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Effects of *Lactobacillus sporogenes* Supplementation on Growth Performance, Survival, Immune Parameters, and Disease Resistance in *Labeo rohita* Challenged with *Aeromonas hydrophila*

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Authors' contributions

This work was carried out in collaboration among all authors. Author KMN took the lead in designing the experiment, conducting the investigation, developing the protocol, and preparing the initial draft of the manuscript. Author VB assisted in sampling and data collection. Author EGJ conceptualize and supervised overall research experiments. Author SN helped in writing and data analysis. Author MAAM wrote the first draft of the article, edited the manuscript and performed the statistical analysis. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

The present study aimed to investigate the effect of *Lactobacillus sporogenes* on the growth performance, immune responses, and susceptibility to *Aeromonas hydrophila* infection in *Labeo rohita*. The diet was prepared with four levels of *L. sporogenes* supplementation, viz.10^4 cfu g^{-1} (F1), 10^6 cfu g^{-1} (F2), 10^8 cfu g^{-1} (F3) and a control diet without supplementation (F0), fed in triplicates for 90 days. Results indicated significant ($p<0.05$) increase in weight performance and decreased feed conversion ratio (FCR) at 10^8 cfu g^{-1} (F3) compared to control group (F0). Probiotic supplementation (F2, F3) led to a noticeable and statistically significant increase ($p<0.05$) in the non-specific immunological reponses including superoxide anion production (SAP), total serum protein, and lysozyme function, compared to the group without supplementation (F0). Furthermore, the probiotic supplemented group exhibited a substantial significant ($p<0.05$) decline in the in the cumulative percent mortality (F1: 23.33±0.5; F2: 26.67±1.15 and F3: 30±1.35) compared to the group without probiotic supplementation (F0: CPM-73.33%) when challenged with *A. hydrophila*. Therefore, overall present study recommended that higher level inclusion of *L. sporogenes* can significantly enhance the growth performance, immune function, and ability to resist diseases in *L. rohita* when challenged with *A. hydrophila* infection.

Keywords: Labeo rohita; lactic acid bacteria; L. sporogenes; non-specific immune parameters; cfu g^{-1}.

1. INTRODUCTION

Due to rising fish consumption and the possibility that capture fishery have been overfished to their capacity, aquaculture has been growing over the past few decades (FAO, 2024). Indian major carps *Catla catla, Labeo rohita and Cirrhinus mrigala* are the most significant economic fish that are considered as highest market priced fish in India. They contribute over 87% of the total production of freshwater fish (Bias 2018). Aquatic animal production at large-scale facilities are subjected to stress, disease, and deterioration of the environment, which results in significant economic losses (Dunshea et al. 2024). Development of infectious diseases including abdominal dropsy, exophthalmia, red spot, tail and fin rot, haemorrhagic septicemia and bacterial gill disease are frequently encountered in *L. rohita* (Padala et al. 2021). According to Harikrishnan and Balasundaram (2005), *A. hydrophila* generally affects freshwater fish species. As a conventional method of controlling fish disease in aquaculture systems, antibiotics have been administered to minimize the disease condition (Prasad et al. 2012). The extensive application of antibacterial drugs in the aquaculture system damages aquatic animals and its gastrointestinal systems resulting unfit for human utilization (Verschuere et al. 2000). This led to the use of beneficial bacteria in aquaculture, which opened the doors to the application of probiotics in aquaculture, which have a positive influence through a variety of actions. Potential benefit of probiotics and its impact on the health and nutrition of some aquaculture animals have been investigated (Merrifield et al. 2010; Al Mamun et al. 2018; Mamun et al. 2019).

The term "probiotic" generally pertains to Gram-positive bacteria from the genus *Bacillus* spp. *Streptococcal* spp. and *Lactobacillus* spp. most commonly used and have been shown to improve the health of aquaculture animal without incurring any noticeable adverse effects (Irianto and Austin 2002). In fish culture, using a particular *Lactobacillus* produced superior outcomes (Barbosa et al., 2011). Probiotic refers to a group of bacteria, including the genus *Lactobacillus*, one of the important probiont that can fermenting a range of carbohydrates mostly into lactate and acetate (Gatesoupe 1999). The environmental function of *Lactobacillus* spp. as a helpful flora in the digestive tract includes, among other things, the synthesis of antimicrobial compounds that improve immune

response and increase fish resistance to bacterial pathogens, increase nutrient availability, additionally utilizing certain carbohydrates that are not digestible (Dimitroglou et al. 2011).

