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Effects of dietary chitosan oligosaccharide complex with rare earth on growth performance and innate immune response of turbot, *Scophthalmus maximus* L.

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Abstract

An 8-week-feeding trial was conducted to investigate the effect of dietary chitosan oligosaccharide complex with rare earth (COS-REE) on growth performance and innate immune response of turbot, *Scophthalmus maximus* L. (Initial average weight was (12.1 ± 0.1) g) as well as disease resistance against *Edwardsiella tarda*. Six practical diets (approximately 53.01% protein and 12.57% lipid) were formulated to contain graded levels (0, 75, 150, 300, 600 and 1200 mg kg^{-1}) of COS-REE. Results of the present study showed that, compared to the control group (0 mg kg^{-1}), the specific growth rate (SGR) was significantly higher in fish fed the diet with 300 mg kg^{-1} COS-REE ($P < 0.05$), while the feed conversion ratio (FCR) significantly decreased ($P < 0.05$). The phagocytic index (PI) and the activity of super oxide dismutase (SOD) of serum in fish fed the diet with 300 mg kg^{-1} COS-REE was significantly higher than fish fed the control diet ($P < 0.05$), but no significant differences were observed in malondialdehyde (MDA) and hepatic metallothionein (MT) concentrations. After 8 weeks, fish were challenged by intraperitoneal injection with *E. tarda*, and COS-REE-treated fish demonstrated increased protection capability. These results suggested that COS-REE could enhance growth, innate immunity and disease resistance in turbot, and the optimum dose was approximately 300 mg kg^{-1}.

Keywords: chitosan oligosaccharide complex with rare earth, turbot, *Scophthalmus maximus* L., growth, immunity

Introduction

The turbot, *Scophthalmus maximus* L., has been recognized by Chinese consumers as one of the most valued species since it was introduced from Europe in 1992. To date, the fish has been widely cultured in Shandong, Liaoning, Jiangsu, and Fujian provinces because of its delicious meat and important commercial value (Irwin, O'Halloran & FitzGerald 1999; Jeannine Person-Le Ruyet 2002). However, disease threats affect the production of turbot. An interesting alternative to disease control is the use of immunostimulants, which could enhance non-specific immune response and build up resistance to diseases (Bricknell and Dalmo Roy 2005). Several immunostimulants such as nisin (Villamil, Figueras & Novoa 2003), vitamin C (Chang, Liang, Wang, Cao & Wang 2005), vitamin E (Gao, Wang, Yang, Qu, Liang, Chang, Zhu & Ma 2008), burdock oligosaccharide (Hao, Sun, Shi, Liang & Li 2006) and Chinese medicinal feed additive (Li 2007) have been used in turbot culture

Rare earth elements (Seishiro & Kazuo 1996) include lanthanoids in group III of the periodic table and scandiumare (Sc) and yttrium (Y), whose chemical and toxicological characteristics were very similar to lanthanoids. In China for more than 40 years, REE are used as feed additives in animal production (Pang, Li & Reng 2001; Qi, Wang, Sun, Luo, Sun & Zhao 2005). Many studies in agricultural science have suggested that low concentrations of REE seemed to favour growth and biomass production of several crops (He & Rambeck 2000; Tyler 2004).

