Fish and Shellfish Immunology 93 (2019) 500–507

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effects of different molar mass chitooligosaccharides on growth, antioxidant capacity, non-specific immune response, and resistance to *Aeromonas hydrophila* in GIFT tilapia *Oreochromis niloticus*

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ARTICLE INFO

Keywords:
GIFT tilapia
Chitooligosaccharides
Growth
Antioxidant capacity
Immune response
Disease resistance

ABSTRACT

A feeding trial was conducted to evaluate the effects of different molar mass chitooligosaccharides (1000 Da, 3000 Da and 8000 Da) on growth, antioxidant capacity, non-specific immune response, and resistance to *Aeromonas hydrophila* in GIFT tilapia (*Oreochromis niloticus*). A total of 600 fish were divided into four treatments with five replicates of thirty fish per tank. The results showed that the supplementation of 1000 Da and 3000 Da COS significantly improved the growth performance and feed utilization in GIFT tilapia. The trend of decreasing total cholesterol, triglyceride, ALT, and ACP activity was observed in fish fed diet supplemented COS. The supplementation of 1000 Da and 3000 Da COS significantly improved the serum TAC activity, and decreased the serum MDA and catalase activities ($P < 0.05$). The lysozyme activity of blood, liver, and gills in fish fed diets supplemented with 1000 Da and 3000 Da COS was significantly higher than that of fish fed control diet after 56 days of feeding ($P < 0.05$). The phagocytic activity and phagocytic index of fish fed diets supplemented with 1000 Da and 3000 Da COS were significantly higher than those of fish fed control diet. Post-challenge test showed that fish mortality in 1000 Da, 3000 Da, and 8000 Da COS groups were significantly lower than that of fish in control group ($P < 0.05$). In conclusion, the present study indicated that dietary 1000 Da and 3000 Da COS supplementation could enhance more performance and immune response of GIFT tilapia than 8000 Da COS.

1. Introduction

GIFT tilapia (*Oreochromis niloticus*) is one of the most important commercial freshwater fish worldwide due to high growth, desirable taste and high market value. With the deterioration of aquaculture environment and the lack of precise nutrition, nutritional diseases pose a great threat to tilapia aquaculture industry. How to maintain the physiological health and enhance the immune defense ability of cultured fish through feed nutrition is an important technical issue. Developing and utilizing new feed additives with pollution-free, residue-free, and non-talerance properties, which can improve the resistance of animal organisms, prevent animal diseases and promote growth, has become an inevitable trend in aquaculture production.

Chitin, known as the sixth life factor of human body and soft gold, is widely distributed in marine products and filamentous fungi, but its development and application are limited, because of the characteristics of poor solubility. Chitosan is a natural nontoxic biopolymer produced by the deacetylation of chitin, a major component of the shells of crustaceans such as crab, shrimp, and crawfish. Chitooligosaccharides (COS) are the degraded products of chitosan or chitin prepared by enzymatic or chemical hydrolysis of chitosan. Recently, COS have been the subject of increased attention in terms of their pharmaceutical and medicinal applications, due to their nontoxic and high solubility properties as well as their positive physiological effects. Administration of COS has been found to be effective against cyclophosphamide induced immunosuppression in mice [1]. The study of Mei et al. [1] also suggested that COS may also be effective in enhancing systemic immune responses and in modulating the functions of immunocompetent

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https://doi.org/10.1016/j.fsi.2019.08.001
Received 14 May 2019; Received in revised form 23 July 2019; Accepted 1 August 2019
Available online 01 August 2019
1050-4648/

cells. Many studies showed that chitosan improves the cellular and humoral immunity through enhancing the respiratory burst, phagocytosis, alternative complement, lysozyme activity and serum bactericidal activity in different fish species, like gilthead sea bream (*Sparus aurata*) [2], olive flounder (*Paralichthys olivaceus*) [3], cobia (*Rachycentron canadum*) [4] and common carp (*Cyprinus carpio*) [5]. The properties of chitosan, such as degree of polymerization, degree of deacetylation and electronic distribution, will greatly affect its biological activity [6]. The lower the molecular weight and the lower the degree of polymerization, the higher the biological activity. Chitooligosaccharides are degraded by chitosan, which are easily absorbed by intestine and quickly enter blood, so they have stronger biological activity [7]. Hu et al. [8] research showed that the bacteriostasis and bactericidal action of chitosan are related to its molecular weight. The smaller the molecular weight, the easier the chitosan can enter the cell wall's interstitial structure, interfere with cell metabolism, and achieve the bactericidal purpose. However, little has been done to incorporate chitooligosaccharides with different molar mass into aquatic animal. Thus, the present study was performed to investigate the effects of different molar mass COS on growth performance, antioxidant capacity, non-specific immune response, and resistance to *Aeromonas hydrophila* in GIFT tilapia. Information regarding the effects of COS supplementation on the enhancement of the growth performance and immunity in tilapia will have potential applications in the aquaculture industry.

2. Materials and methods

2.1. COS preparation

Chitooligosaccharides with molar mass of 1000 Da, 3000 Da, and 8000 Da were selected in the experiment. The deacetylation degree of chitooligosaccharides was more than 90%, which was provided by Dalian Institute of Chemical Physics, Chinese Academy of Sciences.

2.2. Experimental diets and design

The experimental fish were domesticated with basic feed for one week before the test began. Twenty aquariums were selected and the size of the aquarium was 0.3 m^3. Four isonitrogenous (gross protein content, 30.02%) and isoenergetic (gross energy content , 16.15 kJ g^{-1}) diets were designed. The control group was fed with basal diet, while the experimental group was fed with basal diet supplemented with 800 mg kg^{-1} chitooligosaccharides of 1000 Da, 3000 Da, and 8000 Da, respectively. The formulation and proximate chemical composition of diets are presented in Table 1.

All ingredients were ground and sieved into a fine powder through a 300 μm mesh. All the ingredients were thoroughly mixed with soybean oil and water. Pellets were prepared by cold extrusion with a twin-screw extruder model, dried in a forced air oven at 50 °C to a moisture content of about 9%. After drying, the diets were broken up and sieved into proper pellet size. The dry pellets were placed in covered plastic containers and stored in a refrigerator at 4 °C. All diets were analyzed in duplicate for proximate analysis.

2.3. Fish and experimental procedure

The GIFT tilapia (*Oreochromis niloticus*) obtained from a local commercial farm was acclimatized to the experimental conditions for 2 weeks before the start of the trial. At the beginning of experiment, all fish (initial average weight 26.61 ± 0.64 g) in good health condition (uniform body weight, size and swimming sensitive) was randomly distributed into 20 water-circulated tanks at a stocking density of 30 fish per tank. Each diet was randomly assigned to five duplicate tanks.

