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IMMUNOMODULATORY EFFECTS OF DIETARY INTAKE OF CHITOSAN ON NON-SPECIFIC IMMUNE RESPONSE OF *MUGIL CEPHALUS* CHALLENGED WITH *AEROMONAS HYDROPHILA*

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ABSTRACT

The aim of this study was to observe the effect of dietary chitosan on *Mugil cephalus* non-specific immunity. *M. cephalus* with an average weight of 45±2 g were fed diet for 70 days containing control, and control diet with 1%, 2%, and 5% chitosan, respectively. On days 30 and 58, fish from each treatment group (1%, 2%, and 5% chitosan group) were challenged with *Aeromonas hydrophila* to study response of chitosan-fed fish to the bacterial challenge. When compared to the control group, the chitosan fed (2%) groups had higher phagocytic index, phagocytic ratio, and serum bactericidal activity ($P \leq 0.05$). When the fish in all treatments were given *A. hydrophila* intraperitoneally, the relative percentage survival (RPS) was higher in the chitosan (2%) category ($P \leq 0.05$) than in the other treatments. The control group performed poorly in all non-specific immune response assays, which was accompanied by a decline in survival and growth rates. Thus, including chitosan at a concentration of 2% in a fish's diet improved non-specific immunity, decreased fish mortality, and improved fish growth.

Keywords: Chitosan, *Mugil cephalus, Aeromonas hydrophila*, Bacterial infection, Immunostimulant.

1. INTRODUCTION

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 [1]. Non-specific defence mechanism plays an important role at all stages of fish infection. Fish, particularly, depend more heavily on these non-specific mechanisms than do mammals [2]. 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 defence mechanisms. With increasing trend of searching different harmonic measures for preventing fish diseases, imunnostimulants are gaining popularity as an attractive and promising alternative to chemotherapeuants in aquaculture practices [3].

Immunostimulants comprise a group of biological or synthetic substances known to stimulate the non-specific immune mechanisms of host on their own or specific immune mechanism when conjugated with some antigen. It is well known that fish rely more profoundly on nonspecific defence mechanism than mammals [4]. Chitosan is used as an immunostimulant in aquaculture to protect salmonids and carps against bacterial diseases [5]. Chitosan is a linear homopolymer of ß-(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 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 [6].

Mugil cephalus, also known as striped mullet or sea mullet, is a widely distributed fish species. Throughout the globe, this species can be found in temperate and tropical waters. Sea mullet is caught for both commercial and recreational uses, and it is not considered a threatened or endangered species of fish. The majority of mullet caught on ocean beaches are spawning run fish, and their catches have increased as a result of an increasing demand for sea mullet roe, a common delicatessen fish commodity. So, Mullet is successfully grown in many countries [7]. *Aeromonas hydrophila* is most widespread in freshwater fish [8]. *A. hydrophila* causes

disease in carp, eels, milkfish, channel catfish, tilapia, and ayu, as well as stress-related diseases in salmonids characterised by ulcerations, exophthalmia, abdominal distension, and other symptoms [9]. However, as previously said, using antibiotics to combat diseases in aquaculture is not a safe technique. Thus, the goal of this study was to study the effect of chitosan on non-specific immunity, growth and survival of *M. cephalus* against the challenge of *A. hydrophila*.

2. MATERIAL AND METHODS

2.1. Collection and acclimatization of fish

Healthy mullet (*M. cephalus*) fish were collected from the Vellar estuary in Parangipettai, Tamil Nadu, India (latitude 11°29' N and longitude 79°46' E). All of the fish were brought to the lab wet and stocked in triplicate in a 200 L circular plastic tank with a continuous flow through system. Weekly reported parameters for water quality included temperature, pH, dissolved oxygen, and free CO_2.

2.2. Experimental feed

Feed ingredients *viz,* groundnut oil cake, rice bran, soybean, fish and wheat meal were obtained, screened and analysed according to standard method. The diet comprised 29.33% crude protein, 2.10% crude lipid, 18.9% ash and 9.0% humidity. All the ingredients were correctly weighed according to their inclusion rates and ground in an electric grinder separately, thoroughly mixed and enough water was added. Chitosan was subsequently added and mixed to the preparation of the dough in all quantities with vitamins and minerals. The following four diets were prepared in order to study Diet D1, which wasn't added with chitosan and D2, D3 and D4 had the same ingredients as D1, but chitosan (Sigma, USA) supplemented at a level of 1%, 2 and 5% respectively of the diet. Twice daily for a period of 70 days, experimental stock was fed into all treatments. The rate of feeding was 5% of the body weight.

