Aquaculture Research

Aquaculture Research, 2017, **48**, 1158–1167 doi: 10.1111/are.12957

# Effect of dietary chitosan oligosaccharide complex with Ce (IV) on growth, immunity and disease resistance against *Vibrio splendidus* of sea cucumber, *Apostichopus japonicas*

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## Abstract

An 8-week feeding trial was conducted to investigate the effects of dietary chitosan oligosaccharide complex with cerium (Ce IV) (COS-Ce) on growth performance, nonspecific immunity and disease resistance of sea cucumber, *Apostichopus japonicas*. Five isonitrogenous (18.6%) and isolipidic (1.1%) practical diets were formulated with graded level of COS-Ce (0, 150, 300, 600 and 1200 mg $kg^{-1}$ dry feed), which were named as COS-Ce/0, COS-Ce/150, COS-Ce/300, COS-Ce/600, COS-Ce/1200 respectively. Each diet was allocated to four replicates of sea cucumbers (Initial weight: 6.72 ± 0.02 g). Sea cucumbers were fed to apparent satiation once daily (19:00 hours) for 56 days. During the experiment, water temperature was kept at 16 ± 0.5°C, pH 7.8–8.2, dissolved oxygen beyond 5 mg $L^{-1}$, ammonia nitrogen below 0.5 mg $L^{-1}$ and salinity from 30‰ to 31‰. Results showed that the specific growth rate of sea cucumbers was significantly higher in COS-Ce/600 than that in other four treatments. Activities of phagocytosis, respiratory burst, acid phosphatase and alkaline phosphatase in COS-Ce/600 were significantly higher than that in COS-Ce/0 ($P < 0.05$) respectively. On the contrary, cumulative mortality was the lowest in COS-Ce/600 following 14 days exposure to *Vibrio splendidus* ($P < 0.05$). In conclusion, these results confirmed that dietary COS-Ce had beneficial effects on growth performance, nonspecific immunity and disease resistance of sea cucumber.

**Keywords:** chitosan oligosaccharide complex with Ce (IV), sea cucumber, *Apostichopus japonicas*, growth, immunity

## Introduction

The sea cucumber (*Apostichopus japonicas*) was characterized by rich collagen protein, active polysaccharide and mineral elements, as well as high medicinal value, which has made it become one of the most important economic marine-culture species in the north of China. The rapid expansion and intensification of sea cucumber farming has led to the frequent occurrence of infectious diseases. Among them, skin ulceration syndrome is highly contagious and lethal to sea cucumber (Deng, Zhou, Wang & Liu 2008; Deng, He, Zhou, Liu, Tan, Wang, Jiang, Gao & Liu 2009). *Vibrio splendidus* has been proven to be responsible for the skin ulceration syndrome of the sea cucumber (Deng *et al.* 2009; Liu, Zheng, Sun, Hong, Dong, Wang, Tang & Wang 2010).

However, abuse of chemotherapeutants and antibiotics for sea cucumber disease control has been criticized for the negative impacts on environment and food supply chains like potential development of antibiotic-resistant bacteria, presence of antibiotic residues in seafood, destruction of microbial populations in the aquaculture environment, and suppression of the aquatic animal's immune system. Therefore, increasing attention is being paid to the use of immunostimulants for the disease control in aquaculture to maintain the sustainable development of this industry.

Immunostimulants are a group of biological and synthetic compounds which can enhance the nonspecific cellular and humoral defence of mammals. Some of them, such as astaxanthin (Jagruthi, Yogeshwari, Anbazahan, Shanthi Mari, Arockiaraj, Mariappan, Learnal Sudhakar, Balasundaram & Harikrishnan 2014), glucan (Ai, Mai, Zhang, Tan, Zhang, Xu & Li 2007; Bonaldo, Thompson, Manfrin, Adams, Murano, Mordenti & Gatta 2009), carotenoids (Bendich 1989), chitin (Esteban, Cuesta, Ortuno & Meseguer 2001), chitosan (Anbazahan, Shanthi Mari, Yogeshwari, Jagruthi, Thirumurugan, Arockiaraj, Krishnamoorthy, Balasundaram & Harikrishnan 2014) and vitamin combinations (Verlhac, Obach, Gabaudan, Schuep & Hole 1998) have been found to be effective in the prevention of disease in aquatic animals. Chitosan oligosaccharide (COS) is a type of oligosaccharides, which is obtained from chitosan using chemical and enzymatic hydrolysis. The COS has higher activity and more physiological functions than chitosan due to its lower molecular weight and its water ready solubility. Chitosan oligosaccharide (COS) could enhance nonspecific immunity and disease resistance of shrimp (Niu, Lin, Jiang, Chen, Wu & Tian 2013), pompano (*Trachinotus ovatus*) (Lin, Mao, Guan, Lin & Luo 2012), koi (*Cyprinus carpio koi*) (Lin, Mao, Guan, Luo, Luo & Pan 2012) and hybrid tilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂) (Qin, Zhang, Liu, Xu, Yang & Zhou 2014).

