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Dietary Probiotic Supplementation on Hematological and Immunological Parameters of Indian Major Carp (*Labeo rohita*)

J. Priyadharshini [a++], I. Viji Margaret [b#*], M. Barakath Nisha [c++] and R. Ajaz Haja Mohideen [d†]

[a] Zoology Department and Research Centre, Sarah Tucker College (Autonomous), Tirunelveli, Affiliated to Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu, India.
[b] PG Department of Zoology, Sarah Tucker College (Autonomous), Tirunelveli, Tamil Nadu, India.
[c] Sri Paramakalyani Centre for Excellence in Environmental Sciences, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu, India.
[d] PG and Research Department of Zoology, C. Abdul Hakeem College (Autonomous), Ranipet, Tamil Nadu, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

A 60-day feeding trial was conducted to evaluate the influence of dietary supplementation of probiotic bacteria (Lactobacillus acidophilus) to improve the hematological and immunological parameters of Indian Major carp Rohu (*Labeo rohita*). The rohu Fingerlings (mean body weight

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[++] Research Scholar (Reg No. 21111242192007);
[#] Assistant Professor and Head;
[†] Assistant Professor;
**Corresponding author: Email: vijisino@gmail.com;*

6.0±0.6g) were randomly distributed into Four treatment groups each having 10 fishes fed with basal diet and a probiotic supplement containing *L. acidophilus* (1.0×107 CFU/g), then challenged with *A. hydrophila* (0.1 × 107 CFU/mL) was injected intraperitoneally with 0.1ml concentration of 107 CFU/ml on 55th day. After 72 hours, the blood samples were collected from five fish from each group. The hematological parameters such as hemoglobin (Hb; g/dL), red blood cell (RBC; ×106/mm3), white blood cell (WBC; ×103/mm3), and Packed corpuscular volume (PCV) along with immunological parameters of total immunoglobulin, serum lysozyme activity, and Aminotransferases were examined and compared with the control group. The fish fed with probiotics showed significant improvement in hematological and Immunological Parameters (P≤0.05) as compared to control. This study proved that probiotic bacteria *L. acidophilus* are resistant to pathogenic *A. hydrophila* and can contribute to improving the immune system, survival rate, and growth of experimental fish against the fish pathogen *A. hydrophila*.

Keywords: Labeo rohita; probiotics; L. acidophilus; hematological parameters; A. hydrophila.

1. INTRODUCTION

"Nutritionally and economically aquaculture has become the fastest-growing sector in the world, accounting for nearly 50 % of the world's current food fish" [1]. "More than 200 species of fish varieties are practiced by aquaculture worldwide for commercial purposes, thereby increasing economic activity in both developed and developing countries. Fish farming and export in India have been growing rapidly for the last two decades. In India, *Catla catla, Labeo rohita,* and *Cirrhinus mrigala* are the major freshwater carp, which are mainly consumed as a source of proteins and beneficial lipids" [2]. *Labeo rohita* is the most important commercial fish in India with maximum market demand and acceptability as food by consumers due to their taste and flesh.

" However, unpredictable mortalities caused by bacterial, fungal, and viral remain a major challenge to the aquaculture industry worldwide and have led to great economic losses in the fish culture industry every year" [3] and [4]. "The common fish pathogen *Aeromonas hydrophila* outbreaks cause high mortality rates in fish farming by hemorrhagic septicemia (acute form), skin ulcers, and underlying necrosis of the musculature (chronic form)" [5].

"Usually, antibiotics and Chemotherapeutic agents are used for disease management in aquaculture worldwide but there is a risk associated with the transmission of resistant pathogenic bacteria from aquatic environments to humans and other animals" [6]. "Therefore, many countries have restricted the use of antibiotics in aquaculture, and it is essential to develop new strategies to control fish diseases while being friendly to the environment. Currently, probiotics are considered a promising alternative to antibiotics when used in aquaculture to control diseases" [7].

"Probiotics have been widely used as dietary additives in aquaculture, with the characteristics of high security, improving growth, promoting nutrient digestion, enhancing host immunity, modulating the balance of intestinal microbiota, and improving the aquaculture environment" [8], [9] and [10].

A wide range of Gram-positive bacteria*, Bacillus, Carnobacterium*, Streptococcus*, Lactobacillus, Lactococcus,* and *Enterococcus* have been applied as probiotics to improve aquatic animal growth, survival, health, and disease prevention. However, several species of lactic acid bacteria can be considered as an important probiotic for the elevation of fish growth performance and immune responses in several studies. Therefore, this current study was conducted to investigate the *Lactobacillus-based* dietary probiotic supplementation influence on improving the hematological and immunological parameters of Indian major carp Rohu (*Labeo rohita*).