2.3. Bacterial stain

A. hydrophila, a virulent fish pathogenic strain, was received in Tryptose agar slants (TSA) from Annamalai University, Medical College, Annamalai Nagar, Tamil-nadu. *A. hydrophila*was sub-cultured and maintained at 4°C in fish immunology lab, CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University. 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 inoculum throughout the study period.

2.4. Experimental Design

After acclimatization, the fish were divided into four treatments of 10 fishes for each group. Group 1 served as a control and received diet D1 throughout the study. Group 2, Group 3 and Group 4 received diet D2 (1% chitosan), diet D3 (2% chitosan) and diet D4 (5% chitosan), respectively. Total experimental period was 70 days. At day 0, blood was collected from the all-experimental group. In all the experimental groups, the first infection with *A. hydrophila* was given on the 30^{th} day and the Second infection was given on 58^{th} day. Blood samples were collected from day 30 and 58 in all the experimental group to study the immunostimulatory effect of chitosan against *A. hydrophila* infection.

2.5. Phagocytosis assay

The phagocytosis assay was conducted with a slight modification according to Siwicki et al. [10] and the Park and Jeong [11]. In 0.1 ml of sterile microplate, 10^7 cells of freshly cultivated *A. hydrophila* was added to each fish in a 0.1 ml blood sample of PBS. After thorough mixing in the well, it was incubated for 30 minutes at 25°C. The plate was removed and the suspension of blood bacteria was gently mixed again after incubation. Three glass slides and smears were put on 50 microliters of this suspension. After drying the air, the smear was redried with the May-Grunwald Giemsa in 95 % ethanol. There were enumerated phagocytic cells and phagocytic bacteria. A microscopical enumeration of 100 phagocytes per slide determined the phagocytic ratio (PR) and the phagocytic index (PI). Three slides have been calculated on average. 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.

2.6. Serum bactericidal activity

Parts of the sera collected were utilized for studying serum bactericidal activity following Kajita et al [12]. Sera samples from each subgroup were pooled to three numbers. Pooled sera samples were diluted three times with 0.1% gelatin-veronal buffer (GVB+2) (pH 7.5, containing 0.5 mM/ml Mg^{2+} and 0.15 mM/ml Ca^{2+}). The bacteria *A. hydrophila* (live, washed cells used earlier) were suspended in the same buffer to make a concentration of 1 × 105 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 were 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.

2.7. Lysozyme activity

Lysozyme activity was measured by adapting the turbidimetric method described [13]. To check the serum with 50µL of pH 5.8 PBS, a hundred microliters of serum was placed in a 96-well plate and then, the serum was serially diluted until the desired concentration was reached. The last well was used to discard 50µL of the sample finally. To each flask, 125µl of Micrococcus was added to the well. The measurement was made in an ELISA reader at room temperature for 450 nm reduced from 0 to 15 min. The lysozyme activity was converted to lysozyme concentration using hen egg white lysozyme as standard.

2.8. Nitroblue tetrazolium (NBT) assay

NBT assays were used to measure the production of oxidative radicals by neutrophils in blood during the respiratory burst, as defined by Anderson and Siwicki [6]. In short, blood and 0.2 percent NBT were combined in equal parts (1:1), incubated at room temperature for 30 minutes, and then 50 litres were collected and dispensed into Eppendorf tubes. 1 mL dimethyl formamide was applied to the reduced formazan product and centrifuged at 2000 r/min for 5 minutes to solubilize it. Finally, using a microreader and a supernatant, the degree of reduced NBT was calculated at an optical density of 540 nm. As a control, dimethyl formamide was used.

2.9. Statistical analysis

The values of each experimental parameter were expressed as mean ± SD and probabilities of $p<0.05$ were considered significant. The effects of the chitosan on immunological parameter were tested and a statistical package origin 18.1 for Windows 10 was used for these statistical analyses.

3. RESULTS AND DISCUSSION

Infectious diseases wreak havoc on an aquaculture facility's biosecurity, resulting in significant financial losses due to mass killings [14]. Immunostimulants are a promising field of control strategies, according to many natural product challenge experiments [15, 16]. The current study found that consuming chitosan in the diet had immune-modulating effects on Mugil cephalus' non-specific immune functions.