Rare earth elements (REEs) were composed of scandium (21), yttrium (39), lanthanum (57) and the fourteen kinds of chemical elements in lanthanoids (58–71). Among them, cerium (Ce) and lanthanum (La), two light REEs, have become the focus of research in recent years. It was reported that appropriate concentration of Ce had positive effects on embryonic development of large yellow croaker (Xu & Jiang 2004). In addition, REEs could cooperate with hydroxyl, amino and amide groups on the COS to form complexes (Wang, Qiu & Ma 2008; Sun 2009), the formation of these complexes could promote animal immunization as well as inhibit the absorption of heavy metals (Li, Xu, Wang, Sun & Zhang 2010; Cui, Xu, Ai, Wang & Mai 2013).

Up to date, little information was available about the physiological effects of COS-Ce in aquatic animals. Recently, COS-Ce has been found to exert effective protection against dietary cadmium accumulation in turbot (*Scophthalmus maximus* L.) (Cui, Xu, Wang, Zuo, Mai & Ai 2015). However, as far as we know, no information was available about the effects of dietary COS-Ce on sea cucumber. Thus, an 8-week feeding trial was conducted to evaluate whether dietary COS-Ce could exert beneficial effects on growth performance, nonspecific immunity and disease resistance of sea cucumber *Apostichopus japonicus*.

## Materials and methods

### Preparation of COS-Ce

Chitosan oligosaccharide complex with cerium (COS-Ce) was made of 2000 Da viscosity-average molecular weight COS (Haidebei Marine Bio-engineering, Jinan, China) and $(NH_4)_2Ce(NO_3)_6$ (Sinopharm Chemical Reagent, Shanghai, China).

Preparation of COS-Ce was carried out according to the method of Wang, Wen, Liu and Jia (2006). Firstly, 500 mL Ce $(NH4)_2(NO_3)_6$ (0.05 mol $L^{-1}$) was supplemented with 1000 mL COS (10 mg $mL^{-1}$). The solution was stirred for 3 h at the room temperature, adjusted to pH 7 with dilute ammonia solution and incubated at 25°C for 12 h. Then the acetone-ethanol media in volume ratio 1:1 was added to the reaction products in drops. Finally, the sedimentation was vacuum dried for about 12 h under the condition of 50°C.

### Experimental design and diets

The basal diet (control diet) was formulated based on the nutrition requirement of *A. japonicas* (Zhu, Mai, Zhang, Wang & Xu 2005). 150, 300, 600 and 1200 mg $kg^{-1}$ COS-Ce were then supplemented to formulate four other diets (Table 1). All the ingredients were thoroughly mixed, pelleted with an experimental feed mill (F-26(II), South

**Table 1** Formulation and proximate composition of experimental diet on a dry matter basis

| Ingredients | Concentration (%) |
|---|---|
| Fish meal* | 5.0 |
| *Sargassum thunbergii* powder† | 65.0 |
| Soybean meal‡ | 3.0 |
| Saccharomyces cerevisiae‡ | 3.0 |
| Wheat meal‡ | 20.1 |
| Lecithin‡ | 1.2 |
| Vitamin C§ | 0.2 |
| $Ca(H_2P0_4)_2$§ | 1.0 |
| Vitamin premix§¶ | 1.0 |
| Mineral premix§·** | 0.5 |
| Crude protein | 18.6 |
| Crude lipid | 1.1 |

