CHAPTER 5

IMMUNOSTIMULATORY EFFECTS OF DIETARY INTAKE OF CHITIN, CHITOSAN AND LEVAMISOLE ON TILAPIA MOSSAMBICUS
5.1. Introduction

Immunostimulants have long played a major role in disease control in aquaculture practices in the world (Austin, 1993). Sakai, (1999) has extensively reviewed the role of immunostimulants in control of fish disease. Substances like bacterial components, chemical agents, extracts of marine animal or plant extracts have been tried as immunostimulants to protect the fish against several diseases caused by the pathogens *Aeromonas salmonicida*, *Vibrio anguillarum*, *Yersinia ruckeri* (Kajita et al., 1990; Raa et al., 1992; Bailny et al., 1996; Mulero et al., 1998; Esteban et al., 2000; Logambal et al., 2000; Esteban et al., 2001; Miles et al., 2001; Ortuno et al., 2001; Villamil et al., 2003; Dautremepuits et al., 2004). Immunostimulants are generally used to enhance the non-specific system of fish.

Immunostimulants of bacterial origin (β-1,3-glucan, peptidoglycan and lipopolysaccharide) and vitamins received major attention (Esteban et al., 2001). However, the natural substance like chitin and chitosan received little attention. They are non-toxic and biodegradable, and can be extracted from prawn and crab shell wastes. Chitin and chitosan have wide applications in pharmaceutical, agriculture and aquaculture. In aquaculture, it is used as an dietary supplement to protect fish / shrimp against bacterial disease (Kono et al., 1987; Anderson and Siwicki, 1994; Siwicki and Dunier, 1994; Siwicki
et al., 1994; Kawakami et al., 1998; Ortuno et al., 2000; Esteban et al., 2000; Esteban et al., 2001) Levamisole an anti-helminthic drug can also stimulate immune responses. Siwicki, (1989) reported that oral administration of levamisole increased the number of leucocytes, lysozyme activities in serum and stimulated NBT reduction and phagocytic index of phagocytic cells. The aim of the present study was to evaluate the effects of chitin, chitosan, and levamisole on immune response and disease protection in *T. mossambicus* against *A. hydrophila*, an opportunistic pathogen in fresh water fish culture systems.

5.2. Materials and methods

5.2.1. Bacterial strain

*A. hydrophila* strain (AH13) utilized was isolated from diseased fish collected from a local fish farm is according to Shome and Shome, (1999). The species level identification of the strain was carried out by biochemical analyses (Joseph and Carnahan, 1994) and polymerase chain reaction (Chilaka, 2001). Subcultures were maintained on Tryptone soy agar slopes (Himedia, Mumbai) at 5°C and routinely tested for pathogenesis (Joseph and Carnahan, 1994) by injecting into *T. mossambicus* (Davis and Hayasaka, 1983). A stock culture in Tryptone soy broth (Hi-media) was stored at -70 °C with 0.85% (w/v) NaCl and 20% (v/v) glycerol to provide stable inocula throughout the study (Chabot and Thune, 1991; Yadav et al., 1992).
5.2.2. Fish

*T. mossambicus* (average weight 50 ± 8 g) collected from a local fish farm near Pondicherry were acclimatized to the laboratory conditions for 3 weeks in 1500 L (1.5 m X 0.87 m breadth and height) circular collapsible plastic tanks (Plastic craft Corporation, Mumbai) at 29 ± 3° C and with a 12 h light and 12 h dark cycle. About 30% of the water was changed every day in all the experimental tanks.

5.2.3. Feed

A basal diet composed of 40% groundnut oil cake, 33% rice bran, 20% soybean meal, 5% fish meal and 2% minerals and vitamins mixture (each 250 g minerals- vitamins mixture provide vitamin A- 500,000 i.u., vitamin D₃ - 100,000 i.u., vitamin B₂ - 0.2 g, vitamin E - 75 units, vitamin K - 0.1 g, calcium pentothenate - 0.25 g, nicotinamide - 0.1 g, vitamin B₁₂ - 0.6 mg, choline chloride - 15 g, calcium - 75 g, manganese - 2.75 g, iodine - 0.1 g, iron - 0.75 g, zinc - 1.5 g, copper - 0.2 g and cobalt - 0.045 g) was prepared.