Table 1: Relative Percentage Survival (RPS) (%) of challenged *Mugil cephalus* fed chitosan supplemented diet and the control diet

Treatment	Survival (%)	Mortality (%)	RPS (%)
Control	27.4 ± 2.70^{a}	76.4 ± 6.07^{a}	---
Chitosan 1%	63.5 ± 5.45^{b}	41.8 ± 3.91^{b}	46.75 ± 4.37^{a}
Chitosan 2%	$89.7\pm2\ 7.95^{c}$	13.59 ± 0.98^{c}	84.82 ± 7.05^{b}
Chitosan 5%	84.25 ± 6.09^{d}	14.62 ± 1.07^{d}	81.93 ± 7.32^{c}

Data represented as mean ± S.D. In each group, means with different superscript letter (a-d) differ significantly at $p<0.05$ (DMRT).

3.1. Serum Bactericidal activity

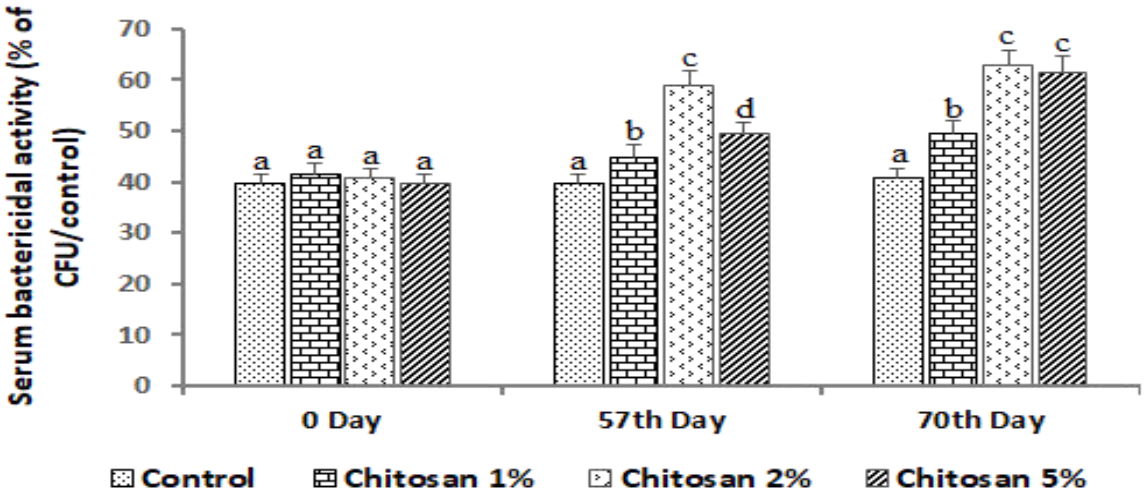
Fig. 1 shows the serum bactericidal activity of the control and chitosan supplemented groups. The bactericidal activity of fish against *A. hydrophila* challenge was substantially increased after 70 days of dietary chitosan feeding as compared to the control group. Fish supplemented with 1% chitosan had a serum bactericidal activity of 48%, 2% chitosan had a serum bactericidal activity of 63%, 5% chitosan had a serum bactericidal activity of 60 %, and fish fed a control diet had a serum bactericidal activity of 37%.

The pathogenic strain of *A. hydrophila* was successfully eradicated by the serum bactericidal activity of 2% chitosan fed classes. Chitosan plays a key role in the stimulation of phagocytic cells' bactericidal activity, owing to its stimulation of the development of reactive oxygen species such as superoxide anion by the affected cells [17]. The increased serum bactericidal activity in the chitosan-treated groups suggests that various humoral factors are involved in innate and/or adaptive immunities that are elevated in the serum to effectively protect the fish from infection [18]. The results of this study showed that 2% Chitosan was effective against *A. hydrophila* infection. This may be due to, and is closely linked to, lower mortality rates (%). Several studies have found that chitosan plays an important role in the

stimulation of bactericidal activity and phagocytic cells, owing to its stimulation of the development of reactive oxygen species such as O_2^- by the affected cells [19, 20]. As a result, Chitosan has improved to be an effective immunostimulant in *M. cephalus*, preventing the organisation of bacterial infection. This may be due to the lower mortality rate and high phagocytic activity, which are both well associated.

