^{c}	17.00 ± 0.26^{c}	17.23 ± 0.12^{c}	17.64 ± 0.29^{c}	18.21 ± 0.29^{c}	20.79 ± 0.18^{c}	18.76 ± 0.13^{c}	19.92 ± 0.12^{c}
Treatment 4[5]	12.66 ± 0.22	17.22 ± 0.06^{c}	18.90 ± 0.23^{d}	19.55 ± 0.06^{d}	18.40 ± 0.40^{c}	18.69 ± 0.40^{c}	22.29 ± 0.34^{c}	19.99 ± 0.06^{c}	20.80 ± 0.15^{d}
Treatment 5[6]	12.33 ± 0.21	15.63 ± 0.24^{bc}	17.51 ± 0.18^{c}	19.90 ± 0.12^{d}	19.80 ± 0.35^{d}	17.01 ± 0.35^{bc}	19.29 ± 0.12^{c}	20.98 ± 0.13^{c}	19.15 ± 0.13^{c}

[1]Sea cucumbers were fed with basal diet continuously.
[2]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) continuously.
[3]Sea cucumbers were fed with diet containing MOS (2000 mg kg^{-1}) continuously.
[4]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 2 days and then with diet containing MOS (2000 mg kg^{-1}) for 5 days alternately.
[5]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 5 days and then with diet containing MOS (2000 mg kg^{-1}) for 2 days alternately.
[6]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 7 days and then with diet containing MOS (2000 mg kg^{-1}) for 7 days alternately.
*Data were expressed as mean ± S.E. Data in the same column with different letters are significantly different ($P < 0.05$).

group after being injected with bacterial suspension and the cumulative mortality was 55.5% in 14 days. Immunostmulants could delay the happening of mortality 1 or 2 days. The cumulative mortality of the sea cucumbers fed with β-glucan or MOS continuously was significantly lower than that in the control group ($P < 0.05$). Sea cucumbers fed with MOS showed less mortality than those fed with β-glucan, however, no significant difference was observed. The cumulative mortalities of sea cucumbers fed by the three alternate strategies (Treatment 3, Treatment 4 and Treatment 5) were significantly lower than those in the groups feeding β-glucan continuously (Treatment 1) or MOS continuously (Treatment 2) ($P < 0.05$). The sea cucumbers fed by the strategy of 2-day β-glucan/5-day MOS alternately (Treatment 3) showed the lowest cumulative mortality (21.1 ± 0.03%) among all the groups (Table 7).

Discussion

In this study, β-glucan and MOS could promote the growth of sea cucumbers. β-glucan has been proved to improve the growth performance in several fish (Sakai 1999) and shrimps (Smith *et al.* 2003). Dalmo and Bøgwald (2008) hypothesized that eaten β-glucan could induce local intestinal inflammatory response and increase resistance against pathogens. The pathogens would otherwise result in decreased weight gain and maybe disease. MOS has been reported to increase the growth of many aquatic animals (Ringø *et al.* 2010), such as rainbow trout *Oncorhynchus mykiss* (Staykov, Spring, Denev & Sweetman 2007), sea bass *Dicentrarchus labrax* (Torrecillas, Makol, Caballero, Montero, Robaina, Real, Sweetman, Tort & Izquierdo 2007), lobster *Panulirus ornatus* (Sang & Fotedar 2010) and shrimp *Panulirus ornatus* (Genc, Aktas, Genc & Yilmaz 2007). The mechanism could be that MOS improves the gut morphology and function (Ringø *et al.* 2010). In this study, when sea cucumbers were fed with dietary β-glucan for 7 days followed by dietary MOS for 7 days alternately, the SGR was significantly higher than those fed with β-glucan or MOS continuously. However, SGR of sea cucumbers fed by the other two alternately feeding strategies was not significantly higher than those fed β-glucan or MOS continuously. It is suggested that even under alternate strategy, one immunostimulant should be used continuously at least 7 days to take

Table 7 Cumulative mortality of *A. japonicus* fed basal diet and diets containing β-glucan or MOS under different feeding strategies after *Vibrio Splendidus* challenge*