The use of probiotics in feed formulation for sustainable aquaculture practices has garnered considerable attention in recent decades. Probiotics that increase fish body enzyme activity can enhance feed digestion and feed utilization, improve fish health and performance (Mohapatra et al. 2012). Application of *Lactobacillus* spp. in several finfishes increased feed utilization, non-specific immunity, and resistant to virulent pathogen (Harikrishnan et al. 2010). Among the *Lactobacillus* spp. *L. sporogenes* considered one of the important probiont used in aquaculture industry and generated several beneficial impacts in finfishes (Sridhar and Joice 2012) and shellfishes (Gupta and Dhawan 2012; Seenivasan et al., 2012). Recent investigation have further emphasized the benefit of probiotics, including *Lactobacillus* spp. in growth modulation, immunity enhancement and disease resistance in *L. rohita*. It has been reported that multi-species probiotic with *B. subtilis* and *Lactobacillus* spp. not only improved the growth performance but also enhanced the immune response, intestinal microbiota and disease resistance in *L. rohita* (Ferdous et al. (2024). Similarly, Maji et al. (2017) reported that supplementation with a consortium of lactic acid bacteria enhanced growth performance, immune responses, and survival rates of *L. rohita* against *Aeromonas hydrophila*. Despite these advancements, there is limited information on the specific effects of *L. sporogenes* in Indian major carps, particularly *L. rohita*. To the best of our knowledge, this is the first study to demonstrate the effects of *L. sporogenes* supplementation on the growth performance, feed consumption, carcass composition, non-specific immunity, and disease resistance in *L. rohita*. Hence, this study was undertaken to investigate the effects of *L. sporogenes* on growth performance feed consumption, carcass composition, non-specific immunity, and its potential for enhancing resistance against *A. hydrophila* infection.

2. MATERIALS AND METHODS

2.1 Research Diet Formulation

The probiotic bacterium *L. sporogenes* was procured from M/S Altech Biotechnology Pvt. Ltd., Bengaluru (Bangalore, Karnataka, India). Three experimental diets, labelled as F1, F2 and F3 were formulated as 10^4 cfu g^{-1}, 10^6 cfu g^{-1}, and 10^8 cfu g^{-1} (F3), of *L. sporogenes*, respectively. The control diet (F0) did not contain any *L. sporogenes*. To achieve the desired texture, the ingredients were mixed and hand-kneaded with an appropriate amount of water (1:0.8 ratio) until a dough was formed. The dough was cooked at the required temperature and cooled rapidly to ambient temperature (29±2 °C) by spreading it onto an aluminum tray. Following the method described by Nesara et al. (2018), *L. sporogenes* was supplemented into the cooled dough to preserve probiotic viability.

The dough was pelletized using a manual pelletizer to produce 3 mm-diameter pellets. The pellets were air-dried in a hot air oven at 60 °C until they reached a moisture content of approximately 10%. Each batch of feed was packaged in thick, labeled polythene bags and stored at 4 °C in a freezer to maintain quality. The experimental diets were evaluated for proximate composition following AOAC (1995) guidelines, and the nutrient composition of dietary components and protein contributions are presented in Table 1. Viability testing of *L. sporogenes* was conducted to ensure the probiotic remained viable after incorporation into the feed. This was evaluated using the plate count method. Serial dilutions of the probiotic-containing feed were prepared and plated onto nutrient agar (Himedia). The plates were incubated, and viable bacterial cells were counted to confirm the retention of probiotic activity in the prepared diets.

2.2 Experimental Design

The *L. rohita* spawn was obtained from the Bhadra Reservoir Project fish seed farm in Shivamogga, Karnataka, India, and reared for 1–2 months at the research and instructional fish farm to achieve the fingerling stage. For the experiment, twelve outdoor cement tanks (1 × 1 × 1 m, bare bottom) were used. Each tank was stocked with 15 uniform-sized *L. rohita* fingerlings (1.17±0.45 cm in length and 0.76±0.15 g in weight). Each diet was tested in triplicate, with three tanks assigned to each of the four dietary treatments: F0 (control), F1 (10^4 cfu g^{-1}), F2 (10^6 cfu g^{-1}), and F3 (10^8 cfu g^{-1}). The duration of the feeding trial was 90 days during the months of April to June, 2017. The fish were fed the experimental diets at 5% of their body weight daily for the duration of the experiment. Physico-chemical parameters of the water, such as temperature, pH, dissolved oxygen, and

ammonia levels, were monitored and maintained within optimal ranges. Parameters were recorded fortnightly during sampling to ensure a consistent environment for the experiment.

