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Dietary Multispecies Probiotic Supplementation Enhances the Immunohematological Responses and Reduces Mortality by *Aeromonas hydrophila* in *Labeo rohita* Fingerlings

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Abstract

A study was conducted to determine the immunomodulatory effect of probiotic mixture on the nonspecific immune response and disease resistance of *Labeo rohita*. Fish were fed with six different test diets with or without probiotics, namely, T1 (basal feed [BF] without probiotics), T2 (BF + *Bacillus subtilis* [BS] + *Lactococcus lactis* [LL]), T3 (BF + LL + *Saccharomyces cerevisiae* [SC]), T4 (BF + BS + SC), T5 (BF + BS + LL + SC), and T6 (BF + heat-killed bacteria of BS + LL + SC). During the prechallenge period, significantly higher ($P < 0.01$) nitroblue tetrazolium assay (NBT), red blood cells (RBC), hemoglobin (Hb), globulin, and albumin content were recorded in the probiotic-supplemented diet group. Feeding all the three probiotics significantly decreased ($P < 0.01$) the serum aspartate aminotransferases (AST), alanine aminotransferases (ALT), acid phosphatase (ACP), and alkaline phosphatase (ALP) activity and increased the myeloperoxidase (MPO) and immunoglobulin M (IgM) value. Significant difference in the NBT, Hb, serum albumin, and MPO was noticed in both prechallenged and postchallenged fish. Lowest survivability was recorded in the nonprobiotic fed group, followed by the group fed with heat-killed probiotics, whereas the highest survivability was noticed in the group fed with all the three probiotics in live form. Thus, a probiotic mixture diet constituting of three probiotics in viable form is found to be optimum to enhance the immunity and postinfection survivability in *L. rohita* fingerlings.

The aquaculture boom in the recent years can be owed to good management practices and improved husbandry. Intensification of aquaculture practices has, however, led to the outbreak of many diseases, thereby necessitating the use of broad-spectrum chemicals, drugs, and antibiotics, for better health management. This indiscriminate usage of antibiotics has led to bacterial resistance (Cabello 2006), toxicity, and bioaccumulation in fish and environment (Vine et al. 2004). Recently, probiotics and immunostimulants have become a useful alternative to chemotherapy and antibiotics in controlling fish diseases. Probiotics are live microbial feed supplements that beneficially affect the host by producing inhibitory compounds, competing for chemicals and adhesion sites, and modulating and stimulating immune function (Giri et al. 2012). Probiotics are also known to enhance the specific and nonspecific immune responses in fish (Nayak 2010). In the aquaculture industry, probiotics species of *Bacillus* (Balcazar et al. 2004; Keysami et al. 2007), *Lactobacillus*

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(Abraham et al. 2007), and *Saccharomyces* (Rumsey et al. 2007), singly or mixed cultures (Salinas et al. 2005; Aly et al. 2008; Mohapatra et al. 2012a, 2012b), are most commonly used.

Bacterial infections are considered to be the most common cause of fish mortality in aquaculture. The motile aeromonads, especially *Aeromonas hydrophila*, affects a wide variety of freshwater as well as marine fish species (Chu and Lu 2005; Zhou et al. 2010a). *A. hydrophila* is known to cause hemorrhagic septicemia in fish along with ulcers, exophthalmia, abdominal distention, and lesions in the gills and opercula (Austin and Austin 1987; Giri et al. 2012).

Probiotics, namely *Bacillus subtilis* (Kumar et al. 2006) and *Saccharomyces cerevisiae* (Pal et al. 2007), when used singly, are known to reduce the diseases caused by *A. hydrophila*. Kumar et al. (2008) documented the immunostimulatory effect of *B. subtilis* (10^8 cfu/g) on being challenged with an intraperitoneal injection of *A. hydrophila*. The probiotic, *Lactococcus lactis* RQ516, was also found beneficial for tilapia in terms of increasing final weight as well as enhancing immune responses by reducing the adhesion capacity of *A. hydrophila* to the host intestinal mucus (Zhou et al. 2010a). Fish fed with the live Baker's yeast (*S. cerevisiae*) showed evidence of growth promotion and low mortalities after an intraperitoneal challenge of *A. hydrophila* (Pal et al. 2007; Tewary and Patra 2011). However, to date, no detailed study has been undertaken to study the effect of a combination of probiotics to fight against *A. hydrophila* infection.

Freshwater aquaculture in India is dominated by the Indian major carps, namely, *L. rohita, Catla catla*, and *Cirrhinus mrigala*, which contribute nearly 87% of the total freshwater production (ICLARM 2001; Das et al. 2005). Among the carps, *L. rohita* is the most widely cultured species in the country (over 70% of the total carp production) and has the highest consumer preference. In order to realize the complete growth potential of fish, it is necessary to prepare a nutritionally balanced supplementary feed, preferably incorporated with probiotics, which not only facilitates faster growth rate but also improves its immune status. Considering these facts, this study was designed to investigate the effects of dietary administration of three probiotic microorganisms, namely, *B. subtilis*, *L. lactis*, and *S. cerevisiae,* in different combinations on the hemato-immunological parameters and histopathology of *L. rohita* and its resistance against *A. hydrophila*.