5.2.4. Experimental feed

The experimental diets were prepared by incorporating the dietary supplements, chitin (poly-1,4-β-N-acetyl-D-glucosamine) 10 g, chitosan (de-N-acetylated chitin) 10 g and levamisole hydrochloride (Sigma, USA) 250 mg / Kg diet in the basal diet. Chitin and chitosan were prepared from shrimp shell waste according to Madhavan and Nair, (1974) with some modification (Gopalakannan et al., 2000).
Fish were divided randomly into four groups of 150 each in triplicate, each receiving one of the above mentioned diets and the control receiving the basal diet without dietary supplements. Fish were fed at a rate of 3% of the body weight each day. The biomass of the fish in each aquarium was measured after each sampling and the daily ration readjusted accordingly.

5.2.5. Sample collection

Five fish from each group were randomly sampled up to 60th day at every 15 days interval. Blood was collected from the caudal vein puncture with a 22-G needle and pooled from a random sample of five fish in each experimental tank after anaesthetizing them with MS-222 (Sigma, USA) once in 15 days. The blood (heparinised 150 iu / ml) collected from each group of fish was tested for the white blood cell count (WBC), lysozyme activity and nitroblue tetrazolium assay (Erdal et al., 1991).

5.2.6. Challenge study

10 fishes in treated as well as control groups were challenged on 30th day by injecting 100 μl of 12 h grown culture of A. hydrophila whole cells at a concentration of 1.5 ± 0.3 X10⁶ cfu / ml intramuscularly into the fish and observation were made for a period of 15 days. Total count of A. hydrophila was determined using Neubauer hemocytometer and total viable count was reconfirmed by spread plate method. Data on relative percentage survival (RPS) was calculated according to (Amend, 1981). Similarly on 60th day the
remaining fish in the tanks of treated and control groups were challenged with *A. hydrophila* and monitored for 15 days.

5.3. Results

5.3.1. Leucocyte count

The dietary supplements significantly enhanced the WBC count to a maximum on 45th day in fishes fed with levamisole supplemented diet, followed by chitosan and chitin (*p*<0.001) (Figure 1). However the leucocytes count started decreasing thereafter but remained higher when compare to control animals on 60th day.

5.3.2. Nitroblue Tetrazolium assay

A study on neutrophil activity clearly shows the positive effect of chitin, chitosan and levamisole on neutrophil respiratory burst activity as evidence from the increased NBT reduction (Figure 2). All the three dietary supplemented immunostimulant significantly (*p*<0.001) enhanced the neutrophil activity on 45th day. While chitosan supplemented diet enhanced the neutrophil activity maximally on the day of 30 (*p*<0.001) when compare to control. But, the control diet did not show any significant reduction of NBT throughout the experimental period.

5.3.3. Lysozyme activity

Significant differences of the serum lysozyme activity was observed in the fishes fed with supplemented diet (*p*<0.001) (Table 7). The maximum serum
Figure 1. Total WBC count of *T. mossambicus* fed with chitin, chitosan and levamisole supplemented diet. Each value (mean ± SD) is the average performance of five fish / treatment for a period of 60 days. Significance at * (P < 0.05).
Figure 2. Effect of chitin, chitosan and levamisole on Neutrophil activity of T. mossambicus. The NBT assay was performed at an interval of 15 days. Each value (mean ± SD) is the average performance of five fish/treatment for a period of 60 days. Significance at * (P < 0.05), ** (P < 0.01), *** (P < 0.001).
Table 7. Serum lysozyme activity (iu / ml) represent in tilapia fed with chitin, chitosan, levamisole and basal diet (control). Each value (mean ± SD) is the average performance of five fish/ treatment for a period of 60 days.