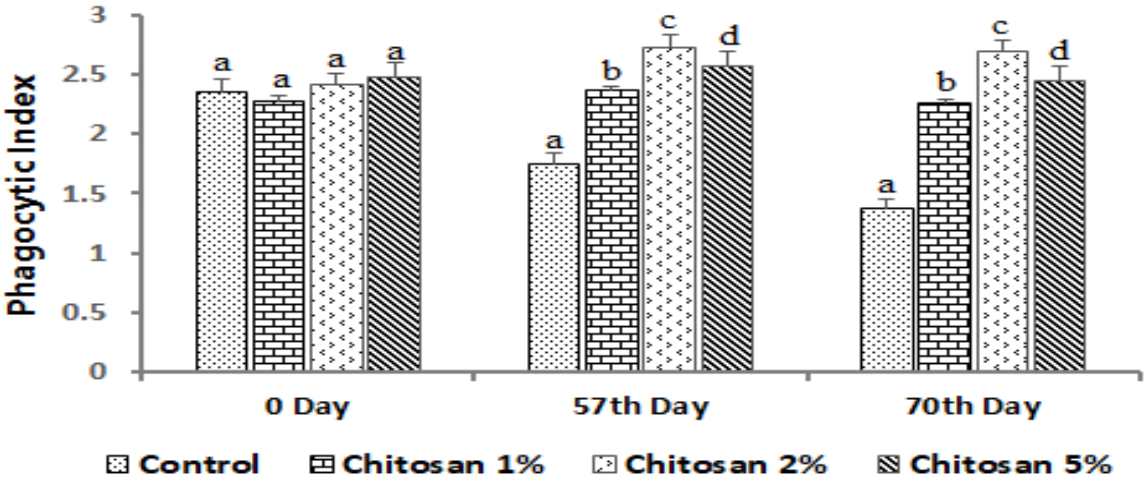
3.2. Phagocytic assay

Fig. 2 (A and B) shows the phagocytic index and phagocytic ratio of immunostimulated, control, and *A. hydrophila*-challenged *M. cephalus*. *M. cephalus* fed chitosan (2%) had a higher phagocytic index and phagocytic ratio than *M. cephalus* fed chitosan (1%), chitosan (5%), and control groups. Both the phagocytic index and the phagocytic ratio were higher in the chitosan supplemented group, particularly in the 2% chitosan group, suggesting that the chitosan at the 2% level was more effective in preventing pathogenic bacteria invasion and increasing phagocytosis.



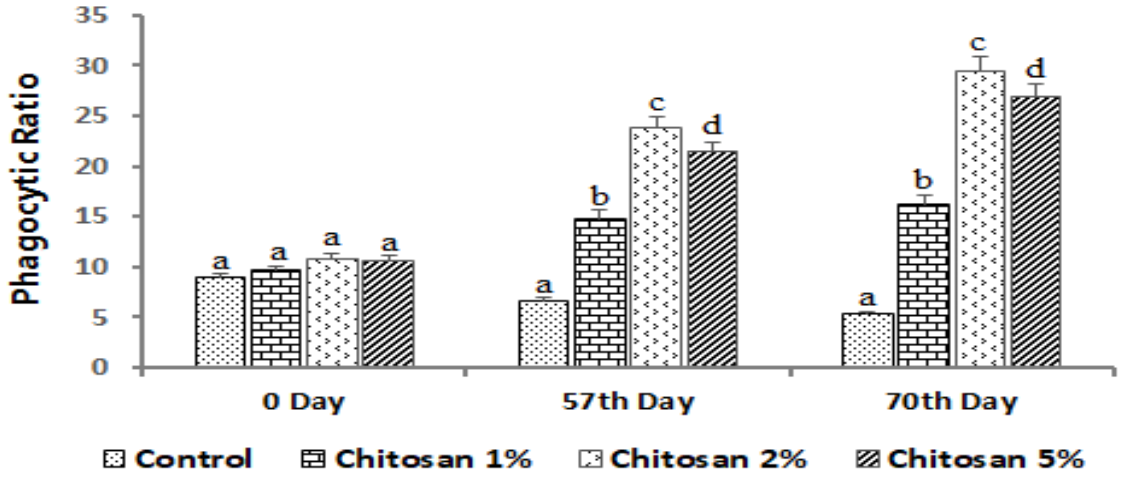
Data represented as mean ± S.D. In each group, means with different superscript letter (a-d) differ significantly at p< 0.05 (DMRT).

