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Effect of dietary chitosan on haematology, innate immunity and disease resistance of Asian seabass *Lates calcarifer* (Bloch)

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Abstract

This study was performed to evaluate the effect of dietary chitosan on haematology, innate immunity and protection against *Vibrio anguillarum* in Asian seabass, *Lates calcarifer*. A basal diet supplemented with 0, 5, 10 and 20 g chitosan kg^{-1} diet was fed to the four different groups for 60 days. The haematological (total erythrocyte count, total leucocyte count, total serum protein, albumin, globulin and albumin-globulin ratio) and innate immune parameters (phagocytic ratio, respiratory burst, serum lysozyme and serum bactericidal activities) were monitored at fortnight interval to assess the effect of chitosan feeding in Asian seabass. All the studied haematological and innate immune parameters were increased significantly ($P \leq 0.05$) in chitosan-fed groups in comparison with control. However, the group fed diet containing 10 g chitosan Kg^{-1} feed showed highest haematological and innate immune parameters on 45th day in comparison with other groups. Moreover, the fish fed the diet containing 10 g chitosan Kg^{-1} feed had significantly higher post-challenge survival (75.56 ± 4.44%) on the 30th day following *V. anguillarum* challenge. Therefore, this study suggests that chitosan at 10 g kg^{-1} diet could be used as prophylactic in Asian seabass culture to enhance the protection against any possible infection by *V. anguillarum*.

Keywords: Asian seabass, chitosan, haematology, innate immunity, *Vibrio anguillarum*

Introduction

Aquaculture is the fastest growing food-production sector, thus helping to meet the growing food demand throughout the world. The recent trend to increase fish production and profitability through intensive or semi-intensive farming using high stocking density and feed supplementation could lead to deterioration of the environmental conditions, thereby resulting in disease outbreaks. Such outbreak of diseases could potentially become a major bottleneck for further development and expansion of aquaculture industry. Traditionally, control and prevention of diseases is often achieved using a wide range of antibiotics, pesticides, disinfectants and other chemicals. The use of chemotherapeutants and antibiotics for controlling diseases has been widely criticized for the negative impacts like residue accumulation in organism tissue and environment, development of drug resistant microorganisms, immunosuppression and reduced consumer preferences for food fish (Anderson 1992; Baulny, Quentel, Fournier, Lamour & Gouvello 1996).

Hence, aquaculture practices have necessitated the development of alternative prophylactic measure to prevent the disease outbreaks. As a consequence, many studies have focused into the modulation of immune system to improve individual's resistance for better health management rather than depending upon antibiotics and chemotherapeutants. To reduce the risk of diseases, the immune mechanism comprising both specific and

non-specific systems in the cultured organisms should be up-regulated by use of probiotics, vaccines, immunostimulants or by selective breeding (Raa, Rørstad, Engstad & Robertsen 1992; Kumar, Mukherjee, Prasad & Pal 2006; Kumar, Mukherjee, Ranjan & Nayak 2008). Although vaccines promise for the prevention of diseases in future, many vaccines are not effective against important diseases (Raa *et al.* 1992), with a few exceptions; many of them are confined to laboratory conditions only. In addition, no commercially effective vaccine is currently available for *Lates calcarifer* against any pathogen in India. Therefore, the use of immunostimulants to enhance the immune system of fish is considered as an attractive and promising area.

Immunostimulants are natural or synthetic substances capable of activating non-specific and/or specific immune responses (Anderson 1992). Immunostimulants are important for better health management as they provide the stimulating factors of defence mechanism, thus protecting against diseases. Several promising adjuvants, natural and synthetic drugs and biological modifiers have been tested *in vivo* and *in vitro* in fish. These substances are effective in stimulating or modulating non-specific defence mechanism and offer protection against viral and bacterial diseases (Cuesta, Meseguer & Esteban 2002; Kumari & Sahoo 2006; Das, Debnath, Patnaik, Swain, Kumar & Misrhra 2009; Rodrıguez, Chamorro, Novoa & Figueras 2009).