2. MATERIALS AND METHODS

2.1 Sample Collection

The experimental fish fingerlings (*Labeo rohita*) were purchased from a commercial aquaculture farm in Kallidaikurichi, Tirunelveli, Tamil Nadu, and immediately brought to the laboratory with an oxygenated polythene bag, were conditioned and acclimatized for 15 days in outdoor tanks (60 cm x 20 cm) under the flow-through condition and were fed with basal diet.

2.2 Experimental Setup

After acclimatization, a total of 40 fish were arbitrarily divided into four groups (average

weight of 6.0 ± 0.6g), each group contained 10 fish and was introduced into plastic troughs with a capacity of 20 liters of unchlorinated water with constant aeration and fed with basal diet and Probiotic supplement for 60 days (Table 1/ Pic.1). Fish was fed thrice daily (10:00 am, 2:00 pm, and 6:00 pm) with a feed amount of approximately 3% of body weight.

2.3 Preparation OF Experimental Diet

Experimental diets were formulated according to the nutritional requirements of *Labeo rohita* [11] and [13]. The percent composition basal diet formulation diet was determined by AOAC [12]: Fish meal- 10%, Soyabean powder-20%, Groundnut cake-20%, Rice bran-35%, wheat flour-10%, Cod liver oil-2.5% and Vitamin and mineral mixture-2.5%. The resultant feed was stored at 4^0C in sealed plastic Ziplock bags until used. To ensure the probiotic level in the supplemented diet, diets were prepared fresh at weekly intervals. The probiotic diet was composed of the basal diet with the inclusion of probiotic *Lactobacillus* at 10^{-7} CFU g−1 and sprayed on feed pellets with a hand sprayer for uniform distribution in the laminar airflow chamber under sterilized conditions. To ensure the probiotic level in the supplemented diet, diets were prepared fresh at weekly intervals [14].

2.4 Experimental Infectivity

On the 55th day, all experimental groups were then challenged with *A. hydrophila* (0.1 × 10^7 CFU/mL), except the control. Each fish in the groups was challenged with the series of serial dilutions against *A. hydrophila* isolate, grown overnight on tryptic soya broth (TSB) at 37°C, and cell suspension was prepared in phosphate buffer saline. Each fish was injected intraperitoneally with a 0.1ml concentration of 10^7 CFU/ml. Control fish were injected with 0.1ml of phosphate-buffered saline. Fish showing clinical symptoms were used for hematological and Immunological studies.

2.5 Collection OF Blood

“After 72 hrs of infection, the blood samples were obtained from the caudal blood vessels (v. caudalis) from 5 fish per treatment using a sterile syringe. A blood sample was divided into two parts; the first part was transferred into a 2-mL sterile test tube with EDTA for hematological assay and the second part was kept in a 2-mL plain Eppendorf tube for serum separation” [15]. “Blood was left to clot at 4°C for 60 min. After that, tubes were centrifuged at 3000 rpm using an Eppendorf centrifuge for 10 min for serum separation. The serum was collected in Eppendorf tubes and stored at -40 °C until analyses”. [15]

2.6 Hematological Examination

“The blood samples were evaluated by the following hematological measurement of hemoglobin (Hb; g/dL), red blood cell (RBC; $\times10^6$/mm^3), white blood cell (WBC; $\times10^3$/mm^3), and Packed corpuscular volume (PCV). A sub-set of blood samples was kept in Eppendorf tubes and added with 20 mM EDTA. Total RBCs and WBCs were counted using a Neubauer Hemocytometer by adjusted under a light microscope along with RBC diluting fluid and WBC diluting fluid respectively” [16]. “Hemoglobin concentration (Hb) was determined via using a hemoglobin reagent set (NICE Chem Diagnostics), according to the cyanomethaemoglobin method of Drabkin” [17]. Packed corpuscular volume (PCV) was determined by centrifugation at 2000 rpm for 20 min. A suitable quantity of whole blood mixed with an anticoagulant is centrifuged in a hematocrit tube until all blood cells are packed at the bottom of the tube. The percentage of the packed cell volume was determined by using the hematocrit tube reader.

$$\mathrm{WBC}(10^3/\mathrm{mm}^3) = \frac{\text{Total no. of cell in1 large square} \times \mathrm{df} \times \mathrm{cf}}{\text{Volume factor}(0.1)}$$

$$\mathrm{RBC}(10^6/\mathrm{mm}^3) = \frac{\text{Total no. of cell in 5 large squares} \times \mathrm{df} \times \text{depth factors}}{\text{No. of small square counted}} \times 16$$

