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Effects of dietary β-1, 3 glucan on innate immune response of large yellow croaker, *Pseudosciaena crocea*

Qinghui Ai*, Kangsen Mai, Lu Zhang, Beiping Tan, Wenbing Zhang, Wei Xu, Huitao Li

The Key Laboratory of Mariculture (Ministry Education of China), Ocean University of China, 5 Yushan Road, Qingdao, Shandong 266003, P.R. China

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Abstract

The present study was conducted to investigate the effects of dietary β-1, 3 glucan on the innate immune response and protection against *Vibrio harveyi* infection in large yellow croaker, *Pseudosciaena crocea*. A basal diet was supplemented with 0% (control), 0.09% (low) and 0.18% (high) β-1, 3 glucan to formulate three experimental diets. Each diet was randomly allocated to triplicate groups of fish in floating sea cages ($1.5 \times 1.5 \times 2.0$ m), and each cage was stocked with 100 fish (initial average weight 9.75 ± 0.35 g). Fish were fed twice daily (05:00 and 17:00) to apparent satiation for 8 weeks. The results of 8 weeks feeding trial showed that low glucan supplementation (0.09%) significantly enhanced fish growth, whereas high supplementation (0.18%) did not. The serum lysozyme activity was significantly increased with the increase of dietary glucan ($P < 0.05$), and fish fed the diet with high glucan had significantly higher lysozyme activity compared with low glucan. There were no significant differences in alternative complement pathway (ACP) activity between fish fed diets with and without supplementation of glucan. The phagocytosis percentage (PP) and respiratory burst activity in fish fed the diet with 0.09% glucan were significantly higher than those in fish fed with the control diet ($P < 0.05$), but both immunological parameters significantly decreased in fish fed the diet with high supplementation compared with low supplementation and no significant difference was observed between the control and high supplementation groups. The challenge experiment showed that fish fed the diet with low glucan had significantly lower cumulative mortality compared with the control and high glucan groups ($P < 0.05$), but no significant difference was observed between the control and high supplementation groups. These results suggested that low glucan could enhance growth and innate immunity of large yellow croaker with an 8-week oral administration, but higher supplementation did not influence growth, or further improve immunity of large yellow croaker.

Keywords: Large yellow croaker; *Pseudosciaena crocea*; β-1, 3 glucan; Immunity; Bacteria challenge; Growth

* Corresponding author. Tel./fax: +86 532 8203 2495.
E-mail addresses: qhai@ouc.edu.cn, qinghuiai@yahoo.com (Q. Ai).

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1. Introduction

β-glucans, which consist of glucose units linked through β-1, 3 or β-1, 6 glycosidic linkage, are one of the most important structural polysaccharides in the cell walls of bacteria, fungi and plants [1]. The β-glucan was first demonstrated to stimulate anti-tumor mechanisms, and increase host resistance to a diverse range of microbial pathogens in mice [2]. Recently, glucans have also been proven to be effective immunostimulants in enhancing immunity and disease resistance for fish [3–8].

Many studies have measured the effects of glucan on immunity of fish. For instance, the *in vitro* culture of macrophages with glucan has been adopted by some authors [6,9], but more were focused on *in vivo* studies [1,4,10,11]. Most of these have used intraperitoneal injection because of efficiency and quickness of the method [1,3,12,13], but it is time-consuming and impractical in aquaculture. Oral administration has been proven to be a suitable procedure for administering glucan [5,14] and some studies have shown that oral administration of glucan enhances the non-specific response and disease resistance of fish [10,11,15]. However, such enhancement varies with dose of glucan, feeding regime and glucan type.

Large yellow croaker (*Pseudosciaena crocea*) is one of the most important cultured marine fish species in China. However, in recent years the culture of this fish is facing disease threats. To date, a preliminary study has been conducted on the nutrition of this fish [16,17], but little information is available on its basal immune response and the increased immunity and protection against pathogen infection which might be stimulated by immunostimulants [18]. The purpose of the present study was to examine the effects of dietary glucan on immunity and disease resistance of this fish.