The chitosan (CTS) biopolymer has been proved to be an effective biopolymer, which received considerable attention for its applications in the agriculture, food and medical industries (Ravi Kumar 2000; Pillai, Willi & Chandra 2009). However, recent studies on chitosan have attracted interest for converting chitosan to its oligosaccharides, whose molecular weights were 10 kDa or less, because of its water-solubility. Many studies have measured the effects of COS on immunological enhancement. For instance, Liang, Chen, Yue and Wang (2007) reported that the hydrolysates of chitisons enhanced immune regulation by inhibiting tumour growth and reducing the survival rate to 34% in 1 day, which was similar to some other studies (Minami, Egawa, Ohira, Okamoto & Matsuhashi 1997; Okamoto, Inoue, Miyatake, Ogihara, Shigemasa & Minami 2003; Dou, Tan, Du, Bai, Wang & Ma 2007). However, recently, several studies have proved that chitosan-metal had better activity than free chitosan and metal salts. Wang, Du, Li, Liu and Hu (2005) showed that chitosan-metal complexes had wide spectra antimicrobial activities than free chitosan and metal salts because of the stronger positive charge after complexation. Guo, Liu and Peng (2003) proved that chitooligosaccharides-Ce and chitooligosaccharides-La had stronger ability of catalytic oxidation than the uncoordinated La and Ce by the experiment of Benzaldehyde oxidation. Sun (2009) and Li, Xu, Wang, Sun and Zhang (2010) reported that COS-REE could significantly remove the Cd in viscera of *Chamys Ferrari* and turbot respectively.

However, knowledge regarding the effect of COS-REE is rather limited in the marine culture. The purpose that the present work was conducted was to investigate the effect of dietary COS-REE on growth, immunity and disease resistance against *Edwardsiella tarda* in turbot (*S. maximus* L.).

Materials and methods

Chitosan oligosaccharide complex with rare earth

Chitosan oligosaccharide complex, COS (viscosity-average molecular weight was 700 ~ 800 Da, degree of polymerization was 4 ~ 5), was provided by College of Food Science and Engineering, Ocean University of China. Rare Element Nitrate was purchased from Shangqiu Rare Earth Weifei Co., LTD, Henan, China.

Preparation of COS-REE was carried out by the method of Sun (2009). In brief, COS (0.6 g) was dissolved in 50 mL of water, adjusted to pH 5 and added Rare Element Nitrate (0.6 g). Stirring was continued for a further 2 h at 50°C until the reaction mixture formed a suspension. Then the suspension was brought to room temperature stationarily and then centrifuged for 15 min at 5199 × *g*. The supernatant was finally dried in a freeze dryer to obtain the product.

The COS-REE was determined using inductively coupled plasma-atomic emission spectrophotometer (ICP-OES; VISTA-MPX, Varian, Palo Alto, CA, USA) after HNO_3-$HClO_4$ mixture digestion. COS-REE contained REE 9.33%, including Dy 2.51%, Ho 2.95%, Lu 0.04%, Sc 0.003% and Tm 3.83%.

Experimental diets

Using fish meal (provided by Qingdao Great Seven Bio-Tech. Co., Ltd, China) as protein source, fish oil and soybean lecithin as lipid sources, the basal diet was formulated. The basal diet was used as the control diet, and 75, 150, 300, 600 and 1200 mg kg^{-1} COS–REE were separately supplemented to formulate five other experimental diets (Table 1). All the ingredients were thoroughly mixed with fish oil, and water was added to produce a stiff dough. The dough was then pelleted with an experimental feed mill (F-26(II), South China University of Technology, China) and dried for about 12 h in a ventilated oven at 50°C. The dietary pellets (1.5 × 3.0 mm) were sealed in a sample bag and stored at −20°C until used.

Feeding experiment

An 8-week growth trial was conducted in a circulating water system. Experimental fish were obtained from a commercial farm in Rizhao, China. Prior to the start of the experiment, the juveniles of turbot were reared and fed with a commercial diet (No.2 feed provided by Qingdao Great Seven Bio-Tech, Qingdao, China) to acclimate to the experimental conditions. At the start of the experiment, the fish were fasted for 24 h and weighed. Fish of similar sizes (12.1 ± 0.1 g) were randomly distributed into 18 500 L cylindrical fibreglass tanks in a circulating system, and each tank was stocked with 40 fish. Each diet was randomly assigned to triplicate tanks. Fish were hand-fed to apparent satiation twice (07:00 and

Table 1 Formulation and chemical composition of the basic diets (% dry weight)

Ingredients	
White fish meal*	65.35
Wheat meal*	25.00
fish oil*	5.50
Soybean lecithin*	1.00
Vitamin premix †‡	0.80
Mineral premix †§	2.00
Choline chlorine (95%)	0.20
Ethoxyquin	0.05
Calcium propionate	0.10
Proximate composition	
Crude protein(% dry matter)	53.01
Crude lipid(% dry matter)	12.57
Ash(% dry matter)	9.50

*Kindly provided by Qingdao Great Seven Bio-Tech. Co., Ltd, China.