2.3 Water Quality Monitoring

Water quality parameters were regularly monitored throughout the experiment to ensure optimal conditions for *L. rohita* culture. The hydrological variables were measured using a digital water quality machine (HI 9828, YSI Inc., Yellow Springs, OH, USA) during fortnightly sampling. The recorded values were as follows: temperature 27±2.0 °C, pH 8.8±1.34, dissolved oxygen (DO) 6.5±1.5 ppm, ammonia (NH_3) 0.05±0.021 ppm, total dissolved solids (TDS) 100±10 ppm, salinity 0.09±0.02 ppt, water pressure 755.60±0.60 mm Hg, and conductivity 153.75±8.84 µS/cm. All parameters were maintained within acceptable ranges to support fish health and growth.

2.4 Proximate Composition Analysis of Test Diets

All feed ingredients used in the test diets underwent proximate composition analysis following standard procedures (AOAC, 1995), as shown in Table 2. Moisture content was determined by heating samples at 105 °C for 30 minutes, allowing them to cool, and then weighing them to a constant weight. Crude protein content was analyzed using the FOSS Kjeltec system, while fat content was measured with the Soxtech system (PELICAN). Fiber content was determined using the Fibretech system (PELICAN). Carbohydrate levels, expressed as nitrogen-free extract (NFE), were calculated using the difference method (Hastings, 1976) with the standard formula:

> NFE (%) =100 − (Moisture % + Crude Protein % + Crude Fat % + Crude Fiber % + Ash %)

2.5 Growth Parameters

Growth performance and feed utilization were evaluated by sampling the fish every 15 days. During each sampling, at least 50% of the stocked fish from each tank were individually weighed and measured. Based on the recorded fish weights, the quantity of feed provided was adjusted to reflect their growth. Growth parameters, including weight gain, feed conversion ratio (FCR), survival rate, and specific growth rate (SGR), were calculated using standard formulae:

i. Weight gain (g) = Mean final weight (g) - Mean initial weight (g)

ii. Specific growth rate (% /day) = [ln final weight - ln initial weight/ Days of experiment] x100

iii. Feed conversion ratio = Feed intake/ Final weight gain

iv. Survival rate (%) = [(Number of fish harvested)/ (Number of fish stocked at the start of the experiment)] × 100

2.6 Sample Collection and Immunological Parameters

2.6.1 Blood sampling

After the completion of a 90-day period, a total of 10 fingerlings from every treatment as well as the control group were selected for physiological investigations. To prepare for blood collection, the fish underwent a 24-hour fasting period during which they were not fed. Using a sterile 2 ml syringe, blood was collected by caudal vein. Subsequently, the obtained blood were carefully transferred into dry EDTA (for NBT assay) and clean 2 ml centrifuge tube. Subsequently, the collected blood samples were centrifuged for ten minutes at 10,000 rpm to divide the serum. After centrifugation, sera obtained from three fish in each treatment group were pooled together to ensure adequate volume for analysis. The pooled sera were kept at -20 °C for further use.

2.6.2 Nitroblue tetrazolium assay (superoxide anion production)

The nitroblue tetrazolium (NBT) assay was assessed according to Anderson and Siwicki (1993). Three fish were sampled, and blood was collected from their caudal veins and transferred to EDTA-coated tubes (EDTAK2). The samples were centrifuged at 5000 rpm for 10 minutes to isolate the leukocyte-containing buffy coat. The buffy coat was carefully transferred to a microtitre plate for cell adhesion. The plate was incubated for 1 hour, after which the cells were treated with 0.3% NBT solution and incubated for an additional hour. Following incubation, the NBT solution was removed, and the cells were treated with a fixing agent, washed thoroughly, and air-dried. To dissolve the formazan produced during the assay, 60 µL of 2N potassium hydroxide

(KOH) and 70 µL of dimethyl sulphoxide (DMSO) were added to each well. The resulting solution, characterized by its distinctive turquoise blue color, was analyzed using a microplate reader at an optical density of 620 nm.