Materials and Methods

Experimental Fish and Design

L. rohita fingerlings were procured from Palghar fish farm, Maharashtra, India, for the experimental study. The fishes were acclimatized to the laboratory conditions for 15 days in five 500-L capacity fiber-reinforced plastic tanks with continuous aeration. During acclimatization, the fish were fed with a fish meal-based practical diet (350 g protein/kg diet and 4200 kcal/kg dietary gross energy). The physicochemical characteristics of the water were as follows: temperature (25.6–26.4 C); hardness (156–185 mg/L) (as $CaCO_3$); pH (7.4–7.6); and dissolved oxygen concentration (5.8–6.9 mg/L) (Mohapatra et al. 2012a). Five hundred and forty fish (average weight 6.00 ± 0.06 g) were equally distributed in six dietary treatment groups (T1 [basal feed without probiotics], T2 [basal feed + *B. subtilis* and *L. lactis*], T3 [basal feed + *L. lactis* and *S. cerevisiae*], T4 [basal feed + *B. subtilis* and *S. cerevisiae*], T5 [basal feed + *B. subtilis*, *L. lactis,* and *S. cerevisiae*], and T6 [basal feed + heat-killed bacteria of *B. subtilis*, *L. lactis*, and *S. cerevisiae*]) with three replicates each (stocking density of 30 fish per tank in 300 L of rearing water), following a completely randomized design (CRD). Fresh feeds were prepared in 15-day intervals to maintain the bacterial count at the desired level. The fish were fed *ad libitum* to apparent satiation four times daily at 0800, 1200, 1500, and 1800 h (Mohapatra et al. 2011, 2012a) at a feeding rate of 2% average body weight for a period of 60 days. The leftover feed and fecal matter were siphoned out daily and 50% water exchange was carried out carefully to avoid stress to the fish.

Preparation of Probiotic Microorganisms and Feed Used for the Study

Pure bacterial strain of *B. subtilis*, *L. lactis*, and *S. cerevisiae* were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India, and maintained at 4 C in the laboratory. These strains were respectively inoculated into test tubes containing brain heart infusion (BHI), de Man Rogosa and Sharpe (MRS), and yeast extract peptone dextrose (YEPD) broth (Himedia, Mumbai, India) and kept in a shaking incubator at 30 C for 24 h. After that, a loopful of the bacterial culture was streaked on the respective Agar media, that is, BHI Agar, MRS Agar, and YEPD Agar (Himedia). The colonies were confirmed as pure isolates of *B. subtilis*, *L. lactis*, and *S. cerevisiae* by performing the essential biochemical tests for confirmation and the cultures were used for mass culture for subsequent use in the experiment. For mass culture, freshly grown pure inoculums of *B. subtilis, L. lactis*, and *S. cerevisiae* was added to 100 mL of BHI, MRS, and YEPD medium, respectively, in a 500-mL conical flask, and incubated at 30 C for 24 h in a shaking incubator. The cultures were centrifuged at 14,000 *g* for 15 min at 4 C. The supernatant was discarded, while the pellets were resuspended in phosphate buffer saline (PBS) (pH 7.2). The bacterial pellets were washed (three times) and resuspended in PBS. The bacterial colony-forming unit (cfu) was calculated by optical density $(OD)_{600}$ and plate count method simultaneously. The data were plotted in graphs, obtaining the relationship cfu vs. OD_{600} vs. time. The concentration of all the three probiotics together was adjusted at 10^{11} cfu/kg feed. The feed was formulated by taking the nutrient requirement of *L. rohita* into consideration (Patra 2011) and prepared as mentioned in Mohapatra et al. (2012a). The different feed ingredients (fish meal [114 g/kg], soyabean oil cake [420 g/kg], rice polish [130 g/kg], wheat bran [110 g/kg], corn flour [100 g/kg], carboxymethyl cellulose [10 g/kg], sunflower oil [40 mL/kg], and cod liver oil [40 mL/kg]) were sieved and mixed properly, steamed for about 20 min, and cooled. The vitamin–mineral mix (30 g/kg), vitamin C (1 g/kg), vitamin B complex (1 g/kg), butylated hydroxytoluene (2 g/kg), glycine (2 g/kg), and the respective probiotic mixture (suspended in PBS) were added into the feed and made into uniform sized pellets.

Bacterial Challenge Study

A virulent strain of *A. hydrophila* was obtained from Aquatic Animal Health and Management Division, CIFE, Mumbai, India. Initially, the bacteria was grown in BHI broth and incubated at 30 C for 24 h in a biochemical oxygen demand (BOD) incubator (Biotechnics, Mumbai, India) and harvested by centrifuging the broth at 5000 *g* for 10 min at 4 C in a cooling centrifuge (Remi C-24, Mumbai, India). The supernatant was discarded and the pellet containing the bacteria was repeatedly washed (three times) with sterile PBS, pH 7.2 and resuspended in PBS. The final bacterial concentration was adjusted to 10^6 cfu/mL. After the prechallenged sampling, the experimental fish were intraperitoneally injected with 0.1 mL of bacterial suspension. Mortalities were recorded daily for 15 days. Dead fish were removed periodically and the tissues were used for bacteriological culture to confirm *A. hydrophila* as the cause of death. Fish in each tank were fed with the same experimental feed during this period as before challenge study.

The relative percent survival (RPS) was calculated in different experimental groups.

$$\text{Relative \% survival} = 1 - (\text{number of mortality in treatment group} / \text{number of mortality in control}) \times 100.$$

Hematological Parameters

At the end of the experimental period of 60 days, six fish from each treatment (two fish per replicate) were anesthetized with clove oil (50 μL/L), and blood was withdrawn from caudal vein using a coated 24-gauge glass syringe with 2.7% ethylenediaminetetraacetic acid (EDTA) solution and transferred to an EDTA-coated test tube. The samples were stored at −30 C until use.

