

## **Effect of dietary chitosan on non-specific immune response and growth of *Cyprinus carpio* challenged with *Aeromonas hydrophila***

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### **Abstract**

This study was conducted to investigate the effect of dietary chitosan on the non-specific immunity, growth and survival of common carp. Common carp with an average weight of  $45 \pm 2$  g and total length of  $31 \pm 2$  cm were fed diets containing 0 (control), 1%, 2% and 5% chitosan for a period of 70 days. Sixty fish in all treatments were challenged with *Aeromonas hydrophila* on day 30 and 58, in order to monitor the response of the chitosan fed fish against the bacterial challenge. Phagocytic index, phagocytic ratio and serum bactericidal activity were increased in the chitosan fed (2 and 5%) groups, compared to the control group ( $P < 0.05$ ). After the fish in all treatments were challenged intra-peritoneally with *Aeromonas hydrophila*, the relative percentage survival (RPS) (82.78 %) was higher in chitosan (2%) group ( $P < 0.05$ ), when compared to other treatments. Feed conversion ratio (1.81) and specific growth rate (SGR) (2.67) were higher in chitosan fed (2%) group, when compared to other treatments. The control group displayed the decreased performance in all the assays of non-specific immune response with the coincidental decrease in the survival and growth rates. Thus, the incorporation of chitosan at a level of 2% in the diet of fish enhanced the non-specific immunity, reduced the fish mortality and enhanced the growth of fish under stress conditions.

**Keywords:** Chitosan, Bacterial infection, Non-specific immune system, Phagocytic activity, Immunostimulant

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### **Introduction**

Non-specific defense mechanism plays an important role at all stages of fish infection. Fish, particularly, depend more heavily on these non-specific mechanisms than do mammals (Avtalion 1981). Hence, in the last decade there has been increasing interest in the modulation of the non-specific immune system of fish, as both a treatment and prophylactic measure against disease. A number of substances including different peptides have been used to increase the resistance of fish by enhancing the non-specific defense mechanisms.

Aquaculture has been expanding with the fast development. However, unmanaged fish culture practices and adverse environmental conditions affect the fish health leading to production losses. Thus, fish farmers have to carry out careful husbandry practices (Sakai 1999). Use of expensive chemotherapeutants and antibiotics for controlling

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disease has widely been criticized for their negative impact like residual accumulation in the tissue, development of the drug resistance and immunosuppression, thus resulting in reduced consumer preference for food fish treated with antibiotics (Anderson 1992). Hence, instead of chemotherapeutic agents, increasing attention is being paid to the use of immunostimulants for disease control measures in aquaculture. Drug resistance and poor growth in farmed fish is a major constraint in aquaculture industry. Growth and immune stimulants holds the promise to improve fish growth and subsequently controls fish diseases in aquaculture.

The use of immunostimulants for the prevention of fish diseases is considered as an attractive and promising area (Anderson 1992; Secombes 1994). Immunostimulants are valuable for the prevention and control of fish diseases in aquaculture as these represent an alternative and supplementary treatment to vaccination. The immunostimulants also have additional advantages, such as growth enhancement and increase in the survival rates of the fishes under stress (Heo et al. 2004). Chitosan is a linear homopolymer of  $\beta$ -(1, 4)-2-amino-deoxy-D-glucose and is prepared by the alkaline deacetylation of chitin, a natural substance obtained from crab shell or any crustacean shell. Anderson and Siwicki (1994) administered chitosan to brook trout *Salvelinus fontinalis* by injection and immersion and found that high levels of protection occurred 1, 2, 3 days afterwards, but protection was greatly reduced by day 14. Chitosan is used as an immunostimulant in aquaculture to protect salmonids and carps against bacterial diseases (Anderson and Siwicki 1994; Siwicki et al. 1994).

Rainbow trout fed with chitosan-oligosaccharides (COS) supplemented diet demonstrated the improved phagocytic activities, respiratory burst activities and the decreased serum cortisol level. Additionally, survival following *Aeromonas hydrophila* challenge was significantly higher among fish fed the COS-supplemented feeds (Luo et al. 2009). Total serum protein, albumin, globulin and packed cell volume showed increasing trend by the injection of chitosan (Sahoo and Mukherjee 1999). *Aeromonas hydrophila* has been reported to cause fin rot disease in hatchery reared *Cyprinus carpio* in Kashmir valley (Hussain et al. 2005). The use of multiple antibiotics as a bath treatment was recommended for controlling fin rot disease. However, as mentioned above, controlling diseases with antibiotics is not a safe procedure in aquaculture practices. Thus, the objective of this study was to study the effect of chitosan on non-specific immunity, growth and survival of common carp against the challenge of *Aeromonas hydrophila*.

