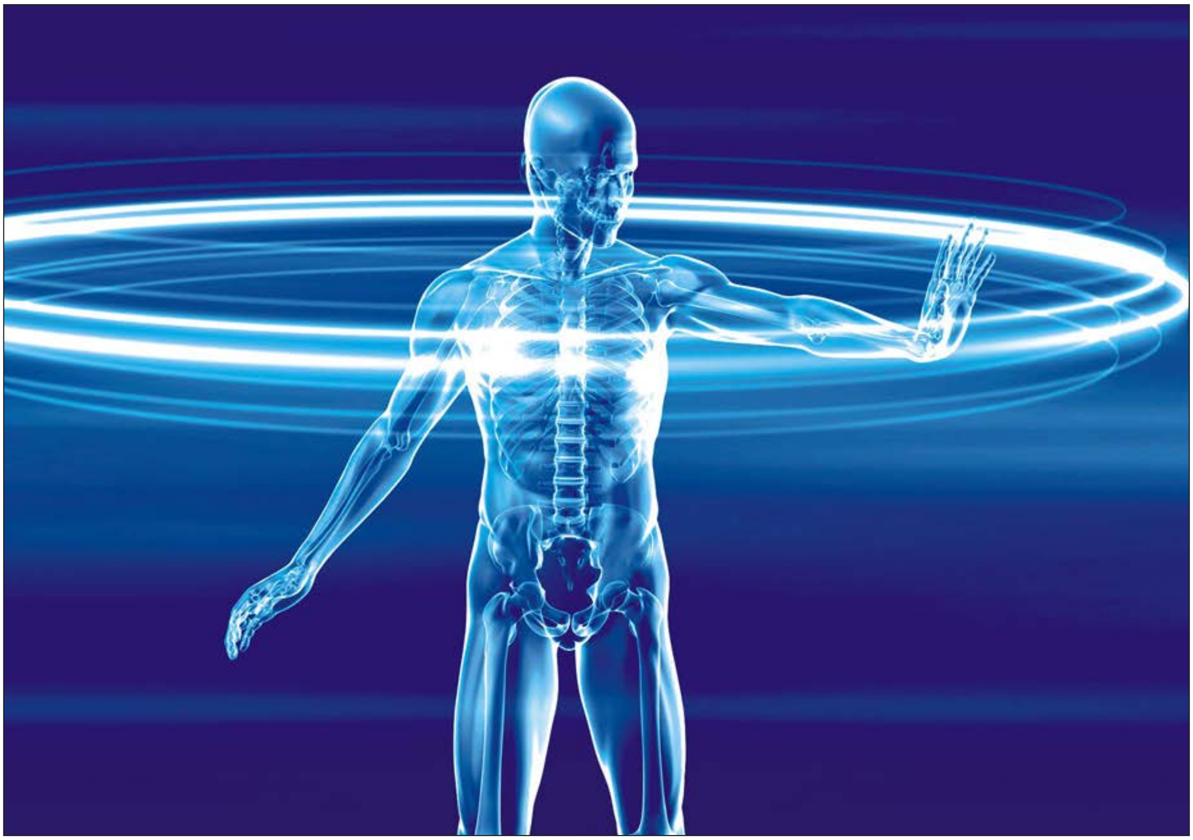

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Evaluation of Immunomodulatory Effect of Dietary Probiotics on the Common Carp, *Cyprinus carpio*

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ABSTRACT

Fish culture is increasing to compensate the shortage of animal protein all over the world. Fish under intensive culture conditions will be badly affected and often fall prey to different microbial pathogens that have been treated with chemotherapeutic substances of which antibiotics were intensively used. The use of natural immunostimulants in fish culture for the prevention of diseases is a promising new development and could solve the problems of massive antibiotic use. Evaluation of immunomodulatory effect of the dietary probiotics on the common carp, *Cyprinus carpio* L. has been studied for the period of one month and the experiments such as, mortality, phagocytic activity and growth rate are conducted through this present study. To prepare the probiotic enriched diet, the colony forming units were found. For *Lactobacillus acidophilus*, the colony forming units were found to be 4.6×10^{7} CFU mL^{-1} for 10^{5} and 2.1×10^{9} CFU mL^{-1} for 10^{7} concentrations. The *Bacillus subtilis* were found to be 3.47×10^{7} CFU mL^{-1} for 10^{5} concentrations and 1.64×10^{9} CFU mL^{-1} for 10^{7} concentrations. Then these bacterial suspensions of 5 mL were sprayed into the 50 g of feed (100 mL kg^{-1} diet) with constant mixing. The experimental group III fed with 10^{5} cells of *Bacillus subtilis* showed minimum mortality rate and maximum mean leucocyte count and growth rate on all days when compared to other experimental groups.