2. Materials and methods

2.1. Experimental diets

Using fish meal and soybean meal as protein sources, fish oil and soybean oil as lipid sources, the basal diet was formulated to contain approximately 43% crude protein and 11% crude lipid. The basal diet was used as the control diet, and 0.5% and 1.0% glucan (containing 18% β-1, 3 glucan, and the 82% of supplementation including protein, cellulose and starch) were separately supplemented to formulate the two experimental diets (including 0.09% and 0.18% β-1, 3 glucan). The glucan, derived from the cell wall of yeast, *Saccharomyces cerevisiae*, was provided by Bioengineering Institute, Ocean University of China (Qingdao, China). Ingredients were ground into fine powder through a 320 μm mesh. All the ingredients were thoroughly mixed with menhaden 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 45 °C. After drying, the diets were broken up and sieved into the appropriate pellet size (2.5 × 5.0 mm), and were stored at −15 °C until used.

2.2. Feeding experiment

Experimental fish were obtained from a commercial farm in Ningbo, China. Prior to the start of the experiment, young large yellow croaker were reared in floating sea cages (3.0 × 3.0 × 3.0 m), and fed the control diet (Diet 1) for 2 weeks to acclimate to the experimental diet and conditions.

At the start of the experiment, the fish were fasted for 24 h and weighed after being anesthetized with eugenol (1:10 000) (Shanghai Reagent Corp., China). Fish of similar sizes (9.75 ± 0.35 g) were randomly distributed into 9 sea cages (1.5 × 1.5 × 2.0 m), and each cage was stocked with 100 fish. Each diet was assigned to triplicate cages. Fish were hand-fed to apparent satiation twice (05:00 and 17:00) daily. The feeding trial lasted for 8 weeks. During the experimental period, the temperature ranged from 19.5 to 25.5 °C, the salinity from 25 to 28‰ and dissolved oxygen content was approximately 7 mg l^{-1}. At the termination of the experiment, the fish were fasted for 24 h before harvest. Total number and mean body weight of fish in each cage were measured.

2.3. Functional immune assay

2.3.1. Sample collection

After being fasted for 24 h, blood samples were collected from the caudal vein of five fish per cage with a 27-gauge needle and 1 ml syringe, and allowed to clot at room temperature for 4 h. Following centrifugation (836 × *g*, 10 min, 4 °C), the serum was removed and frozen at −80 °C until use. Head-kidney macrophages from five fish in each cage were isolated as described by Secombes [19] with some modifications. Briefly, the head kidney was excised, cut into small fragments and transferred to RPMI-1640 (Gibco, USA) medium supplemented with 10IU/ml heparin (Sigma, USA), 100 IU/ml penicillin (Amresco, USA), 100 μg/ml streptomycin (Amresco, USA) and 2% foetal calf serum (FCS) (Gibco, USA). Cell suspensions were prepared by forcing the head-kidney through a 100 μm steel mesh. The resultant cell suspensions were enriched by centrifugation (836 × *g* for 25 min at 4 °C) on 34%/51% Percoll (Pharmacia, USA) density gradient. The cells were collected at the 34–51% interface and washed twice. Cell viability was determined by the trypan blue exclusion method and the cell density was determined in a haemocytometer. Then additional RPMI 1640 medium was added to adjust the cell concentration (1×10^7 ml^{-1}) for analysis.

2.3.2. Lysozyme assay

The lysozyme activity in serum of five fish in each cage was determined as described by Ellis [20]. Results were expressed in units of lysozyme ml^{-1} serum. One unit is defined as the amount of sample causing a decrease in absorbance of 0.001 min^{-1} at 530 nm compared to the control (*Micrococcus lysodeikticus* suspension without serum).

2.3.3. Alternative complement pathway (ACP) activity

Serum ACP activity for five fish in each cage was assayed according to Yano [21]. Briefly, a series of volumes of the diluted serum ranging from 0.1 to 0.25 ml were dispensed into test tubes and the total volume made up to 0.25 ml with barbitone buffer in presence of ethyleneglycol-bis (2-aminoethoxy)-tetraacetic acid (EGTA) and Mg^{2+}, then 0.1 ml of rabbit red blood cells (RaRBC) was added to each tube. After incubation for 2 h at 22°C, 3.15 ml 0.9% NaCl was added. Following this, the sample was centrifuged at 836 × *g* for 5 min at 4 °C to eliminate unlysed RaRBC. The optical density of the supernatant was measured at 414 nm. The volume of serum producing 50% haemolysis (ACH50) was determined and the number of ACH50 units ml^{-1} was obtained for each group.