During the experimental period (56 days), the fish were fed the experimental diet at a rate of 3% of the live body weight daily (supply food according to fish demand; otherwise, the excess feed was collected within 30 min after meal by fishing nets, then drying). To prevent the waste of dietary pellets, fish was slowly hand-fed little by little to apparent satiation on the basis of visual observation of fish feeding behavior, three times (08:00, 12:00, and 17:00) daily. Each tank was constructed with an air stone connected to an electric compressor. The water quality parameters in each tank were measured weekly and included temperature (via a thermometer), pH-value (using Loviband Ltd., Model ET7919 pH-meter) and dissolved oxygen (using Loviband Ltd., Model ET7919 dissolved oxygen meter). The water temperature was 26.0 ± 3 °C, pH-value 7.3 ± 0.2, dissolved oxygen content approximately 6.5 ± 0.3 mg l^{-1}, ammonia-N less than 0.08 mg l^{-1}, nitrite-N less than 0.02 mg l^{-1}, and the photoperiod 12 L: 12 D. At the termination of the feeding trial, the fish was fasted for 24 h before harvest.

The total number, individual body length and weight of fish from each tank were measured to calculate the survival, growth performance, and feed utilization of fish fed the test diets according to the following formulae:

Survival (%) = 100 × (final no. of fish / initial no. of fish).

Weight gain rate (WGR, %) = (final body weight–initial body weight) × 100/initial body weight.

Specific growth rate (SGR %, d^{-1}) = 100 × [(Ln (final body weight) - Ln (initial body weight)) / duration (56 days)].

Table 1
Formulation and proximate composition of the experiment diets (g kg^{-1}) fed to GIFT tilapia for 56 days.

Items	COS molar mass (Da)			
	Control	1000	3000	8000
Ingredient Wheat (CP 13.58%) [a]	379.0	378.2	378.2	378.2
Fish meal (CP 65.36%) [b]	100.0	100.0	100.0	100.0
Soybean meal (CP 44.62%) [c]	150.0	150.0	150.0	150.0
Rapeseed meal (CP 36.15%) [d]	150.0	150.0	150.0	150.0
Cotton seed meal (CP 41.33%) [e]	150.0	150.0	150.0	150.0
Corn oil [f]	50.0	50.0	50.0	50.0
Calcium dihydrogen phosphate [g]	15.0	15.0	15.0	15.0
Choline chloride (50%) [h]	2.0	2.0	2.0	2.0
Vitamin premix [i]	2.0	2.0	2.0	2.0
Mineral premix [j]	1.0	1.0	1.0	1.0
NaCl	1.0	1.0	1.0	1.0
Chitooligosaccharide (1 ku)	0	0.8	0	0
Chitooligosaccharide (3 ku)	0	0	0.8	0
Chitooligosaccharide (8 ku)	0	0	0	0.8
Total	1000	1000	1000	1000
Proximate composition (g kg − 1 dry matter)				
Crude protein	30.02	30.02	30.02	30.02
Total lipid	7.32	7.32	7.32	7.32
Ash	5.13	5.13	5.13	5.13
Gross energy (kJ g^{-1})	16.15	16.15	16.15	16.15

[a] Wheat flour (Taishan Industrial Co., Ltd., China).
[b] Fish meal (Tianpu Feedstuff Company, Shandong, China).
[c] Soybean meal (Tianpu Feedstuff Company, Shandong, China).
[d] Rapeseed meal (Tianpu Feedstuff Company, Shandong, China).
[e] Cotton seed meal.
[f] Corn oil (Xiwang Industrial Co., Ltd., China).
[g] Calcium dihydrogen phosphate (Fuda Feedstuff Company, Shandong, China).
[h] Choline chloride (Fuda Feedstuff Company, Shandong, China).
[i] Vitamin premix (mg kg-1 diet): retinol acetate 30 mg; cholecalciferol 5 mg; alpha-tocopherol 60 mg; ascorbic acid 600 mg; vitamin K3 7 mg; thiamin 20 mg; riboflavin 20 mg; pyridoxine HCL 12 mg; vitamin B12 0.05 mg; inositol 100 mg; pantothenic acid 50 mg; niacin acid 35 mg; folic acid 8 mg; biotin 0.06 mg.
[j] Mineral premix (mg or g kg-1 diet): KI (1%) 60 mg; CoCl2·6H2O (1%) 7 mg; CuSO4·5H2O 20 mg; FeSO4·H2O 300 mg; ZnSO4·H2O 200 mg; MnSO4·H2O 60 mg; Na2SeO3·5H2O (1%) 60 mg; MgSO4·7H2O 2600 mg.

Feed efficiency rate (FER) = (final body weight - initial body weight) / feed intake

Protein efficiency ratio (PER, %) = 100 × live weight gain (g) / dry protein intake (g).

2.4. Sample collection and analysis

2.4.1. Sample collection and chemical analysis

All of the sample fish was anesthetized with MS-222 (solution concentration at 10 mg l^{-1}) , some blood samples were collected and pooled from the caudal vessels onto 100 IU ml^{-1} sodium heparin to estimate the phagocytic activity of leucocytes. The other blood sample were centrifuged at 4,000g for 10 min to collect serum, which was used for biochemistry parameters, antioxidant related parameter , lysozyme , and phagocytic activity assays. Plasma samples were collected from the caudal vein of four fish per tank with a heparinized syringe and transferred into a heparinized tube. All samples were pooled by tank for analysis.

Gill and liver tissue sample collection: While fish is under anesthesia, open abdominal cavity of fish, dissect approximately 0.5–1 g piece of liver and place into a bag, five fish samples were pooled in one bag. Then gently pull back the operculum with rounded forceps, using a fine pointed scissors, remove 4–6 filaments just above the septum from a fish and place into a bag, five fish samples were pooled in one bag. Label every bag with sample number and date. Keep samples on ice while in the field.

2.4.2. Blood biochemical parameters

The concentrations of total protein, blood glucose, total cholesterol, triglyceride, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AKP), and acid phosphatase (ACP) activities in serum were determined by the colorimetric enzymatic method using commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). One unit of ALT is defined as the amount of enzyme that generates 1 mmol of pyruvate per min at 37 °C. One unit of AST is defined as the amount of enzyme that will generate 1 mmol of glutamate per min at 37 °C. One unit of AKP activity was defined as the amount of enzyme that reacted with the matrix and produced 1 mg phenol in 15 min at 37 °C. One unit of ACP activity was defined as the amount of enzyme in 100 ml serum necessary to produce 1 mg nitrophenol for 30 min at 37 °C.

2.4.3. Antioxidant-related parameters assay

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase, and total antioxidant capacity (TAC) activities in serum were determined with a spectrophotometer (UV-2300, Shanghai Zike Technology Instrument Co., Ltd., Shanghai, China). Antioxidant-related parameter detection kits (SOD, GSH-Px, catalase, and TAC) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). One unit of SOD, expressed as unit ml^{-1}, was defined as the amount of enzyme that produced a 50% inhibition in colour formation measured at 550 nm. One unit of GSH-Px activity was defined as the amount of enzyme that reduced the GSH concentration in the reaction system at 1 μmol l^{-1} per min. One unit of catalase activity was defined as the amount of enzyme that catalysed the decomposition of 1 μmol of H_2O_2 per min. One unit of TAC was defined as a 0.01 increment of absorbance of the reaction system caused by serum per milliliter reacting at 37 °C for 1 min. Malondialdehyde (MDA) was determined using the procedure of the thiobarbituric acid (TBA) test [9]. A sample (1.0 ml) containing the biological material is mixed with 2.0 ml of a solution 15% w/v of trichloroacetic acid (TCA) and 0.375% w/v of TBA prepared in 0.25 N HC1. The mixture of the reagent with the sample is heated for 15 min in a boiling-water bath, and then cool and centrifuged at 1500 g to remove the precipitate of the tissue sample. The MDA concentration of the supernatant can be determined directly from the molar extinction coefficient of the pink pigment.