Key words: Antimicrobial agents, intensive finfish aquaculture, immunostimulants, dietary probiotics, *Lactobacillus acidophilus, Bacillus subtilis, Cyprinus carpio*

INTRODUCTION

Fish culture is an age-old practice in India and India is one of the second largest fish producers in the world. One of the major issues in the aquaculture is the loss associated with diseases. So far there have been a number of approaches have been applied in an attempt to address this problem including sanitary prophylaxis, disinfection and chemotherapy. The alternative approaches have been applied now days to boost or stimulate the innate immune system of cultured fish by using various compounds. These compounds are termed as immunostimulants which include bacteria and bacterial products (Chakrabarthy, 1998). Aquaculture productivity constitutes significant portion of in many countries of Asia. Large scale mortalities of fish often occur in ponds and loss is due to environmental pollution stress followed by microbial infection (Michael, 1997). The aquaculture and fisheries have a promising role to play in social development by providing nutritional security for the Indian population and contributing to the economic advancement of the farmers and fishery workers as more than 14.66 million fisher man and fish farmers are totally different on fisheries for the livelihood in India (Phale *et al.*, 2009).

The major components of the innate immune system are macrophages, monocytes, granulocytes and humoral elements, such as lysozymes or the complement system. Immunostimulants can be administered by injection, bathing or orally, with the later appearing to be the most practicable and most commonly used now a days (Yin *et al.*, 2006). The parallel use of biological products either the probiotic or in combination with prebiotics is recently the goal of the disease biocontrol strategy in aquaculture as they improve the fish health and modify the fish associated microbial community (Gibson and Roberfroid, 1995). Ravichandran *et al.* (2010) studied the antimicrobial peptides from the marine fishes and reported that the marine fishes possessed antimicrobial peptides as a part of their defence system. Their studies also emphasize the role of bioactive peptides in marine fishes. Immunostimulants include a wide range of chemical agents, bacterial components, polysaccharides, animal or plant extracts, nutritional factors and cytokines. The term applies to any compound that modulates the immune system by increasing the host's resistance to disease. Immunostimulants mainly facilitate the function of phagocytic cells and increase their bactericidal activities. Several immunostimulants also stimulate lysosomes and the antibody responses of fish (Bagheri *et al.*, 2008). A promising alternative approach for controlling fish diseases is the use of probiotics or beneficial bacteria which control pathogens through a variety of mechanisms. The uses of probiotics, in humans and animal nutrition, are well documented and recently, have been applied to aquaculture. *Bacillus subtilis* has been shown to possess antitumor and immunomodulatory activities. Some studies have demonstrated that *B. subtilis* and spores of *B. subtilis* act as probiotics by promoting the growth and viability of the beneficial lactic acid bacteria in the intestinal tracts of humans and some animals (Aly *et al.*, 2008). Common carp is an omnivores fish, feeds mainly in the benthic region of the pond. The ecological spectrum of crop is broad. Best growth is obtained when water temperature ranges between 23 and 30°C. The fish can survive cold winter periods (Milstein *et al.*, 2002). Today domesticated common carp is one of the most important species in freshwater fish culture mostly raised for human consumption especially in Asia (Khalili and Amirkolaie, 2010). Hence, the present study has been undertaken to evaluate the immunomodulatory effect of dietary probiotics on the common carp, *Cyprinus carpio*. The following parameters such as mortality, phagocytic activity and leucocyte count were studied.