†Kindly provided by Qingdao Master Biotechnology, China.

‡Vitamin premix (mg kg^{-1}diet): Vitamin D, 5 mg; Vitamin K, 10 mg; Vitamin B_{12}, 10 mg; Folic acid, 20 mg; Vitamin B_6, 20 mg; Vitamin B_1, 25 mg; Vitamin A, 32 mg; Vitamin B_2, 45 mg; Calcium pantothenate, 60 mg; Biotin, 60 mg; Nicotinic acid, 200 mg; Vitamin E, 240 mg; Inositol, 800 mg; Vitamin C, 2000 mg; Microcrystalline cellulose, 4292.54 mg;

§Mineral premix (mg kg^{-1} diet): $CuSO_4 \cdot 5H_2O$, 10 mg; Na_2SeO_3, 20 mg; $MnSO_4 \cdot H_2O$, 45 mg; $CoCl_2 \cdot 6H_2O(1\%)$, 50 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $Ca(IO_3)_2$, 60 mg; $FeSO_4 \cdot H_2O$, 80 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; Zeolite Powder, 18485 mg.

18:00) daily. During the experimental period, the water temperature fluctuated from 19 to 20°C, pH from 7.5 to 7.8 and salinity from 24‰ to 27‰. Air stones in each tank maintained the dissolved oxygen concentration at 7 mg L^{-1} or more. Turbot were reared under natural light from July 10 to September 4 2010, and accumulation of feed and faeces at the bottom of tank were siphoned daily.

At the termination of the experiment, all treatment groups were fasted for 24 h. All fish in each tank were weighed to determine the final body weight. Total number of fish in each tank was also measured.

Analysis and measurement

Sample collection and analysis

At the termination of feeding experiment, three fish per tank were randomly collected and stored frozen (−20°C) for determination of proximate carcass composition. Proximate composition analysis on fish body was performed using standard methods of Association of Official Analytical Chemists (1995). Samples of fish were dried to a constant weight at 105°C to determine moisture. Protein was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method; lipid by ether extraction using Soxhlet; ash by combustion at 550°C.

The feeding trial was from July 10 to September 4, 2010. After 56 days of feeding, the fish were fasted for 24 h. Five fish per tank were randomly collected and then immersed in a solution of 100 mg L^{-1} of eugenol to anaesthetize fish. Blood samples were collected from the caudal vein of fish with a 1 mL syringe. The first aliquot of blood was added by sodium heparin and transferred to an Eppendorf as anticoagulant and used for the measurement of phagoctic activity. The second aliquot was allowed to clot at 4°C for 12 h. Serum was separated and stored at −70°C until used. The ventral belly surface of the fish was opened to expose the abdominal cavity. The liver was separated for metallothionein analysis.

Phagocytic activity

Phagocytic activity for five fish in each tank was determined by a modified method of Wang (2009). *Staphylococcus aureus* was cultured on nutrient bouillon and incubated at 37°C for 12 h. Following this, the preparation was centrifuged (4°C at 3327 × *g*) for 10 min and re-suspended in sterile saline solution at a concentration of 1.7×10^9 cfu mL^{-1}. Samples (100 μL) of the anticoagulant were placed in microtitre wells and incubated with 100 μL of the *S. aureus* suspension. The plates incubated for 0.5 h at 20°C, were shaken every other 10 min during the incubation. Then a drop of the mixture was taken in a clean slide and pushed into flakes. After air-drying, the slides were stained with Giemsa solution for 2 min. In all cases triplicate wells were used for each tank. Then, 100 cells in each smear were examined under the microscope to determine phagocytic index.