## Materials and methods

### *Fish and rearing conditions*

Healthy and disease free advanced fingerlings of common carp *Cyprinus carpio* having an average weight of  $45 \pm 2$  g and total length of  $31 \pm 2$  cm were procured from Fish Farm, Faculty of Fisheries (SKUAST-K). The experiment was conducted at the fish farm, Faculty of Fisheries, SKUAST-K, India. The experimental stock was acclimatized for a period of 2 weeks in concrete ponds containing same source of water which was used for conducting the experimental trial and the stock was fed on control diet (D<sub>1</sub>). Twelve experimental concrete ponds with proper inlets and outlets and measuring  $6.0 \times 6.0 \times 1.5$  m were used for conducting the experiment. These were thoroughly treated with quick lime and disinfected with KMnO<sub>4</sub>. The ponds were then filled with the spring water and a uniform water column of 1.2 m was maintained through out the experiment. Pond water replenishment was carried out every week by replacing 40-50% of the pond water. After acclimatization, the fish were divided into four treatments of 60 specimens each and each treatment was further divided into three replications containing 20 specimens each.

Water quality parameters including temperature, dissolved oxygen, pH and free CO<sub>2</sub> were recorded on weekly basis. Water and air temperature were recorded by a standard quality thermometer. Dissolved oxygen and pH were recorded with digital DO and pH meter, respectively. Free CO<sub>2</sub> was determined titrimetrically following the standard procedures (APHA 1998). The dissolved oxygen (DO) content of water throughout the experimental period ranged between 7 and 10 mg/l; pH ranged between 7 and 8.5; temperature of the water in the ponds ranged between 31 °C during August and 22 °C during the end of October. The free CO<sub>2</sub> content of the pond water ranged between 0 and 5 mg/l. The source of water used for conducting the experimental trial was natural spring water.

### *Experimental feed*

Feed ingredients viz, groundnut oil cake (GOC), rice bran, soybean meal, fish meal and wheat flour were procured, screened and subjected to proximate analysis following standard procedure (AOAC 2006). The diet was composed of 29.33% crude protein, 2.10% crude lipid, 18.9% ash, and 9.0% moisture. All the ingredients were properly

weighed as per their inclusion rates in the four experimental diets (Table 1) and were ground separately in an electric grinder and thoroughly mixed and water was added in sufficient quantity. The whole mixture was steam-cooked for 20-25 minutes. Thereafter, chitosan along with vitamin and mineral mixture at the required quantities were incorporated and mixed thoroughly and evenly to the prepared dough. The resultant dough of desired consistency was passed through a hand pelletizer with a die of 3 mm and the pellets were air-dried under shade. The dried pelleted diets were packed in air tight polythene bags and stored at -20 °C. Diet D<sub>1</sub> served as the control diet as it was not supplemented with chitosan, whereas diets D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> comprised the same ingredients as that of D<sub>1</sub> but these were supplemented with chitosan (Sigma, USA) at a level of 1%, 2% and 5% of diet, respectively.

Experimental stock in all treatments was fed twice daily for a period of 70 days. The feeding rate was 5% of their body weight. The feed was offered in the feeding trays, which were immersed in the ponds at a depth of 0.7 m and inspected to monitor the consumption of feed which was always found to be consumed in full within an hour.

Table 1. Ingredient composition of experimental diets\*

Ingredients	Inclusion rate (%)			
	D <sub>1</sub> (control)	D <sub>2</sub>	D <sub>3</sub>	D <sub>4</sub>
GOC	32.00	32.00	32.00	32.00
Rice bran	26.10	26.10	26.10	26.10
Wheat flour	20.05	20.05	20.05	20.05
Soybean meal	15.90	15.90	15.90	15.90
Fish meal	03.95	03.95	03.95	03.95
Vitamin & mineral mixture	02.00	02.00	02.00	02.00
Chitosan	-	1%	-	-
Chitosan	-	-	2%	-
Chitosan	-	-	-	5%

\*Crude protein: 29.33%, crude lipid: 2.10%, ash: 18.9%, and moisture: 9.0%.