2.6.3 Lysozyme activity

Lysozyme activity was assessed applying a customized turbidimetric technique reported by Parry et al. (1965). Two replicates of 50 µl serum were dispensed into each well of a 96-well plate, along with 50 µl of Phosphate Buffered Saline (PBS). The serum was serially diluted across the wells, with 50 µL being transferred from one well to the next, effectively halving the concentration with each dilution. After the dilutions, 125 µL of a *Micrococcus luteus* suspension was added to each well. The plate was incubated at room temperature, and the decrease in absorbance at 450 nm was recorded over a period of 0 to 15 minutes using an ELISA reader. Lysozyme activity was calculated based on the rate of reduction in absorbance per minute (0.001 min^{-1}). One unit of lysozyme activity was defined as the amount of enzyme required to cause this rate of decrease in absorbance.

2.6.4 Total plasma protein

Serum plasma protein levels were determined using the Lowry method with a GeNei™ protein analysis kit. For the assay, 50 µL of distilled water and 50 µL of serum were mixed and dispensed into each well of a 96-well plate. Subsequently, 200 µL of Lowry reagent was added to each well, and the plate was incubated at room temperature for 10 minutes. Following this initial incubation, 200 µL of Folin-Ciocalteu reagent was added to each well, and the plate was incubated for an additional 30 minutes at room temperature. After the reaction, absorbance was measured at 650 nm using a spectrophotometer. The absorbance values were used to quantify serum protein concentration based on a standard curve prepared with known protein concentrations.

2.7 Determination of LD_{50}

A virulent strain of *A. hydrophila* (ATCC 36562) was purchased from Pune, India's National Collection of Industrial Microorganisms (NCIM). The dried bacterial cells in the ampule were revive in trypton soya broth at 37 °C and cultured further to keep in agar slants at chill temperature (4°C). Ten fish fingerling (6.89±1.55g) per tank were used to determine the LD_{50}. Virulent pathogen, *A. hydrophila* were inoculated ranging from 10^2 to 10^8 cfu ml^{-1} was injected intraperitoneally to each fish. Physiological saline 0.1 ml injections into the fish group considered as the control. Daily mortality rates were documented for 240 hours. Based on Reed and Muench's (1938) estimations, the degree of pathogenicity (LD_{50}) was determined.

2.8 Challenge Study

Following a 90-day period of experimental feeding, a sample size of ten fish were selected from both the control and treatment groups in order to assess their disease resistance. Fishes were challenged (intramuscular) with 0.1 ml of *A. hydrophila* (2.3×10^7 cfu ml^{-1}. One negative control fish group received a dose of 0.1 ml of phosphate buffered saline. Percent survival, was calculated (Amend, 1981) and utilized as a measure of protection against diseases. Ethical permission were taken before sacrificing the fishes (No. FCM/ AEC/DFK 1501-2015-2017/01).

> RPS = 100 × (1- % mortality in *L. sporogenes* fed fishes / % mortality in control (F0) fishes)

2.9 Data Analysis

One-way analysis of variance (ANOVA) was performed to analyze the data from the experimental and control groups. To determine the significance of differences between treatments, Duncan's multiple range test was employed as a post hoc analysis. All statistical analyses were conducted using SPSS software (version 26). The results were expressed as mean ± standard error, and a significance level of $p<0.05$ was considered for all comparisons.

3. RESULTS AND DISCUSSION

3.1 Experimental Ingredients and Diets

In the present study, Table 1 outlines the proximate analysis of the dietary ingredients used. Higher and lower protein content had recorded in fish meal (67.45 %) and tapioca flour (2.34 %) respectively. Moreover, data showed that maximum level of fat, fibre and ash content was present in groundnut oil cake (16.92 %) wheat bran (10.31%) and in fish meal (25.21%). Crude protein contents in all the experiments (F_0, F_1, F_2 and F_3) were ranging from 27.09 % to 27.14% are shown in Table 2.