Measurement of serum super oxide dismutase and malondialdehyde

The SOD and MDA kits were provided by Nanjin Jiancheng Bioengineering Institute, China. The SOD content was measured using the xanthine oxidase technique based on the spectrophotometric monitoring of SOD-mediated reduction of DTNB at 550 nm. One unit of SOD activity was defined as a point where a sample gives 50 inhibition of a colorimetric reaction between reactive dye and superoxide anion. SOD activity was expressed as U mL^{-1} serum.

The concentration of MDA was quantified using thiobarbituric acid reaction. The level of lipid peroxidation was indicated by the concentration of MDA in serum. MDA content was expressed as nmol per millilitre of serum.

Measurement of hepatic metallothionein

Parts of liver tissue were homogenized in 9 volumes of 20 mM Tris-HCl buffer, pH 8.0, supplemented with 250 mM saccharose. The homogenates were centrifuged at 105 000 *g* for 60 min at 4°C, and the resulting supernatant, as cytosol, was used for MT quantification using Ellman's reaction. The metallothionein-containing solution (50 μL) was re-suspended in 10 μL 1.2N HCl and subsequently 200 μL 0.1 mM EDTA. After the reaction for 10 min, a volume of 200 μL Ellman's solution containing 1 mM EDTA, 5 mM DTNB and 6N guanidine hydrochloride was added to the sample. After 3 min, the sample was added with 0.1 M PBS pH 7.3 buffer solution until the volume was 5 mL. Finally the absorbance of the sample was evaluated at 412 nm.

Challenge test

The *E. tarda* was obtained from College of Life Science, Ocean University of China. The *E. tarda* strain was originally isolated from *Scophthalmus maximus* L. At the termination of the feeding experiment, 10 fish of each tank were randomly selected for the challenge test. Each fish was injected intraperitoneally with 0.2 mL *E. tarda* suspension at 10^5 cfu mL^{-1} (Concentration and dose determined by pre-experiment).

Calculations and statistical analysis

The following variables were calculated:

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

$$\text{Feed conversion ratio (FCR)} = \text{Dry feed fed in g/wet weight gain in g}$$

$$\text{Survival rate (\%)} = 100 \times (N_t/N_0)$$

$$\text{Protection capability (\%)} = (1 - D_t/D_o) \times 100$$

Where W_t and W_0 were final and initial body weight, respectively, t was duration of experimental days. N_t and N_0 were final and initial number of fish respectively. D_t was the mortality of groups treated with COS-REE, and D_o was the mortality of the control group.

All data were subjected to one-way analysis of variance in SPSS 18.0 for Windows. Levene's test for homogeneity was used, while square-root arcsine data transformation was used for all percentage data. Differences between the means were tested by Tukey's multiple range tests. When overall differences were significant ($P < 0.05$), Tukey's test was used to compare the means among individual treatments. The results were presented as means ± SEM (standard error of the mean).

Results

Survival and growth performance

After the 8-week feeding trial, the survival of fish increased from 86.0% to 97.0% with increasing dietary COS-REE (Table 2), however, no significant differences were observed among dietary treatments ($P > 0.05$). At 300–1200 mg kg^{-1} supplement COS-REE, fish had significantly higher SGR ($2.3\%d^{-1}$) than those fed the diets supplemented with COS-REE below 300 mg kg^{-1} ($P < 0.05$), and no significant differences were observed among fish fed the diets with higher than 600 mg kg^{-1} ($P > 0.05$). With increasing dietary

Table 2 Effects of COS-REE on specific growth rate, feed conversion ratio and survival rate of *Scophthalmus maximus* L.*