Bacteria (cfu sea cucumber^{-1})	Treatment	No. of sea sea cucumber	Cumulative mortality (%), number of dead sea cucumbers (in each group) and time after challenge (Day)													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1×10^8	Control[1]	3×30	0	0	0	8.9 ± 0.01	20.0 ± 0.02	35.5 ± 0.04^{c}	45.5 ± 0.02^{c}	47.8 ± 0.01^{d}	51.1 ± 0.01^{e}	52.2 ± 0.01^{d}	55.5 ± 0.01^{c}	55.5 ± 0.01^{c}	55.5 ± 0.01^{c}	55.5 ± 0.01^{c}
			(0,0,0)	(0,0,0)	(0,0,0)	(3,2,3)	(6,7,5)	(10,9,13)	(15,13,13)	(15,14,14)	(15,16,15)	(15,16,16)	(16,17,17)	(16,17,17)	(16,17,17)	(16,17,17)
1×10^8	Treatment 1[2]	3×30	0	0	0	0	0	0^{a}	7.8 ± 0.01^{a}	12.2 ± 0.01^{ab}	15.6 ± 0.01^{bc}	24.4 ± 0.3^{c}	30.0 ± 0.02^{b}	42.2 ± 0.05^{b}	43.3 ± 0.04^{b}	43.3 ± 0.04^{b}
			(0,0,0)	(0,0,0)	(0,0,0)	(0,0,0)	(0,0,0)	(0,0,0)	(2,2,3)	(4,4,3)	(5,5,4)	(6,7,9)	(8,9,10)	(13,10,15)	(13,11,15)	(13,11,15)
1×10^8	Treatment 2[3]	3×30	0	0	0	0	5.5 ± 0.01	12.2 ± 0.03^{b}	17.8 ± 0.02^{b}	21.1 ± 0.02^{c}	23.3 ± 0.02^{d}	26.7 ± 0.02^{c}	28.9 ± 0.03^{b}	34.4 ± 0.01^{b}	34.4 ± 0.01^{b}	34.4 ± 0.01^{b}
			(0,0,0)	(0,0,0)	(0,0,0)	(0,0,0)	(2,1,2)	(2,5,4)	(4,6,6)	(5,7,7)	(6,8,7)	(7,8,9)	(7,9,10)	(10,11,10)	(10,11,10)	(10,11,10)
1×10^8	Treatment 3[4]	3×30	0	0	0	0	0	2.2 ± 0.01^{ab}	4.4 ± 0.01^{a}	6.6 ± 0.02^{a}	8.9 ± 0.01^{a}	12.2 ± 0.01^{a}	14.4 ± 0.02^{a}	14.7 ± 0.01^{a}	20.0 ± 0.02^{a}	21.1 ± 0.03^{a}
			(0,0,0)	(0,0,0)	(0,0,0)	(0,0,0)	(0,0,0)	(0,1,1)	(2,1,1)	(3,1,2)	(3,3,2)	(4,3,4)	(5,3,5)	(5,4,6)	(6,5,7)	(6,5,8)
1×10^8	Treatment 4[5]	3×30	0	0	0	0	0	4.4 ± 0.01^{ab}	7.8 ± 0.01^{a}	11.1 ± 0.01^{a}	12.2 ± 0.01^{ab}	14.4 ± 0.01^{ab}	20.0 ± 0.02^{a}	21.1 ± 0.03^{a}	22.2 ± 0.03^{a}	23.3 ± 0.02^{a}
			(0,0,0)	(0,0,0)	(0,0,0)	(0,0,0)	(0,0,0)	(1,2,1)	(2,2,3)	(3,4,3)	(3,4,4)	(4,4,5)	(5,6,7)	(5,6,8)	(5,7,8)	(6,7,8)
1×10^8	Treatment 5[6]	3×30	0	0	0	0	0	7.8 ± 0.01^{ab}	12.2 ± 0.02^{ab}	18.9 ± 0.01^{bc}	21.1 ± 0.01^{cd}	22.2 ± 0.02^{bc}	22.2 ± 0.02^{ab}	22.2 ± 0.02^{ab}	22.2 ± 0.02^{a}	22.2 ± 0.02^{a}
			(0,0,0)	(0,0,0)	(0,0,0)	(0,0,0)	(0,0,0)	(2,2,3)	(3,3,5)	(5,6,6)	(6,6,7)	(6,6,8)	(6,6,8)	(6,6,8)	(6,6,8)	(6,6,8)
0(PBS)	Blank	108	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1]Sea cucumbers were fed with basal diet continuously.
[2]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) continuously.
[3]Sea cucumbers were fed with diet containing MOS (2000 mg kg^{-1}) continuously.
[4]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 2 days and then with diet containing MOS (2000 mg kg^{-1}) for 5 days alternately.
[5]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 5 days and then with diet containing MOS (2000 mg kg^{-1}) for 2 days alternately.
[6]Sea cucumbers were fed with diet containing β-glucan (1250 mg kg^{-1}) for 7 days and then with diet containing MOS (2000 mg kg^{-1}) for 7 days alternately.
*Data were expressed as mean ± S.E. Data in the same column with different letters are significantly different ($P < 0.05$).