The total erythrocyte count (TEC) and the total leukocyte count (TLC) were determined using a

Neubauer-type hemocytometer (LO-Laboroptic, Lancing, UK), using Toission's solution and Turk's solution as the respective diluting solution. Blood (20 µL/L) was mixed with 3980 µL of the corresponding diluting fluid in a clean test tube and shaken well to suspend the cells uniformly in the solution. The cells were counted using a Neubauer-type hemocytometer.

$$\text{Number of cells/mL} = (\text{number of cells counted} \times \text{dilution}) / (\text{area counted} \times \text{depth of fluid}).$$

The amount of hemoglobin (Hb) present in the blood was determined by estimating cyanmethemoglobin using Darbkin's Fluid (Qualigens Diagnostics Kit, Mumbai, India). Five milliliters of Drabkin's working solution was taken in a clean and dry test tube and 20 µL of blood was added to it. The absorbance was measured using a spectrophotometer (Merck, Gurgaon, India) at a wavelength of 540 nm. The final concentration was calculated by comparing with standard cyanmethemoglobin (Qualigens).

The nitroblue tetrazolium assay (NBT) was performed following modified standard protocol (Stasiak and Baumann 1996) to measure the superoxide ion production. Fifty microliters of blood was placed into the wells of "U" bottom microtitre plates and incubated at 37 C for 1 h to facilitate adhesion to the cells. Then the supernatant was removed and the loaded wells were washed three times in PBS. After washing, 50 µL of 0.2% NBT was added and was incubated for another 1 h. The cells were then fixed with 100% methanol for 3 min and again washed thrice with 30% methanol. The plates were then air-dried. Then 60 µL 2 N potassium hydroxide and 70 µL dimethyl sulfoxide were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue colored solution was then read in enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 540 nm.

Immunological Parameters

For serum analysis, another six fish from each group were anesthetized, and blood was collected without using any anticoagulant. The blood was allowed to clot for 2 h and centrifuged at 5000 *g* for 5 min at 4 C. The serum was collected in another dry eppendorf and kept at −20 C until use. Qualigens kit was used for the analysis of the different serum parameters. Total protein, albumin, alkaline phosphatase (ALP), acid phosphatase (ACP), aspartate aminotransferases (AST), and alanine aminotransferases (ALT) were some of the parameters that were analyzed.

The serum myeloperoxidase (MPO) activity was measured according to Quade and Roth (1997) with slight modification. About 10 µL of serum was diluted with 90 µL of Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in 96-well plates. Then, 35 µL of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB) (Himedia) and 5 mM hydrogen peroxide (H_2O_2) (Qualigens) (both substrates of MPO and prepared on same day) were added. The color change reaction was stopped after 2 min by adding 35 µL of 4 M sulfuric acid (H_2SO_4). The OD was read at 450 nm in a microplate reader (µQuant, Universal Microplate Spectrophotometer, Northstar Scientific, Leeds, UK).

The immunoglobulin level in the serum was determined by the ELISA assay (Swain et al. 2007). The serum samples in serial dilution with 1× tris buffered saline (TBS), pH 7.4 were coated into the 96-well flat-bottomed ELISA plates (Tarsons, Kolkata, India) at 50 µL/well. After 3 h of incubation at room temperature (25 ± 1 C), the plates were washed three times with TBST (TBS with 0.05% Tween 20) at 5 min interval. Blocking agent (5% skim milk powder in TBS) was added at 100 µL per well and incubated at 4 C over night. The plates were washed again with TBST for three times at 5 min interval. Anti-rohu Ig rabbit serum was then added at 1:5000 dilution (determined earlier by checkerboard titration method) in 50 µL volume to all the wells and incubated for 2 h at room temperature. The plates were washed again with TBST as mentioned above. Subsequently, goat anti-rabbit IgG HRPO (horseradish peroxidase) conjugate (Genei, Bangalore) was added to the wells at 1:2000 dilution as specified by the manufacturer. After 2 h of incubation at room temperature and washing with TBST, 100 µL of

TABLE 1. *Dietary probiotics combinations on RBC, WBC, Hb content, and NBT of different experimental groups.*[a]