2.4.4. Lysozyme activity assay

The lysozyme activity in serum of five fish in each tank was determined as described by Ellis [10] and Meng et al. [11]. Weigh up liver or gill tissue sample 5g, place it into the plate, break up with steril scissors, homogenate with same volume of 0.1 M potassium phosphate buffer. The homogenate fluid were centrifuged at 4,000g for 15 min to collect supernatant solution, which was used for lysozyme activity assays. The lysozyme substrate was a 0.2 mg ml^{-1} freeze-dried *micrococcus lysodeikticus* (Sigma, USA) suspension in 0.05 M Phosphate Buffer Solution (PBS), pH 6.2. Serum (100 μl) was added to 1.9 ml of the bacterial suspension and the reduction in absorbance at 530 nm was measured after 0.5 min and 4.5 min at room temperature. Results were expressed in units of lysozyme activity ml^{-1} serum or liver/gill tissue suspension. One unit is defined as the amount of sample causing a decrease in absorbance of 0.001 min^{-1} at 530 nm.

2.4.5. Phagocytic activity assay of leucocytes

The phagocytic activity was adapted from the method described by Esteban et al. [12] with slight modification. Five heparinized 3 ml volume blood samples/replicate were carefully overlaid onto an equal volume of a Histo-paque medium (1.077 g ml^{-1}, Sigma-Aldrich, St. Louis, MO, USA) on a polystyrene tube. The sample was centrifuged at 3000 g for 10 min at 4 °C for separation of viable leucocytes from the peripheral blood. The leukocytes at the interface were collected and washed twice with RPMI-1640 medium supplemented with 100 IU ml^{-1} penicillin and 1 mg ml^{-1} streptomycin and adjusted to 4×10^7 ml^{-1} using the culture medium. One mL of the cell suspension was placed onto a 1 ml volume of a 1×10^6 *Saccharomyces serevisae* suspension and incubated at 37 °C for 1 h. Ten ml of the mixture were spread onto the clean slide and stained with Giemsa stain. Under the oil immersion lens of an Olympus CX22 bright-field biological microscope, approximately 200 phagocytic cells were counted.

Phagocytosis percentage = no. of ingesting phagocytes/total no. of phagocytes

Phagocytic index = no. of ingested yeast cells/no. of ingesting phagocytes

2.4.6. Challenge test

To investigate the resistance of tilapia to *Aeromonas hydrophila*, 30 fish from experimental and control groups were used. After 56 days of feeding and blood samplings, the fish was injected intraperitoneally with 0.1 ml of a 1.5×10^6 CFU ml^{-1} *A. hydrophila* which was suspended in phosphate buffered saline. The fish were checked regularly with eyes for any overt signs of disease including behavioural abnormalities and dead fish taken slowly from aquariums without creating stress factors. Cumulative mortality was noted in all the groups for 6 days of post infection.

2.5. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA followed by the use of Tukey's method to determine significant differences among treatment groups. Probabilities of $P < 0.05$ were considered significant. Statistical analysis was performed using SPSS 18.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Growth performance

Growth performance and feed utilization data of GIFT tilapia are

Table 2
Growth performance and feed utilization in GIFT tilapia fed test diets for 56 days[a].

Items	Molar mass of COS (Da)			
	Control	1000	3000	8000
Initial body weight (g)	26.61 ± 0.78	26.56 ± 0.78	26.63 ± 0.59	26.58 ± 0.66
Final body weight (g)	72.96 ± 1.13^{a}	80.96 ± 1.36^{bc}	82.67 ± 1.29^{c}	76.02 ± 1.34^{ab}
Weight gain rate (%)	174.91 ± 6.72^{a}	203.79 ± 5.88^{bc}	210.44 ± 6.57^{c}	186.25 ± 4.39^{ab}
Specific growth rate (% day $^{-1}$)	1.81 ± 0.04^{a}	1.99 ± 0.03^{bc}	2.02 ± 0.03^{c}	1.88 ± 0.04^{ab}
Feed efficiency rate	0.66 ± 0.01^{a}	0.72 ± 0.02^{b}	0.73 ± 0.02^{b}	0.68 ± 0.01^{a}
Protein efficiency rate (%)	220.71 ± 8.63^{a}	241.78 ± 7.29^{b}	242.59 ± 6.98^{b}	225.75 ± 11.02^{a}
survival (%)	100	100	100	100

[a] Data represent means ± SD. Values with different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments ($P > 0.05$).

shown in Table 2. The results showed that the supplementation of different molecular mass COS improved the growth performance and feed utilization in GIFT tilapia. The final body weight, weight gain rate, specific growth rate, feed efficiency rate, and protein efficiency ratio of fish fed diets supplemented with 1000 Da or 3000 Da COS were significantly higher than those of fish fed control diet after 56 days of feeding ($P < 0.05$). There were no significant differences between 1000 Da and 3000 Da COS ($P > 0.05$).

3.2. Blood biochemical parameters

Table 3 represents the blood biochemical parameters of GIFT tilapia after 56 days of feeding. The supplementation of different molar mass COS did not significantly affect the content of total protein, blood glucose, total cholesterol, triglyceride, alanine transaminase, aspartate transaminase, alkaline phosphatase and acid phosphatase activity ($P > 0.05$), but the trend of decreasing total cholesterol, triglyceride, alkaline phosphatase, and acid phosphatase activity was observed in fish fed diet supplemented COS.

3.3. Antioxidant-related parameters

The supplementation of 1000 Da and 3000 Da COS significantly improved the serum TAC activity (Table 4). Conversely, 1000 Da and 3000 Da COS inclusion generally decreased the serum MDA and catalase activities, which were significantly lower in fish fed the 1000 Da and 3000 Da COS diet compared to fish fed the control diet. No significant differences were observed in the serum SOD and GSH-Px activities among the dietary treatments.

3.4. Lysozyme activity

The lysozyme activity of blood (A), liver (B), and gills (C) in tilapia are shown in Fig. 1A, B, 1C. The results showed that the supplementation of different molar mass COS improved the lysozyme activity in GIFT tilapia. The lysozyme activity of blood, liver and gills in fish fed diets supplemented with 1000 Da and 3000 Da COS was significantly higher than that of fish fed control diet after 56 days of feeding ($P < 0.05$). There were no significant differences between 8000 Da COS and control diet ($P > 0.05$).

3.5. Phagocytic activity of leucocytes

Phagocytic activity (A) and phagocytic index (B) of leucocytes in GIFT tilapia are shown in Fig. 2A and B. The results showed that the supplementation of different molar mass COS improved the phagocytic activity in GIFT tilapia. The phagocytic activity and phagocytic index of fish fed diets supplemented with 1000 Da and 3000 Da COS were significantly higher than those of fish fed control diet after 56 days of feeding ($P < 0.05$).

3.6. Challenge test

The post-challenge mortality was recorded daily after the fish was challenged with *A. hydrophila* for 6 days (Fig. 3.). Fish mortality in 1000 Da, 3000 Da, and 8000 Da COS groups were significantly lower than that of fish in the control group ($P < 0.05$). No significant differences were observed in mortality between fish fed 1000 Da and 3000 Da COS diets.