Table 1. Ingredients used to prepare various experimental diets and their composition

	Feed composition			
	Fish meal	**Ground nut oil cake**	**Wheat bran**	**Tapioca flour**
Moisture (%)	9.75±1.06	9.65±0.02	8.02±0.10	7.20±0.07
Dry matter (%)	90.25±1.07	90.35±0.01	91.98±0.08	92.80±0.08
Crude protein (%)	67.45±0.50	38.30±0.42	18.30±0.27	2.34±0.26
Ether extract (%)	13.55±0.07	16.92±0.56	8.54±0.23	4.60±0.14
Crude fibre (%)	1.55±0.05	3.92±0.13	10.31±0.02	2.31±0.05
Ash (%)	25.21± 0.12	7.32± 0.17	4.50± 0.70	5.27± 0.31
Nitrogen free extract (%)	17.51 ± 0.55	23.89 ± 0.76	50.33 ± 0.28	78.28 ± 0.44
Protein contribution (%)	13.59	7.72	5.18	0.68

Table 2. Proximate composition of formulated diet

	F_0	F_1	F_2	F_3
Moisture (%)	8.17± 0.03	7.76±0.01	7.58±0.03	7.36±0.02
Dry matter (%)	91.82±0.03	92.23±0.01	92.42±0.03	92.63±0.02
Crude protein %)	27.09±0.31	27.14±0.17	27.13±0.01	27.14±0.01
Ether extract (%)	6.07±0.10	6.10±0.21	6.37±0.17	6.35±0.10
Crude fibre (%)	6.88±0.02	6.86±0.09	6.34±0.04	7.17±0.04
Ash (%)	10.75±0.35	11.00±0.70	11.25±0.35	11.25±0.35
Nitrogen free extract (%)	41.04±0.33	41.14±0.29	41.33±0.18	40.73±0.24

Table 3. Comparison of the growth performance of *L. rohita* after 90 days of feeding with a control diet and a diet supplemented with probiotic *L. sporogenes* (mean of 3 replicates± standard error)

	F_0	F_1	F_2	F_3
Initial body weight	$0.76±0.02^a$	$0.76±0.02^a$	$0.76±0.02^a$	$0.76±0.02^a$
Final body weight	$5.45±0.50^b$	$6.35±0.39^a$	$6.21±0.35^a$	$6.52±0.49^a$
Weight gain	$717.10±72.05^b$	$835.52±86.22^a$	$817.10±86.09^a$	$857±81.99^a$
Feed conversion ratio	$2.58±0.32^a$	$2.17±0.11^b$	$2.20±0.13^b$	$2.16±0.50^b$
Specific growth rate	$2.17±0.11^b$	$2.35±0.11^a$	$2.23±0.02^{ab}$	$2.37±0.10^a$
Survival (%)	95.55^a	95.35^a	95.55^a	84.44^a

3.2 Growth Metrics and Feed Utilization

The observed data demonstrated that dietary supplementation with probiotics significantly enhanced fish biomass (weight gain) compared to the non-probiotic group (F0) ($p < 0.05$). Over the 90-day trial period, the highest growth was observed in the F3 group, followed by F1, F2, and F0 (Table 3). However, no statistically significant differences ($p > 0.05$) were noted among the probiotic-supplemented groups. The F3 group exhibited the lowest and most optimal feed conversion ratio (FCR), with F1, F2, and F0 following in that order. Specific growth rate (SGR) results indicated that the F1 and F3 groups achieved the highest values, 2.35±0.11 and 2.37±0.10, respectively, which were not significantly different ($p > 0.05$) from each other. The F2 group displayed an intermediate SGR of 2.23±0.02, which was significantly higher than the non-supplemented control group F0 ($p< 0.05$) but did not differ significantly from F1 and F3. The control group (F0) exhibited the lowest SGR of 2.17±0.11, significantly lower than all probiotic-supplemented groups ($p< 0.05$). These results indicate that the inclusion of *L. sporogenes* in the diet significantly improves specific growth rates (SGR) compared to the control group, underscoring the growth-promoting benefits of probiotic supplementation in aquaculture.

Evidence of previous works done by few authors the optimal dietary supplement of *L sporogenes* range was decided at levels of 10^4 cfu g^{-1}, 10^6 cfu g^{-1}, and 108 cfu g^{-1}. Our results showed no significant differences in weight gain between the F1 (10^4 cfu g^{-1}) and F2 (10^6 cfu g^{-1}) treatment groups. However, fish in the F3 group (10^8 cfu g^{-1}) exhibited the highest weight gain, feed

efficiency, and growth performance, suggesting this supplementation level meets the nutritional and physiological requirements of *L. fingerlings*. These findings align with those of Ferdous et al. (2024), who demonstrated that multi-species probiotics, including *B. subtilis* and *Lactobacillus* spp., significantly enhanced growth, feed utilization, and disease resistance in *L. rohita* larvae. Similarly, Maji et al. (2017) reported that a lactic acid bacteria consortium improved growth and immune response in *L. rohita*, validating the efficacy of probiotics in enhancing feed efficiency and reducing FCR. Mamun et al. (2020) further emphasized the role of probiotics in improving gut health and nutrient uptake. In their study, *L. rohita* fed a diet containing *L. rhamnosus* (10^8 cfu g⁻1) for 60 days exhibited significant improvements in intestinal morphology and gut immunity compared to fish fed the basal diet.