Parameters		Treatments					
		T1	T2	T3	T4	T5	T6
RBC ($\times 10^6$ cells/μL)	Prechallenge	1.08 ± 0.05^{dA}	1.08 ± 0.03^{dA}	1.31 ± 0.06^{cA}	1.35 ± 0.05^{cA}	2.01 ± 0.03^{a}	1.68 ± 0.02^{bA}
	Postchallenge[b]	0.57 ± 0.03^{dB}	0.44 ± 0.07^{dB}	0.79 ± 0.07^{bcB}	0.89 ± 0.11^{bB}	1.81 ± 0.14^{a}	0.78 ± 0.03^{bcB}
WBC ($\times 10^6$ cells/μL)	Prechallenge	2.39 ± 5.85^{B}	2.56 ± 5.13^{B}	2.52 ± 5.60^{B}	2.53 ± 0.65^{B}	2.43 ± 0.85^{B}	2.46 ± 1.65^{B}
	Postchallenge[b]	3.00 ± 1.8^{eA}	3.07 ± 1.0^{deA}	3.77 ± 5.25^{bA}	3.58 ± 3.30^{cA}	4.49 ± 1.95^{aA}	3.13 ± 1.55^{dA}
Hemoglobin (g/dL)	Prechallenge	2.85 ± 0.05^{dA}	2.65 ± 0.15^{d}	4.40 ± 0.10^{bA}	4.50 ± 0.10^{bA}	6.05 ± 0.05^{a}	3.85 ± 0.05^{cA}
	Postchallenge[b]	1.85 ± 0.05^{eB}	2.40 ± 0.10^{d}	3.40 ± 0.20^{cB}	3.80 ± 0.10^{cB}	5.85 ± 0.05^{a}	3.45 ± 0.05^{bB}
Respiratory burst activity (NBT) (A_{540})	Prechallenge	0.20 ± 0.01^{c}	0.35 ± 0.03^{bA}	0.36 ± 0.02^{bA}	0.47 ± 0.09^{aA}	0.48 ± 0.03^{aA}	0.20 ± 0.01^{c}
	Postchallenge[b]	0.16 ± 0.02^{d}	0.25 ± 0.01^{bcB}	0.16 ± 0.02^{dB}	0.28 ± 0.02^{bB}	0.35 ± 0.01^{aB}	0.21 ± 0.01^{cd}

Hb = hemoglobin; NBT = nitroblue tetrazolium assay; RBC = red blood cells; WBC = white blood cells.

[a]Values with different lowercase superscripts (a, b, c, d, e) in the same row are significantly different ($P < 0.01$). Values with different uppercase superscripts (A, B) in the pre- and postchallenge groups in the same column for each parameter of the treatments are significantly different ($P < 0.01$).

[b]Postchallenge: challenged the fish with *Aeromonas hydrophila* in the last 15 days of experiment (from 60 to 75 days of feeding).

substrate, TMB/H_2O_2 (Genei, Bangalore, India) was added to all the wells. The color reaction was stopped by adding 1 N H_2SO_4 immediately to all the wells at 50 μL per well. The absorbance was read at 450 nm in an ELISA reader.

Histological Analysis

The gill and intestinal sections of three fish from each replicate were dissected and immediately fixed in neutral-buffered formalin (NBF). The tissue samples were embedded in paraffin wax, cut at 5 μm, and stained with hematoxylin and eosin (H&E), as described by Mohapatra et al. (2011). Stained slides were examined and photographed under a light microscope.

Statistical Analysis

Data were analyzed by one-way ANOVA and the significant difference between the treatments was determined by Duncan's Multiple Range Test (DMRT) using SPSS (Version 16.0). A significance level of $P < 0.01$ was used.

Results

Hematological Parameters

The TEC, TLC, and Hb content of *L. rohita* fingerlings are shown in Table 1. There was a significant difference ($P < 0.01$) in the TEC among the different treatment groups before the challenge with *A. hydrophila*. Significant reduction in erythrocyte count in the postchallenged fish was noticed in the control group. Significantly higher count was recorded in the T5 group fed with the diet supplemented with all the three probiotics in the pre- and postchallenge period. Similar to erythrocyte count, Hb also showed a similar trend.

Dietary probiotics had no significant effect on the TLC in the prechallenged fish. However, a significant increase in the leukocyte count was observed in the postchallenged fish than its prechallenged counterparts.

Respiratory Burst Activity

The respiratory burst activity was significantly influenced by the probiotic supplementation in the diet. The NBT activity of the neutrophils of *L. rohita* fingerlings was significantly ($P < 0.01$) higher in the treatment group T5, during both the pre- and postchallenge period (Table 1). No significant difference was observed in the treatment groups T1 and T6 in both the pre- and postchallenged fish.

Immunological Parameters

There were significant differences in the serum total protein and globulin content among the different experimental groups before and after the challenge study (Table 2). The probiotics fed groups showed significantly higher total protein and globulin content in both the pre- and postchallenge period. A significant decrease in the serum total protein and globulin was noticed after the *A. hydrophila* challenge in both the pre-

TABLE 2. *Serum chemistry of* Labeo rohita *fingerlings fed with different combination of probiotics.*[a]