2.3.4. Phagocytic activity

Phagocytic activity for five fish in each cage was determined by a modified method of Pulsford et al. [22]. The 100 μm cell suspensions of head kidney leucocytes (1×10^7 cells ml^{-1}) was placed into a sterile slide and the cells allowed to attach for 30 min at 25 °C. Following attachment, 100 μl yeast suspension (Bakers yeast, Type II, Sigma, USA, 1×10^8 cells ml^{-1}) was added to the cell monolayer, and the slide was incubated for 45 min at 25 °C. Then unattached cells were washed off with phosphate buffered saline. After air-drying, the slides were fixed in ethanol, redried and stained with Giemsa. Then, 200 cells were examined by microscopy to determine the percentage of cells with phagocytic activity.

2.3.5. Respiratory burst activity

Production of intracellular superoxide anion (O_2^-) was evaluated using nitroblue tetrazolium (NBT) (Shanghai Reagent Corp., China) reduction following the method of Secombes [19] with some modifications, for five fish from each cage. A 100 μl cell suspension was stained with 100 μl 0.3% NBT and 100 μl Phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) (1 μg ml^{-1}) for 30 min. Absolute methanol was added to terminate the staining. Each tube was washed three times with 70% methanol and air-dried. Then 120 μl 2 M KOH and 140 μl dimethyl sulfoxide (DMSO, Sigma, USA) were added and the colour was subsequently measured at 630 nm with a spectrophotometer using KOH/DMSO as a blank.

2.3.6. Challenge test

The *Vibrio harveyi* strain was originally isolated from infected large yellow croaker. The seven day LD_{50} was determined by intraperitoneal injection of 48 fish with graded doses of *Vibrio harveyi* (10^6, 10^7, 10^8 and 10^9 cfu/fish) at 23 °C, and the result showed that the LD_{50} on day 7 was 10^8 cfu/fish.

At the termination of the feeding experiment, 12 fish of each cage (each dietary treatment has 36 fish) were transferred into 300 l tanks (76 × 55 × 60 cm) for the challenge test. The water temperature was maintained at about 23 °C. After 5 days adaptation, the fish were injected intraperitoneally with 0.2 ml PBS containing 1.6×10^8 live *Vibrio harveyi* from a 24 h culture in 2216E medium at 25 °C. The mortality was recorded for 6 days.

2.4. Calculations and Statistical analysis

The following variables were calculated:

Specific growth rate (SGR) = (Ln W_t − Ln W_0) × 100/t

Survival rate = $N_t \times 100/N_0$

Phagocytosis Percentage (PP) = (Number of cells ingesting yeast/Number of adherent cells observed) × (Number of yeast ingested/Number of adherent cells observed).

Where W_t and W_0 were final and initial fish weight, respectively; N_t and N_0 were final and initial number of fish, respectively; t is duration of experimental days.

All data were subjected to analysis of variance in SPSS 10.0 for Windows. Differences between the means were tested by Tukey's multiple range test. The level of significance was chosen at $P < 0.05$ and the results are presented as means ± S.E.M. (standard error of the mean).

3. Results

3.1. Survival and growth

The survival rate increased from 89.3 to 92.0% with increasing dietary glucan, but no significant differences were observed among dietary treatments. Fish fed the diet with 0.09% glucan had significantly higher SGR (1.73% day^{-1}) compared with the control group (1.51% day^{-1}) ($P < 0.05$). However, the SGR (1.58 day^{-1}) in fish fed the diet with 0.18% glucan was not significantly different from the control group (Table 1, $n = 300$).

3.2. Serum lysozyme and ACP activity

The serum lysozyme activity in fish fed diets with both glucan levels were significantly higher than the control group (97.00 unit ml^{-1}), and the value in fish fed with 0.18% glucan (118.60 unit ml^{-1}) was significantly higher compared with 0.09% glucan (131.87 unit ml^{-1}) ($P < 0.05$). No significant differences in serum ACP activity (from 113.87 to 116.26 unit ml^{-1}) were observed among dietary treatments (Table 2, $n = 15$).