### Bacterial strain and challenge study

A virulent strain of *Aeromonas hydrophila* received in Tryptose Soya Agar Slants (TSA) from IMTECH (Institute of Microbial Technology), Chandigarh was maintained at 4 °C in the Division of Veterinary Microbiology and Immunology, SKUAST-K, Shuhama. From this slant culture, sub-cultures were maintained on Tryptose Soya Agar (TSA) slants (Hi-media, Mumbai) at 5 °C. A stock culture in Tryptose Soya Broth (TSB) (Hi-media, Mumbai) was maintained at -40 °C with 0.85% NaCl (w/v) and 20% (v/v) glycerol to provide stable inocula throughout the study period as followed by Chabot and Thunne 1991 and Yadav et al. 1992.

The fingerlings in all groups were challenged with 100 µl of *Aeromonas hydrophila* at a concentration of  $1.5 \pm 0.3 \times 10^6$  CFU/ml in phosphate buffer saline (PBS) as a medium. The bacterial suspension in PBS was inoculated intra-peritoneally in all specimens of all groups by 1 ml insulin syringe on day 30 and the specimens were re-challenged on day 58. Due care was taken to avoid any injury while challenging the experimental stock with *A. hydrophila*. All the challenged specimens were released back into their respective ponds and were observed for their response against the injected bacterial strain.

### Experimental regime

The experimental stock was released of 20 specimens in each replication of each treatment in experimental ponds P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub> and P<sub>4</sub> and fed on diets D<sub>1</sub>, D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub>, respectively.

### **Sampling schedule**

0 day: Blood sample collection.

1st to 70th day: Feeding with chitosan supplemented diet (Only for treatment groups).

30th day: 1st infection with *A. hydrophila* (In all the groups).

57th day: Blood sample collection.

58th day: 2nd infection with *A. hydrophila* (In all the groups).

70th day: Blood sample collection.

Length and weight of 9 randomly selected fish, 3 from each replication of each treatment were recorded at fortnightly intervals and ration was adjusted accordingly on the basis of fish biomass. The fortnightly recorded data was used for calculating the feed conversion ratio (FCR), specific growth rate (SGR) and net fish production.

Six specimens, two from each replication, were sampled at random and blood samples were collected on day 57 and 70 from the caudal vein. After blood collection, the fish were released back in their respective treatments ponds. Mortality was recorded throughout the period of study and Relative Percentage Survival (RPS) was calculated as per the method of Baulny et al. 1996.

### **Blood and serum collection**

The blood sampling was carried out for the analysis of the phagocytosis assay and serum bactericidal activity in the blood. Two fish from each replicate with a total of six fish were sampled randomly from each treatment and were anaesthetized with MS<sub>222</sub> (100 ppm) (Sigma). Blood was collected from the caudal vein using a syringe, which was previously rinsed with 2.7% EDTA solution. The blood was then transferred immediately to an Eppendroff tube containing a pinch of EDTA powder, shaken gently and kept at 4 °C. The blood was used for determination of hemoglobin content, hematocrit value, total erythrocyte and leucocyte count.

For serum, another six fish from each treatment were anaesthetized and blood was collected in 5 ml test tube without anticoagulant and allowed to clot for 2 h at room temperature in a slanting position. The tubes were centrifuged at 2360 × g for 15 min by using Optic technology centrifuge (New Delhi, India) and the supernatant serum was separated and collected in screw cap Eppendroff tubes and stored at -40 °C for further analysis.

### **Phagocytosis assay**

The phagocytosis assay was performed according to the method of Siwicki et al. (1994) and Park and Jeong (1996) with a slight modification. 10<sup>7</sup> cells of freshly grown *A. hydrophila* in 0.1 ml of PBS were added to 0.1 ml of blood sample of each fish in sterile microplate. This was then incubated for 30 min at 25 °C after thorough mixing in the well.

After incubation, the plate was removed and blood bacteria suspension was mixed gently again. Fifty microlitres of this suspension were put on three glass slides and smears were made. After air drying, the smear was fixed in 95% ethanol, re-dried and stained with May–Grunwald Giemsa. The phagocytic cells and phagocytosed bacteria were enumerated. Phagocytic ratio (PR) and phagocytic index (PI) were determined by enumerating 100 phagocytes per slide under a microscope. The average of three slides was calculated.