*Where, df = dilution factor and cf = counting factor

Hematocrit (PCV) = (Height of RBCs in mm/Height of RBC and plasma) X 100

2.7 Iimmunological Parameters

2.7.1 Total Immunoglobulin (IG)

Total Immunoglobulin (Ig) assay was conducted in fish serum according to the procedure of Milla [18]. Estimation of total immunoglobulin in serum. 0.1 ml of serum was placed into an Eppendorf vial and added 0.1 ml of 12% polyethylene glycol that had been suspended in deionised water. Incubation was done at room temperature for 2 h under constant mixing followed by centrifugation at 5000 rpm for 10 min, taking out the

supernatant, and protein concentration was determined.

Total immunoglobulin was calculated using the following formula:

> Total immunoglobulin (g dl-1) = Total protein in individual sample plasma -Total protein taken out by absorption to polyethylene glycol.

2.8 Aminotransferases

"The aminotransferases including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicators of hepatocyte damage were estimated by the method of the International Federation of Clinical Chemistry (IFCC)" [19]. These enzymes are present in hepatocyte cytosol and during episodes of altered plasma membrane permeability, they leak into the extracellular fluid

2.9 Lysozyme Activity

"The lysozyme activity was determined by lysing the gram-positive bacteria that were sensitive to the lysozyme enzyme of *Micrococcus lysodeikticus*, according to the method described by Clerton" [20].

2.10 Statistical Analysis

Data was collected, documented, and saved on a computer spreadsheet for statistical analysis during the experiment. The statistical variance was calculated among the treatment groups using a one-way analysis of variance ($p \leq 0.05$). The mean, as well as standard deviation, were used to depict all the data (SD).

3. RESULTS

3.1 Clinical Findings

After 72 hours of the injection (on the 58th day), Slight swelling, and lesions on the skin of the injection sites were observed in all the experimental fish. Red blood spots were observed on the skin and fins of the experimental fish (T2, T3, and T4), the fish was weak and tired, and 10% mortality was recorded in all experimental groups except control(T1).