Table 1
Growth response and survival of large yellow croaker (*Pseudosciaena crocea*) fed the diets with graded levels of β-1, 3 glucan (means ± S.E.M, $n = 300$)

Diet No. Supplementation level	β-1, 3 glucan level (%)	Growth response	
		Survival %	SGR*% d^{-1}
Diet 1 (0.0%)	0	89.3 ± 0.7	1.51 ± 0.05^{b}
Diet 2 (0.5%)	0.09	92.0 ± 0.6	1.73 ± 0.03^{a}
Diet 3 (1.0%)	0.18	90.3 ± 1.5	1.58 ± 0.05^{ab}
ANOVA**			
F value		1.882	5.953
P value		0.232	0.038

Data are expressed as mean ± S.E.M. Means in the same column sharing the same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$). The significant differences between experimental groups were determined by one-way analysis of variance (ANOVA). *SGR: specific growth rate. **ANOVA: one-way analysis of variance.

Table 2
Effects of dietary β-1, 3 glucan levels on the activities of serum lysozyme and alternative complement pathway of large yellow croaker (*Pseudosciaena crocea*) (means ± S.E.M., $n = 15$)

Diet No. Supplementation level	β-1, 3 glucan level	Immune response	
		Lysozyme (unit ml^{-1})	ACP* (ACH50* unit ml^{-1})
Diet 1 (0.0%)	0	97.00 ± 3.90^{c}	113.87 ± 7.13
Diet 2 (0.5%)	0.09	118.60 ± 3.14^{b}	116.26 ± 11.73
Diet 3 (1.0%)	0.18	131.87 ± 1.55^{a}	115.75 ± 7.91
ANOVA**			
F value		33.789	0.019
P value		0.010	0.981

Data are expressed as mean ± S.E.M. Means in the same column sharing the same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$). The significant differences between experimental groups were determined by one-way analysis of variance (ANOVA). *ACP means Alternative Complement pathyway. ACH50 means 50% haemolysis. **ANOVA: one-way analysis of variance.

3.3. Phagocytosis and respiratory burst activity of head kidney macrophages

The PP (7.2%) in fish fed the diet with 0.09% glucan was significantly higher than the control (4.8%) and 0.18% glucan groups (5.0%). There was no significant difference in PP between fish fed the diets with the control and 0.18% glucan groups (Fig. 1, $n = 15$).

The respiratory burst activity of head-kidney macrophages was significantly increased by 0.09% glucan ($OD_{630} = 0.35$) compared with the control and 0.18% glucan groups ($OD_{630} = 0.26$) ($P < 0.05$) (Fig. 2, $n = 15$). However, no significant differences in this parameter were observed between the control ($OD_{630} = 0.26$) and high glucan groups.

3.4. Challenge test

The challenge test ($n = 36$ for each dietary treatment) showed that long-term oral administration of glucan enhanced the protection against bacterial infection (Fig. 3). The cumulative mortality rate in fish fed the diet with 0.09% glucan (55.6%) was significantly lower than the control (88.9%) and 0.18% glucan groups (82.6%) after injecting with *Vibrio harveyi*, but the 0.18% glucan group was not significantly different from the control group.

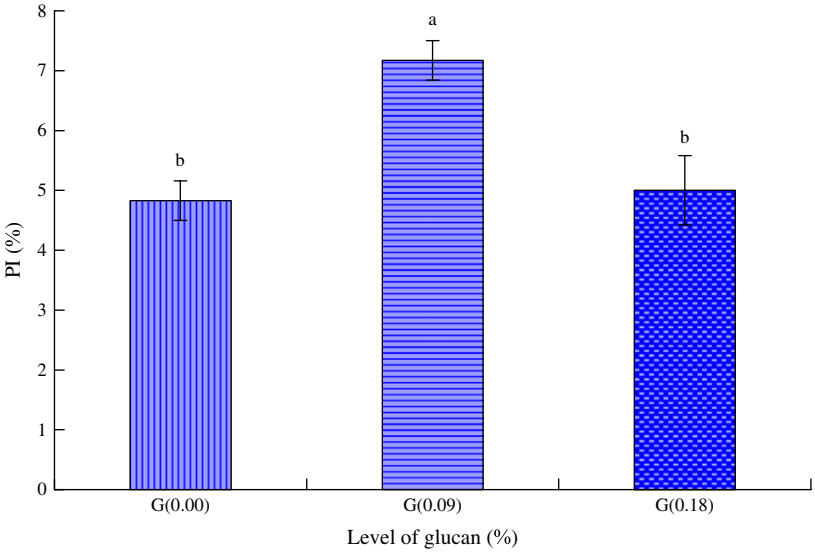

Fig. 1. Effects of dietary β-1, 3 glucan on phagocytosis percentage (PP) of large yellow croaker (*Pseudosciaena crocea*). Data are expressed as mean (S.E.M.). Means in the same column sharing the same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$). $n = 15$.