<table>
<thead>
<tr>
<th>Duration (days)</th>
<th>Control</th>
<th>Chitin</th>
<th>Chitosan</th>
<th>Levamisole</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>927 ± 175</td>
<td>2459 ± 307***</td>
<td>2610 ± 73***</td>
<td>1971 ± 204***</td>
</tr>
<tr>
<td>30</td>
<td>854 ± 239</td>
<td>3147 ± 278***</td>
<td>3196 ± 112***</td>
<td>2357 ± 141**</td>
</tr>
<tr>
<td>45</td>
<td>922 ± 136</td>
<td>2654 ± 141**</td>
<td>2810 ± 122**</td>
<td>2196 ± 234*</td>
</tr>
<tr>
<td>60</td>
<td>868 ± 209</td>
<td>1942 ± 302***</td>
<td>2215 ± 209***</td>
<td>1732 ± 273**</td>
</tr>
</tbody>
</table>

Note: * p<.05; ** p <0.01; *** p <0.001
lysozyme activity was found on day 30 in all the groups fed with chitin, chitosan and levamisole supplemented diets. Fish fed with non supplemented diet have no change in the serum lysozyme activity throughout the experimental period.

5.3.4. Challenge experiment

The results of disease resistance revealed that the fish fed with chitosan, levamisole and chitin attained significant relative percentage survival (RPS) value of 77%, 66% and 55% respectively when they are challenged with *A. hydrophila* 30 days after feeding with supplemented diet (*p*<0.05) (Table 8). However marked decrease in the relative percentage survival was observed on the day of 60 when they are challenged with *A. hydrophila* in all the groups fed with supplemented diet viz., chitosan (61%), levamisole (51%) and chitin (39%) (*p*<0.01).

5.4. Discussion

Prevention of fish disease using such immunostimulants is a prospective area upcoming area. Spectrum of immunostimulants like β-glucans, peptidoglycan, lentinan, chitin and chitosan has been tested in fish (Sakai et al., 1992; Samuel et al., 1996). Chitin and Chitosan are natural polysaccharide and is main component of insect cuticle and crustacean exoskeleton. Few studies on chitin and chitosan immunostimulant property and protection against infection in fish have been reported. Chitin was found to be stimulating macrophage activities in rainbow trout (Sakai et al., 1992) and innate immune responses in sea bream (Esteban et al., 2001). Rainbow trout and yellow tail
Table 8. Mortality and relative percentage survival (RPS) after challenge in *Tilapia mossambicus* with *A. hydrophila*.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of challenged fish</th>
<th>Mortality to <em>A. hydrophila</em></th>
<th>Survival (%)</th>
<th>Relative Percentage Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>30th Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitin</td>
<td>10</td>
<td>4 (40)*</td>
<td>60</td>
<td>55.56</td>
</tr>
<tr>
<td>Chitosan</td>
<td>10</td>
<td>2 (20)*</td>
<td>80</td>
<td>77.78</td>
</tr>
<tr>
<td>Levamisole</td>
<td>10</td>
<td>3 (30)*</td>
<td>70</td>
<td>66.67</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>9 (90)</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>60th Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitin</td>
<td>18</td>
<td>9 (50)**</td>
<td>50</td>
<td>39.28</td>
</tr>
<tr>
<td>Chitosan</td>
<td>19</td>
<td>6 (31.58)**</td>
<td>68.42</td>
<td>61.65</td>
</tr>
<tr>
<td>Levamisole</td>
<td>19</td>
<td>8 (42.11)**</td>
<td>57.89</td>
<td>51.14</td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>14 (82.35)</td>
<td>17.65</td>
<td>-</td>
</tr>
</tbody>
</table>

Significant: * p<0.05; ** p<0.01

"Fish were challenged by intramuscular injection with the *A. hydrophila* strain.

"Relative percent of survival = 1 - [% mortality in the immunostimulants fed group / % mortality in the control group] X 100. RPS values over 50 indicate positive effect of the immunostimulants (Amend, 1981)"
injected with chitin showed increased resistance to *V. anguillarum* (Sakai et al., 1992) and *Pasteurella piscida* (Kawakami et al., 1998) respectively. However this is the first time that the use of chitosan as dietary supplementation has been studied in fish. Chitin and levamisole also used in this study to find out the better immunostimulant to protect the fish.