4. Discussion

The results of the present study clearly show that the supplementation of chitooligosaccharides (average molar mass 1000 or 3000 Da) significantly improved the growth performance and feed utilization in GIFT tilapia. Chitin, poly-β-1,4-N-acetyl-D-glucosamine, is a cellulose-like biopolymer. It is highly acetylated (15–21% acetyl content) and is insoluble in common solvents. Chitosan, is acetylated only to the extent of 3–5% and is prepared from chitin by deacetylation with alkali [13]. Both chitin and chitosan are acetylated

Table 3
Blood biochemical parameters in GIFT tilapia fed test diets for 56 days.

Parameters	Molar mass of COS (Da)			
	Control	1000	3000	8000
Total protein (g l^{-1})	36.88 ± 2.11	38.33 ± 2.51	39.15 ± 3.28	37.92 ± 4.13
Blood glucose (mmol l^{-1})	3.95 ± 0.21	4.12 ± 0.18	4.15 ± 0.17	4.06 ± 0.23
Total cholesterol (mmol l^{-1})	3.07 ± 0.22	2.88 ± 0.31	2.76 ± 0.27	2.67 ± 0.33
Triglyceride (mmol l^{-1})	2.89 ± 0.16	2.46 ± 0.14	2.47 ± 0.22	2.59 ± 0.31
Alanine transaminase (u l^{-1})	87.67 ± 8.36	87.57 ± 5.11	93.12 ± 5.19	83.36 ± 4.66
Aspartate transaminase (u l^{-1})	727.36 ± 28.32	722.23 ± 3731	657.44 ± 34.52	713.45 ± 42.17
Alkaline phosphatase (u l^{-1})	32.59 ± 1.46	27.53 ± 2.59	28.64 ± 3.12	29.61 ± 1.63
Acid phosphatase (u l^{-1})	4.48 ± 0.45	3.82 ± 0.36	3.75 ± 0.22	3.92 ± 0.24

*Data represent means ± SD. Values with different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments ($P > 0.05$).

Table 4
Antioxidant-related parameters of GIFT tilapia fed the experimental diets for 56 days.

Parameters	Molar mass of COS (Da)			
	Control	1000	3000	8000
Superoxide dismutase (U ml^{-1})	22.31 ± 1.56	25.75 ± 1.33	26.91 ± 2.15	23.78 ± 1.79
Glutathione peroxidase (U μl^{-1})	0.14 ± 0.02	0.15 ± 0.03	0.17 ± 0.03	0.16 ± 0.02
Catalase (U ml^{-1})	58.76 ± 4.21^{b}	48.91 ± 3.36^{a}	47.28 ± 3.79^{a}	54.78 ± 2.35^{ab}
Total antioxidant capacity (U ml^{-1})	16.34 ± 1.02^{a}	21.75 ± 1.15^{ab}	25.81 ± 1.08^{b}	20.33 ± 1.21^{ab}
Malondialdehyde (nmol ml^{-1})	25.33 ± 1.24^{b}	17.97 ± 1.45^{a}	18.39 ± 1.55^{a}	23.12 ± 1.29^{b}

*Data represent means ± SD. Values with different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments ($P > 0.05$).

aminopolysaccharides, the differences being the extent of acetylation [14]. The molecular weight of chitin or chitosan varies from hundreds of thousands to millions, and there are a lot of hydrogen bonds between and inside molecules, so it is insoluble in some common solvents such as water, which greatly limits the application of chitosan. However, chitooligosaccharide (COS), which is one type of the oligosaccharides, is produced from chitin or chitosan by chemical or enzymatic decomposition methods [15]. The COS has a higher activity and more physiological functions than chitin and chitosan due to its lower molecular weight or its ready solubility in water. Shiau and Yu [16] showed that both chitin and chitosan supplementation depresses tilapia growth regardless of the supplementation level. With regard to COS research, Lin et al. [17] showed that both the final weight and specific growth rate of Ovate Pompano (*Trachinotus ovatus*) were significantly higher with increasing dietary chitooligosaccharides (average molecular mass < 5000 Da) levels up to 4 g kg^{-1}, and Zhang et al. [18] also researched that 500 mg kg^{-1} body weight of COS (average molecular mass 1000 Da) supplemented in feed enhanced the growth of turbot. According to Li et al. [15], dietary supplementation with 50 or 100 mg kg^{-1} chitooligosaccharides (average molecular mass 1500 Da) also improved the growth rate of broilers. However, According to Qin et al. Research [19], supplementation with 1000 mg kg^{-1} feed of commercial chitooligosaccharides as recommended dose had no effect on the growth parameters of tilapia, but improved intestinal health, changed autochthonous gut bacteria and improved resistance to infection by *A. hydrophila*. Some studies suggested that the growth enhancing effect of immunostimulants depends on the dosage, molecular weight, duration of feeding, environmental temperature, route of administration, and species [17,20].

The supplementation of different molar mass COS did not significantly affect the content of serum biochemical parameters, but the trend of decreasing total cholesterol, triglyceride, alkaline phosphatase, and acid phosphatase activity was observed in fish fed diet supplemented COS. Choi et al. [21] showed that COS can reduce blood lipid.