These findings are further supported by recent studies highlighting the role of probiotics and related supplements in improving growth, immunity, and disease resistance in *L. rohita*. Baisakhi et al. (2024) demonstrated that a combination of *B. subtilis* and *Saccharomyces cerevisiae* significantly enhanced hemato-immunological indices, digestive enzyme activities, and disease resistance in *L. rohita*. Similarly, Priyadharshini et al. (2024) reported that dietary supplementation with *L. acidophilus* significantly improved survival, growth, and hematological parameters, including WBC, RBC, Hb, and Hct, in *L. rohita*. In another study dietary feeding of *B. amyloliquefaciens* in *L. rohita* significantly enhanced the immune parameters and disease resistance against *A. hydrophila* (Barui et al. 2024). Additionally, Kumar et al. (2023) explored the synergistic effects of prebiotics and probiotics on *L. rohita*. The combination of *L. plantarum* (10^8 cfu g^{-1}) with β-glucan or mannan-oligosaccharide resulted in significant improvements in growth, hematological indices, and carcass composition. Several authors have reported positive outcomes in various commercial aquaculture fish species fed with single- or multi-strain probiotic bacteria, significantly enhancing growth, feed efficiency, and immunity (Mamun et al., 2020; Tachibana et al., 2020; Abdel-Latif et al., 2023; Balami et al., 2022; Muhammad et al., 2022). The results of the present study align with this body of evidence, particularly demonstrating the importance of optimal probiotic dosages, such as 10^8 cfu g^{-1}, in achieving superior growth and immunity in *L. rohita*.

3.3 Non-specific Immune Parameters

The production of superoxide anion, total serum protein, and lysozyme activity in *L. rohita* under different treatments and control groups are presented in Table 4. Significant differences ($p<0.05$) were observed among the groups for all parameters. Superoxide anion production was highest in the F2 (10^6 cfu g^{-1}) group at 0.232 ± 0.057 U/mL, significantly higher than the control (F0) and F1 (10^4 cfu g^{-1}) but not significantly different from F3. Total serum protein levels were also significantly elevated in the F2 group (55.40 ± 0.66 mg/L), followed by F3 (47.07 ± 0.62 mg/L), while the F0 and F1 groups showed lower values. Lysozyme activity was significantly higher in all probiotic-supplemented groups (F1, F2, and F3), with the F3 group recording the highest value (1258.00 ± 4.35 U/mL). In contrast, the control group (F0) exhibited the lowest lysozyme activity (910.33 ± 2.60 U/mL), indicating the beneficial impact of probiotic supplementation on immune parameters in *L. rohita*.

Serum respiratory burst is considered as one of the vital bactericidal mechanisms in fish and often indicates the health status of fish (Hampton et al. 2020; Erfanmanesh et al. 2024). The observed enhancements in superoxide anion production and lysozyme activity in *L. rohita* following probiotic supplementation are consistent with findings from previous studies. Baisakhi et al. (2024) reported that administering *B. subtilis* and *Saccharomyces cerevisiae*, either individually or in combination, significantly improved hemato-immunological indices and disease resistance in *L. rohita*. Moreover, dietary intake of *B. amyloliquefaciens* in *L rohita* not only modulated the immune responses but also provide resistance when challenged with virulent *A. hydrophila*. Singh et al. (2024) further corroborated these findings by showing that supplementation with an autochthonous probiotic strain of *B. subtilis* improved immune-biochemical responses and increased resistance to *A. hydrophila* in *L. rohita*. Many studies have reported that incorporating probiotics into fish diets enhances respiratory burst activity of phagocytes, which is a critical component of the innate immune system (Chen et al., 2015; Hajirezaee et al., 2024).