Immunostimulants administration found to affect the growth in few studies (Mulero et al., 1998; Siwicki et al., 1994). In the present study all the three dietary supplements have no effect on the growth of fish. Similar effect was also reported in gilthead seabream (Esteban et al., 2001). However in the field study, chitosan and levamisole supplemented diet enhanced the growth of common carp (Gopalakannan and Arul, 2006).

The WBC levels in fish fed with chitin, chitosan and levamisole significantly increased from control level to a maximum on 45 day whereas in control it remained unaffected during the same period. The highest value was observed in the levamisole fed fishes, followed by chitosan and chitin. This is an indication for the enhancement of non-specific immune system.

The increased leukocytes probably represented the inflammatory response against bacteria (Roberts, 1978). Harikrishnan et al. (2003) reported that the increased WBC counts in *C. carpio* after herbal treatment. However, Wedemeyer et al. (1983) reported no effect on leukocyte values in Steel head trout (*Salmo gairdneri* Richardson) after challenging with *Y. ruckeri*. Where as, coho salmon (*Onchorhynchus kisutch* Walbaum) challenged with *A. salmonicida* demonstrated the depressed leukocyte values.
NBT assay is a quick and inexpensive test focusing on the ability of phagocytes to reduce the dye by the production of oxygen radicals. In the animals (in vivo) the oxygen radicals are aimed at the destruction of bacterial invaders. The ability of macrophages to kill pathogenic microbes is probably one of the most important mechanisms of protection against diseases among fishes. Increased respiratory burst activity can be correlated with increased killing activity (Sharp and Secombes, 1993). The neutrophil activity was higher in chitosan and chitin treated fish. This may be due to the enhancement of immune system by immunostimulation of chitosan. Esteban et al., (2001) have observed a similar enhancement of respiratory burst activity gilthead sea bream fed with chitin supplemented diet. The injection of Ocimum sanctum L extract into the T. mossambicus also enhanced the higher neutrophil activity (Logambal et al., 2000). However NBT gradually decreased in all the treatment except the control after 45th day treatment. This may be due to the overdose of immunostimulant or immunosuppressive effect of the immunostimulants.

Lysozyme is a cationic enzyme that breaks the β-1,4-glycosidic bond between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls. This action is known to attack mainly gram positive bacteria and also in conjunction with complement even some gram negative bacteria. However different results have been reported regarding the influence of immunostimulants on serum lysozyme activity. Oral administration of yeast glucan plus vitamin C (Verlhac and Gabaudan, 1994), chitin (Esteban et al., 2001) and intraperitoneal injection of chitin (Sakai et al., 1992) did not affect the serum lysozyme activity. On contrary, the increased lysosome activity was
observed in salmon (Engstad et al., 1992), trout (Jorgensen et al., 1993; Thompson et al., 1995) and Yellowtail (Matsuyama et al., 1992) after an intraperitoneal injection of yeast glucan or schyzophyllan. The present observation demonstrates that the supplementation of chitosan, chitin and levamisole in the diet enhances the lysozyme activity in the serum.

The results revealed that in general fish fed with chitin, chitosan and levamisole supplemented diet enhanced the survival compared to control. Similarly dietary macrogard and vitastim enhanced resistance against a protozoan disease in juvenile dentex (*Dentex dentex*), another fish of the family Sparidae (Efthimiou, 1996). Vitastim has also induced an increase in protection against bacterial infection in other fish species (Jeney et al., 1997; Nikl et al., 1993).

Based on the results of present study it may be concluded that the administration of chitosan supplemented diet (1%) enhances *Tilapia* immune activity through the non specific modulation of WBC, respiratory burst activity and serum lysozyme. Natural immunostimualnts like chitin and chitosan are biocompatible, biodegradable and safe for the environment and human health.