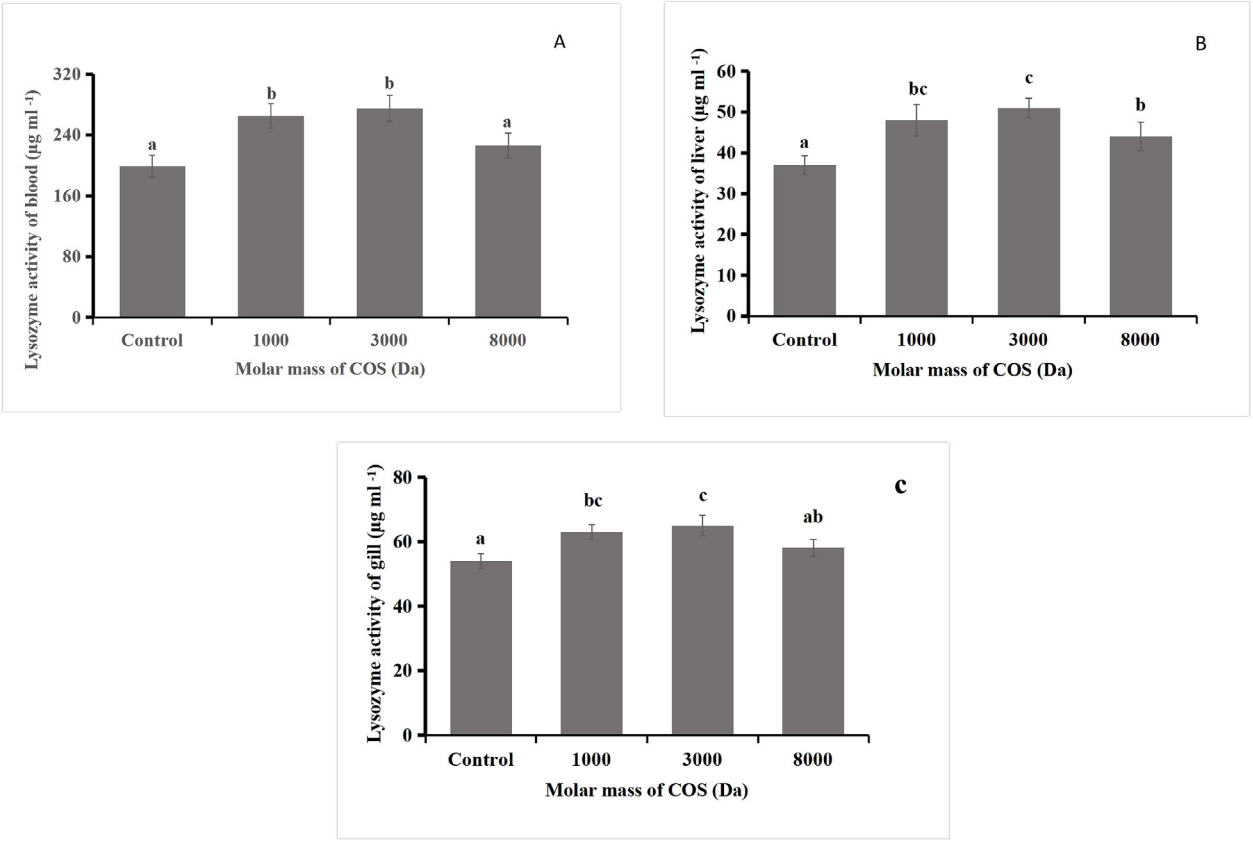

Fig. 1. A. Lysozyme activity of blood (μg ml −1) in tilapia, Bars with different letters are significantly different ($P < 0.05$) B. Lysozyme activity of liver (μg ml −1) in tilapia, Bars with different letters are significantly different ($P < 0.05$). 1C. Lysozyme activity of gill (μg ml − 1) in tilapia, Bars with different letters are significantly different ($P < 0.05$).

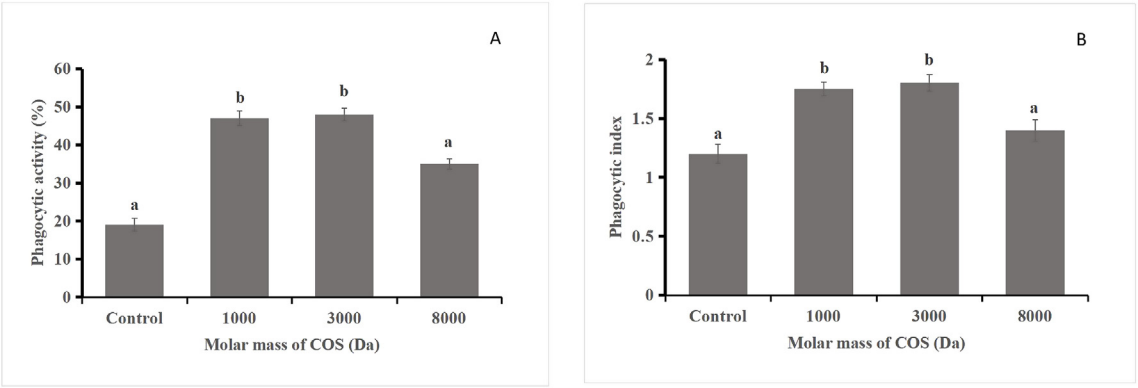

Fig. 2. A. Phagocytic activity of leucocytes in tilapia, Bars with different letters are significantly different (P < 0.05). B. Phagocytic index of leucocytes in tilapia, Bars with different letters are significantly different ($P < 0.05$).

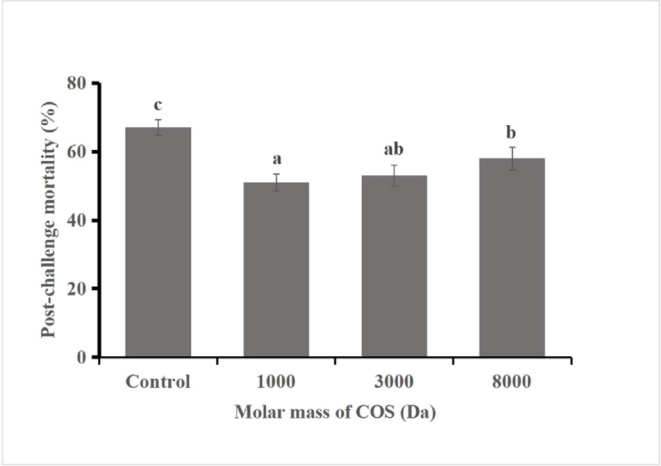

Fig. 3. Post-challenge mortality of tilapia after infection with A. Hydrophila for 6 days. Bars with different letters are significantly different (P < 0.05).

In study of mice, COS can increase the excretion of triglycerides and total cholesterol, and then effectively reduce the serum lipid content, so that the animal body can maintain a healthy level. Similar result were obtained for early-weaned pigs [22] and broilers [23]. The reason for this results might be that COS can form a gel complex with gastric acid in the gastrointestinal tract where the gel complex cannot be degraded under the high pH environment in the intestine. This gel complex can absorb bile acid and cholesterol and the gel, bile acid, cholesterol mixtures are discharged in feces, thus the absorption of fat and cholesterol is decreased [23]. AKP and ACP are the two important phosphatase enzymes taking part in several metabolic functions such as permeability, growth and cell differentiation, protein synthesis, as well as uptake and transport of nutrients [24]. In this study, although COS did not significantly affect ACP and AKP activity, there was a downward trend in data indicators. It has been demonstrated that serum AKP activity is linked with the availability and absorption of minerals into blood stream [25,26]. Kaya et al. [25] showed that enhancement of serum AKP in poultry is associated with elevated blood calcium and phosphorous concentrations. Many of the above mechanisms come from other domestic animal, but due to the particularity of aquatic animals, further study is needed to evaluate the effect of COS on blood characteristics of aquatic animal.

Chitooligosaccharides have been identified as potent antioxidant agents [27]. It has been proved that chitooligosaccharides is capable of lowering MDA concentrations, and enhancing activity of antioxidant enzymes such as SOD, GPX and catalase. Likewise, the results of both in vitro and in vivo studies have revealed the potential antioxidant activity of COS [27]. Although the exact mechanism of action of COS is still rather unclear, its radical scavenging activity has been ascribed to the reaction of unstable free radicals with amino and hydroxyl groups at the C-2, C-3, and C-6 positions of the pyranose ring which results in formation of stable macromolecule radicals [28]. Zou et al. [29] showed that COS exerts definite effects on hydroxyl and ABTS radical scavenging. In the present study, COS did not significantly affect the serum SOD and GPX activity, but COS inclusion in diets significantly affect the serum TAC and catalase activities. Similarly, Niu et al. [30] found the significant elevation of total antioxidant status and GPX activity in digestive glands of *P. monodon* offered a COS containing diet. Also, we detected a significant reduction in MDA concentration. However, Samad et al. [28] showed no significant changes in MDA concentration. The observed differences could be due to the variations in degree of deacetylation and molecular weight of COS used in the studies which have been identified as the factors influencing antioxidant activity of COS [31,32].