advantage of the synergistic effect of β-glucan and MOS on growth.

In this study, continuous oral administration of β-glucan caused immunity fatigue in *A. japonicus*. Compared with those in the control group, TCC and superoxide anion production of the sea cucumbers fed with β-glucan continuously were significantly higher on the 4th day. However, after the 18th day, there were no significant differences in TCC and superoxide anion production between these two groups. Similar finding has also been reported in a previous experiment on *L. vannamei*, in which β-glucan and glycyrrhizin were tested (Bai *et al.* 2010). The decrease in TCC may be due to the formation of brown body, which is the encapsulation structure of echinoderms formulated by coelomocytes (Canicatti, D'Ancona & Farina-Lipari 1989). As an important part of sea cucumber immune system, brown body can surround and digest non-self substance (Coteur, Warnau, Jangoux & Dubois 2002). However, the formation of brown body could decrease the amount of coelomocytes and reduce the source of superoxide anion. By contrast, in this study, the SOD activity and phagocytosis of the sea cucumbers fed with β-glucan were significantly higher than those of the control group during the 4-week experiment. The results of phagocytosis revealed that β-glucan could increase the proportion of cells, which were activated to phagocytize pathogen. Even when the TCC decreased, this proportion of cells in sea cucumbers fed with β-glucan continuously was still higher than that in the control group.

In this study, the changing trend of TCC and superoxide anion of the sea cucumbers fed with MOS continuously is different compared with those fed with β-glucan continuously. Compared with those in the control group, TCC and superoxide anion production of sea cucumbers fed with β-glucan continuously were significantly higher on the 4th day. With the experiment prolonging, these two parameters were not significantly different after the 18th day. For the sea cucumbers fed with MOS continuously, these two immune parameters were not significantly higher than those in the control group until the 15th day. The different changing trend of immune parameters may be due to different mechanisms of β-glucan and MOS to activate the immune system. Studies have shown that immune system of sea cucumber *Holothuria glaberrima* responds differentially to different pathogen-associated molecular patterns (PAMPs) (Ramírez-Gómez, Aponte-Rivera, Méndez-Castaner & García-Arrarás 2010). In the sea cucumber *A. japonicus*, mannose-binding lectin (MBL), the pattern-recognition proteins (PRPs) for mannan, has already been isolated (Bulgakov, Eliseikina, Petrova, Nazarenko, Kovalchuk, Kozhemyako & Rassbazov 2007). MBL are known to activate the lectin pathway of the complement system, which is thought to play an important role in host defence in lower animals (Fujita 2002), and were proved to be related with phagocytosis in echinoderms (Bulgakov, Nazarenko, Petrova, Eliseikina, Vakhrusheva & Zubkov 2000). β-glucan can be identified by β-glucan binding protein (βGBP) or LPS and β-glucan binding protein (LGBP) and trigger the activation of prophenoloxidase (proPO) system as shown in invertebrates like shrimp (Dalmo & Bøgwald 2008) and scallop (Su, Song, Xu, Wu, Li & Xiang 2004). This difference in recognition and activation pathway for β-glucan and MOS may account for the difference of immune parameters in this study.