Phagocytic ratio (PR; i.e. percentage of cell with engulfed bacteria) = No. of phagocytic cells with engulfed bacteria/No. of phagocytic cells.

Phagocytic index (PI; i.e. number of engulfed bacteria per cell) = No. of engulfed bacteria/No. of phagocytic cells.

### **Serum bactericidal activity**

Parts of the sera collected were utilized for studying serum bactericidal activity following Kajita et al. (1990). Sera samples from each subgroup were pooled to three numbers. Pooled sera samples were diluted three times with 0.1% gelatin-veronal buffer (GVB<sup>+</sup>) (pH 7.5, containing 0.5 mM/ml Mg<sup>2+</sup> and 0.15 mM/ml Ca<sup>2+</sup>). The bacteria *A. hydrophila* (live, washed cells used earlier) were suspended in the same buffer to make a concentration of 1 × 10<sup>5</sup> CFU/ml. The diluted sera and bacteria were mixed at 1:1, incubated for 90 min at 25 °C and shaken.

One control group containing bacterial suspension in same buffer was also incubated for 90 min at 25 °C. The numbers of viable bacteria was then calculated by counting the colonies from the resultant incubated mixture on TSA plates in duplicate (two plates per sample) after 24 h incubation. The bactericidal activity of test serum was expressed as percentage of colony forming units in test group to that in the control group.

**Growth parameters**

The recorded data on weight was used for calculation of feed conversion ratio (FCR) and specific growth rate (SGR). On each sampling day, the SGR or percent body weight increase per day and FCR for all the experimental groups were calculated according to Ricker (1979) as follows:

$$\text{SGR} = [\text{Ln (Final weight)} - \text{Ln (Initial weight)}] / t \text{ (time interval in days)} \times 100.$$

$$\text{FCR} = \text{Feed given (dry weight)} / \text{Weight Gain (wet weight)}.$$

**Relative Percentage Survival**

Recorded mortality data was used for calculating Relative Percentage Survival (RPS) following Amend (1981).  
 $\text{RPS} = 1 - [(\text{Mortality (\%)} \text{ in treated group}) / (\text{Mortality (\%)} \text{ in control group})] \times 100$

**Statistical analysis of the experimental data**

The experimental data was subjected to the statistical analysis following the (Completely Randomized Design) CRD. The statistical difference between the treatment means and within the treatment means was assessed by two way analysis of variance (ANOVA) techniques followed by Duncan's multiple range test using statistical package (SPSS 14.0) to find out the significant difference at 5% level ( $P < 0.05$ ) of significance.

**Results and discussion****Phagocytic assay**

Phagocytic index and phagocytic ratio of immunostimulated and the control common carp challenged with *Aeromonas hydrophila* are shown in Fig. 1 (A and B) respectively. Phagocytic index and phagocytic ratio of common carp fed on chitosan (2%) were higher than those of the control and chitosan (1%) group ( $P < 0.05$ ). However, no significant difference was found between the phagocytic index of chitosan fed (2%) and chitosan (5%) throughout the challenge study. Both phagocytic index and phagocytic ratio were increased in chitosan supplemented group, particularly in 2% chitosan group, indicating that the chitosan at 2% level was more effective in combating the invasion of pathogenic bacteria and increased phagocytosis.

The enhancement of phagocytosis might be due to binding of chitosan by its receptors on the phagocytic cells. Phagocytosis and elimination of pathogens by macrophages are essential mechanisms for the elimination of invading pathogens and reflect the immune status of the fish (Sweet and Zelikoff 2001). Chitosan can increase non-specific immunity by either enhancing the number of phagocytes or activating phagocytes or by increasing the synthesis of molecules involved in the innate immunity such as the complement lysozyme antiprotease (Cha et al. 2008). Similar finding was reported by Wang and Chen (2005), who found that phagocytic activity was significantly higher for the shrimp that received chitosan at 2 and 4  $\mu\text{g/g}$  than that of shrimp that received saline, and the control shrimp after 1 day. Phagocytic activity of shrimp that received chitosan at 4  $\mu\text{g/g}$  was significantly higher than that of the control shrimp (Wang and Chen 2005).