Parameters		Treatments					
		T1	T2	T3	T4	T5	T6
Serum protein (mg/dL)	Prechallenge	1.95 ± 0.15^{cA}	2.55 ± 0.15^{bA}	2.60 ± 0.20^{b}	2.55 ± 0.25^{bc}	3.25 ± 0.25^{aA}	2.20 ± 0.10^{cA}
	Postchallenge[b]	1.40 ± 0.10^{cdB}	1.50 ± 0.10^{cB}	2.10 ± 0.20^{b}	2.10 ± 0.15^{b}	2.48 ± 0.15^{aB}	1.40 ± 0.20^{dB}
Serum albumin (mg/dL)	Prechallenge	0.68 ± 0.04^{b}	0.72 ± 0.07^{b}	0.89 ± 0.05^{aA}	0.71 ± 0.03^{bA}	0.87 ± 0.03^{aA}	0.73 ± 0.01^{bA}
	Postchallenge[b]	0.50 ± 0.06^{c}	0.66 ± 0.01^{a}	0.65 ± 0.05^{abB}	0.58 ± 0.03^{bcB}	0.65 ± 0.01^{aB}	0.44 ± 0.01^{cdB}
Serum globulin (mg/dL)	Prechallenge	1.12 ± 0.28^{dA}	1.81 ± 0.22^{cA}	1.71 ± 0.15^{c}	2.03 ± 0.29^{bA}	2.55 ± 0.23^{aA}	1.45 ± 0.19^{dA}
	Postchallenge[b]	0.57 ± 0.09^{dB}	0.83 ± 0.11^{cB}	1.40 ± 0.25^{b}	1.52 ± 0.19^{abB}	1.75 ± 0.08^{aB}	0.82 ± 0.19^{cdB}
Serum A : G ratio	Prechallenge	0.64 ± 0.06^{aB}	0.40 ± 0.09^{cB}	0.52 ± 0.02^{ab}	0.35 ± 0.08^{c}	0.33 ± 0.03^{c}	0.50 ± 0.03^{a}
	Postchallenge[b]	0.87 ± 0.09^{aA}	0.80 ± 0.11^{aA}	0.41 ± 0.10^{b}	0.38 ± 0.02^{c}	0.37 ± 0.03^{c}	0.53 ± 0.08^{b}
Serum AST (IU/L)	Prechallenge	40.25 ± 4.30^{aB}	26.70 ± 1.60^{bB}	39.91 ± 1.05^{aB}	40.12 ± 1.05^{aB}	16.00 ± 4.85^{dB}	18.69 ± 0.10^{cB}
	Postchallenge[b]	65.65 ± 6.50^{bA}	40.62 ± 8.05^{eA}	52.95 ± 1.50^{dA}	61.52 ± 5.45^{cA}	26.29 ± 7.38^{fA}	68.39 ± 5.75^{aA}
Serum ALT (IU/L)	Prechallenge	20.70 ± 0.62^{aB}	18.07 ± 0.17^{bB}	14.77 ± 0.21^{cdB}	13.82 ± 0.26^{dB}	10.43 ± 0.34^{eB}	15.76 ± 0.22^{bcB}
	Postchallenge[b]	26.21 ± 0.23^{bcA}	25.20 ± 0.45^{cA}	28.06 ± 0.13^{aA}	26.55 ± 0.47^{bA}	21.38 ± 0.41^{dA}	21.18 ± 0.31^{dA}
Serum ACP (IU/L)	Prechallenge	27.80 ± 1.49^{B}	25.18 ± 0.85^{B}	22.91 ± 2.35	22.22 ± 0.72^{B}	21.01 ± 1.46	27.46 ± 1.75^{B}
	Postchallenge[b]	44.99 ± 3.42^{aA}	36.44 ± 2.10^{bcA}	32.24 ± 0.34^{c}	30.15 ± 1.08^{cdA}	23.85 ± 2.02^{d}	42.76 ± 1.49^{abA}
Serum ALP (IU/L)	Prechallenge	134.36 ± 4.52^{bB}	165.04 ± 0.45^{a}	156.40 ± 4.47^{a}	172.44 ± 6.47^{a}	123.37 ± 7.23^{b}	167.37 ± 9.49^{a}
	Postchallenge[b]	182.23 ± 3.31^{bA}	179.87 ± 3.64^{b}	171.13 ± 4.58^{b}	179.12 ± 6.67^{b}	137.89 ± 2.29^{c}	197.14 ± 1.81^{a}
Serum MPO ($OD_{450/nm}$)	Prechallenge	0.71 ± 0.02^{eB}	1.00 ± 0.01^{dB}	1.79 ± 0.03^{bB}	1.68 ± 0.03^{bB}	4.00 ± 0.11^{aB}	1.44 ± 0.11^{cB}
	Postchallenge[b]	1.00 ± 0.01^{eA}	1.67 ± 0.11^{dA}	2.68 ± 0.01^{cA}	3.15 ± 0.17^{bA}	5.08 ± 0.08^{aA}	3.17 ± 0.05^{bA}
Serum IgM	Prechallenge	0.113 ± 0.001^{eA}	0.194 ± 0.005^{cA}	0.160 ± 0.004^{dA}	0.218 ± 0.002^{bA}	0.283 ± 0.003^{aA}	0.191 ± 0.002^{cA}
	Postchallenge[b]	0.066 ± 0.002^{eB}	0.162 ± 0.003^{cB}	0.123 ± 0.001^{dB}	0.176 ± 0.001^{bB}	0.239 ± 0.007^{aB}	0.151 ± 0.001^{cB}

ACP = acid phosphatase; ALT = alanine aminotransferases; ALP = alkaline phosphatase; AST = aspartate aminotransferases; IgM = immunoglobulin M; MPO = myeloperoxidase.

[a]Values with different lowercase superscripts (a, b, c, d, e) in the same row are significantly different ($P < 0.01$). Values with different uppercase superscripts (A, B) in the pre- and postchallenge groups in the same column for each parameter of the treatments are significantly different ($P < 0.01$).

[b]Postchallenge: challenged the fish with *Aeromonas hydrophila* in the last 15 days of experiment (from 60 to 75 days of feeding).

and postchallenge groups. Globulin content of the postchallenged fish was significantly lower than the prechallenged fish fed with probiotics, both the active and heat-inactivated probiotic. The A : G ratio was increased in the postchallenge period than the prechallenged period. The A : G ratio differed significantly ($P < 0.01$) on probiotic feeding in both pre- and postchallenged fish, with maximum value being recorded in T1 and minimum in T5.

Significant effect ($P < 0.01$) of probiotic supplementation was found on the serum AST and ALT in both pre- and postchallenged fish. Increased serum AST and ALT values were recorded in the postchallenged groups with the least value in T5. Probiotic inclusion resulted in a lower serum ACP and ALP level in all the prechallenged probiotic fed groups; however, after bacterial infection, the postchallenged fish showed higher values than the prechallenged fish. There was no significant difference between the pre- and postchallenged fish in the serum ALP except in the nonprobiotic fed group (T1).