One such potential immunostimulant is chitosan, which is a de-acetylated product of chitin, commercially manufactured from exoskeleton of shrimp and crab. It has multiple applications in the fields of medicine, agriculture and aquaculture. Chitosan has been found to stimulate the main cellular innate immune function in mammals (Suzuki, Okawa, Hashimoto, Suzuki & Suzuki 1984; Peluso, Petillo, Ranieri, Santin, Ambrosio, Calabro, Avallone & Balsamo 1994; Shibata, Foster, Metzger & Myrvik 1997a; Shibata, Metzeger & Myrvik 1997b). It is used as an immunostimulant to protect salmonids against bacterial diseases (Anderson & Siwicki 1994; Siwicki, Anderson & Rumsey 1994) and also to enhance the respiratory burst and phagocytic activities in various fish species (Siwicki *et al.* 1994; Gopalakannan & Arul 2006; Cha, Lee, Song, Lee & Jeon 2008; Kledmanee 2010). Asian seabass, *L. calcarifer*, fed chitosan-coated diet resulted in increased innate immune activities (Kledmanee 2010), but he has not reported challenge study to know the possible protection against pathogens. Special attention must be paid to its dose and duration for sustainable enhancement, so that the non-specific defence mechanism of fish can be stimulated without risking harmful effects including immunosuppression and increased susceptibility as seen in other immunostimulants (Anderson, Siwicki, Dixon & Lizzio 1989; Kajita, Sakai, Atsuta & Kobayashi 1990).

Asian seabass is a candidate culture species in diversified Indian aquaculture system and its culture is a growing enterprise. This fish species is susceptible to various pathogens of parasitic, bacterial and viral origin (Wong & Leong 1989; Anderson & Norton 1991; Subhasinghe & Shariff 1992; Soltani, Munday & Burke 1996; Azad, Thirunavukkarasu, Kailasam & Rajan 2004; Kumar, Parameswaran, Ahmed, Musthaq & Hameed 2007). Bacterial diseases mainly caused by *Vibrio anguillarum* are a major problem in seabass farming industries (Toranzo & Barja 1990). Keeping this in view, this study was undertaken to evaluate the effect of chitosan on haematology, innate immunity and diseases resistance in Asian seabass.

Materials and methods

Fish and husbandry

The Asian seabass fingerlings weighing 2 ± 0.1 g were procured from commercial fish seed farm (Meenakshi Aquafarm, Tanjavur District, Tamil Nadu, India). Fish were reared further and the fingerlings having an average weight of 15 ± 2 g were used for the study. The fingerlings were weaned on the artificial diet and were acclimatized to feed on experimental diet without chitosan. Filtered seawater was used throughout the experimental period and round the clock aeration was provided to all tanks having experimental fish.

Experimental design

Four rectangular tanks, each with one tonne capacity, were taken for the experiment. All the tanks were partitioned with the help of 1-mm mesh-size nylon net and partitions were kept as replicates for a group. Then, the fish were randomly distributed into all eight partitioned chambers, each containing 15 seabass fingerlings. All the tanks were filled with 800-L filtered sea water of salinity 30–32 g L^{-1}. During the experimental period, the temperature ranged between 27 and

30°C and dissolved oxygen content was varied from 5–7 mg L^{-1}.

The control group (T_1) fish were fed normal diet without chitosan. The treatment groups T_2, T_3 and T_4 were fed feed containing chitosan @ 5, 10 and 20 g kg^{-1} feed respectively. The experiment was conducted for a period of 60 days.

Preparation of experimental diets

The different feed ingredients such as dextrin, carboxy methyl cellulose (CMC) were procured from Himedia, India; cod liver oil and vitamin-mineral mixture (each 100 g mineral-vitamin mixture provided Vitamin A 200 000 IU; Cholecalciferol 40 000 IU; Vitamin B_2 80 mg; Vitamin E 30 IU; Vitamin K 40 mg; Calcium pantothenate 100 mg; Nicotinamide 400 mg; Vitamin B_{12} 240 mg; Choline chloride 6 g; Calcium 30 g; Manganese 1.1 g; Iodine 40 mg; Iron 300 mg; zinc 600 mg; copper 80 mg; cobalt 18 mg) were brought from a local veterinary drug store and fish meal was procured from local market for the preparation of experimental diets (Table 1). Chitosan was obtained from Regional Research Center of Central Institute of Fisheries Technology, Visakhapatnam. Chitosan was derived from the crustacean shells. The required quantities of all the ingredients were taken and mixed thoroughly with water to make dough of required consistency. Then dough was steam cooked for 20 min, cooled and required quantity of vitamin & mineral mixture and chitosan were mixed thoroughly. The cellulose was replaced with equal amount of required chitosan in the different treatment diets. Then, pellets were made using hand pelletizer. The pellets were initially fan dried and then kept under sunlight for complete drying. The dried pellets were packed in polythene bags and kept in refrigerator.