Table 4. Production of super oxide anion, total serum protein, and lysozyme activity of *L. rohita* under treatments and control group (mean of 3 replicates± standard error)

Treatments	F_0	F_1	F_2	F_3
Super oxide anion production	0.175±0.070[b]	0.186±0.033[b]	0.232±0.057[a]	0.197±0.035[ab]
Total serum protein(mg/l)	38.34±0.271[b]	41.69±0.52[b]	55.40±0.66[a]	47.07±0.62[ab]
Lysozyme activity (U/ml)	910.33±2.60[b]	1242.66±1.45[a]	1253.00±4.48[a]	1258.00±4.35[a]

In our study fishes fed with probiotics exhibited stronger immune responses in contrast to the group not supplemented dietary *L. sporogenes*. Total protein in serum is a non-specific humoral components indicated higher immunological state when fish possessed higher amount in blood (Mamun et al., 2022a). Meanwhile lower level of total protein in serum lead to microbial infections, organ failure and nutritional imbalance in aquaculture organisms (Rathore et. al., 2021). Notably, major improvements in serum protein levels was noticed in the F2 group, which had a probiotic count of 10^6 cfu g^{-1}. A consistent pattern of positive effects stemming from probiotic supplementation on fish health and immunity. This trend is particularly evident in the significant enhancement of lysozyme expression and increased populations of beneficial bacteria like *Lactobacillus* sp. resulting from dietary lactic acid (LA) supplementation in common carp (Hoseini et al. 2023). This phenomenon aligns with the findings of Abdel-Latif et al. (2023), who reported elevated serum lysozyme activity and total immunoglobulin levels in *P. hypophthalmus* fingerlings following multispecies probiotic supplementation. Importantly, the positive impacts of *Bacillus* sp. supplementation extend beyond just lysozyme activity. As reported by Ji et al. (2023), dietary supplementation of *B. subtilis* significantly enhanced disease resistance in Chinese perch (*Siniperca chuatsi*), resulting in heightened resistance against the pathogen *A. hydrophila*. Additionally, El-Son et al. (2022) observed that Sanolife® PRO-F probiotic supplementation in Nile tilapia led to positive effects on antioxidant capacity and lysozyme activity. These multifaceted improvements in diverse immune response parameters further underline the comprehensive benefits of probiotics in promoting overall fish health and immune function. It is worth noting that the effects of probiotic interventions can be species-specific, as evidenced by the study conducted by Keereelang et al. (2022). Their research involving *L. plantarum* (LP) supplementation in black shark minnow (*L. chrysophekadion*) exhibited species-specific improvements in immune responses and disease resistance. This was reflected in the enhancement of lysozyme and peroxidase activities, alongside increased survival rates against *A. hydrophila* infection. These findings underscore the importance of probiotic interventions to suit the unique requirements of different fish species. Moreover, the combination of probiotics with other dietary additives has showcased promising outcomes. Yousefi et al. (2023) demonstrated that the supplementation of Gum Arabic (GA) and/or *L. helveticus* (LH) yielded multiple improvements, including enhanced superoxide dismutase and catalase activities. These results suggest potential synergistic effects achievable by integrating probiotics with other dietary components, further enhancing fish health and immune responses.

Lysozyme activity, an important component of the nonspecific immune response in fish, is often used as an indicator of fish health status (Biller et al. 2021). According to Liu et al. (2012) feeding fish *E. coioides* with *B. subtilis* at a concentration of 10^8 cfu g^{-1} resulted in significantly higher lysozyme activity compared to lower concentrations and control diets. On the contrary, compared to the control fish, rainbow trout, *O. mykiss*, fed with a greater concentration of *L. rhamnosus* (JCM 1136) at 10^{11} cfu g^{-1} had considerably increased serum lysozyme activity (Ezabi et al. 2005). Mahmoudzadeh et al. (2016) supplemented *O. mykiss* diets with *B. subtilis* for the period of 44 days and observed enhanced lysozyme activity and total antibody levels, which aligns with the findings of El-Boshy et al. (2010), who supplemented *S. cerevisiae* to *O. niloticus* for around 21 days and observed similar results. In another study by Sîrbu et al. (2022), lysozyme activity significantly increased in the treatments including probiotics, prebiotics and synbiotics groups compared to the control group. Additionally, Opiyo et al. (2019) found that Nile tilapia grown in ponds with minimal input and supplemented with either *Saccharomyces*

cerevisiae or *Bacillus subtilis* at different levels exhibited substantially higher lysozyme activity than the untreated group.