MPO Activity

The polynuclear granulocyte content in serum was measured in terms of MPO activity (Table 2). Significantly higher ($P < 0.01$) MPO activity was recorded in all the treatment groups than the control group. Surprisingly, the fishes fed heat-inactivated probiotics also showed similar increase in MPO activity along with the other probiotic-supplemented groups.

Production of Immunoglobulin (IgM)

The total serum IgM was significantly higher ($P < 0.01$) in all probiotic-supplemented groups in comparison with control in the prechallenged fish (Table 2). After bacterial infection, reduced IgM production was recorded in all the groups. However, the treatment group T5 showed the highest IgM production among all other treatment groups.

Histo-Architectural Analysis of Pre- and Postchallenged Fish

To understand the role of active/inactive probiotics on fish health, histological analysis of the intestine and gill of both pre- and postchallenged fish were performed. Among prechallenged fish, the intestinal sections of the both control and probiotic (active) fed fishes showed no apparent alterations in the lamina propria (Fig. 1A, B). However, after the bacterial challenge, there were marked histological changes in the intestinal tissues. The intestinal sections of T1 (Fig. 1D) and T6 (Fig. 1F) showed extensive damage in the intestine with abundance of bacterial colonies. But the severity of infection was less in probiotic fed fish (T5). The intestines had distended lumen and large amount of vacuolation in the lamina propria (data not shown).

On the other hand, gills from T1 and T6 (Fig. 1G, I) demonstrated mild to moderate degeneration of secondary gill filament, whereas the other groups appeared to be normal in the prechallenge group. Similar to intestinal data, noticeable changes were observed in postchallenged fish. The fish from T1 and T6 showed complete loss of primary and secondary gill filaments but other groups, especially T5 (Fig. 1K) had moderate loss of secondary gill filaments.

Survivability

No mortality was observed during the feeding trial (60 days) before the challenge study. The first mortality was observed on the third day of *A. hydrophila* infection. The relative percentage survival is presented in Fig. 2. Mortality was recorded up to 7 days after injection. The highest survivability was recorded in the group fed with active form of probiotics in different combinations and the lowest survival in the control.

Discussion

In recent years, the use of beneficial probiotic bacteria has been gaining recognition for controlling pathogens within the aquaculture industry (Irianto and Austin 2002). In this study, we have characterized the hemato-immunological effect of dietary addition of different probiotic microorganisms used in various combinations (either live or heat-killed form) in *L. rohita* fingerlings. We observed a significantly better hemato-immunology with increased NBT and MPO activity and enhanced