Dietary administration of chitosan in rainbow trout *Oncorhynchus mykiss*, was reported to increase its phagocytic index of *Staphylococcus aureus* (Siwicki et al. 1994). Enhanced or elevated phagocytic activity was reported in carp after i.p. injection of different glucans such as schizophyllan, scleroglucan and lentinan 3 and 6 days prior to challenge (i.p.) by *Edwardsiella tarda* (Yano et al. 1989). Non-specific defense mechanisms are rapidly activated by immuno-stimulants like chitosan and these systems are quickly mobilized to protect the fish against pathogens. This is why, in this study, we observed an enhancement of innate immune responses. Therefore, chitosan showed the positive effects on phagocytic activity in fish against *A. hydrophila* infection, proving its immunostimulatory effect.

**Serum bactericidal activity**

Serum bactericidal activity of common carp fed chitosan supplemented and control diet is shown in Fig. 2. Serum bactericidal activity of chitosan fed common carp increased with increasing time of exposure to the chitosan supplemented diet and was always higher than the control group ( $P < 0.05$ ). The serum bactericidal activity of fish supplemented with 2% chitosan was 55% and in fish fed on the control diet was 38%. Serum bactericidal activity of 2% and 5% chitosan fed groups did not differ significantly throughout the experimental period, indicating that

chitosan at the level of 2% and 5% was equally effective against the pathogenic strain of *A. hydrophila*. This could be attributed to and correlated well with the reduced mortality percentage and high phagocytic activity.

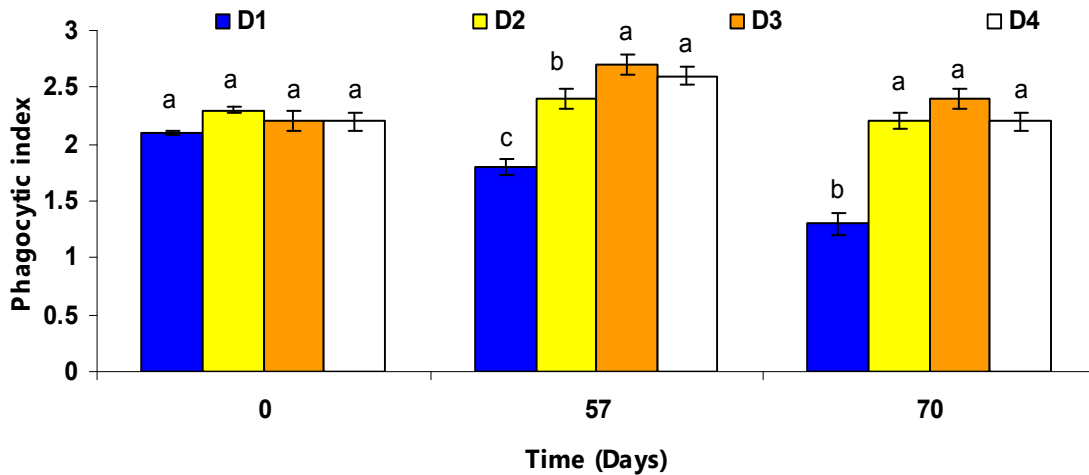


Figure 1 (A). Change in phagocytic index observed in challenged *Cyprinus carpio* fed chitosan supplemented and control diet. Bars represents the standard deviation (n=3). Same small letters on the bar indicate no significant difference between the different treatment and the control group on the particular sampling day ( $P < 0.05$ ). D1: Control group, D2: Chitosan (1%) fed group, D3: Chitosan (2%) fed group, D4: Chitosan (5%) fed group.