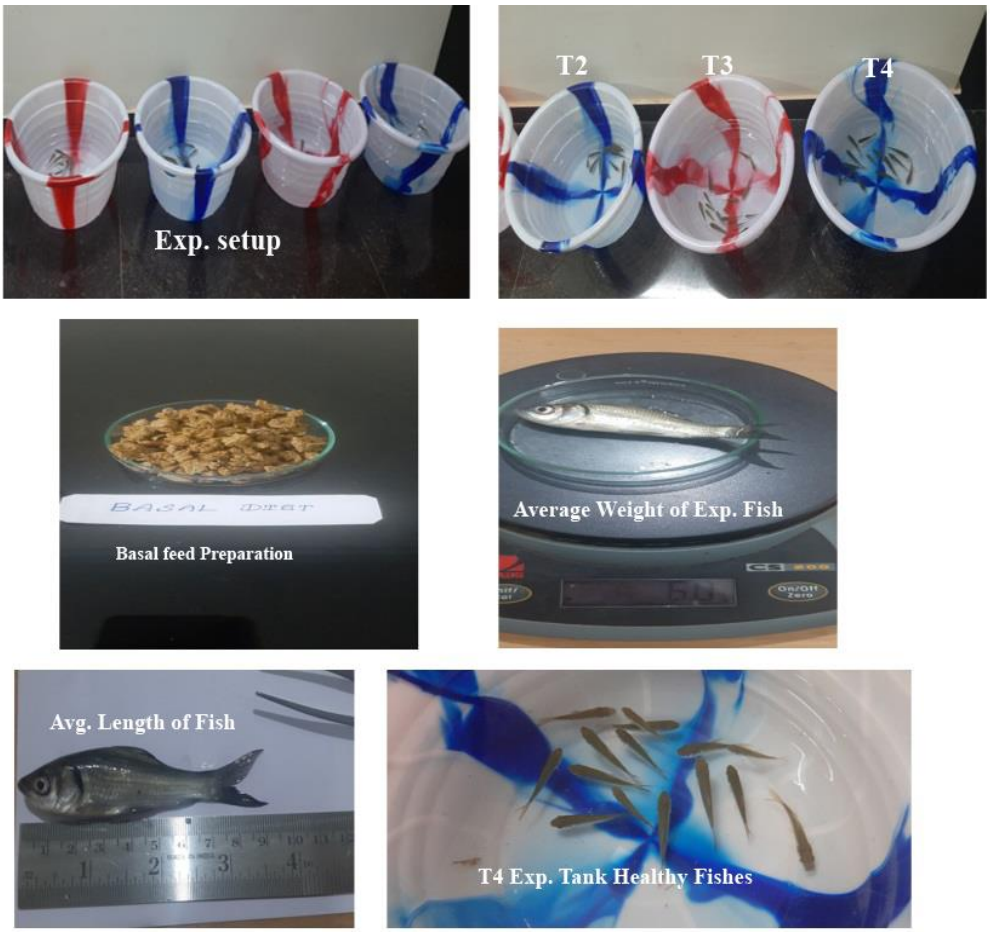
3.2 Hematological Parameters

The hematological parameters were measured, and the results are summarized in Table 2. Probiotic-supplemented groups (T3 and T4) showed no significant ($p < 0.05$) difference between the control (T1) in hemoglobin levels, WBC, and RBC contents than the experimental groupT2. Hemoglobin level was found to be almost equal in experiment groups T3 and T4 (5.11 ± 0.02 and 5.66 ± 0.05) and Control(T1) (5.24 ± 0.02). In T2 group was mostly affected and the fish in the test tank were found to have less value in Hb, RBCs, and WBCs levels (Table 2). RBC content was observed between 4.26 ± 0.07 and 4.75 ± 0.11(cells × 10^6 /mm^3) in probiotic supplemented group T3 and T4 and, the lesser RBC value was recorded in the T2 group (3.26 ± 0.02). Besides, the highest WBC value (cells × 10^3 /mm^3) was observed in T4, and the lowest was in T2. However, no significant ($p > 0.05$) difference was recorded for Packed corpuscular Volume (PCV) among the probiotic treatment groups T3 and T4.

3.3 Immunological Parameters

In the present study, to evaluate the influence of the challenge test with *Aeromonas hydrophila*, the biochemical and immunological parameters of Indian Major carp rohu *(L. rohita)* the probiotic-supplemented groups increased as compared to the control and T2 groups (Table 3). Total Immunoglobulin (IG) (µg/mL) content increased significantly ($p \leq 0.05$) in the fish fed with a probiotic-supplemented group (T3 and T4) compared to the fish fed without Basal feed (T2). The increase in total Immunoglobulin content in the probiotics-treated groups was dependent on the highest protein concentration in T4 (93.53 ± 1.04 ^{a}) and the lowest in T2 (81.05 ± 1.48 ^{a}). In the control group, T1 also found an increased amount of total Ig when compared with probiotic-treated groups.

There was a significant increase in the values of Lysozyme activity (0.67 ± 0.12) in all the experimental groups between 0.62 ± 0.17^a to 0.67 ± 0.12^a. However, in the probiotic-fed fish, T4 was significantly higher ($p \leq 0.05$) than all the experimental groups. Significantly higher ($p \leq 0.05$) Aminotransferases (IUl-1) activity in the blood serum of *L. rohita* fingerling was observed in fish fed with probiotic-supplemented diets when compared to the basal diet (Table 3). Fish fed with a diet supplemented with the highest dose of *L. acidophilus* (10^8 cfu g-1) exhibited significantly higher aminotransferase activity among all the treatments. This current research results confirm that probiotics application in aquaculture plays a vital role in fish health and the promotion of non-specific immune response and resistance against pathogens.



Picture 1. Experimental setup

Table 1. Experimental setup

Treatments			
Control	**A basal diet with and without probiotic bacterial culture**		
T1	**T2**	**T3**	**T4**
Control Basal diet* (Without *Lactobacillus*)	Basal diet + 0.1 ×10^7 ml of *A. hydrophila*	Basal diet + 0.1 ×10^7 ml of *A. hydrophila* + *Lactobacillus* 1.0×10^7CFU/g feed	Basal diet + 0.1 ×10^7 ml of *A. hydrophila* + *Lactobacillus* 1.0×10^8 CFU/g feed

Table 2. Hematological parameters of *L. rohita* challenged with *A. hydrophila* (0.1 × 10^7 CFU/mL)

Bood Parameters	**Experimental Group (A basal diet with and without probiotic bacterial culture)**			
	T1	**T2**	**T3**	**T4**
RBC (x10^6 µL)	4.12 ± 0.03	3.26 ± 0.02	4.26 ± 0.07	4.75 ± 0.11
WBC (x 10^3 mm^3)	120.11 ± 5.55	110.35 ± 7.32	124.29 ± 5.88	126.30 ± 6.40
Haemoglobin (Hb) (g/dl)	5.24 ± 0.02	4.30 ± 0.07	5.11 ± 0.02	5.66 ± 0.05
Packed corpuscular volume (PCV-%)	26.4±0.587	21.3±0.256	24.2±0.384	24.4±0.334