MATERIALS AND METHODS

In the present study, the fresh water fish common carp, *Cyprinus carpio* collected from Devi fish form Madurai, Tamil Nadu. The collected fishes were transported to the laboratory in polythene bags containing oxygenated water. The present work has been carried out during September 2010-March 2011. They were acclimated to laboratory conditions for 20 days in non-chlorinated water in glass tanks. Proper aeration was maintained and fed *ad libitum*. The two probiotic bacteria used in these experiments are *Lactobacillus acidophilus* and *Bacillus subtilis* were obtained from the Microbiology Department of Ayya Nadar Janaki Ammal college, Sivakasi. Heat killed *Aeromonas hydrophila* were used as an antigen. This bacterial antigen was injected into the fish body through intraperitoneal injection.

Probiotic supplementation: Two probiotic strains namely *Lactobacillus acidophilus* and *Bacillus subtilis* were selected as the dietary supplementation along with the feed. In order to obtain the bacterial suspensions, the probiotic strains namely *Lactobacillus acidophilus* and *Bacillus subtilis* were grown in Man-Rogosa-Sharpe medium (MRS) broth and nutrient broth in shaking incubator at 30°C for overnight. To prepare the probiotic enriched diet, the colony forming units were found (Kannan, 2000). For *Bacillus subtilis* the colony forming units was found to be 3.47×10^{7} CFU mL^{-1} for 10^{5} concentration (Exp I) and 1.64×10^{9} CFU mL^{-1} for 10^{7} concentration (Exp II). The colony forming units for *Lactobacillus acidophilus*, was found to be 4.6×10^{7} CFU mL^{-1} for 10^{5} (Exp III) and 2.1×10^{9} CFU mL^{-1} for 10^{7} concentration (Exp IV).Then these bacterial suspension of 5 mL were sprayed into the 50 g of feed (100 mL kg^{-1} diet) with constant mixing and stored in the refrigerator for further offering to fishes.

The clinical signs and mortalities were monitored for seven days of post challenge. The percentage of mortality was calculated by Balasubramanian (2006). The numbers of the phagocytic and unphagocytic leucocytes were counted under the microscope (Michael *et al.*, 1998). A manual method of Stoskopf (1993) has been used for counting the leucocytes using a Neubaer's haemocytometer counting chamber.

Statistical analysis: The data obtained from the experiments were analyzed by a one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Evaluation of immune response in common carp, *Cyprinus carpio* to two different probiotic bacteria *Lactobacillus acidophilus* and *Bacillus subtilis* were used against the pathogenic bacteria *Aeromonas hydrophila* was carried out in the present investigation. The fishes in the control group showed maximum mortality (66.66±0.577%) when compared to other experimental groups Table 1. This may be due to the supplementation of probiotic bacteria along with the feed in the experimental groups than control groups. These results confirmed the immunomodulatory effect of *Lactobacillus acidophilus* and *Bacillus subtilis* in common carp, *Cyprinus carpio* and also their inhibitory effect to *Aeromonas hydrophila*. The variation in the mortality ratios in different experimental groups shows that the experimental group III is more potent than other experimental groups in protecting the *Aeromonas hydrophila* infection. These results were supported by Ehab (1991), Sahu *et al.* (2007) reported that the survival rate after challenging the fish, *Labeo rohita* with *Aeromonas hydrophila* was increased, when the fishes were fed with the diets containing *Magnifera indica* kernel as feed additive with their normal diet.

Table 1: Cumulative percentage mortality of fingerlings of common carp, *Cyprinus carpio* intraperitoneally challenged with 0.1 mL of 10^{8} CFU mL^{-1} of *Aeromonas hydrophila*

		Cumulative percentage mortality in different days after treatment (%)			
Fish groups	Total No. of fishes	0	10	20	30
Control	10	0	36.66±0.577	56.66±0.577	66.66±0.577
Exp I	10	0	13.33±0.577	23.33±0.577	26.66±0.577
Exp II	10	0	10.00±0	40.00±0	43.33±0.577
Exp III	10	0	3.33±0.577	10.00±1	10.00±1
Exp IV	10	0	6.66±0.577	13.33±0.577	13.33±0.577

Table 2: The mean leucocyte count ($\times 10^3$ mm^{-3}) of fingerlings of *C. carpio* after administration of different concentrations of probiotic feed