Diet No.(mg kg^{-1} COS-REE)	Diet 0 (0)	Diet 1 (75)	Diet 2(150)	Diet 3 (300)	Diet 4 (600)	Diet5 (1200)
Initial body weight/g	12.2 ± 0.0	12.4 ± 0.2	11.5 ± 0.7	12.0 ± 0.2	12.3 ± 0.2	12.2 ± 0.2
Final body weight/g	35.6 ± 1.9^{a}	41.8 ± 1.7^{b}	39.1 ± 0.7^{ab}	43.2 ± 1.0^{b}	43.7 ± 1.4^{b}	40.7 ± 1.1^{ab}
Specific growth rate/%/d	1.9 ± 0.1^{a}	2.2 ± 0.1^{ab}	2.2 ± 0.1^{ab}	2.3 ± 0.1^{b}	2.3 ± 0.1^{b}	2.2 ± 0.0^{ab}
Feed conversion ratio	1.6 ± 0.1^{a}	0.8 ± 0.0^{b}	0.8 ± 0.0^{b}	0.7 ± 0.0^{b}	0.8 ± 0.0^{b}	0.8 ± 0.0^{b}
Survival rate/%	86.0 ± 2.1	94.3 ± 2.3	97.0 ± 1.0	96.0 ± 4.0	97.0 ± 2.1	95.3 ± 1.5

*Data presented as means ± SEM ($n = 3$,); Data within the same column with the same superscripts were not significantly different determined by Tukey's multiple test ($P > 0.05$).
COS-REE, chitosan oligosaccharide complex with rare earth.

Table 3 The whole-body composition of turbot fed experimental diets for 8 weeks*

Diet No.(mg kg^{-1} COS-REE)	Diet 0(0)	Diet 1 (75)	Diet 2(150)	Diet 3 (300)	Diet 4 (600)	Diet 5 (1200)
Moisture (%)	78.2 ± 0.2	76.3 ± 1.6	78.3 ± 0.7	77.3 ± 0.2	77.5 ± 0.8	77.5 ± 0.6
Crude protein (% w.w.)	53.1 ± .2.6	52.1 ± 1.7	51.6 ± 4.2	48.6 ± 0.3	52.8 ± 1.3	45.9 ± 0.8
Crude lipid (% w.w.)	10.1 ± 1.0	11.6 ± 0.9	10.6 ± 0.7	9.1 ± 0.7	10.9 ± 0.9	12.2 ± 2.3
Ash (% w.w.)	12.9 ± 0.1	12.4 ± 0.5	12.2 ± 0.3	13.0 ± 0.4	12.3 ± 0.7	12.7 ± 0.5

*Data presented as means ± SEM (n = 3); Values of crude protein, crude lipid and ash were expressed on a wet weight basis. Data within the same column with the same superscripts were not significantly different determined by Tukey's multiple test ($P > 0.05$).

COS-REE, the FCR significantly decreased ($P < 0.05$), and then reached a plateau at 75 – 1200 mg kg^{-1} supplement COS-REE.

Body composition

There were no significant differences in body composition (protein, lipid, ash and moisture) among fish fed the diets with graded levels of COS-REE ($P > 0.05$) (Table 3). The whole-body protein contents decreased with the increase of dietary COS-REE, but no significant differences were observed among dietary treatments ($P > 0.05$). The whole-body lipid content (ranging from 9.1% to 12.2%) and whole-body ash content (ranging from 12.2 to 13.0%) were not significantly different ($P > 0.05$).

Immune parameters

The PI activity increased significantly from 1.18 to 1.38 when dietary COS-REE increased from 0 to 300 mg kg^{-1} and decreased with further increase of dietary COS-REE ($P < 0.05$) (Table 4). Fish fed 300 mg kg^{-1} COS-REE showed a significantly higher SOD activity than those fed 150 or 600 mg kg^{-1} COS-REE diet ($P < 0.05$), but no significant difference was detected between fish fed 300 mg kg^{-1} COS-REE and those fed the control diet or 1200 mg kg^{-1} COS-REE. The MDA concentration in fish fed the diets with COS-REE was not significantly different from those fed the control diet ($P > 0.05$). To some extent, however, some positive correlations were observed between dietary COS-REE level and the control diet. Fish fed the diet with COS-REE had less MDA concentration than those fed the control diet, but there was no significant difference ($P > 0.05$). In hepatic MT concentration, fish fed 150 and 600 mg kg^{-1} COS-REE had significantly less MT than those fed the control diet ($P < 0.05$),but there was no difference between fish fed 300 mg kg^{-1} COS-REE and the control diet ($P > 0.05$).