Table 1 Percentage (%) inclusion of the feed ingredients in the experimental diet

Ingredients	Percentage
Fish meal	60
Dextrin	25
Cod-liver oil	5
Vitamin-mineral mixture	5
CMC	2
Cellulose*	3

*Cellulose was replaced with an equal quantity of different dose of immunostimulant required in different treatment feed.

Feeding and maintenance of experimental tanks

Feeding was done @ 5% of the fish biomass per day. The daily ration was divided into two equal parts to feed morning at 7.30 am and evening at 5.30 pm. The manual cleaning and siphoning the excess feed pellets and faecal matter was done everyday in the morning. Everyday, about 40–50% water of the experimental tanks was exchanged with filtered seawater. This was done throughout the experimental period.

Sampling and analysis

Samples were collected on 15th, 30th, 45th and 60th days of the experiment for different studies. Blood was drawn from the caudal peduncle region using a sterile syringe (Discardit), which was pre-rinsed with 2.7% EDTA (Qualigens) solution. Then, it was collected in small glass vials wherein 20 µL of 2.7% EDTA solution was coated by drying the vials in hot air oven (Newtronic). For serum, blood was drawn without rinsing the syringe with anticoagulants and collected in clean and dry eppendrof tubes. The blood was allowed to clot for 45 min in inclined position at room temperature followed by 30 min incubation at 4°C and then centrifuged at 4000 *g* for 5 min at 4°C. The serum was collected in sterilized eppendrof tube and analysed for different serum parameters.

Haematological studies

For the estimation of total erythrocyte count and total leucocyte count (TEC, Schaperclaus, Kulow & Schreckenbach 1991), 20 µl of blood was mixed with 3980 µL of red blood cell (RBC) diluting fluid and white blood cell (WBC) diluting fluid (Dacies fluid) in clean glass vials respectively. The mixture was shaken well to suspend the cells uniformly in the solution. The cells were counted using a haemocytometer (Feinoptik, Blakenburg, Germany) and expressed as:

$$Number of RBC/mm3 = Nr \times 10000$$

$$Number of WBC/mm3 = Nw \times 500$$

where, N_r is the total number of red blood cells counted in five squares of the haemocytometer and 10 000 is the factor obtained after taking the initial dilution factor into consideration and N_w denotes the total number of white blood cells

counted in 4 squares of the haemocytometer and 500 is the factor obtained after taking the initial dilution factor into consideration.

Serum chemistry

Serum was collected into sterilized eppendrof tubes and analysed for the different serum parameters in AR. 601, Semi Automatic analyzer (Qualigens Diagnostic kit) using Qualigens kits. The parameters that were analysed using this instrument were total protein (biruet method using buffered dye reagent and biuret reagent, Qualigens Diagnostic kits) and albumin (bromocresol green binding method, Qualigens Diagnostic kits). The globulin content was calculated by subtracting albumin content from total protein content.

Determination of cellular immune parameters

Respiratory burst activity

The Nitroblue tetrazolium or respiratory burst assay was carried out following the method of Secombes (1990) and modified by Stasiack and Bauman (1996). 50 μL of blood was pipetted into the wells of 'U' bottom microtitre plates and incubated at37°C for 1 h to facilitate adhesion of cells. Then, the supernatant was gently removed and the adhered cells were washed three times with PBS. After washing, 50 μL of 0.2% (w/v) Nitro blue tetrazolium in PBS was added to the wells and incubated for 1 h at room temperature. The supernatant was removed and the cells were fixed with 100% (v/v) methanol for 3 min and then washed thrice with 30% (v/v) methanol. The plates were air dried and 60 μL 2N potassium hydroxide and 70 μL dimethyl sulphoxide were added to each well to dissolve the formazon blue crystal. The optical density of the turquoise blue coloured solution was read in microplate reader at 540 nm against a potassium hydroxide/dimethyl sulfoxide (KOH/DMSO) blank.