*Purchased from Qingdao Great Seven Biotechnology.
†Purchased from Shandong Jinpai Feed, China.
‡Purchased from Shandong Liuhe Group, China.
§Kindly provided by Qingdao Master Biotechnology, China.
¶Vitamin premix (mg $kg^{-1}$ diet): thiamin 90 mg, riboflavin 150 mg, pyridoxine HCl 210 mg, vitamin $B_{12}$ 0.03 mg, vitamin $K_3$ 50 mg, inositol 600 mg, calcium pantothenate 150 mg, niacin acid 600 mg, folic acid 15 mg, biotin 1.20 mg, retinol acetate 32 mg, cholecalciferol 12 mg, α-tocopherol 120 mg, ethoxyquin 150 mg.
**Mineral premix (mg $kg^{-1}$ diet): KI 0.8 mg, $CoCl_2{\cdot}6H_2O$ (1%) 40 mg, $CuSO_4{\cdot}5H_2O$ 100 mg, $FeSO_4{\cdot}7H_2O$ 450 mg, $ZnSO_4{\cdot}H_2O$ 250 mg, $MnSO_4{\cdot}H_2O$ 60 mg, $MgSO_4{\cdot}7H_2O$ 4000 mg.

China University of Technology, Guangzhou, China), and dried in an oven at 40°C. The pellets were broken up and sieved into 0.3–0.45 mm length and stored at −20°C until use.

### Experimental animals and culture condition

Experimental sea cucumbers were obtained from a commercial farm (Qingdao, China). After 2 weeks acclimation, sea cucumbers (initial average weight: 6.72 ± 0.02 g) were randomly distributed into 20 glass tanks (60 L) with recirculating seawater and each tank was stocked with 17 sea cucumbers. Each diet was allocated to four tanks of sea cucumber to apparent satiation once daily (19:00 hours). During the 8-week feeding trial, water temperature was kept at 16 ± 0.5°C; pH 7.8–8.2, dissolved oxygen more than 5 mg $L^{-1}$; ammonia nitrogen less than 0.5 mg $L^{-1}$, and salinity 30–31‰.

### Sampling procedure

At the end of the feeding experiment, sea cucumbers were fasted for 24 h and weighed by tank to monitor growth. Five sea cucumbers of each replicate were dissected using the tail cutting method. Then, 1 mL of coelomic fluids from each sea cucumber was collected. Coelomic fluids in one replicate (5 mL) were thoroughly mixed for the assay of immune parameters. The coelomic fluids (3 mL) of each replicate was instantly mixed with equal volume of sterile anticoagulant solution (0.02 mol $L^{-1}$ EGTA (Sangon Biotech, Shanghai, China), 0.48 mol $L^{-1}$ NaCl, 0.019 mol $L^{-1}$ KCl and 0.068 mol $L^{-1}$ Tris-HCl, pH 7.6) according to the method of Xing, Leung and Chia (1998).

The remaining coelomic fluids (2 mL) were used for the analysis of total coelomocytes counts (TCC), phagocytosis activity and respiratory burst activity. Briefly, coelomic fluids were centrifuged (4°C, 3000 *g*) for 10 min to collect coelomocytes. After that, coelomocytes were resuspended in the same volume of cold PBS (0.01 mol $L^{-1}$, pH 7.4). Cell lysate supernatant (CLS) was prepared according to the method of Klanian (2013). First, coelomocytes above were sonicated at 22 kHz for 25 s at 0°C. Cell lysate supernatant (CLS) was separated by centrifugation (4000 *g*, 4°C, 10 min) and stored at −80°C for the assay of immune related enzymes. Twelve juveniles from each replicate were intraperitoneally injected with the pathogen bacteria *V. splendidus* ($10^8$ CFU $mL^{-1}$).

### Immune parameters

#### *Total coelomocytes counts*

Coelomocytes were counted and calculated as cells per milliliter using a hemocytometer (Qiujing, Shanghai, China) under light microscope at 400× magnification.

#### *Phagocytosis assay*

Phagocytosis activity was determined by neutral red method (Long, Wang, Liu, Zhou, Cui & Jiang 2005; Chen, Zhang, Shen & Wang 2010) with some modification. In brief, 100 μL coelomic fluids were injected into each well of the microplate and incubated at 25°C for 30 min for adhesion. Then, the supernatant was carefully removed and 100 μL of 0.03% neutral red was added into each well and incubated at 25°C for 30 min. Cells were then washed with PBS for three times and incubated with cell lysis buffer (acetic acid: ethanol = 1:1) for 20 min. The results were measured by a universal microplate spectrophotometer (Bio Tek, Vermont, USA) at 540 nm. The $OD_{540}$ of

  

$10^6$ cells represents the capability of coelomocytes phagocytosing neutral red.

