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The Influence of *Bacillus subtilis* and Ascorbic Acid on the Immune Response and Serum Factors of *Cyprinus carpio*

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Abstract: The immunostimulatory effect of probiotics and vitamin C has been established in many systems including fish. The current investigation was carried out to study the effect of dietary supplementation of a probiotic bacterium ''*Bacillus subtilis*'', vitamin C in the form of ascorbyl polyphosphate and their combination on the immune response of Common carp (*Cyprinus carpio*) fingerlings fed for a period of 60 days. The total serum protein and globulin content was significantly higher ($p<0.05$) in probiotic (*B. subtilis* @ 10^8 CFU/g of the feed) fed group while the respiratory burst activity of blood neutrophils was significantly high in vitamin C (ascorbyl polyphosphate @100 mg per kg diet) fed group. The antibody level was significantly high in *Bacillus subtilis* treated group followed by the probiotic (*B. subtilis*@10^8 CFU/g of the feed) and ascorbyl polyphosphate (ascorbyl polyphosphate @100 mg per kg diet) combined group. The least percentage of mortality was recorded in *B. subtilis* treated group (25%) followed by 35 and 40% in ascorbyl polyphosphate treated and *B. subtilis* and ascorbyl polyphosphate combined groups, respectively.

Key words: Common carp · *Bacillus subtilis* · *Cyprinus carpio* · Probiotics · vitamin C

INTRODUCTION

The success of modern aquaculture requires good management practices. Advancement of intensive aquaculture practices often have a negative impact on the pond environment leading to outbreaks of infectious diseases [1]. A wide range of antibiotics, drugs and chemicals are routinely used to control diseases. These agents not only cause a deterioration of the pond environment but also adversely affect the health status of fishes.

Aquaculture faces serious problems due to various adverse effects of antibiotics such as accumulation in the tissue and immunosuppression. Moreover, due to the availability of limited vaccines in few countries and their pathogenspecific protective action [2], much attention has been directed towards the use of probiotics and immunostimulants in aquaculture to control infectious diseases [3, 4].

Stimulation of the immune response by dietary supplementation of immunostimulants has already been proven in aquaculture [5]. Among various immunostimulants, the immunostimulatory effect of vitamin C has already been established in several fish species [6-8] but not hitherto in Common carp. Similarly, probiotics are used as dietary supplementations in aquaculture and their role in intestinal microbial balance, growth, nutrition, health status and resistance against infectious agents are already established [9]. Although, some reports are available on the activation of the immune response by dietary supplementation with probiotics in fish and shellfish [10] their direct involvement in the immune response is not well established [11]. In India, despite severe economic losses due to disease outbreaks in Indian major carps, very few attempts have been made to find suitable immunostimulants and/or probiotic for effective improvement of health conditions of Indian major carps [12].

Therefore, the present investigation was carried out to study the effect of dietary supplementation with the probiotic bacterium, *Bacillus subtilis*, vitamin C in the form of ascorbyl polyphosphate and their combination on the immune response and disease resistance of Common carp.

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Table 1: The percentage composition of the basal diet used in the experiment trial study in common carp fingerlings.

S1. No.	Lngredients	Conrtol group	Probiotic treated group	Vitamin C treated group	Vitamin C and probiotic combined group
1.	Groundnut oil cake	40	40	40	40
2.	Rice bran	25	25	25	25
3.	Fish meal	10	10	10	10
4.	Soybean oi	201	20	20	20
5.	Vegetable oil	3	3	3	3
6.	Vitamin and mineral mixture	15	1.5	1.5	1.5
7.	Cellulose	0.5	0.4	0.4	0.3
8.	Test supplement	-	B.subtilis @ 10^8 CFU/g	Ascorby polyphosphate @ 10mg	Ascorby polyphosphate @ 10mg + B.subtilis @ 10^8 CFU/g

composition vit A 500,000 I.U., vit D_3 100,000 I.U., vit D_2 vit E - 75 units, vit K-0.1 g, calcium pantothenate - 0.25 g, nicitinamide - 1.0 g, v it B_{12} - 0.6 g, choline - 15 choride - 15 g, calcium - 75 g, mangenesse - 2.75 g, iodine - 0.1 g, iron - 0.75 g, zine - 1.5g.

[a]Vitamin and mineral mixture: commercially obtainted from sigma, St Louis, MO, USA.

MATERIALS AND METHODS

Four different types of feed were prepared for the experimental trial. Out of four types of feed, one was control feed with no additional supplements, its composition is shown in table 1. A probiotic treated feed was prepared with all the ingredients of control feed supplemented with the probiotic bacterium, *B. subtilis* isolated from the gastrointestinal tract of *Cirrhinus mrigala* @ 10^8 CFU/g of the feed (based on the dose required for colonization in the gut of rohu [13]), while a vitamin C treated feed contained ascorbyl polyphosphate @ 100 mg per kg diet. A probiotic and vitamin C combined feed was prepared by mixing both *B. subtilis* @ 10^8 CFU/g and ascorbyl polyphosphate @ 100 mg per kg diet (Table 1).