Phagocytic ratio

The phagocytic assay was performed following Siwicki *et al.* (1994) and Park and Jeong (1996) with slight modification. *V anguillarum* (10^7) cells in 0.1 ml of PBS was added to 0.1 ml of pooled blood samples in sterile microplate. After thorough mixing in the well, the plates were incubated for 30 min at 25°C. After incubation, the plate was removed and blood bacteria suspension was mixed gently again. 50 μL of this suspension was put on three glass slides and smears were made. After air drying, the smear was fixed in 95% ethanol, air dried and stained with May-Grunwald Giemsa stain. The Phagocytic cells and phagocytized bacteria were enumerated. Phagocytic ratio (PR) was determined by enumerating 100 phagocytes per slide under the microscope. The average of three slides was calculated. Phagocytic ratio (i.e. percentage of cells with engulfed bacteria)PR = Number of of Phagocytic cells with engulfed bacteria/Number of Phagocytes

Determination of humoral immune parameter

Serum lysozyme activity

A turbidimetric assay utilizing lyophilized *Micrococcus luteus* ATCC 49732 (DIFCO, BBL-Qualis lab) was used to determine lysozyme activity in serum following the method of Sankaran and Gurnani (1972) using hen egg white lysozyme (HEWL) (Sigma, USA) as standard with partial modification. Thus, 150 μL of *M. luteus* at a concentration of 0.2 mg mL^{-1} (w/v) in 0.02 M acetate buffer was mixed with 15 μL of serum sample and then it was pipetted into 96-well U bottom microtitre plates (Tarson, India) and initial optical density was read at 450 nm in microplate reader. The final optical density was read after 1 h of the incubation at 25°C. A standard curve was prepared using HEWL. Serum lysozyme values were expressed as μg mL^{-1} equivalent to HEWL activity.

Serum bactericidal activity

The bacterial killing assay was performed according to Rainger and Rowley (1993). *Vibrio anguillarum* bacterial culture was centrifuged and the pellet was washed and re-suspended in PBS. Then, the bacterial number was calculated by measuring optical density (O.D) in spectrophotometer and also confirmed using plate count method. This bacterial suspension was serially diluted (1:10) with PBS upto five times. Bacterial killing assay was determined by incubating 2 μL of this diluted *V. anguillarum* suspension with 20 μL of serum in a micro-vial for 1 h at 37°C. In the control group, serum was replaced with PBS. After incubation, the number of viable bacteria was determined by counting the colonies grown on TCBS (Thiosulphate citrate bile sucrose) agar plate for 24 h at 37°C. The bactericidal activity of test serum was expressed as percentage of colony forming units in the test group to that in the control.

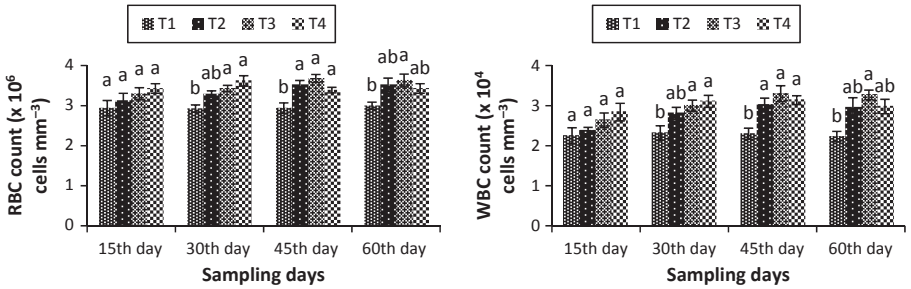

Figure 1 Effect of dietary supplemented graded levels of the chitosan on haematological parameters of *L. Calcarifer* (Mean ± SEM). Means in the column bearing different superscripts letter on the same sampling day vary significantly ($P \leq 0.05$).