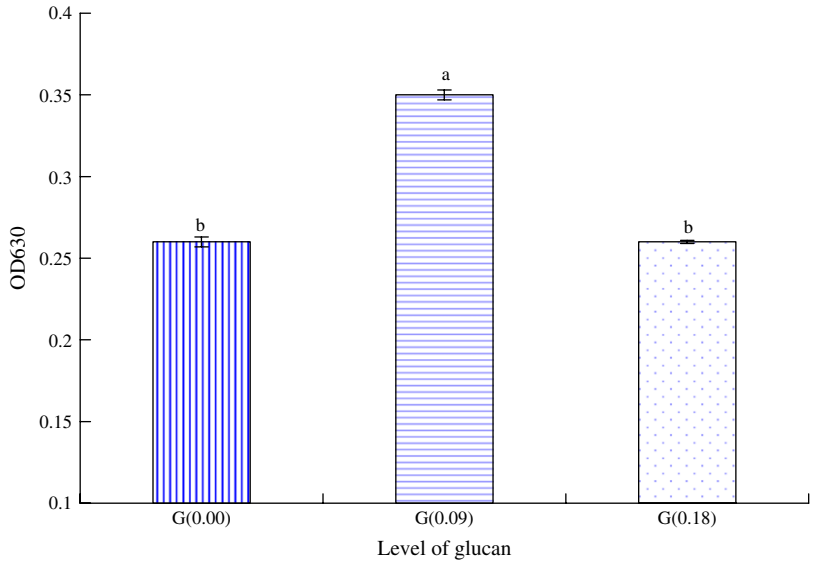

Fig. 2. Effects of dietary β-1, 3 glucan on respiratory burst of activity of large yellow croaker (*Pseudosciaena crocea*). Data are expressed as mean (S.E.M). Means in the same column sharing the same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$). $n = 15$.

4. Discussion

The present study showed that growth was increased by feeding a low dose of β-1, 3 glucan for 8 weeks. However, the growth was not further enhanced by feeding of 0.18% β-1, 3 glucan in this study, suggesting low supplementation (0.09% β-1, 3 glucan) was optimal for the growth of large yellow croaker. To date, there is no exact explanation on how glucan works to enhance growth rate. Some workers [23,24] presumed that increased survival and growth by glucan probably comes from disease resistance of shrimp larvae, whose immune system was activated by β-glucan, thus preventing infection by pathogenic bacteria. Others [25] observed that *Penaeus monodon* was well equipped to digest

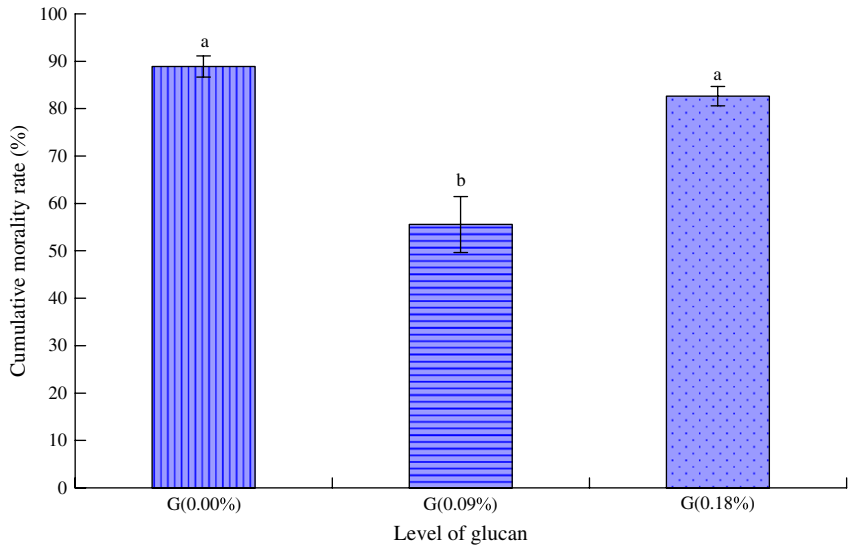

Fig. 3. Effects of dietary β-1, 3 glucan on cumulative mortality of large yellow croaker (*Pseudosciaena crocea*) after infection with *Vibrio harveyi*. Data are expressed as mean (S.E.M). Means in the same column sharing the same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$). $n = 36$.