Common carp (*Cyprinus carpio*) fingerlings of average weight 60 (±0.19) gm were acclimatized for 15 days prior to the start of the experiment. Twenty Common carp fingerlings were maintained in 1000/plastic pool, with constant aeration and daily two-thirds water exchange. Four separate sets of experiments containing 20 animals in duplicate were fed with test feed separately @ 3% body weight/day for a period of 60 days. During the investigation, different physico-chemical parameters such as temperature, DO and pH were routinely monitored. The pH and temperature of the rearing pools varied from 7.4-7.8 and 26-29 °C, respectively. After sixty days feeding the diets, blood was collected through cardiac puncture from 10 Common carp fingerlings in duplicate from each group with heparin (50 IU/ml of blood) and without heparin to evaluate different haematological and non-specific parameters. After the feeding trial, ten Common carp fingerlings in duplicate from each group were intraperitoneally challenged with 0.1 ml of virulent Edwardsiella tarda bacterium ($10^{5.5}$ CFU/ml) and the percentage mortality in each group was recorded for 10 days post challenge. Among the haematological parameters, the total leucocytes and packed cell volume were counted by standard haematological procedures. The serum protein concentration was estimated by the method of Bradford [14] while the albumin content was estimated spectrophotometrically using a standard kit (Glaxo, Iran). The globulin content was estimated by subtracting the albumin content from total protein content. Finally, the A:G ratio was calculated.

The respiratory burst activity was measured by the reduction of NBT by intracellular superoxide radicals by the method of Anderson *et al.* [15] with slight modifications. Briefly, 100 ml of heparinized blood from fish of each group were placed on a cover slip (22 mm square) in duplicate and were incubated in a moist chamber for 90 min at 30 °C.

After incubation, the cover slips were rinsed in PBS (pH 7.2) and covered with 0.02% NBT dissolved in DMSO. The cover slips were then incubated for 1 h at 15 °C. After incubation dark blue stained NBT positive cells were examined at 100 × magnification in a compound microscope to determine the activated cells. At least 100 cells per sample at three different sites were examined for each test.

At the end of the experimental trial, ten Common carp fingerlings in duplicate from each group were immunized with an *E. tarda* bacterin (made from a virulent strain with LD_{50} of $10^{5.5}$ CFU/ml) intraperitoneal by injecting 0.1 ml of bacterin suspension (10^9 CFU/ml). After 10 days post-immunization, blood was collected from all the twenty immunized fish through cardiac puncture up to 45 days post-immunization with one week interval and serum was collected to evaluate the antibody level by indirect ELISA as per the method of Swain *et al.* [16].

Statistical analyses were done by Duncan's Multiple Range Test to test the significant difference among different serum components and antibody level by using statistical analysis system (SAS), version 6.12 [17].

RESULTS

Dietary supplementation of various types of feed treatments affected the blood parameters (Table 2). The total leukocyte count was least in the control group while the count was significantly higher in *B. subtilis* and *B. subtilis* and ascorbyl polyphosphate combined group. The percentage of packed cell volume in the different feed treatment groups did not differ significantly. Among the serum parameters, the serum protein content was significantly higher in the *B. subtilis* treated group (1.89 ± 0.04 mg/dl) while it was lowest in the control group (1.74 ± 0.05 mg/dl). No significant difference ($p>0.05$) in the serum albumin content between control, *B. subtilis* treated and the ascorbyl polyphosphate treated groups was recorded while it was significantly higher (0.71 ± 0.02 mg/dl) in *B. subtilis* and ascorbyl polyphosphate combined group. The mean serum globulin content was significantly higher (1.28 ± 0.04 mg/dl) in the *B. subtilis* treated group but there was no significant difference ($p>0.05$) between the ascorbyl polyphosphate treated and *B. subtilis* and ascorbyl polyphosphate combined group although they were significantly higher than the control group. The albumin: globulin ratio was significantly highest ($p<0.05$) in *B. subtilis* and ascorbyl polyphosphate combined group and lowest in the *B. subtilis* treated group (Table 2). The production of oxygen radicals as measured by the percentage of NBTþ cells was significantly higher in the ascorbyl polyphosphate group (82.66 ± 1.69) but no significant difference ($p>0.05$) from the control group was recorded in any other feed treated groups (Fig. 1).

In indirect ELISA, the mean OD value of the non-immunized control Common carp sera was 0.074 and this was used as the cut off value. The highest antibody level with mean (±SD) OD values of 0.17 (±0.02), 0.23 (±0.01), 0.19 (±0.02) and 0.21 (±0.03) in the control, *B. subtilis* treated, ascorbyl polyphosphate treated and *B. subtilis* and ascorbyl polyphosphate combined groups, respectively, were recorded at 17 days post-immunization. The antibody level was significantly highest in the *B. subtilis* treated group and while no difference was found between the *B. subtilis* and ascorbyl polyphosphate combined group and the ascorbyl polyphosphate treated group, both the groups were significantly higher

Tanle 2: Different blood and serum parameters (values were expressed as mean ± SD in various groups.