In this study, all assayed four immune parameters of the sea cucumbers in alternate groups were significantly higher than those in the control group on the 4th day. This is same as the sea cucumbers fed with β-glucan alone. After the 18th day, the TCC and superoxide anion production of the sea cucumbers fed with β-glucan alone decreased. However, these two immune parameters in the three alternate groups were still significantly higher than those in the control group. Furthermore, phagocytosis of sea cucumbers in the three alternate groups was significantly higher than those fed with β-glucan or MOS continuously. These results showed that alternate administration of β-glucan and MOS is better to stimulate the immune system. It was implied that when the sea cucumbers were fed with β-glucan, part of immune system was activated and the coelomocytes were consumed for the formation of brown body. With the subsequent feeding of MOS, sea cucumber's coelomocytes may have been compensated and other part of immune system was activated. This hypothesis could explain, in this study, that the immune parameters of the sea cucumbers in the alternate patterns were significantly higher than those in the control group along the experiment. Furthermore, it could also explain that the phagocytosis of the sea cucumbers fed with β-glucan and MOS alternately (Treatment 3, Treatment 4 and Treatment 5) was significantly higher than

those fed with β-glucan continuously (Treatment 1) or MOS continuously (Treatment 2). Further work is needed to confirm this hypothesis.

The results of susceptibility to the *V. splendidus* confirmed and can be explained by the differences of the immune parameters among groups. At the end of the feeding experiment, the SOD activity and phagocytosis of the sea cucumbers fed with β-glucan continuously were significantly higher than those in the control group. And these significantly higher two immune parameters could explain the significantly lower mortality rate of the sea cucumbers fed with β-glucan continuously compared with those in control group. For the sea cucumbers fed with MOS continuously, the assayed four parameters were all significantly higher than those in the control group, which explain the lower cumulative mortality compared with control. TCC and superoxide anion of sea cucumbers fed with dietary MOS continuously were significantly higher than those fed with dietary β-glucan continuously at the beginning of the challenge experiment. These two higher immune parameters can explain the lower cumulative mortality of the sea cucumbers fed with dietary MOS continuously. All immune parameters of sea cucumbers in three alternate groups were significantly higher than those in the control group, and the cumulative mortality of the alternate groups was only half of that in the control group. The TCC and phagocytosis of sea cucumbers in the alternate group were significantly higher than those fed with MOS continuously. This could be used to explain the significantly lower mortality rate of the sea cucumbers in the alternate groups compared with those in the MOS group.

In conclusion, alternate administration of β-glucan and MOS is better than continuously pattern to lead a final promoting growth, immune responses and resistance to infection. On the whole, among all the experimental treatment, the strategy alternately feeding β-glucan 7 days then MOS 7 days is most suitable for sea cucumbers, because it shows the highest SGR and the second lowest cumulative mortality.

Acknowledgments

This study was supported by national high-tech research and development program of China (2006AA100313). We appreciate Mr. Mingzhuang Zhu for his assistance in the operation of flow cytometry.

References

Bai N., Zhang W.B., Mai K.S., Wang X.J., Xu W. & Ma H.M. (2010) Effects of discontinuous administration of β-glucan and glycyrrhizin on the growth and immunity of white shrimp *Litopenaeus vannamei*. *Aquaculture* **306**, 218–224.

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Bricknell I. & Dalmo R.A. (2005) The use of immunostimulants in fish larval aquaculture. *Fish & Shellfish Immunology* **19**, 457–472.

Bulgakov A.A., Nazarenko E.L., Petrova I.Y., Eliseikina M.G., Vakhrusheva N.M. & Zubkov V.A. (2000) Isolation and properties of a mannan-binding lectin from the coelomic fluid of the holothurian *Cucumaria japonica*. *Biochemistry* **65**, 933–939.