*Respiratory burst activity*

Respiratory burst activity of coelomocytes was evaluated using nitroblue tetrazolium (NBT; Sigma, St. Louis, MO, USA) according to the method of Anderson, Brubacher, Calvo, Unger and Burreson (1998) with minor modifications. 50 μL 0.2% poly-D-lysine (Sigma) was added to a 96-well microplate to improve coelomocytes adhesion. Three replicates of 100 μL aliquot of coelomic fluids from sea cucumbers in each aquarium were added to wells and centrifuged (300 *g*, 4°C) for 10 min. The supernatant was carefully discarded and 100 μL phorbol 1, 2-myristate 1, 3-acetate (Sigma; 1 μg $mL^{-1}$) was added to each well, and then the plates were incubated at 37°C for 30 min. The cells in each well were then stained with 100 μL 0.3% NBT at 37°C for 30 min. Absolute methanol was added to terminate the staining. Each well was washed three times with 70% methanol and air-dried. Then 120 μL KOH (2 mol $L^{-1}$) and 140 μL dimethyl sulfoxide (DMSO; Amresco, Solon, OH, USA) were added and the colour was subsequently measured at 630 nm with a universal micro plate spectrophotometer using KOH/DMSO as a blank. The absorbance of $10^6$ cells represents the capability of coelomocytes respiratory burst activities.

*Total superoxide dismutase activity*

Superoxide dismutase (SOD) activity was measured spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture consisted of 50 mmol $L^{-1}$ potassium phosphate buffer (pH 7.8), 0.1 mmol $L^{-1}$ EDTA (Sangon Biotech), 0.1 mmol $L^{-1}$ xanthine, 0.013 mmol $L^{-1}$ cytochrome C and 0.024 IU $mL^{-1}$ xanthine oxidase. The reaction was triggered by the addition of the xanthine oxidase. Results were expressed in units of SOD per milligram soluble protein and each unit was defined as the amount of enzyme necessary to produce 50% inhibition of the ferricytochrome C reduction rate measured at 550 nm (Ōyanagui 1984).

*Total nitric oxide synthase activity*

Total nitric oxide synthase (T-NOS) activity was determined by its ability to convert L-arginine to NO using a commercial kit (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China). The optical density was measured at 530 nm following the operation manual. One unit of T-NOS activity was defined as the amount of T-NOS producing 1 nmol NO per minute. Specific activity was expressed as T-NOS units per mg protein.

*Acid phosphatase and alkaline phosphatase activities*

Acid phosphatase (ACP) and alkaline phosphatase (AKP) activities were determined with disodium phenyl phosphate as substrate by using a commercial kit (Nanjing Jiancheng Bioengineering Institute). The optical density was measured at 520 nm following the operation manual. Results were expressed in units of ACP or AKP per gram soluble protein. One unit of ACP was defined as the amount of enzyme necessary to produce 1 mg nitrophenol in 30 min at 37°C. Each unit of AKP was defined as the amount of enzyme necessary to produce one miligram phenol in 15 min at 37°C.

*Total soluble protein*

To standardize the levels of enzyme activities, total soluble protein of CLS was determined according to Bradford (1976) using a commercial Kit (Nanjing Jiancheng, Bioengineering Institute), using bovine serum albumin as the standard.

## Challenge test

The *V. splendidus* associated with sea cucumber skin ulceration disease (Zhang, Wang & Rong 2006) was provided by Yellow Sea Fisheries Research Institute, Chinese Academy Fishery Sciences (Qingdao, China). The bacteria pathogen was grown in trypticase soy broth medium at 28°C for 24 h. The $LD_{50}$ was determined prior to challenge and the $LD_{50}$ for 7 days was $10^8$ CFU $mL^{-1}$.

At the end of the feeding experiment, 12 sea cucumbers from each aquarium were injected intraperitoneally with 0.1 mL PBS containing $10^8$ CFU live *V. splendidus*. The sea cucumbers were fed their respective diets once daily, and mortality was monitored for 14 days.

## Calculations and statistical analysis

The following variables were calculated:

$$\text{Specific growth rate (SGR)}(\%d^{-1}) = (\ln W_t - \ln W_0) \times 100/t$$

$$\text{Accumulatively mortality}(\%) = D_T/D_0 \times 100\%$$

where $W_t$ and $W_0$ were final and initial sea cucumber weight during the 56-day feeding experiment, respectively; $t$ was duration of feeding experiment; $D_0$ was the number of sea cucumbers at the beginning of the challenge test; $D_T$ was the number of sea cucumbers at the end of the challenge test.