Challenge test

The challenge test showed that oral administration of COS-REE enhanced the protection against bacterial infection (Fig. 1). No significant difference in the protection capability was detected between the control group and those with COS-REE ($P > 0.05$), but the highest value for protection against *E. tarda* was found in fish fed the diet with 300 mg kg^{-1} COS-REE.

Discussion

The present study showed that dietary COS-REE levels could significantly increase the growth of turbot. When the diets were supplied with 300

Table 4 Effects of COS-REE on PI, SOD, MDA and MT of *Scophthalmus maximus* L.*

Diet No. (mg kg^{-1}COS-REE)	Diet 0(0)	Diet 1 (75)	Diet 2(150)	Diet 3 (300)	Diet 4 (600)	Diet 5 (1200)
PI	1.18 ± 0.04^{a}	1.28 ± 0.03^{ab}	1.33 ± 0.02^{b}	1.38 ± 0.01^{b}	1.37 ± 0.01^{b}	1.32 ± 0.02^{b}
SOD	53.6 ± 4.6^{ab}	39.3 ± 9.3^{a}	37.5 ± 4.0^{a}	67.9 ± 0.5^{b}	38.5 ± 1.0^{a}	47.6 ± 2.9^{ab}
MDA	24.6 ± 8.2	11.0 ± 2.3	12.9 ± 1.5	14.3 ± 1.6	15.5 ± 2.8	17.4 ± 2.1
MT	1.9 ± 0.2^{b}	1.5 ± 0.3^{ab}	1.0 ± 0.1^{a}	1.4 ± 0.2^{ab}	0.8 ± 0.1^{a}	1.0 ± 0.1^{ab}

*Data presented as means ± SEM (n = 3); Data within the same column with the same superscripts were not significantly different determined by Tukey's multiple test ($P > 0.05$).

COS-REE, chitosan oligosaccharide complex with rare earth; MDA, malondialdehyde; MT, metallothionein; PI, phagocytic index; SOD, super oxide dismutase.

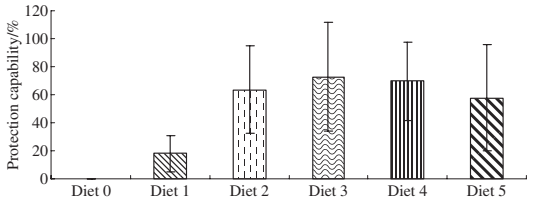

Figure 1 Effects of chitosan oligosaccharide complex with rare earth (COS-REE) on protection capability of *Scophthalmus maximus* L. after challenged with *Edwardsiella tarda.*

and 600 mg kg^{-1} COS-REE, the SGR was significantly higher than that in other groups. Based on SGR, the minimum supplement of dietary available COS-REE for the optimal growth of turbot was 300 mg kg^{-1}.This agreed well with the results of some previous studies in red sea bream, Japanese eel and yellowtail (Michiko, Takashi & Chiaki 1987), carp and rainbow trout (Tang, Zhang, Wang & Li 1997), weaning pig (Liu, Piao, Kim, Wang, Shen, Lee & Li 2008; Chen, Kim, Cho, Yoo, Wang, Huang, Kim & Shin 2009) and broilers (Li, Piao, Kim, Liu, Wang, Shen, Jung & Lee 2007).