Challenge test

The *V. anguillarum* was isolated from a diseased Asian seabass and was grown on Luria Bertani broth (Himedia, Mumbai) at 37°C for 24 h in a BOD incubator. The culture was centrifuged at 10 000 *g* for 10 min. The supernatant was discarded and the pellet was re-suspended in the sterile phosphate buffer saline (PBS, pH 7.4). Then, the bacterial number was calculated by measuring optical density (O.D) in spectrophotometer and also confirmed using plate count method. The final bacterial suspension was adjusted to 1×10^6 cfu mL^{-1} by serial diluation. Fish were challenged intraperitoneally with the bacterial suspension of 0.1 ml (1×10^6 cfu mL^{-1}) after 60 days of feeding trial. The cause of death was ascertained by re-isolating *V. anguillarum* from kidney of the moribund fish. The percentage survival in each of the experimental group was recorded up to the 30th day after challenge. The percentage survival was calculated using the following equation: Survival (%) = (Number of fish survived after challenge/Initial number of fish) × 100

Statistical Analysis

All the data were analysed using one-way ANOVA using SPSS V. 16 Software (SPSS, Chicago, Illinois, USA). The means of the studied heamatological and innate immune parameters were compared using Duncan's multiple range tests to find the difference at 5% ($P \leq 0.05$) level.

Results

Total erythrocyte counts (TEC) and total leucocyte counts (TLC) were significantly increased in chitosan-fed group of fish compared with control from 30th day onwards till 45th day after which it started declining (Fig 1). A significantly higher ($P \leq 0.05$) TEC ($3.68 \pm 0.10 \times 10^6$ cells/mm^3) and TLC ($3.30 \pm 0.20 \times 10^4$ cells/mm^3) were recorded in T_3 on 45th day in comparison to other treatments. Total serum protein (Fig 2a), albumin (Fig 2b) and globulin (Fig 2c) contents significantly increased in fish fed chitosan incorporated diets till 45th day; afterward, it started decreasing. Albumin-globulin ratio was significantly higher ($P \leq 0.05$) in T_4 (0.77 ± 0.05) on 15th day in comparison with other treatment groups (Fig 2d).

The cellular immune parameters i.e. respiratory burst activity (Fig 3a) and phagocytic ratio (Fig 3b) were significantly increased in fish fed chitosan incorporated diets compared with control group of fish. However, this increasing trend was observed up to 45th day and then onwards, it decreased. A significantly higher ($P \leq 0.05$) respiratory burst activity (0.56 ± 0.03) and phagocytic ratio (57.11 ± 2.53) were recorded in T_3 on 45th day in comparison with other treatments and control groups. The humoral immune parmeters, serum lysozyme (Fig 4a) and serum bactericidal (Fig 4b) activities increased after the feeding of chitosan-supplemented diet. The maximum activities of these parameters were observed on 45th day in fish fed 10 g chitosan kg^{-1} feed. The chitosan-fed group (T_2, T_3 and T_4) showed higher survival (57.78 ± 5.88%; 75.56 ± 4.44% and 68.89 ± 5.88% respectively) in comparison with control (46.67 ± 3.85%) group (Fig 5). Among treatment groups, medium-dose fed group (T_3) recorded significantly ($P \leq 0.05$) highest survival compare with other treatment.

Discussion

Nowadays, the use of immunostimulants is up-coming in aquaculture practices. Several studies