All data were subjected to one way analysis of variance in SPSS 16.0 for windows. The results were presented as means ± SEM. Data were analysed by one-way ANOVA. When overall differences were significant at less than 5% level, tukey's multiple range tests were used to compare the means among individual treatments.

## Results

### Growth performance

The specific growth rate (SGR) of sea cucumber increased significantly from 0.90 to 1.21% $d^{-1}$ as dietary COS-Ce increased from 0 to 600 mg $kg^{-1}$ ($P < 0.05$), and then decreased to 0.96% $d^{-1}$ with further increase in COS-Ce (Table 2).

### Immune responses

*Phagocytosis*

Phagocytosis activity of coelomocytes increased significantly from 0.39 to 0.71 $OD_{540}/10^6$ as dietary COS-Ce increased from 0 to 600 mg $kg^{-1}$, and then decreased to 0.45 $OD_{540}/10^6$ with further increase in COS-Ce ($P < 0.05$) (Fig. 1A).

**Table 2** Effects of dietary COS-Ce on the growth of sea cucumber, *Apostichopus japonicas* (means ± SEM, $n = 4$)*

| COS-Ce level (mg $kg^{-1}$) | Initial weight (g) | Final weight (g) | Specific growth rate (% $d^{-1}$) |
|---|---|---|---|
| 0 | 6.74 ± 0.03 | 11.18 ± 0.17$^b$ | 0.90 ± 0.03$^b$ |
| 150 | 6.68 ± 0.04 | 11.16 ± 0.36$^b$ | 0.92 ± 0.05$^b$ |
| 300 | 6.70 ± 0.04 | 12.10 ± 0.38$^b$ | 1.05 ± 0.05$^b$ |
| 600 | 6.78 ± 0.04 | 13.36 ± 0.16$^a$ | 1.21 ± 0.02$^a$ |
| 1200 | 6.70 ± 0.04 | 11.48 ± 0.18$^b$ | 0.96 ± 0.04$^b$ |
| ANOVA | | | |
| *F*-value | 1.27 | 12.33 | 10.08 |
| *P*-value | 0.32 | 0.00 | 0.00 |

*Data presented as means ± SEM ($n = 4$); data within the same column with the same superscripts were not significantly different determined by Tukey's multiple test ($P > 0.05$); data in the same column with different letters are significantly different by Tukey's test ($P < 0.05$).

ANOVA, one-way analysis of variance.

*Respiratory burst*

Respiratory burst activity of coelomocytes increased significantly from 0.52 to 0.89 $OD_{630}/10^6$ as dietary COS-Ce increased from 0 to 600 mg $kg^{-1}$, and then decreased to 0.61 $OD_{630}/10^6$ with further increase in COS-Ce ($P < 0.05$). However, no significant differences were observed in this parameter between COS-Ce/0 and COS-Ce/1200 treatment ($P > 0.05$) (Fig. 1B).

*Total superoxide dismutase*

Total SOD (T-SOD) activities of CLS significantly increased from 31.69 to 66.76 U $mg^{-1}$ protein as dietary COS-Ce increased from 0 to 300 mg $kg^{-1}$, and then decreased to 40.18 U $mg^{-1}$ protein with further increase in COS-Ce ($P < 0.05$; Fig. 1C).

*Total nitric oxide synthase*

Total nitric oxide synthase activities of CLS ranged from 2.36 to 3.07 U $mg^{-1}$ proteins with the increase in dietary COS-Ce. However, there were no significant differences in T-NOS among dietary treatments ($P > 0.05$; Fig. 1D).

*Acid phosphatase and AKP*

Acid phosphatase activities of CLS significantly increased from 42.41 to 58.27 U $g^{-1}$ protein as dietary COS-Ce increased from 0 to 600 mg $kg^{-1}$ ($P < 0.05$), and then decreased with further increase in COS-Ce ($P > 0.05$, Fig. 1E). Alkaline phosphatase (AKP) activity showed similar trend to ACP activity with the increase in dietary COS-Ce. AKP activities of sea cucumber fed diet with 600 mg $kg^{-1}$ was 9.73 U $g^{-1}$ protein, significantly higher than that in COS-Ce/0 (4.18 U $g^{-1}$ protein) and COS-Ce/1200 (6.22 U $g^{-1}$ protein) ($P < 0.05$, Fig. 1F).