In fish, the non-specific immune system is more important for disease resistance than specific immune system as the latter needs a longer time for antibody production and specific cellular activations [33]. Thus, we have focused on several indicators of humoral (serum lysozyme) and cellular (phagocytic activity, phagocytic index) innate immunity in this study, which has been shown to be the first line of defense of fish [34]. COS are ideal candidates for therapeutics involving immunomodulatory activity. The present study demonstrated that the dietary application of 1000 Da and 3000 Da COS had beneficial effects on the some non-specific immune parameters (lysozyme activity, phagocytic activity and phagocytic index) of GIFT tilapia. Similar findings have also been reported in other animals including pigs [35], broilers [15], *Trachinotus ovatusor* (molar mass less than 5000 Da COS) [17], and juvenile rainbow trouts (average molar mass 1200 Da COS) [36]. Liu et al. [17] reported that the respiratory burst activity, phagocytic capacity, lysozyme and superoxide dismutase activity were signifificantly increased with the increased levels of dietary COS (average molecular weight lower than 5000 Da) ($P < 0.05$). Luo et al. [36] reported that supplementation of COS in diets did not affect production performance and body composition of rainbow trout. However, fish fed the 40 mg kg^{-1} COS diet demonstrated improved phagocytic activities, respiratory burst activities and decreased serum cortisol level. Also, Suzuki et al. [37] found that COS could enhance the activity of complement C3, and the increase of activity was positively correlated with the length of COS main chain. The COS could enhance the immune defense function of organism by enhancing the ability of complement C3 lysis and clearing immune complexes. In the present study, those immunological parameters in fish fed 8000 Da COS remained relatively unchanged compared with control group. The biofunctionalities of COS are highly related to its molecular weight. The beneficial effects of low molecular COS (molar mass < 5000 Da) had been proved in

preventing negative mineral balance [38], whereas the molecular weight of COS is critical for microorganism inhibition [39]. In addition, the effect of COS administration on animal immunity greatly depended on their degree of polymerization (DP) [40]. Wei et al. [41] found that COS with the DP equal to or greater than 6 had greater bioactivities. These differences may be due to the type of immunostimulants used and experimental species, which resulted in different availability of immunostimulants.

The mechanism of how chitooligosaccharides enhance growth and immune responses is not very clear to date. The intestinal flora of fish is composed of beneficial and harmful flora. The beneficial flora usually covers the surface of gastrointestinal mucosal epithelium and prevents the colonization of pathogenic microorganisms on intestinal mucosal tissue to protect the integrity of intestinal flora. Oligosaccharides can optimize the intestinal microflora of fish by regulating the balance between the two microbial flora [42]. Therefore, we speculate that the growth-promoting mechanism of COS may be that COS can regulate the microflora of fish gastrointestinal tract, maintain the integrity of intestinal mucosa, optimize the intestinal environment, improve the digestibility of feed nutrients, and thus improve the growth performance of fish. Some results of previous studies on diffffferent fish species have shown the beneficial effects of dietary COS supplementation on growth performance, nutrient digestibility and small intestinal morphology [43–45]. It is assumed that COS mediates changes in digestive enzymes activity due to its low molecular weight, thus improving apparent digestion of most nutrients and intestinal health [46]. Oligosaccharides can promote the synthesis of vitamins and increase the absorption of trace elements, thus indirectly improving the body's immunity. COS are generally defined as complexes, which bind specifically to the cell surface receptor proteins of phagocytes or lymphocytes and activate the nonspecific immune system of animals through the synergy of cytokines, thus stimulate the production of effective immune responses [47].

Bacterial challenge test has often been used as a final indicator of fish health status after nutrition trial [48]. *A. hydrophila* is a ubiquitous Gram-negative bacterium that has been wildly used in fish immunonutrition studies due to the fact that it is responsible for hemorrhagic septicemia and ulcerative diseases, and causes high levels of mortality in freshwater fish [49]. Chitooligosaccharides are nontoxic and water-soluble compounds obtained by enzymatic degradation of chitosan, which is derived from chitin by a deacetylation process. Chitooligosaccharides possess broad range of activities such as antitumour, antifungal, antibacterial activities. Numerous studies have shown that oral administration of immunostimulants can effectively improve resistance of fishes to pathogenic bacteria or virus [36,50,51]. The present study showed that the oral administration of COS significantly reduced cumulative mortality of GIFT tilapia after injecting with *A. hydrophila*. Moreover, 1000 Da COS significantly reduced post-challenge mortality more. The increase in resistance against *A. hydrophila* in GIFT tilapia fed with COS can be possibly explained on the basis of increasing non-specific immune response. As far as chitooligosaccharides was concerned, a similar result was obtained by Lin et al. [17], who showed that dietary inclusion of 4 g kg^{-1} COS (average molar mass less than 5000 Da) improve fish's resistance to infection by *V. harveyi*. Xia et al. [52] showed that the bacteriostasis of *E. coli* increased with the decrease of molar mass of chitooligosaccharide, especially when the molecular weight was about 1500 Da. Moreover, Kittur et al. [53] and Joaoc et al. [54] also found that the bacteriostatic effect of chitooligosaccharides was related to the molecular weight, deacetylation degree and concentration of chitooligosaccharides. The mechanism of how chitooligosaccharides reduce pathogenic bacteria is unclear. Previous studies have suggested that N-acetyl glucosamine is a basic component of the structure of chitooligosaccharides [55] and is also a component of intestinal mucins [56] that serve as receptors when bacteria bind to the gut of the host [57]. Therefore chitooligosaccharides comprised of N-acetyl glucosamine may bind to certain types of bacteria [57] and possibly interfere with their adhesion to mammalian cells [58]. In addition, chitooligosaccharides can serve as a fermentable substrate for beneficial intestinal bacteria [15,59], which may induce production of organic acids and thus reduce intestinal pH; this decrease in pH may minimize the prevalence of intestinal pathogens [60].

In conclusion, the present study indicated that dietary COS supplementation could enhance the performance and the immune response of GIFT tilapia. The results suggested that low molar mass COS at 1000 Da and 3000 Da were better than 8000 Da COS. However, further research needs to be conducted to clarify molecular mechanisms of low molar mass COS bacteriostasis and the effect of COS precise dose.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Grant No. 31472288) to Jiting Wang; Shandong Science and Technology Development Plan Project to Jiting Wang (Grant No. 2014GGH210010); The Key R&D Projects of Shandong Province to Jiting Wang (Grant No. 2019GNC106078); and Funds of Shandong “Double Tops” Program.