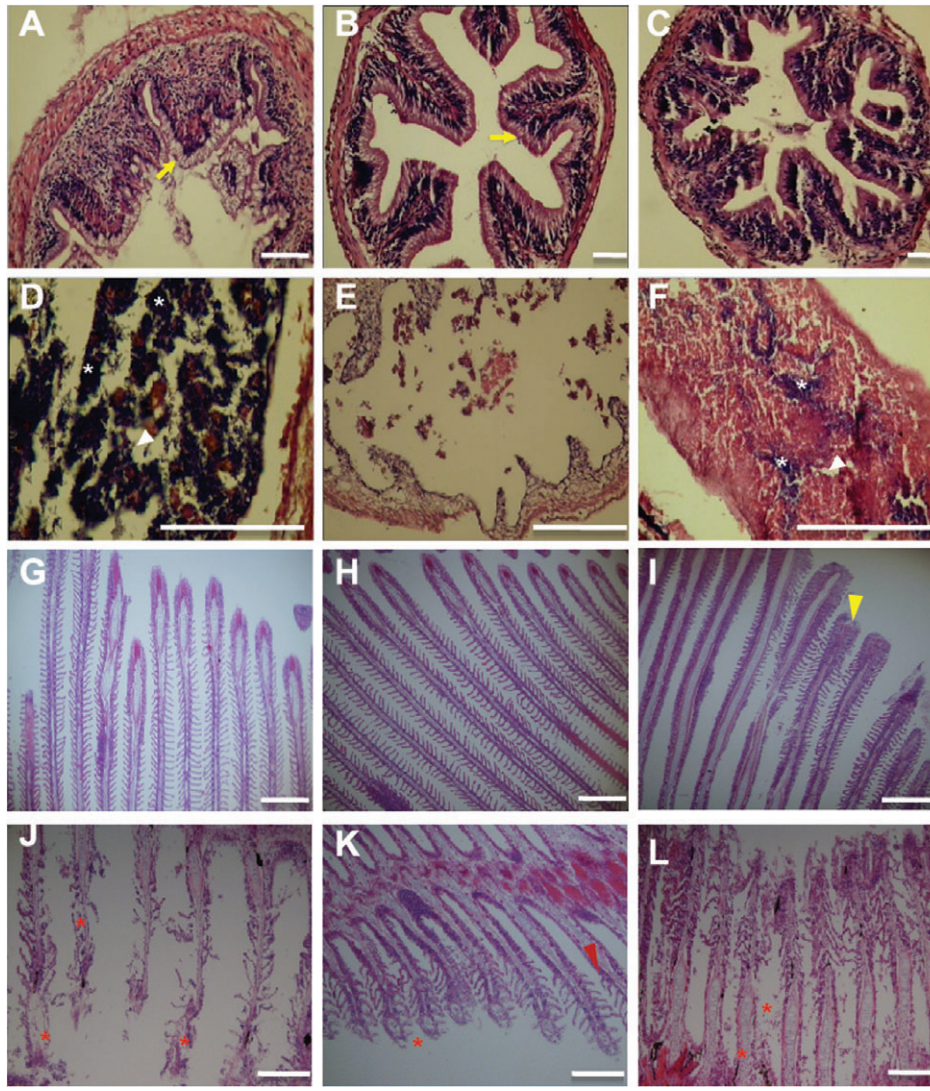

FIGURE 1. *Intestinal and gill architecture of* Labeo rohita *fed with different composition of probiotics. Prechallenged intestine: T1 (A), T5 (B), T6 (C); postchallenged intestine T1 (D), T5 (E), T6 (F); prechallenged gill: T1 (G), T5 (H), T6 (I); postchallenged gill: T1 (J), T5 (K), T6 (L). Before bacterial infection, the intestine of T1 (A) and T5 (B) had active lamina propia (yellow arrows) and abundant goblet cell. After challenge, both T1 (D) and T6 (F) had severe necrosis (white arrowhead) with probable pathogenic bacteria prevailing in intestine (white asterisk), but T5 (E) had comparatively less effect (vacuolation in lamina propia and distension of lumen). On the other hand, in the prechallenged groups (G–I) and stumpy looking secondary lamellae (yellow arrowheads) in gills of T1 (G) and T6 (I). Severe necrosis and complete loss of primary and secondary gill filament were prevalent in T1 (J) and T6 (L) after infection (red asterisk). But T5 showed better protective characteristics and several secondary filaments remained intact (red arrowhead). Bar = 50 μm.*

immunoglobulin production in probiotic fed fishes when compared to control group, even after pathogenic infection. The probiotic treated fish also showed better intestinal and gill histology than the nontreated groups.

Hematological and physiological parameters are typically used to assess the health status of fish and detect physiological changes (Atamanalp and Yanik 2003; Reyes-Becerril et al. 2011). Fish phagocytes, upon activation, are

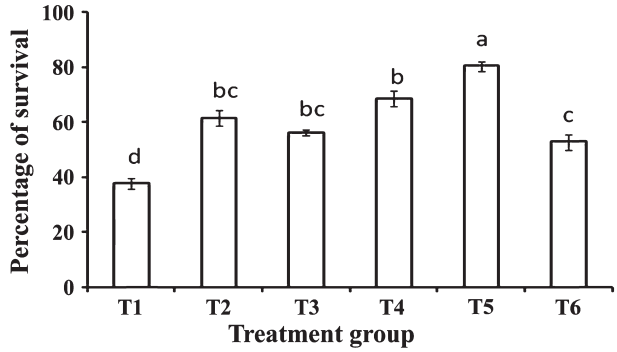

FIGURE 2. *Relative percent survival of* Labeo rohita *fingerlings following an* Aeromonas hydrophila *postchallenge infection. Values in graph indicating the mean ± SE (*N = *3). Similar letters in the graphs denote nonsignificance (*P < *0.01).*

able to produce superoxide anion (O_2^{-1}) and its reactive derivates (H_2O_2 and hydroxyl radicals) during a period of intense oxygen consumption (popularly known as NBT activity) (Secombes 1996; Zhou et al. 2010b). These reactive oxygen species (ROS) are considered to be toxic to fish pathogens and can be correlated with increased bacterial pathogen killing activity of phagocytes (Sharp and Secombes 1993; Zhou et al. 2010a). Nikoskelainen et al. (2003) found that dietary lactic acid bacterium *L. rhamnosus* at a level of 8×10^4 cfu/g feed can enhance the NBT activity in rainbow trout. The highest NBT value was observed in the group fed with three live probiotics supplementation suggesting better phagocytic activity and higher protection against pathogenic infection. Highest NBT value was also observed in T5 group among pathogen-contaminated groups. Salinas et al. (2006) reported an increased NBT activity in *Sparus aurata* fed with heat-inactivated *L. delbrueckii*.

Although no changes were observed in phagocytic activity, we observed a significant difference in bloodlets (white blood cells [WBC] and red blood cells [RBC] numbers) in fish fed with heat-inactivated microbes, suggesting the enhancement of nonspecific immune response (Christybapita et al. 2007). This hypothesis was further supported by the data obtained from T5 group, where the postchallenged fish showed highest number of WBC. Moreover, during oxidative respiratory burst, the azurophilic granules of neutrophils release the MPO, to utilize one of oxidative radicals and produce hypochlorous acid with ability to kill pathogens. In our study, MPO activity was increased after probiotic addition, which was further accelerated by pathogen infection. A similar pattern of increased MPO activity was also recorded in both pre- and post-*A. salmonicida* infection periods in rainbow trout fed with yeast-supplemented (*S. cerevisiae*) diet (Siwicki et al. 1994). This indicates that the modulation of MPO activity was one of the most immediate and key effects produced by probiotic bacteria on the host immune system (Zhou et al. 2010b). On the other hand, increased RBC count and Hb contents are also associated with probiotic supplementation-related disease resistance (Marzouk et al. 2008). In this study, increased amount of leukocyte and Hb was observed after multispecies probiotic supplementation, indicating an elevated immune status and disease resistance (Kumar et al. 2006). Taken together, our hematological data suggest that multispecies probiotic supplementation, depending on type of organism used (dead/alive and species of microorganism), can induce both specific and nonspecific immune system in order to enhance the protection against pathogens.