Fig. 1: Change in serum bactericidal activity observed in challenged *Mugil cephalus* fed chitosan supplemented and control diet



Data represented as mean ± S.D. In each group, means with different superscript letter (a-d) differ significantly at p< 0.05 (DMRT).

Fig. 2(A): Change in phagocytic index observed in challenged *Mugil cephalus* fed chitosan supplemented and control diet



Data represented as mean ± S.D. In each group, means with different superscript letter (a-d) differ significantly at p< 0.05 (DMRT).

Fig. 2(B): Change in phagocytic ratio observed in challenged *Mugil cephalus* fed chitosan supplemented and control diet

The increased phagocytosis may be due to chitosan binding to its receptors on phagocytic cells. Phagocytosis and pathogen removal by macrophages are important mechanisms for removing invading pathogens and represent the fish's immune status [21]. Chitosan can boost non-specific immunity by raising the number of phagocytes, activating phagocytes, or increasing the synthesis of innate immunity molecules like the complement lysozyme antiprotease [22]. These findings are consistent with those of Harikrishnan et al [23] and Mari et al [24], who discovered that phagocytic activity was significantly increased in fish fed a 1% chitin and chitosan diet on weeks 2 and 4. All groups supplemented with 0.5%, 1%, and 2% chitosan had significantly higher phagocytic indexes than the control group, while the fish group supplemented with 2% chitosan had significantly lower phagocytic indexes than the fish group supplemented with 1 percent chitosan. According to Gopalakannan and Arul [25] and Luo et al [26] this may be due to the long-term administration of a high dose of chitosan, which could have depleted the cells. Immunostimulants like chitosan easily activate non-specific protection mechanisms, which are quickly mobilised to defend the fish against pathogens. This is why we saw an increase in innate immune responses in this research. As a result, chitosan had a positive impact on phagocytic activity in fish infected.

3.3. Serum lysozyme activity

Fig. 3 shows *M. cephalus* serum lysozyme activity after a 70-day Chitosan feeding trial and a challenge with *A. hydrophila*. The 2% chitosan fed group had the most activity, followed by the 5% chitosan fed group, 1% chitosan fed group, and the control group had the least activity.

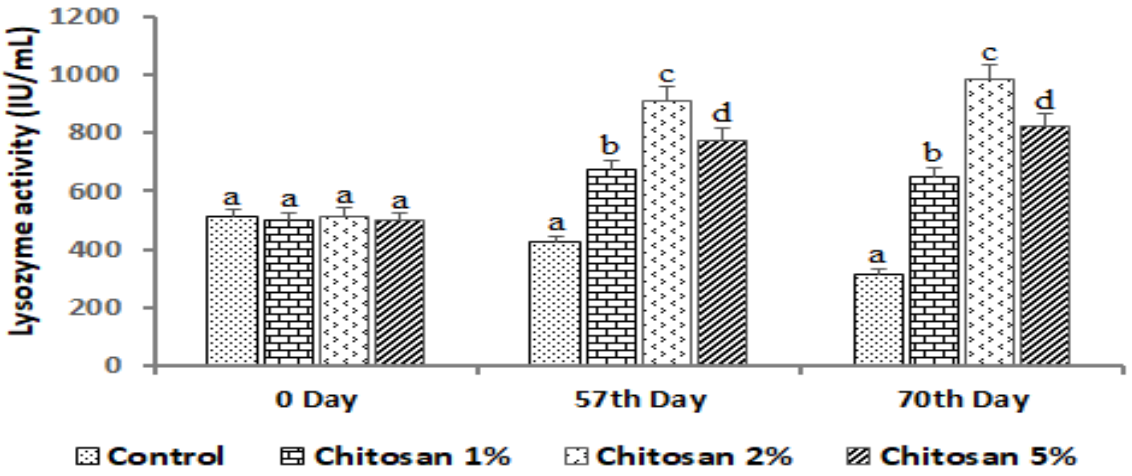
Lysozyme is a hydrolytic enzyme that catalyses the hydrolysis of 1, 4-beta-linkages between N-acetyl-muramic acid and N-acetyl glucosamine in the peptidoglycan of bacteria's cell wall. Both Gram-negative and Gram-positive bacteria are believed to be attacked by it. The increased levels of lysozyme and bactericidal activity suggested improved bacterial infection resistance [27]. In this analysis, supplemented feeds increased lysozyme activity in all treatments, with the 2% chitosan fed group having the highest activity. Ashouri et al [28] observed a substantial increase in lysozyme production in chitosan supplemented groups in Asian seabass *Lates calcarifer* on day 60 when compared to the control group. According to the findings of this study, Chitosan should be included in the diet to promote immune response and protect against *A. hydrophila* infection.