### Challenge test

The cumulative mortality of sea cucumber decreased significantly from 58.46% to 33.33% as dietary COS-Ce increased from 0 to 600 mg $kg^{-1}$, and then increased to 61.54% as dietary COS-Ce increased from 600 to 1200 mg $kg^{-1}$ ($P < 0.05$; Fig. 2).

## Discussion

Dietary COS has been shown to improve the growth performance of pigs (Zhou, Cho & Kim 2012),

  

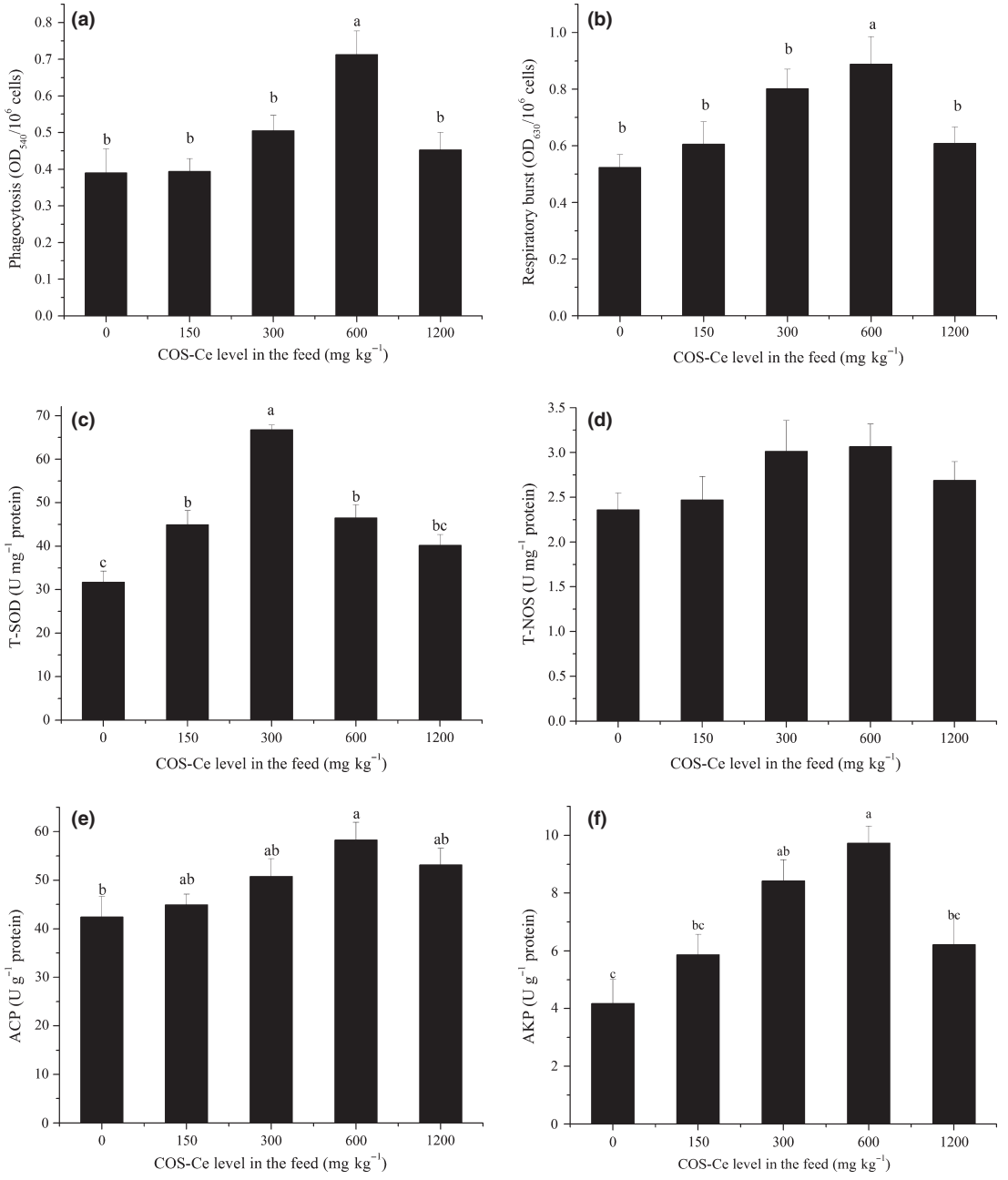


**Figure 1** Phagocytosis (A), respiratory burst (B), total superoxide dismutase (T-SOD) activity (C), total nitric oxide synthase (T-NOS) activity (D), acid phosphatase (ACP) activity (E) and alkaline phosphatase (AKP) activity (F) of *Apostichopus japonicas* fed with graded doses of dietary COS-Ce for 8 weeks. Values are means ± SEM ($n$ = 4). Bars bearing with different letters are significantly different by Tukey's test ($P < 0.05$).