Group	Total leucocyes count (10^3 cells/ml)	Eacked cell volume (%)	Protein (mg/dl)	Globulin (mg/dl)	A:G
Control group	17.07 ± 0.33^c	27.57±1.5	1.74 ± 0.05^a	0.64 ± 0.08^a	0.59 ± 0.13^b
Probiotic treated group	19.12 ± 0.45^a	28.12±1.3	1.89 ± 0.04^b	0.60 ± 0.03^a	0.46 ± 0.03^a
Vitamin C treated group	18.72 ± 0.63^b	27.62±0.75	0.67 ± 0.065^a	0.67 ± 0.06^a	0.58 ± 0.05^b
Vitamin Cand probiotic combined group	19.2 ± 0.03^a	28.12±1.43	0.71 ± 0.08^c	0.71 ± 0.025^b	0.63 ± 0.08^c

Different superscripts indicate the significant difference ($p>0.05$) among the groups.

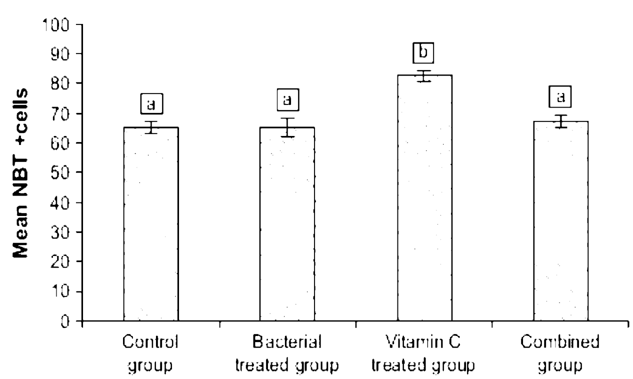

Fig. 1: The respiratory burst activity as measured by NBT assay in *Cyprinus carpio* fingerlings fed with probiotic and vitamin C for a period of sixty days (*different superscripts indicate the significant difference ($p< 0.05$) among the groups).

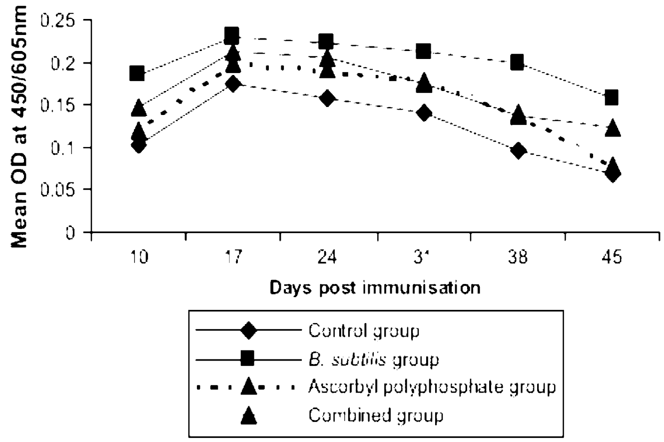

Fig. 2: The antibody response in Common carp fingerlings fed with different types of feed to *E. tarda* bacterin.

than the control group. The antibody titre persisted up to 45 days post-immunization in the *B. subtilis* treated and *B. subtilis* and ascorbyl polyphosphate combined groups while it persisted up to 38 days post-immunization in the ascorbyl polyphosphate treated group (Fig. 2). The percentage of mortality was highest (60%) in the control group followed by the *B. subtilis* and ascorbyl polyphosphate combined group (40%) and ascorbyl polyphosphate treated group (35%). The least percentage of mortality (25%) was recorded in *B. subtilis* treated group.

During the present investigation the probiotic bacterium *B. subtilis* was found to trigger the immune system of Common carp. The significant rise ($p<0.05$) in total serum protein, globulin and the antibody response against *E. tarda* bacterin confirmed the involvement of *B. subtilis* in triggering the immune system following dietary administration. On challenge with virulent *E. tarda*, there was a high survival in the *B. subtilis* group compared to other groups.

DISCUSSION

Although, the dietary requirement of vitamin C varies from species to species, the type and the source of vitamin C often affects the bioavailability and subsequent responses. The involvement of vitamin C in both the non-specific and specific immune responses of rohu as evident from increased serum parameters, respiratory burst activity and antibody titre are mainly due to the stable nature of ascorbyl polyphosphate compared to the non-derivatives form of vitamin C [18, 19]. These results of immune enhancement are in accordance with the earlier findings of Hardie *et al.* [20]. However, the present study differs from the findings of earlier workers where high levels of vitamin C were required to enhance antibody levels [6, 8]. In the present work, even at 100 mg/kg diet, ascorbyl polyphosphate was able to enhance the antibody level.

Interestingly, in the probiotic and ascorbyl polyphosphate combined group the level of various immune parameters was not significantly enhanced compared to the individual probiotic and ascorbyl polyphosphate treated groups. This might be due to the poor establishment of the *B. subtilis* in the gastrointestinal tract of Common carp fingerlings because the co-administration of vitamin C along with probiotic bacterium might have promoted the growth of other gut bacteria, which either utilized or reduced the ascorbyl polyphosphate.

Therefore, dietary supplementation of *B. subtilis* was found to be better in comparison to other types of treatments for use in aquaculture.

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