References

[1] Y.X. Mei, H.X. Chen, J. Zhang, X.D. Zhang, Y.X. Liang, Protective effect of chitooligosaccharides against cyclophosphamide induced immunosuppression in mice, Int. J. Biol. Macromol. 62 (2013) 330–335.
[2] A. Cuesta, M.A. Esteban, J. Meseguer, In vitro effect of chitin particles on the innate cellular immune system of gilthead seabream *(Sparus aurata L.)*, Fish Shellfish Immunol. 15 (2003) 1–11.
[3] S.H. Cha, J.S. Lee, C.B. Song, K.J. Lee, Y.J. Jeon, Effects of chitosan coated diet on improving water quality and innate immunity in the olive flounder *Paralichthys olivaceus*, Aquacult 278 (2008) 110–118.
[4] X. Geng, X.H. Dong, B.P. Tan, Q.H. Yang, S.Y. Chi, H.Y. Liu, X.Q. Liu, Effects of dietary chitosan and Bacillus subtilis on the growth performance, non-specific immunity and disease resistance of cobia, *Rachycentron canadum*, Fish Shellfish Immunol. 31 (2011) 400–406.
[5] S. Maqsood, P. Singh, M.H. Samoon, A.K. Balange, Effect of dietary chitosan on non-specific immune response and growth of *Cyprinus carpio* challenged with *aeromonas hydrophila*, Int. Aquat. Res. 2 (2010) 77–85.
[6] R.A.A. Muzzarelli, Chitosan-based dietary foods, Carbohydr. Polym. 29 (1996) 309–316.
[7] Z.P. Hu, Research advances in chitooligosaccharides, Chin. J. Biochem. Pharm. 24 (2003) 210–212.
[8] Y. Hu, Z. Du, H. Liu, Study on the correlation between chitosan antimicrobial activity and molecular weight and environmental media, J. Anal. Sci. 19 (2003) 308.
[9] J.A. Buege, S.D. Aust, Microsomal lipid peroxidation, Methods Enzymol. 52 (1978) 302–310.
[10] A.E. Ellis, Lysozyme assays, in: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Robertsen, W.B. van Miswinkel (Eds.), Techniques in Fish Immunology, SOS Publications, N. J. air Haven, 1990, pp. 101–103.
[11] X. Meng, J. Wang, W. Wan, M. Xu, T. Wang, Influence of low molecular weight chitooligosaccharides on growth performance and non-specific immune response in Nile tilapia *Oreochromis niloticus*, Aquacult. Int. 25 (2017) 1265–1277.
[12] M.A. Esteban, V. Mulero, A. Cuesta, J. Ortuno, J. Meseguer, Effects of injecting chitin particles on the innate immune response of gilthead seabream (*Sparus aurata L.*), Fish Shellfish Immunol. 10 (2000) 543–554.
[13] L.T. Fillar, M.G. Wirick, Bulk and solution properties of chitosan, in: A.A. Muzzarelli, E.R. Pariser (Eds.), International Conference on Chitin and Chitosan at MIT, Proc. 1st, 1978, pp. 169–181.
[14] R.A.A. Muzzarelli, Enzymatic synthesis of chitin and chitosan. Occurrence of chitin, in: R.A.A. Muzzarelli (Ed.), Chitin, Pregamon Press, New York, NY, 1977, pp. 5–44.
[15] X.J. Li, X.S. Piao, S.W. Kim, P. Liu, L. Wang, Y.B. Shen, Effects of chitooligosaccharide supplementation on performance, nutrient digestibility, and serum composition in broiler chickens, Poult. Sci. 86 (2007) 1107–1114.
[16] S.Y. Shiau, Y.P. Yu, Dietary supplementation of chitin and chitosan depresses growth in tilapia, *Oreochromis niloticus* × *O. aureus*.Aquacult. 179 (1999) 439–446.
[17] S.M. Lin, S.H. Mao, Y. Guan, X. Lin, L. Luo, Dietary administration of chitooligosaccharides to enhance growth,innate immune response and disease resistance of *Trachinotus ovatus*, Fish Shellfish Immunol. 32 (2012) 909–913.
[18] Y. Zhang, W. Xu, L.Q. Cui, D.F. Wang, Effects of chitooligosaccharides in diets of turbot (*Scophthalmus maximus*) on the growth and immune performance, J. Ocean Univ. China 42 (2012) 71–74.
[19] C.B. Qin, Y.T. Zhang, W.S. Liu, L. Xu, Y.L. Yang, Z.G. Zhou, Effects of chito-oligosaccharides supplementation on growth performance, intestinal cytokine expression, autochthonous gut bacteria and disease resistance in hybrid tilapia *Oreochromis niloticus* × *Oreochromis aureus*, Fish Shellfish Immunol. 40 (2014) 267–274.
[20] R.A. Dalmo, J. Bøgwald, Beta-glucans as conductors of immune symphonies, Fish Shellfish Immunol. 25 (2008) 384–396.