Serum proteins and A : G ratio are measures of various humoral elements of the nonspecific immune defense system (Gupta et al. 2008). Nayak et al. (2007) reported the positive role of *B. subtilis* on increased serum protein and globulin contents when challenged against *Edwardsiella tarda,* thereby suggesting the

immune-enhancing effects of *B. subtilis*. In this experiment, increase in serum protein and globulin was recorded in all fish fed with different live probiotics in both the pre- and postchallenge period. Decreased A : G ratio (indicator of fish immunity) value was recorded in the fish fed with different combination of probiotics, suggesting uplifted immune status of fish. Immunoglobulins, especially IgM, play an important role in pathogen recognition and activation of innate immune system via the classical pathway system of complement activation (Shoemaker et al. 2005; Reyes-Becerril et al. 2011).

In this study, we observed higher IgM value in the probiotic supplemented with all three probiotics, even after the *A. hydrophila* challenge. In agreement with our result, Salinas et al. (2008) have also reported that *B. subtilis* and *L. delbrueckii lactis* in combination can increase the numbers of IgM+ cells in *S. aurata* juvenile within 3 weeks of supplementation while individually both the probiotic strains failed to induce any such change. This uplifted IgM level may also be associated with immunostimulants produced (e.g., β-glucan from yeast) from both the viable and nonviable form of probiotics (Panigrahi et al. 2005; Donate et al. 2010). Hence, it can be very well said that probiotics act as immunomodulators as they can enhance the IgM levels in fish (Nikoskelainen et al. 2003; Reyes-Becerril et al. 2011). Enhanced immune conditions or elevated immunomodulators can subsequently reduce the stress in fish (Nayak 2010). We also observed a significant decrease in AST and ALT activity, which suggests reduced cortisol-induced protein catabolism (Freeman and Idler 1973), hence less stress condition in the probiotic-supplemented group.

The intestine is the primary target organ of orally administered probiotics (Picchietti et al. 2009). Destruction of goblet cell reduces the mucus production and further weakens the barrier in the intestine, which may further proceed to necrosis and death. The major role of probiotics is to compete with the pathogens for attachment in these sites (Vine et al. 2004) and enhance the mucosal immunity. Our histology data from intestine depict an altered lamina propria and other damages (probably necrotic) after pathogenic infection suggesting variable pathogenic bacteria colonization in the intestine. In our study, we also obtained comparatively lesser damage because of pathogenic bacteria in probiotic (active) fed fish than the control group. It is quite evident from our data that activated forms of probiotics can effectively obstruct the attachment of the pathogen to the intestinal wall.

Unlike Ouwehand et al. (2000), our data suggest that heat-inactivated probiotics failed to restrict the cell damage after pathogenic infection, which might be due to the loss of useful characteristics particularly the colonization capacity of the microbes (Mohapatra et al. 2012a). Panigrahi et al. (2005) also reported that the probiotic strain of *L. rhamnosus* in viable form is a better immune inducer compared to its heat-inactivated form. Gill is one of the major portals for pathogenic invasion in fish (Ringø et al. 2007; Cerezuela et al. 2012) because of its direct exposure to the external environment. Our histological data show that even control fishes in prechallenged group have some secondary lamellae degeneration, which might be associated with other contaminants (Mohapatra et al. 2011) or increased localized stress-induced cell damage (Mohapatra et al. unpublished data). Several workers have reported that oral administration of *B. subtilis* (Irianto and Austin 2002), *L. acidophilus* (Aly et al. 2008), and yeast (Siwicki et al. 2010) is helpful in providing sufficient protection to the vital organs against the toxins produced by *A. hydrophila*. However, further detailed analysis of probiotic-induced necrosis resistance mechanism might shed more light on this matter.

Interestingly, after *A. hydrophila* infection, we documented highest survivability in T5 (fed with all three probiotics) group, while lowest in T1 (no probiotic control diet). This difference in survivability might be associated with elevated hemato-immunological condition in the probiotic fed fish. This is in agreement with the findings of Nayak et al. (2007) and Kumar et al. (2008) who obtained highest survivability in probiotic fed *L. rohita* after being administered with a virulent strain of *A. hydrophila*. However, other dietary combinations of probiotics (T2,

T3, T4, and T6) did not affect the survivability, although significant differences were observed in the hemato-immunological parameters. This might be associated with maintaining survivability threshold by varying levels of immune enhancement and further health improvement.

This result demonstrates that incorporation of multispecies probiotics (at equal concentration) in the diet of *L. rohita* is more effective in enhancing the health status of the animal in comparison to the monospecies probiotic incorporation in the diet as the different probiotics complement each other by occupying different niches within the gut microflora environment, resulting in better survival, growth, viability, and adhesion capacity (Timmerman et al. 2004; Salinas et al. 2005; Mohapatra et al. 2012a).

From this study, it is concluded that the use of combination of two bacteria and one yeast (1:1:1 ratio) as probiotic in the live form with a final concentration of 10^{11} cfu/kg resulted in better immunity and postinfection survivability of *L. rohita*. The results suggest that a variety of different probiotic microorganisms in the live form when used in combination elicit better immune response in fish. However, further studies are required to optimize the exact dose of each probiotic to be incorporated in combination in the diet for better health and immunity of fish.

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