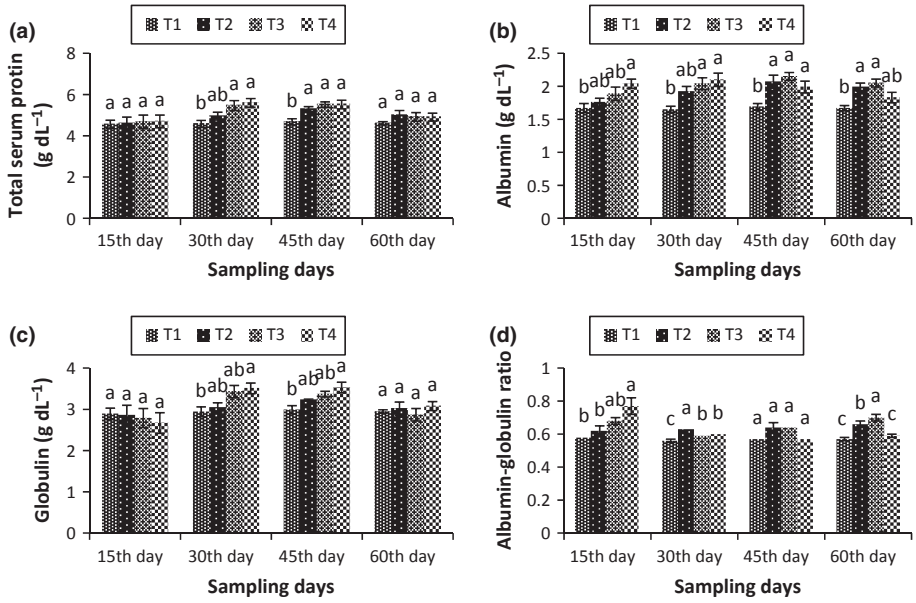

Figure 2 Biochemical profile (a: total serum protein; b: albumin content; c: globulin content; d: albumin-globulin ratio) of *L. Calcarifer* fed with different doses of chitosan-supplemented diet (Mean ± SEM). Means in the column bearing different superscripts letter on the same sampling day vary significantly ($P \leq 0.05$).

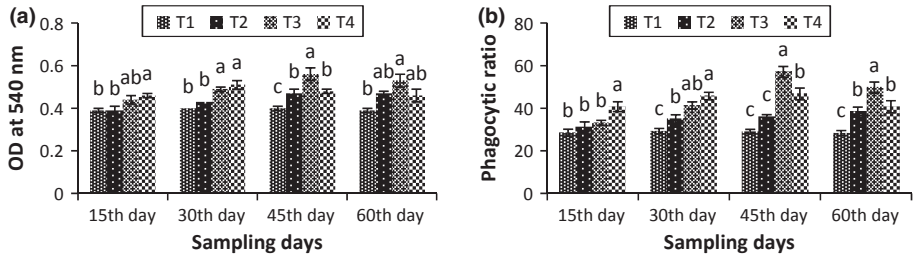

Figure 3 Effect of dietary supplemented graded levels of chitosan on cellular immune parameters (a: respiratory burst activity; b: phagocytic ratio) of Asian seabass (Mean ± SEM). Means in the column bearing different superscripts letter on the same sampling day vary significantly ($P \leq 0.05$).

have reported about the beneficial effects of using immunostimulants in terms of improving the health status of fish and their protection from disease causing organisms (Raa 1996; Sakai 1999). In this study, an attempt has been made to evaluate the effects of dietary supplementation of chitosan on the haematlogical and innate immune parameters of Asian seabass (*Lates calcarifer*) and protection against *V. anguillarum*.

The total erythrocyte counts (TEC) were significantly increased in chitosan-fed groups compared with control group. A similar finding was reported by Duncan and klesius (1996) that the percentage of erythrocytes was significantly higher in fish fed diets containing *S. cervisiae.* The other authors had also reported increase in total erythrocyte counts in *Labeo rohita* fed with different immunostimulants (Misra, Das, Mukherjee & Pattnaik 2006; Misra, Das & Mukherjee 2009). Red blood cells significantly increased in *Epinephelus bruneus* fed with 2% of chitin and chitosan diets from weeks 1 to 4 against *V. alginolyticus* (Harikrishnan, Kim, Balasundaram & Heo 2012). However, in this study, increase in TEC in the highest dose fed group (T_4) was recorded up to 30th day, whereas in medium (T_3) and low dose (T_2), it was up to 45th day. The increased level of TEC in chitosan-fed group might be due to increased erythropoiesis.

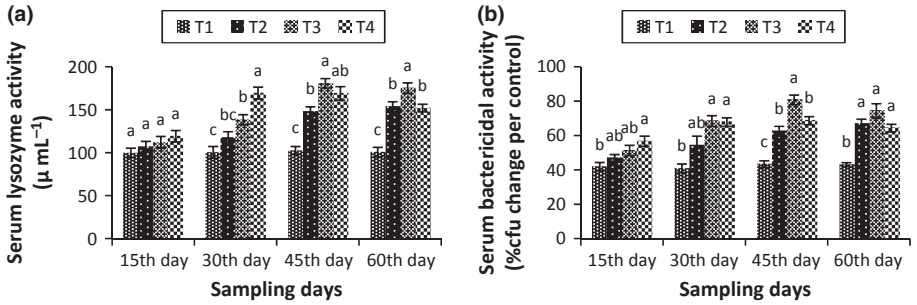

Figure 4 Humoral immune response (a: serum lysozyme activity; b: serum bactericidal activity) of *L. Calcarifer* fed with different doses of chitosan-supplemented diet (Mean ± SEM). Means in the column bearing different superscripts letter on the same sampling day vary significantly ($P \leq 0.05$).