[21] B.K. Choi, K.Y. Kim, Y.J. Yoo, In vitro antimicrobial activity of a chitoologosaccharide mixture against *Actinobacillusa ctinomycetemcomitans* and *Streptococcus mutans*, Int. J. Antimicrob. Agents 18 (2001) 553–557.
[22] Z.R. Tang, Y.L. Yin, C.M. Nyachoti, R.L. Huang, T.J. Li, C.B. Yang, X.J. Yang, J. Gong, J. Peng, D.S. Qi, J.J. Xing, Z.H. Sun, M.Z. Fan, Effect of dietary supplementation of chitosan and galacto-mannan-oligosaccharide on serum parameters and the insulin-like growth factor-I mRNA expression in early-weaned pigs, Domest. Anim. Endocrinol. 28 (2005) 430–441.
[23] X.Z. Ma, Y. Yang, X.D. Xie, Y.L. Feng, Effect of chitosan on growth performance and lipo-metabolism of broiler chickens (male), J. Fujian Agricul. U. (China) 16 (2001) 30–34.
[24] N. Ram, Mercuric chloride, cythion and ammonium sulfate induced changes in the brain, liver and ovarian alkaline phosphatase content in the fish *Channa punctatus*, Environ. Ecol. 3 (1985) 263–268.
[25] H. Kaya, A. Kaya, M. Gul, S. Celebi, S. Timurkaan, B. Apaydin, Effects of supplementation of different levels of organic acids mixture to the diet on performance, egg quality parameters, serum traits and histological criteria of laying hens, Eur. Poult. Sci. 78 (2014), https://doi.org/10.1399/eps.2014.46.
[26] W. He, S. Rahimnejad, L. Wang, K. Song, K. Lu, C. Zhang, Effects of organic acids and essential oils blend on growth, gut microbiota, immune response and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) against *Vibrio parahaemolyticus*, Fish Shellfish Immunol. 70 (2017) 164–173.
[27] W. Xia, P. Liu, J. Zhang, J. Chen, Biological activities of chitosan and chitooligosaccharides, Food Hydrocolloids 25 (2011) 170–179.
[28] S. Rahimnejad, X. L Yuan, L. Wang, K. Lu, K. Song, C.X. Zhang, Chitooligosaccharide supplementation in low-fish meal diets for Pacific white shrimp *(Litopenaeus vannamei*): effects on growth, innate immunity, gut histology, and immune-related genes expression, Fish Shellfish Immunol. 80 (2018) 405–415.
[29] P. Zou, X. Yang, W.W. Huang, H.T. Zhao, J. Wang, R.B. Xu, X.L. Hu, S.Y. Shen, D. Qin, Characterization and bioactivity of polysaccharides obtained from pine cones of *Pinus koraiensis* by graded ethanol precipitation, Molecules 18 (2013) 9933–9948.
[30] J. Niu, H.Z. Lin, S.G. Jiang, X. Chen, K.C. Wu, Y.J. Liu, S. Wang, L.X. Tian, Comparison of effect of chitin, chitosan, chitosan oligosaccharide and -acetyl-dglucosamine on growth performance, antioxidant defenses and oxidative stress status of *Penaeus monodon*, Aquacult 372 (2013) 1–8.
[31] J.Y. Je, P.J. Park, S.K. Kim, Free radical scavenging properties of hetero -chitooligosaccharides using an ESR spectroscopy, Food Chem. Toxicol. 42 (2004) 381–387.
[32] P.J. Park, J.Y. Je, S.K. Kim, Free radical scavenging activities of differently deacetylated chitosans using an ESR spectrometer, Carbohydr. Polym. 55 (2004) 17–22.
[33] D.P. Anderson, Immunostimulants, adjuvants and vaccine carrier in fish: application to aquaculture, Annu. Rev. Fish Dis. 2 (1992) 281–307.
[34] R.A. Dalmo, K. Ingebrigtsen, J. Bogwald, Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES), J. Fish Dis. 20 (1997) 241–273.
[35] K.N. Han, I.K. Kwon, J.D. Lohakare, S. Heo, B.J. Chae, Chitooligosaccharides as an alternative to antimicrobials in improving performance, digestibility and microbial ecology of the gut in weanling pigs, AJAS (Asian-Australas. J. Anim. Sci.) 20 (2007) 556–562.
[36] L. Luo, X.F. Cai, C. He, M. Xue, X.F. Wu, H.N. Cao, Immune response, stress resistance and bacterial challenge in juvenile rainbow trouts *Oncorhynchus mykiss* fed diets containing chitosan-oligosaccharides, Curr. Zool. 55 (2009) 416–422.
[37] Y. Suzuki, K. Miyatake, Y. Okamoto, E. Muraki, S. Minami, Influence of chain length of chitosan on complement activation, Carbohydr. Polym. 54 (2003) 465–469.
[38] W.K. Jung, S.H. Moon, S.K. Kim, Effect of chitooligosaccharides on calcium bioavailability and bone strength in ovariectomized rats, Life Sci. 78 (2006) 970–976.
[39] Y.J. Jeon, P.J. Park, S.K. Kim, Antimicrobial effect of chitooligosaccharides produced by bioreactor, Carbohydr. Polym. 44 (2001) 71–76.
[40] Y.G. Du, M.J. Zhang, H. Zhang, X.F. Bai, Study of new preparation technology and anticancer activity on chitooligosaccharide, Chin. J. Microecol. 1 (2001) 5–7.
[41] X.L. Wei, Y.F. Wang, J.B. Xiao, W.S. Xia, Separation of chitooligosaccharides and the potent effects on gene expression of cell surface receptor CR3, Int. J. Biol. Macromol. 45 (2009) 432–436.
[42] H. Wang, M. He, P. Liu, T. Hu, X. Chen, Study on normal intestinal flora of carp, Acta Hydrobiol. Sin. 18 (1994) 354–359.
[43] X. Meng, J. Wang, W. Wan, M. Xu, T. Wang, Influence of low molecular weight chitooligosaccharides on growth performance and non-specifific immune response in Nile tilapia *Oreochromis niloticus*, Aquacult. Int. 25 (2017) 1265–1277.
[44] Y. Chen, X. Zhu, Y. Yang, D. Han, J. Jin, S. Xie, Effffect of dietary chitosan on growth performance, haematology, immune response, intestine morphology, intestine microbiota and disease resistance in gibel carp (*Carassius auratus gibelio*), Aquacult. Nutr. 20 (2014) 532–546.
[45] M.K. Najafabad, M.R. Imanpoor, V. Taghizadeh, A. Alishahi, Effffect of dietary chitosan on growth performance, hematological parameters, intestinal histology and stress resistance of Caspian kutum (Rutilus frisii kutum Kamenskii, 1901) fifingerlings, Fish Physiol. Biochem. 42 (4) (2016) 1063–1071.
[46] W. Le, L. Ting-Ting, W. Xi-Long, Y. Gui-Ping, Y. Shi-Bin, Chitosan supplementation may improve the digestive physiology and health of captive *Leiothrix lutea*, Avian Biol. Res. 8 (2015) 221–226.
[47] B. Lambrecht, M. Gonze, D. Morales, G. Meulemans, T.P. van den Berg, Comparition of biological activities of natural and recombinant chicken interferon-gamma, Vet. Immunol. Immunopathol. 70 (1999) 257–267.
[48] C. Lim, M. Yildirim-Aksoy, M.H. Li, T.L. Welker, P.H. Klesius, Influence of dietary levels of lipid and vitamin E on growth and resistance of Nile tilapia to *Streptococcus iniae* challenge, Aquacult 298 (2009) 76–82.
[49] J. Vivas, B. Carracedo, J. Riãn, B.E. Razquin, P. Lopez-Fierro, F. Acosta, Behavior of an *Aeromonas hydrophila* aroA live vaccine in water microcosms, Appl. Environ. Microbiol. 70 (2004) 2702–2708.
[50] A. Gopalakannan, V. Arul, Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of *Cyprinus carpio* and control of *Aeromonas hydrophila* infection in ponds, Aquacult 255 (2006) 179–187.
[51] M. Sakai, Current research status of fish immunostimulants, Aquacult 172 (1999) 63–92.
[52] W.Y. Xiag, Y.N. Wu, Functional properties of chitooligosaccharides, J. Jiangnan U. 15 (1996) 297–302.
[53] F.S. Kittur, A.B.V. Damar, M.C. Varadaraj, R.N. Tharanathan, Chitooligosaccharides preparation with the aid of pectinase isozyme from *Aspergillus niger* and their anti bacterial activity, Carbohydr. Res. 340 (2005) 1239–1245.
[54] F. Joaoc, T. Frenik, C.S. Jos, Antimicrobial effects of chitosan and chitooligosaccharides, upon *Staphlococcus aureus* and *Escherichia coli*,in food model system, Food Microbiol. 25 (2008) 922–928.
[55] S.K. Kim, N. Rajapakse, Enzymatic production and biological activities of chitosan oligosaccharides (COS): a review, Carbohydr. Polym. 62 (2005) 357–368.
[56] D.K. Podolsky, Oligosaccharide structures of human colonic mucin, J. Biol. Chem. 260 (1985) 8262–8271.
[57] P. Klemm, M.A. Schembri, Bacterial adhesins: function and structure, Int. J. Med. Microbiol. 290 (2000) 27–35.
[58] J. Rhoades, G. Gibson, K. Formentin, M. Beer, R. Rastall, Inhibition of the adhesion of enteropathogenic *Escherichia coli* strains to HT-29 cells in culture by chitooligosaccharides, Carbohydr. Polym. 64 (2006) 57–59.
[59] H.W. Lee, Y.S. Park, J.S. Jung, W.S. Shin, Chitosan oligosaccharides, dp 2-8,have prebiotic effect on the Bifidobacterium bifidium and *Lactobacillus sp*, Anaerobe 8 (2002) 319–324.
[60] L.L. Mikkelsen, M. Jakobsen, B.B. Jensen, Effects of dietary oligosaccharides on microbial diversity and fructo-oligosaccharide degrading bacteria in faeces of piglets post-weaning, Anim. Feed Sci. Technol. 109 (2003) 133–150.