β-glucan and suggested that growth rate was enhanced by the energetic benefits obtained through β-glucan. However, no such reports were related to fish. Therefore, the mechanism of growth-promotion by β-1, 3 glucan remains to be identified in fish.

Relatively lower phagocytosis (PP) was observed in the present study compared with some other studies [26,27]. The differences are probably due to different species, animal size, study method or the particles used. Yeast was used in the present study, which is consistent with some previous studies [28,29], but differed from other studies using bacteria [26,27] or latex beads as engulfed materials [30].

Feeding a low dose of β-1, 3 glucan to large yellow croaker significantly enhanced serum lysozyme activity, PP, respiratory burst activity of head-kidney and disease resistance in the present study. These immunological parameters, however, decreased significantly in fish fed the diet with high glucan compared with low supplementation, although lysozyme activity continued to increase with the increase of dietary glucan. The serum ACP activity was not enhanced by feeding glucan at either of the levels, which was in agreement with other studies [11,31], but was different from the result of Engstad et al. [32], who found the ACP activity was strongly activated by the injection of glucan. The protection against infection was significantly increased by low supplementation of glucan, but the high supplementation did not influence the protection, which correlated well with the change of the above immunological parameters. The result suggested that the enhanced protection against bacteria was, or at least in part, due to increased non-specific immunity in this fish, which was similar to the results of some previous studies after glucan treatment [3,6,33]. Therefore, adequate dose of glucan was important in promoting immunity and disease resistance in long-term oral administration of glucan.

The mechanism by which glucan enhance immunity of fish is still not completely elucidated to date. In mammals, the existence of glucan receptors on macrophages and neutrophils has been revealed, and the first step for interaction between glucan and phagocytes involves the binding of glucan to the receptor [34,35]. In fish, glucan receptors have also been reported to exist on macrophages [36,37]. Some studies have shown that glucan enhances non-specific immunity through direct activation of macrophages [5,38]. The enhanced PP and respiratory burst activity of head-kidney macrophages by feeding a low dose of β-1, 3 glucan in the present study provided good support for the notion that dietary glucan supplementation influences the macrophage, which probably further influences the immunity of fish. However, the exact mechanism of the effect of glucan on immunity of fish needs further studies.

Feeding high dose of β-1, 3 glucan resulted in immunosuppression or feedback regulation in the present study, which is similar to some other studies [33,39]. This suggested that the high level of β-1, 3 glucan directly induced the respiratory burst, which, after a period, can exhaust the cells. However, lysozyme activity in fish fed the high glucan level (0.18%) was further enhanced by increasing glucan, suggesting different immunological parameters respond differently to β-1, 3 glucan.

The effect of glucan administration on immunity depends on administration route, dosage and feeding regime [5]. Results after 8-weeks of feeding in the present study showed that feeding of low glucan (0.09%) enhanced the immunity and protection against infection. This agreed well with the results of other workers [11], who found yeast supplemented diets (including glucan) enhanced non-specific immunity after a 4-week feeding experiment, but is different from a previous study in *Labeo rohita* fingerlings [26], where the immunostimulatory effect of glucan peaked at day 42 after feeding with the diet containing 250 mg kg^{-1} β-glucan and subsequently decreased at the end of the 56 day feeding trial. These differences may be due to the type of glucan used and experimental species, which resulted in different availability of glucan and transfer time to macrophages.

In conclusion, feeding a low dose (0.09%) of β-1, 3 glucan significantly increased growth, innate immunity and protection against infection for large yellow croaker, but high β-1, 3 glucan level (0.18%) had no significant effect on immunity and protection against bacterial infection compared with the control diet. These results suggest that β-1, 3 glucan level should be taken into account when a long term oral administration is conducted.

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