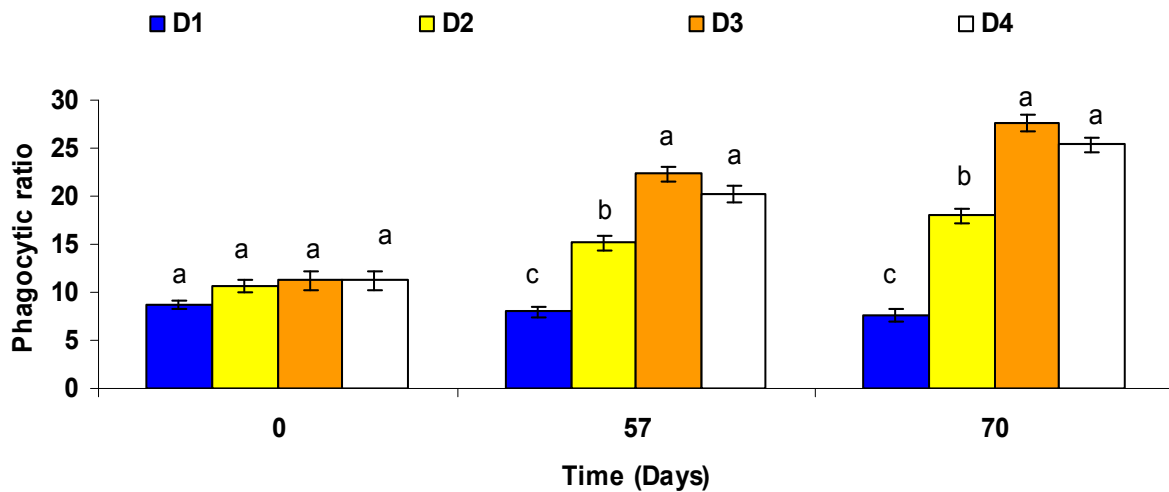


Figure 1 (B). Change in phagocytic ratio observed in challenged *Cyprinus carpio* fed chitosan supplemented and control diet. Bars represents the standard deviation (n=3). Same small letters on the bar indicate no significant difference between the different treatment and the control group on the particular sampling day ( $P < 0.05$ ).

Chitosan plays an important role in the stimulation of bactericidal activity of phagocytic cells that stems mainly from its stimulation of the generation of reactive oxygen species like superoxide anion by the affected cells (Misra et al. 2006). The increased serum bactericidal activity in chitosan treated groups indicates that various humoral factors are involved in innate and/or adaptive immunities which are elevated in the serum to protect the fish effectively from infection (Das et al. 2009). Thus, chitosan proved to be as an effective immunostimulant in preventing the establishment of bacterial infection in common carp.

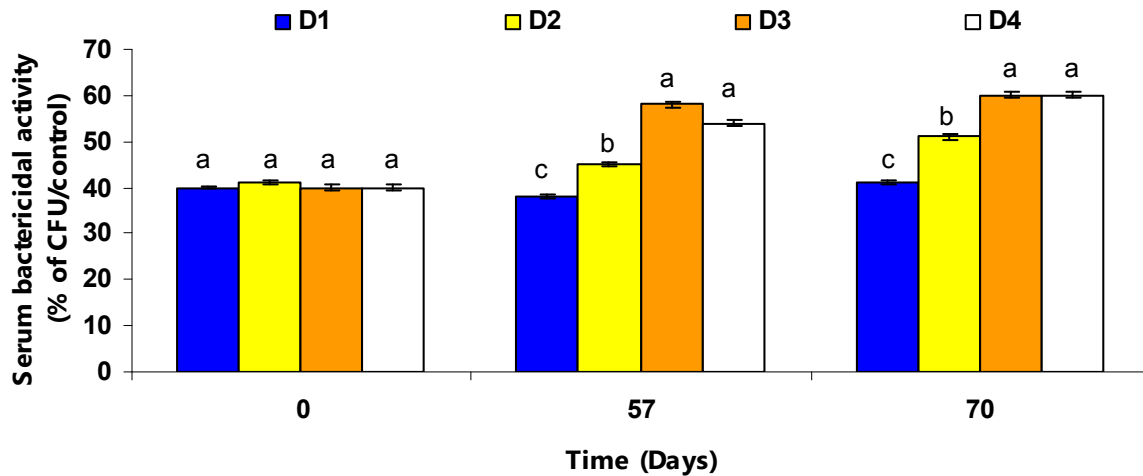


Figure 2. Change in serum bactericidal activity observed in challenged *Cyprinus carpio* fed chitosan supplemented and control diet. Bars represents the standard deviation (n=3). Same small letters on the bar indicate no significant difference between the different treatment and the control group on the particular sampling day ( $P < 0.05$ ).

Table 2. Feed conversion ratio (FCR) and specific growth rate (SGR) of challenged *Cyprinus carpio* fed chitosan supplemented diet and the control diet at the end of experiment. Data presents mean  $\pm$  SD from triplicate determination (n=3). Values with the same superscript in a column do not differ significantly ( $P > 0.05$ ).