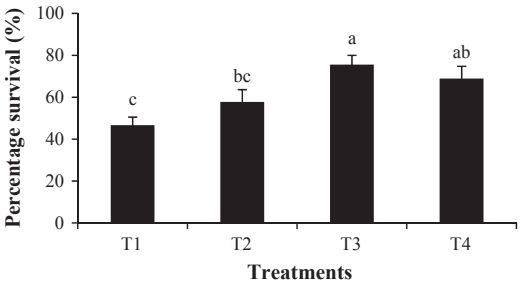

Figure 5 Percentage survival (%) of *L. Calcarifer* fed with different doses of chitosan-supplemented diet. Means in the column bearing different superscripts letter on the same sampling day vary significantly ($P \leq 0.05$).

However, decrease in TEC after 30th day in T_4 might be due to stress caused by the higher content of chitosan in the diet for longer period.

Total leucocyte count (TLC) in fish increased with the administration of chitosan-supplemented diet. Similar to the present investigation, elevated level of TLC was reported in common carp (Gopalakannan & Arul 2006), *E. bruneus* (Harikrishnan *et al.* 2012) and rainbow trout (Meshkini, Tafy, Tukmechi & Pajuh 2012) after feeding chitosan-supplemented diet. However, the increase in TLC was observed up to 45th day in medium and low dose and 30th day in higher dose after which it was started decreasing. The significantly highest TLC was observed in medium-dose fed group (T_3) of fish on 45th day. Meshkini *et al.* (2012) observed significantly increased leucocyte count up to 6 week in fish fed with 0.5% chitosan-supplemented diet, whereas 0.25% supplementation showed significantly enhanced TLC up to 11 weeks. Similarly, many authors have also reported decrease in leucocyte count with high dose of chitosan (Kledmanee 2010; Meshkini *et al.* 2012) and levamisole (Siwicki 1989; Kajita *et al.* 1990; Misra *et al.* 2009) and β-glucan (Misra *et al.* 2006). The higher dose of immunostimulant might negatively influence the release of factors that support the synthesis of leucocytes from haemopoetic organs (Misra 2004) into the blood. Thus, the administration of 20 g chitosan kg^{-1} diet beyond 30 days and 10 g chitosan kg^{-1} diet beyond 45 days may not be suitable as leucocytes, main scavenger cells, get suppressed.

Among the serum proteins, albumin and globulin are the major proteins, which play a significant role in the immune response. Globulins like gamma-globulin are absolutely essential for maintaining a healthy immune system and contain all the immunoglobulins in the blood. Increase in serum protein, albumin and globulin levels are thought to be associated with a stronger innate response in fish (Wiegertjes, Stet, Parmentier & Van Muiswinkel 1996). In this study, total serum protein, albumin and globulin contents increased in all treatment groups, which indicate the enhancement of innate immunity of the fish. Similar results have been reported in olive flounder (Cha *et al.* 2008), common carp (Dautremepuits, Paris-Palacios, Betoulle & Vernet 2004) and *E. bruneus* (Harikrishnan *et al.* 2012) after feeding chitosan-supplemented diet. The increase in total serum protein could be attributed to serum lysozyme activity, serum bactericidal activity, globulin content and probably some other peptides (Misra *et al.* 2006). However, Bagni and Archetti (2000) found no significant difference in protein content or in albumin/globulin ratio in seabass (*Dicentrarchus labrax*) fed with feed containing β-glucan, alpha-tocopherol and ascorbic acid. This might be due to different immunostimulants and the change

in their feeding pattern (2-week period in every 3 months for 40 weeks). In this study, the increase in the A/G ratio in chitosan-fed groups compared with control is also in agreement with Misra *et al.*(2006) and Mondal, Chakraborty, Pramanik, Rout and Islam (2004).