	Days after probiotic administration of feed			
Fish groups	0	10	20	30
Control	38.61±0.244	39.30±0.488	42.12±0.151	44.48±0.050
Exp I	38.88±0.160*	39.78±0.244*	44.48±0.234*	47.04±0.012*
Exp II	38.98±0.244*	40.00±0.576*	48.28±0.150*	53.72±0.088*
Exp III	39.09±0.333*	40.58±0.244*	59.36±0.138*	65.52±0.013*
Exp IV	39.09±0.514*	40.58±0.333*	51.80±0.096*	56.12±0.098*

*Value are statistically significant at $p<0.05$

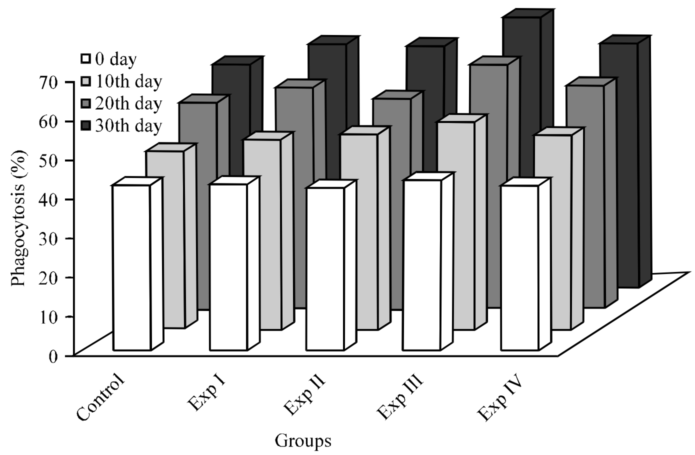

Fig. 1: Diagrammatic representation of the phagocytic activity of *Cyprinus carpio* treated with 0.1 mL of 10^8 CFU mL^{-1} of *Aeromonas hydrophila*

Concerning the non-specific immune stimulation in *Cyprinus carpio*, different experimental groups received diets supplemented with different concentrations of probiotics. The increase in leucocytes could be attributed that the probiotics used increased the blood parameter values as a result of haemopoietic stimulation. It was clear that high non-specific immunity was developed as manifested by increased number of leucocytes. In the present investigation the leucocyte counts were significantly higher in experimental group III (65.52±0.013) supplemented group when compared to the control group (Table 2) and the values were significantly higher ($p>0.05$) when compared to the control values.

These agreements are also similar to those of Gopalakannan and Arul (2006) who reported that there was an increase in leucocytes count after feeding the common carp with immunostimulants like chitin.

The phagocytic index was higher in the experimental group III (65.33±2.081%) in the thirtieth day of the experiment (Fig. 1). These could be attributed to the different components of bactericidal compounds released by the probiotic bacteria which activates the macrophages of the immune system and that can lead to the phagocytosis. Similar kind of results were also reported by Ortuno *et al.* (2002) when they fed the gilthead sea bream with the **β**-glucans and found that the feed stimulated the phagocytic function. Pavaraj *et al.* (2011) also reported similar kind of result when they fed the *Cyprinus carpio* with *Ocimum sanctum* and they found that the plant treated fishes no mortality was seen. Increase in weight of the fish observed in the plant extract treated groups and phagocytic activity in the low dose of 10 ppm. Salinas *et al.* (2004) reported that the dietary administration of *Lactobacillus delbrueckii* and *Bacillus subtilis*, either singly or in combination, increased phagocytic activity in the fish gilthead sea bream, *Sparus aurata*. Diaz-Rosales *et al.* (2006) observed a higher phagocytic ability in gilthead sea bream, *Sparus aurata* when they were fed with the two inactivated probiotic bacteria supplemented diet. Tatina *et al.* (2010) studied the effects of different levels of dietary vitamins C and E on some of hematological and biochemical parameters of sterlet (*Acipenser ruthenus*) and the results reveal that fish fed diets containing 100 mg kg^{-1} vitamin E and 400 mg kg^{-1} vitamin C (diet 7) had the highest WBC ($p>0.05$).

CONCLUSION

The probiotic strain *Bacillus subtilis* of concentration 10^5 cells was found to be best probiotic strain that would be considered as the best probiotic strain that would be considered as the best concentration for the aquaculture, from this study.

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