Bulgakov A.A., Eliseikina M.G., Petrova I.Y., Nazarenko E.L., Kovalchuk S.N., Kozhemyako V.B. & Rassbazov V.A. (2007) Molecular and biological characterization of a mannan-binding lectin from the holothurian *Apostichopus japonicus*. *Glycobiology* **17**, 1284–1298.

Canicatti C., D'Ancona G. & Farina-Lipari E. (1989) The *Holothuria polii* brown bodies. *Italian Journal of Zoology* **56**, 275–283.

Chang C.F., Chen H.Y., Su M.S. & Liao I.C. (2000) Immunomodulation by dietary β-1,3 glucan in the brooders of the black tiger shrimp *Penaeus monodon*. *Fish & Shellfish Immunology* **10**, 505–514.

Chen J.X. (2003) Overview of sea cucumber farming and sea ranching practices in China. *SPC Beche-de-Mer Information Bulletin* **18**, 18–23.

Coteur G., Warnau M., Jangoux M. & Dubois P. (2002) Reactive oxygen species (ROS) production by amoebocytes of *Asterias rubens* (Echinodermata). *Fish & Shellfish Immunology* **12**, 187–200.

Dalmo R.A. & Bøgwald J. (2008) β-glucans as conductors of immune symphonies. *Fish & Shellfish Immunology* **25**, 384–396.

Deng H., He C.B., Zhou Z.C., Liu C., Tan K.F., Wang N.B., Jing B., Gao X.G. & Liu W.D. (2009) Isolation and pathogenicity of pathogens from skin ulceration disease and viscera ejection syndrome of the sea cucumber *Apostichopus japonicus*. *Aquaculture* **287**, 18–27.

Esteban M.A., Rodríguez A., Cuesta A. & Meseguer J. (2005) Effects of lactoferrin on non-specific immune responses of gilthead seabream (*Sparus auratus* L.). *Fish & Shellfish Immunology* **18**, 109–114.

Fujita T. (2002) Evolution of the lectin-complement pathway and its role in innate immunity. *Nature Reviews Immunology* **2**, 346–353.

Genc M.A., Aktas M., Genc E. & Yilmaz E. (2007) Effects of dietary mannan oligosaccharides on growth, body composition and hepatopancreas histology of *Penaeus semisulcatus* (de Haan 1844). *Aquaculture Nutrition* **13**, 156–161.

Gu M., Ma H.M., Mai K.S., Zhang W.B., Wang X.J. & Bai N. (2010) Immune response of sea cucumber *Apostichopus japonicus* coelomocytes to several immunostimulants *in vitro*. *Aquaculture* **306**, 49–56.

Gu M., Ma H.M., Mai K.S., Zhang W.B., Bai N. & Wang X.J. (2011) Effects of dietary β-glucan, mannan oligosaccharide and their combinations on growth performance, immunity and resistance against *Vibrio splendidus* of sea cucumber, *Apostichopus japonicus*. *Fish & Shellfish Immunology* **31**, 303–309.

Hultmark D. (2003) *Drosopjila* immunity: paths and patterns. *Current Opinion in Immunology* **15**, 12–19.

Li J.Y., Sun X.Q., Zheng F.R. & Hao L.H. (2009) Screen and effect analysis of immunostimulants for sea cucumber, *Apostichopus japonicus*. *Chinese Journal of Oceanology and Limnology* **27**, 80–84.

Ramírez-Gómez F., Aponte-Rivera F., Méndez-Castaner L. & García-Arrarás J.E. (2010) Changes in holothurian coelomocyte populations following immune stimulation with different molecular patterns. *Fish & Shellfish Immunology* **29**, 175–185.

Ringø E., Olsen R.E., Gifstad T.Ø., Dalmo R.A., Amlund H., Hemre G.-I. & Bakke A.M. (2010) Prebiotics in aquaculture: a review. *Aquaculture Nutrition* **16**, 117–136.