All values are presented as mean ± SD. The alphabetical superscripts in the values indicate significant ($p < 0.05$) differences among different treatments in each row

Table 3. Immunological parameters of *L. rohita* challenged with *A. hydrophila* (0.1×10^7 CFU/mL)

Parameters	Experimental Group (A basal diet with and without probiotic bacterial culture)			
	T1	T2	T3	T4
Total Immunoglobulin (IG) (µg/mL)	90.84 ± 3.44 [a]	81.05 ± 1.48 [a]	90.25 ± 2.55 [a]	93.53 ± 1.04 [a]
Aminotransferases (IUl-1)	24.2± 0.12 [a]	23.2± 0.25 [a]	24.4± 0.86 [a]	25.3± 0.08 [a]
Lysosome activity unit/ml	0.64 ± 0.15[a]	0.62 ± 0.17[a]	0.65 ± 0.10[a]	0.67 ± 0.12[a]

All values are presented as mean ± SD. The alphabetical superscripts in the values indicate significant ($p < 0.05$) differences among different treatments in each row

4. DISCUSSION

Over the decades, in the aquaculture industry, Antibiotics and chemical therapeutics used to protect fish from diseases, recently increasing the activity of pathogens and resistant strains affecting the fish culture and environment, so creating an alternative to them is needed to consider sustainable aquaculture, so probiotics have been used in recent times as an alternative and eco-friendly approach to aquaculture [21]. "Many studies support probiotics are recognized to improve the growth performance of fish and shellfish species through modification of microbial community, exclusions of the pathogen, upgradation of a non-specific immune response, and disease resistance" [22], [23], [24-27].

"The current study expressed that the freshwater fish *L. rohita* after being challenged *with A. hydrophila* in the period of a 60-day experimental study, supplementation of *Lactobacillus* probiotics significantly increased the Hematological and immunological parameters than the control and basal feed treatment group. Several authors reported that *Lactobacillus spp.* improved the bacterial community in the intestine of aquatic organisms, especially in fish, by destroying harmful bacteria and facilitating the colonization of beneficial bacteria" [28] and Yu et al [29]. "Furthermore, some similar findings were reported that WBC and RBC count improved due to the usage of *Bacillus spp*. in *O. niloticus* and the application of *Lactobacillus spp*. enhanced the intestinal LAB (lactic acid bacteria) for common carp" [25], [30] and [31].

In the present study, fish fed with a probiotic-supplemented diet showed significantly higher values of total immunoglobulin, serum lysozyme activity, and Aminotransferases in 60 days of experimental study against the challenge with fish pathogen *A. hydrophila.* the findings of the present study indicate that the administration of probiotics increased the immunity and enzyme activity of fish under stressful conditions.

Some of the previous studies supported to this current research that probiotics-treated fish groups significantly improved growth performance, Immunity, and maximum enzyme activity [32]. The outcomes of probiotics probiotics-supplemented group might result from the reflection of enriched blood parameters and serum protein levels in experimental fish against challenged with fish pathogens. Ramos et al [33] reported that "dietary supplements with a probiotic mixture (*Bacillus sp., Lactobacillus sp., Pediococcus sp., Enterococcus sp*.) improved digestion, nutrient absorption, and immune response by stimulating digestive enzymes and improving intestinal morphology of *O. niloticus"*. A similar observation was reported by Salam et al [34]. The result of the present study suggests that *L. acidophilus* supplementation could increase the hematological parameters and non-specific immunity of Indian major carp rohu, resulting in resistance of fish to *A. hydrophila* infection.

5. CONCLUSION

The major findings from the study, probiotics supplement diet with *Lactobacillus acidophilus* can enhance the hematological Parameters and Immunological parameters of fish Indian major carp (*Labeo rohita*) against the infection caused by the pathogenic bacteria *Aeromonas hydrophila.* This appears to be achieved by increasing the blood parameters of RBC, WBC, PCV, and Haemoglobin levels. The higher dose of *L. acidophilus* (10^8 cfu g^{-1}feed) resulted in improved non-specific immune response in the form of increased lysozyme and aminotransferase activities and enhanced resistance to bacterial infection with significantly reduced mortality against the pathogenic *A. hydrophila*, which further

indicated improvement in the overall health status of fish. Therefore, a probiotic diet supplemented with *Lactobacillus acidophilus* is recommended to improve the fish growth and survival rate by enhancing immunity against pathogenic bacteria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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