In this study, enhanced respiratory burst activity was observed in chitosan-fed groups in comparison with the control group of fish. Similar to this, Siwicki *et al.* (1994) had reported increase in respiratory burst activity in rainbow trout treated with chitosan. Enhanced respiratory burst activity was observed till 45th day in low- and medium-dose chitosan-fed groups and 30th day in high-dose fed group. However, respiratory burst activity was maintained significantly higher in fish fed 10 g chitosan kg^{-1} diet even after 45 days. Similar to this, Gopalakannan and Arul (2006) reported increase in respiratory burst activity in *Cyprinus carpio* fed with 10 g chitosan kg^{-1} feed up to 60 days and afterwards, it started decreasing. Kledmanee (2010) also reported enhanced respiratory burst activity up to 47 days in Asian seabass fed 1% chitosan in diet. However, Cha *et al.* (2008) reported significantly stimulated neutrophil respiratory burst activity even after 12 weeks of feeding chitosan-coated diet to *P. olivaceus.* The increased respiratory activities in chitosan-fed group might be due to the immunostimulatory effect of chitosan.

Phagocytic activity, measured by phagocytic ratio (PR), was enhanced by feeding different doses of chitosan. Similar to this study, increased phagocytic activity after chitosan treatment was reported by different authors in different fish species (Sakai, Kamiya, Ishii, Atsuta & Kobayashi 1992; Siwicki *et al.*1994; Esteban, Mulero, Cuesta, Ortuno & Meseguer 2000; Kledmanee 2010). Enhanced PR was observed till 45th day in medium- and high-dose chitosan-fed groups. However, PR was significantly high in medium-dose fed group upto 60th day. Similarly, Kledmanee (2010) reported significantly stimulated phagocytosis percentage (PP) and phagocytic index (PI) in *L. calcarifer* when fed 1% chitosan by pellet weight for 47 days. The result suggests that to allow the phagocytic cells to engulf more number of pathogens, it is better to restrict the administration of chitosan after 45 days in medium-dose fed and 30 days in high-dose fed fish.

Lysozyme is an important hydrolytic enzyme with a protein character in the non-specific defence system. Lysozyme is detected in the phagocytic cells, mucus and sera of several fish species (Studnicka, Siwicki & Ryka 1986). An increased level of serum lysozyme activity has been considered to be a natural protective mechanism in fish (Ingram 1980; Robertsen, Ehgstad & Jørgensen 1994). This study showed elevated level of serum lysozyme activity in fish fed different doses of chitosan. Similarly, Cha *et al.* (2008) reported significantly stimulated mucus lysozyme activity in *P. olivaceus* after feeding chitosan-coated diet. However, significant increase in serum lysozyme activity was recorded from 30th day onwards with a peak on 45th day. Thereafter, a decreasing trend was observed in medium- and high-dose fed groups, but remained significantly higher in comparison with control throughout the experiment. Similar to this study, enhanced serum lysozyme activity was found with a peak on 30th day in *C. carpio* fed with 10 g chitosan kg^{-1} feed (Gopalakannan & Arul 2006).

In this experiment, serum bactericidal activity was enhanced in different groups of fish fed with different doses of chitosan. This showed that chitosan-fed fish serum has enhanced bactericidal properties *in vitro*. The higher bactericidal activities can possibly be due to the higher concentration of lysosomal enzymes. Similar views were put forth by Nikoskelainen, Ouwehand, Bylund, Salminen and Lilius (2003) who observed significant increase in complement bactericidal activity of rainbow trout fed with *Lactobacillus rhamnosus*. Many authors have reported increase in serum bactericidal activity after treatment with different immunostimulants (Chen & Ainsworth 1992; Jørgensen, Lunde & Robertsen 1993; Misra *et al.* 2006, 2009).

Immunostimulants have shown their protective effect against many bacteria such as *Vibrio anguillarum*, *V. salmonicida*, *Aeromonas salmonicida*, *Yersinia rukeri* and *Streptococcus* spp. and to parasitic infections such as white spot disease (Sakai 1999). Similarly, this study showed increased percentage survival of fish fed with three different doses of chitosan, when challenged with *V. anguillarum*. The increase in resistance against *V. anguillarum* in chitosan-fed group can be explained on the basis of increase in non-specific immune system of fish. Similarly, Gopalakannan and Arul (2006) and Siwicki *et al.* (1994) had also reported increased protection against pathogenic bacteria in chitosan-fed fish. However, the degree of protection depends

upon the dose of chitosan as seen in this study. The group fed with diet containing 10 g of chitosan kg^{-1} feed showed significantly higher survival among the chitosan-fed groups.

These findings collectively suggest that chitosan can be effectively used as immunomodulator in Asian seabass. However, further studies are needed to be carried out to ascertain the probable protection by dietary chitosan against the wide range of pathogens in the same fish.

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