broilers (Huang, Yin, Wu, Zhang, Li, Li & Nie 2005), pompano (*T. ovatus*) (Lin, Mao, Guan, Lin *et al.* 2012), koi (*C. carpio*) (Lin, Mao, Guan, Luo *et al.* 2012) and American white shrimp (*Litopenaeus vannamei*) (Niu, Liu, Lin, Mai, Yang, Liang & Tian 2011). Oral administration of REEs could enhance the growth of some animals, such as pigs (He, Ranz & Rambeck 2001) and poultry (He, Wehr & Rambeck 2010). Cui *et al.* (2013) found that the survival rate and SGR of turbot were significantly enhanced when 300 mg kg$^{-1}$ COS-REE was included in the diets. To our knowledge, little is

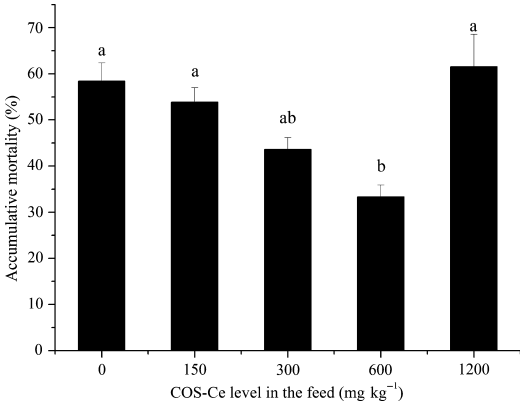


**Figure 2** Cumulative mortality during 14 days *Vibrio splendidus* challenge of sea cucumbers fed diets supplemented with graded level of COS-Ce. Values are means ± SEM ($n$ = 4). Bars bearing with different letters are significantly different by Tukey's test ($P < 0.05$).

known about the effect of COS-Ce on growth performance in sea cucumber or other aquatic species, and this is the first study to determine the effect of dietary COS-Ce supplementation on the growth and immune response in sea cucumber. Our results indicate that the SGR in sea cucumbers fed the 600 mg kg$^{-1}$ of COS-Ce diet was significantly higher than those fed the 0 mg kg$^{-1}$ of COS-Ce diet.

COS-REE could promote growth performance by direct effects of the gastrointestinal tract and indirect effects of the intermediate metabolism. Within the scope of direct effects, attention has been paid to the antibacterial properties of REEs (Zhang, Feng, Zhu, Liu & Gu 2000; Zhao, Liu, Xie, Shen & Qu 2002; Liu, Yi, Lu, Zhu, Dong & Qu 2004). It might be due to REEs influences the microbial environment in the gut. Furthermore, it was suggested that rare earths could improve nutrient digestibility and utilization, and thereafter enhance the growth performance of animals (He, Wang, Xu, Chen & Rambeck 2003; He, Yang, Hidari & Rambeck 2006). On the other hand, cerium and lanthanum were resembled to calcium, which could inhibit coagulation (Jakupec, Unfried & Keppler 2005) and regulate hormonal release or responses (He & Loh 2000; Forster, Berk, Hoppen, Rambeck & Flachowsky 2008). However, the exact mechanism of COS-REE affected aquatic animal remains incompletely explained and more efforts should be made in further studies.