Ringø E., Olsen R.E., Gonzalez Vecino J.L., Wadsworth S. & Song S.K. (2012) Use of immunostimulants and nucleotides in aquaculture: a review. *Journal of Marine Science Research & Development* **1**, 104–126. doi:10.4172/2155-9910.1000104.

Sajeevan T.P., Philip R. & Singh I.S. (2008) Dose/frequency: a critical factor in the administration of glucan as immunostimulant to Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture* **287**, 248–252.

Sakai M. (1999) Current research status of fish immunostimulants. *Aquaculture* **172**, 63–92.

Sang M.M. & Fotedar R. (2010) Effects of mannan oligosaccharide dietary supplementation on performances of the tropical spiny lobsters juvenile (*Panulirus ornatus*, Fabricius 1798). *Fish & Shellfish Immunology* **28**, 483–489.

Smith V.J., Brown J.H. & Hauton C. (2003) Immunostimulation in crustaceans: dose it really protect against infection? *Fish & Shellfish Immunology* **15**, 71–90.

Song Y.L. & Hsieh Y.T. (1994) Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. *Developmental & Comparative Immunology* **18**, 201–209.

Staykov Y., Spring P., Denev S. & Sweetman J. (2007) Effect of a mannan oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture International* **15**, 153–161.

Su J.G., Song L.S., Xu W., Wu L.T., Li H.L. & Xiang J.H. (2004) cDNA cloning and mRNA expression of the lipopolysaccharide-and beta-1,3-glucan-binding protein gene from scallop *Chlamys farreri*. *Aquaculture* **239**, 69–80.

Torrecillas S., Makol A., Caballero M.J., Montero D., Robaina L., Real F., Sweetman J., Tort L. & Izquierdo M.S. (2007) Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. *Fish & Shellfish Immunology* **23**, 969–981.

Wang S.H. & Chen J.C. (2005) The protective effect of chitin and chitosan against *Vibrio alginolyticus* in white shrimp *Litopenaeus vannamei*. *Fish & Shellfish Immunology* **19**, 191–204.

Wang T.T., Sun Y.X., Jin L.J., Xu Y.P., Wang L., Ren T.J. & Wang K.L. (2009) Enhancement of non-specific immune response in sea cucumber (*Apostichopus japonicus*) by *Astragalus membranaceus* and its polysaccharides. *Fish & Shellfish Immunology* **27**, 757–762.

Xing J., Leung M.F. & Chia F.S. (1998) Quantitative analysis of phagocytosis by amebocytes of a sea cucumber, *Holothuria leucospilota*. *Invertebrate Biology* **117**, 67–74.

Zhang C.Y., Wang Y.G. & Rong X.J. (2006) Isolation and identification of causative pathogen for skin ulcerative syndrome in *Apostichopus japonicus*. *Journal of Fishery Sciences of China* **30**, 118–123 (in Chinese with English abstract).

Zhang Q., Ma H.M., Mai K.S., Zhang W.B., Liufu Z.G. & Xu W. (2010) Interaction of dietary *Bacillus subtilis* and fructooligosaccharide on the growth performance, non-specific immunity of sea cucumber, *Apostichopus japonicus*. *Fish & Shellfish Immunology* **29**, 204–211.

Zhao Y.C., Ma H.M., Zhang W.B., Ai Q.H., Mai K.S., Xu W., Wang X.J. & Liufu Z.G. (2011) Effects of dietary β-glucan on the growth, immune responses and resistance of sea cucumber, *Apostichopus japonicus* against *Vibrio splendidus* infection. *Aquaculture* **315**, 269–274.

Zheng F.R., Liu H.Z., Sun X.Q., Qu L.Y., Dong S.L. & Liu J.L. (2010) Selection, identification and application of antagonistic bacteria associated with skin ulceration and peristome tumescence of cultured sea cucumber *Apostichopus japonicus* (Selenka). *Aquaculture* **334–337**, 24–29.

Zhu W., Mai K.S., Zhang B.G., Wang F.Z. & Xu G.Y. (2005) Study on dietary protein and lipid requirement for sea cucumber. Stichopus japonicus. *Mariculture Science* **29**, 54–58 (in Chinese with English abstract).