3.4. NBT assay

There was a significantly higher respiratory burst of cell activity in the treated group than in the control group (fig. 4). The highest significant reduction in NBT was observed in chitosan fed fish of 2% followed by chitosan fed fish of 5% and chitosan fed fish of 1%.

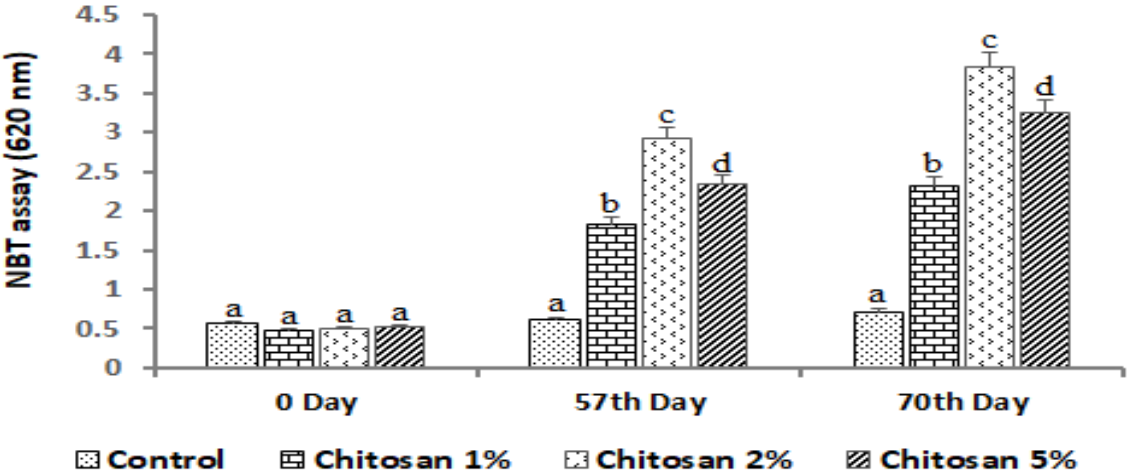
The NBT test is a fast and cost-effective test that focuses on the ability of phagocytes to reduce colouration through producing oxygen radicals that destroy *in vivo*

bacterial invaders. Macrophages are probably one of the key mechanisms for the protection of fish diseases in order to kill pathogenic microbes [26]. In this study, 2% of the chitosan fed group was able to observe the higher optical density of the NBT test. These results were consistent with Harikrishnan et al [23], as well as Vahedi and Ghodratizadeh [29], where it was found that the administered Chitin Diet (10, 25, 50 Mg/kg). Gopalakannan & Arul [23] for *Cyprinus carpio* produced similar results. Sharp & Secombes [30] suggested that increased activity with respiratory explosion may be linked to the increased activity to kill phagocytes by bacterial disease.



Data represented as mean ± S.D. In each group, means with different superscript letter (a-d) differ significantly at p< 0.05 (DMRT).

Fig. 3: Lysozyme activity observed in challenged *Mugil cephalus* fed chitosan supplemented and control diet



Data represented as mean ± S.D. In each group, means with different superscript letter (a-d) differ significantly at p< 0.05 (DMRT).

Fig. 4: NBT observed in challenged *Mugil cephalus* fed chitosan supplemented and control diet

4. CONCLUSION

Based on the findings of this study, it can be concluded that chitosan can be included in the diet to improve immune function and protect against *A. hydrophila* infection, which causes severe losses to fish stocks in hatcheries and ponds. To stimulate *M. cephalus* immune function and provide a high level of defence against the invading bacterial pathogen, a dose of 2% chitosan is ideal. The fish farmers will benefit greatly from this baseline knowledge.

5. ACKNOWLEDGEMENT

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Conflict of interest

We declare that we have no conflict of interest.

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