Like most invertebrates, non-specific immunity plays a dominant role in the process of immune defence. Coelomocytes of echinoderms have been found to be directly involved with the immune response of sea cucumber (Ramírez-Gómez, Aponte-Rivera, Méndez-Castaner & García-Arrarás 2010). Activated coelomocytes could improve the disease resistance by producing sterilization substances like $H_2O_2$ and $O_2^-$, which are capable of killing invasive pathogens (Coteur, Warnau, Jangoux & Dubois 2002). $O_2^-$ concentration was considered to be an important index to quantify respiratory burst activity since it was the primary production during the respiratory burst of coelomocyts (Sritunyalucksana, Sithisarn, Withayachumnarnkul & Flegel 1999). Previous studies have showed that the immune system of animals could be activated by chitosan, COS and REEs (Kawagoe, Hirasawa, Wang, Liu, Ueno & Sugiyama 2005; Chen, Kim, Cho, Yoo, Wang, Huang & Shin 2009; Shanthi Mari, Jagruthi, Anbazahan, Yogeshwari, Thirumurugan, Arockiaraj & Harikrishnan 2014), but not much studies of the dietary COS-REE regulated sea cucumber immune response. Results of present study suggested that the phagocytic activity and respiratory burst activity of coelomocytes could be significantly enhanced by using 600 mg kg$^{-1}$ COS-Ce in sea cucumber compare with 300 mg kg$^{-1}$ COS-Ce in turbot (Cui *et al.* 2013). It was assumed that COS specifically combined the receptor proteins of phagocytes on fish cell surface, improved the activity of phagocytes and stimulated the immune response (Kim & Rajapakse 2005; Dang, Li, Wang, Wang, Zou, Guo & Zhang 2011).

Superoxide dismutase played an important role in the balance of superoxide anion production and clearance (Fridovich 1989). Jiao (2009) confirmed that the SOD activity of pancreas of oriental weatherfish (*Misgurnus anguillicaudatus*) was significantly improved after exposure to $Ce^{3+}$ (0.025 mg L$^{-1}$) for 10 days. In this study, T-SOD activity of coelomocytes in sea cucumber increased significantly with the supplementation of 300 mg kg$^{-1}$ COS-Ce in the diets. Meanwhile, COS-Ce could protect organism against radiation-induced damage, both by acting as free-radical scavengers and by increasing the T-SOD production. NOS was an important part of non-specific immune system in the organism. Under the action of pathogen (bacteria, viruses and parasites) and cytokines (IFN, IL and TNF), inducible nitric oxide synthase (iNOS) was induced to produce large amounts of NO which make NO free radicals stimulate phagocytic cells by increasing

  

inflammation response and then kill microbe infected cells (Vital, Gonçalo, Cruz, Figueiredo, Duarte & Lopes 2003). Although COS has been found to possess the ability to activate iNOS activity of phagocytic cells, no significant differences were observed in T-NOS activity in response to graded dietary COS-Ce in this study. Alkaline phosphatase (AKP) was a zinc-containing glycoprotein widespread in the living tissue. ACP was an important enzyme of animal lysosomes (Blasco, Puppo & Sarasquete 1993). When fed the sea cucumber were fed the diet with 600 mg $kg^{-1}$ COS-Ce, AKP and ACP activity in coelomocytes were significantly higher than other sea cucumber groups, however, the AKP and ACP activity were decreased with further increase in dietary COS-Ce. These results consisted with the findings of Schmidlin and Wang (Schmidlin, Tchouboukov, Wegehaupt & Weber 2012; Wang, Li, Lu, Jin, Deng & Zeng 2012), who found that high concentration of Ce exerted an inhibitory effect on the enzyme activity.

In this study, sea cucumber fed the diet with 600 mg $kg^{-1}$ COS-Ce had significantly lower accumulative mortality after intraperitoneal injection with *V. splendidus*. This suggested that improved disease resistance of sea cucumber in COS-Ce/600 could be largely attributed to the enhanced activity of phagocytosis and respiratory burst, and the immune related enzymes (T-SOD, ACP and AKP). Some studies had reported that COS and rare earth effectively enhanced the immune system of animals and improved the resistance to pathogenic bacteria infection (Huang, Deng, Yang, Yin, Xie, Wu & Guo 2007; Moon, Kim, Koo, Joo, Nam, Park & Kang 2007; Lin, Mao, Guan, Lin *et al.* 2012). Cui *et al.* (2013) had proved that suitable level of COS-REE could significantly increase the growth, innate immunity and disease resistance of turbot (*Scophthalmus maximus*).

In conclusion, under the experimental conditions, dietary 600 mg $kg^{-1}$ COS-Ce could significantly improve growth, innate immunity and disease resistance against *V. splendidus* for sea cucumber. Following experiments are needed to elucidate the mechanisms about how dietary COS-Ce exerts the beneficial effects on growth and immunity in sea cucumber.

## Acknowledgments

The study was financially supported by the National Natural Science Foundation of China (NSFC, no. 31371731), Shandong Provincial Natural Science Foundation of China (ZR2009DM026).

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