Treatments	Mean FCR	Mean SGR
Control (D1)	2.32 $\pm$ 0.09 <sup>a</sup>	1.82 $\pm$ 0.16 <sup>a</sup>
D2	2.02 $\pm$ 0.12 <sup>b</sup>	2.20 $\pm$ 0.18 <sup>b</sup>
D3	1.81 $\pm$ 0.10 <sup>c</sup>	2.67 $\pm$ 0.19 <sup>c</sup>
D4	1.85 $\pm$ 0.06 <sup>c</sup>	2.38 $\pm$ 0.09 <sup>d</sup>

### Growth Parameters

#### Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR)

SGR and FCR of immunostimulated and the control common carp at the end of experimental trail are shown in Table 2. The results showed that the dietary chitosan (2% and 5%) supplementation significantly enhanced the SGR of *Cyprinus carpio* when compared with the fish fed on the control diet ( $P < 0.05$ ). SGR in the chitosan (2%) fed group and the control diet fed group were 2.67 and 1.82, respectively. FCR values of chitosan (2%) fed group and the control diet fed group were 2.32 and 1.81, respectively.

Similar findings were reported by Gopalakannan and Venkatesan (2006) showing that chitosan (1%) had the positive effect on growth of common carp when compared with the control. Present findings contradict with the results of Kono et al. (1987) and Shiau and Yu (1999). Feeding of supplemented diet containing 10% chitin, chitosan or cellulose did not affect the growth of red sea bream, Japanese eel and yellow tail (Kono et al. 1987). On the contrary, Shiau and Yu (1999) observed the depressed growth in tilapia after feeding chitin and chitosan at 2, 5 and 10% level. They also speculated that the depressed growth in tilapia may be due to interference of chitosan and chitin in the absorption of nutrients. However, in the present study, fishes were fed on chitosan (1, 2 and 5%) supplemented diet, which was lower than the dosage or levels used by Kono et al. (1987) and Shiau and Yu (1999). Chitosan may play a crucial role in enhancing the digestion and absorption of nutrients when incorporated at lower levels (Gopalakannan and Venkatesan 2006). Thus, the chitosan especially at the levels of 2 and 5% was effective in enhancing the growth of common carp in the pond conditions.

Table 3. Relative Percentage Survival (RPS) (%) of challenged *cyprinus carpio* fed chitosan supplemented diet and the control diet. Values with the same superscript in a column do not differ significantly ( $P > 0.05$ ).

Treatment	Survival (%)	Mortality (%)	RPS (%)
Control (D1)	26.5 <sup>d</sup>	73.5	---
D2	62.2 <sup>c</sup>	37.8	48.5
D3	87.35 <sup>a</sup>	12.65	82.78
D4	85.00 <sup>b</sup>	15.00	79.59

### Relative Percentage Survival (RPS)

Mortality and survival percentage and RPS of common carp fed on chitosan supplemented diet and the control diet after challenging with *A. hydrophila* is presented in Table 3. The mortality percentage was highest (73.5%) in the control (infected) group and was lowest (12.65%) in Chitosan (2%) group. The relative percentage survival was highest (82.78%) in Chitosan (2%) group and was lowest (48.50%) in Chitosan (1%) group. This might be due to the enhancement of the non-specific immune system of the fish by chitosan. Gopalakannan and Venkatesan (2006) also reported that the RPS in the chitosan supplemented group of common carp challenged with *Aeromonas hydrophila* was significantly higher than the control and chitin supplemented group. Rairakhwada et al. (2007) reported the highest RPS (100%) was recorded in 0.5% levan fed and the lowest RPS was recorded in 1% levan fed fish.

### Conclusion

From the results of the present investigation, it can be concluded that chitosan can be incorporated in diet in order to increase immune function and protection against infection of *Aeromonas hydrophila* which is prevalent mostly during summer season leading to heavy losses to the fish stock in hatchery and ponds. A dose of 2% chitosan is optimum to stimulate the immune function of common carp and confer a high degree of protection against the invading bacterial pathogen. Same doses of chitosan were equally effective in stimulating the growth of common carp. This base line information will of immense importance to the fish farmers of the temperate climatic areas.

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