In the present study, dietary COS-REE at the dose of 300 mg kg^{-1} significantly improved phagocytic activity. The compounds did not show a linear relationship between dose and phagocytic index, but have a maximum effect at intermediate doses. The relationship between stimulant dose and phagocytic activity has been studied in previous works (Esteban, Mulero, Cuesta, Ortuño & Meseguer 2000). The enhancement of phagocytosis was due to binding of COS to its receptors on the phagocytic cells, which had COS binding sites on the cell membrane (Yu, Zhao & Ke 2004; Kim and Rajapakse 2005; Liénart, Gautier & Domard 1991). When COS elicitor binds to receptors on the membrane of phagocytes, it results in increased concentration of free Ca^{2+} in the cytoplasm and may induce activation of Ca^{2+}/calmodium-dependent protein kinase II (CaMK II). Moreover, activated CaMK II could phosphorylate and activate Toll-like receptor (TLR), then promote TLR agonist-induced inflammatory factors such as IL-6, TNF-α and IFN-1 (Liu 2006). Furthermore, REE have been found to disturb the metabolism of calcium (Lin, Kadono, Yoshizuka, Furuichi & Kawano 2006). However, the exact mechanism by which COS-REE affect the free Ca^{2+} concentration and immunity of fish is still not elucidated to date and needs further studies.

The SOD, MDA and MT played key role in the balance between production and removal of endogenous ROS in the body and reflected the toxic effect of superoxide radical. Relating to the immune system, free radicals damage immune cells. The present study found a rapid elevation of SOD activity when fish fed the diet with 300 mg kg^{-1} COS-REE, while SOD activity decreased at lower and higher COS-REE inclusion. This suggested that suitable COS-REE level would be helpful to immunity of turbot. Cao, Yan, Luo and Yu (2010) proved that allogyogenetics silver crucian carp fed the diet with 10g kg^{-1} chotisan showed a higher SOD activity of serum than those fed 5 or 15g kg^{-1} chotisan diet. SOD was one of the metal enzymes possessing Cu/Zn or Mn. Chen, Cao, Lu and Wang (2000) showed that the inhibition of rare earth on SOD activity may be due to the alteration of distribution or shape of electric-charges in enzyme molecular, and not due to the replacement of metal ions because rare earth were not the similarities of substrate and could not lie in the site of Cu/Zn which should be occupied by substrate. Lower or higher COS-REE level would change the alteration of distribution or shape of electric-charges in enzyme molecular of SOD, and subsequently reduced SOD activity. Compared to the control group, SOD activity increased in fish fed with 300 mg kg^{-1} COS-REE, while MDA and MT concentration descended. The results revealed that COS-REE decreased oxidative damage. This was in agreement with the findings of Park, Je & Kim 2003; who reported that COS had a high potential to scavenge radicals. However, Kawagoe, Hirasawa, Wang, Liu, Ueno and Sugiyama (2005) showed that orally administrated cerium increased hepatic MT concentration and generated reactive oxygen species in the mouse liver. In the present study, MT concentration descended when the fish fed with COS-REE, which demonstrated that the reactive oxygen species was not increased. Thus, from the present experiment, it is clear that oral administration of COS-REE at the dose of 300 mg kg^{-1} has an increasing effect on scavenging radicals and non-specific immunity.

The swollen abdomen was one of the most important diseases of turbot, and its agent was the *E. tarda* (Castro, Toranzo, Barja, Núñez & Magariños 2006; Feng 2008). Direct selection of immunostimulants based on challenge test that fish is subjected to infection with a specific pathogen on common environment, is the most intuitional way to prove the effect of immunostimulants. In the

present study, oral administration of COS-REE increased protection capability challenge with the bacterial pathogens *E. tarda*, and the highest protection capability was observed in the challenged fish pre-immunostimulated with 300 mg kg^{-1} COS-REE. This was consistent with the previous results of PI and SOD, which had the highest value when fish fed the diet with 300 mg kg^{-1} COS-REE. The result suggested that the enhanced protection against bacteria was, or at least in part, due to increased innate immunity in this fish. The protective effect of oral administration with immunostimulants against several infectious pathogens has also been reported in many works (Jorge, Haruhisa, Toshiro & Hidetsuyo 2006).

In conclusion, feeding a dose (300mg kg^{-1}) of COS-REE significantly increased growth, innate immunity and protection against bacterial infection for turbot. These results suggest that COS-REE should be taken into account when a period of term oral administration is to be conducted. Furthermore, experiments need to be conducted to clarify the action mechanisms of COS-REE.

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