3.4 Body Composition of *L. rohita*

The influence of *L. sporogenes* on the proximate whole-body composition is depicted in Table 5. Observed data showed no remarkable ($p>0.05$) difference in all the composite analysis (moisture, dry matter, protein, fat, ash and carbohydrate) of fish muscle either in the probiotic fed groups or without probiotic group despite the fact that fish fed probiotics showed greater levels. According to the current data of fish body composition, there is no noticeable difference between both the treatment and control groups. Likewise, Eid and Mohamed (2008) found no statistically significant variation in the proximate compositions of in tilapia fingerlings when they were supplemented with various levels of commercially produced feed additives. Meanwhile, carcass content were significantly higher in probiotic mixed feed given to the juveniles of common carp, *Cyprinus carpio* (Mohsen et al. 2016).

Table 5. Comprehensive analysis of the proximate composition (%) of fish muscle considering multiple treatment conditions

Treatments	F_0	F_1	F_2	F_3
Moisture (%)	77.55±0.052[a]	77.98±0.037[a]	77.55±0.037[a]	78.16±0.037[a]
Dry matter (%)	22.44±0.052[a]	22.01±0.037[a]	22.44±0.037[a]	21.84±0.037[a]
Protein (%)	15.08±0.17[a]	15.01±0.23[a]	15.52±0.11[a]	15.30±0.33[a]
Ether extract (%)	2.73±0.024[a]	2.75±0.061[a]	3.02±0.037[a]	3.14±0.011[a]
Ash (%)	2.10±0.017[a]	2.09±0.018[a]	2.07±0.014[a]	2.10±0.020[a]
NFE (%)	2.52±0.04[a]	2.14±0.05[a]	1.82±1.11[a]	1.35±0.22[a]

Nitrogen free extract (mean of 3 replicates± standard error)

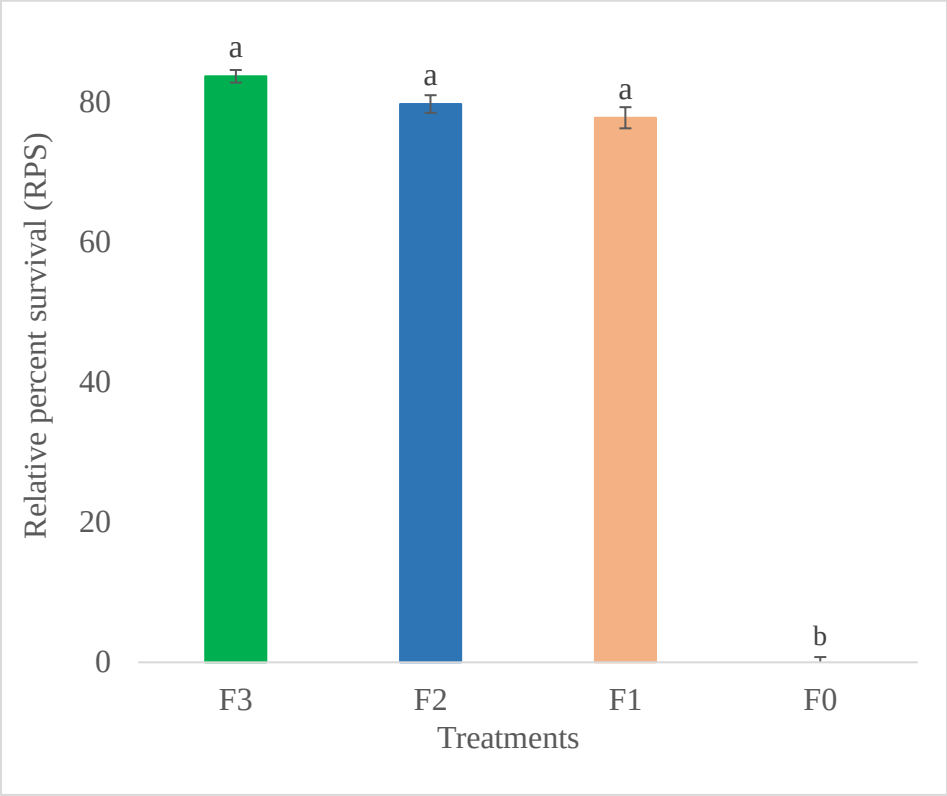

Fig. 1. The determination of the relative percentage of survival (RPS) in *L. rohita* was conducted across a range of treatments and the control group following a challenge with A. *hydrophila*

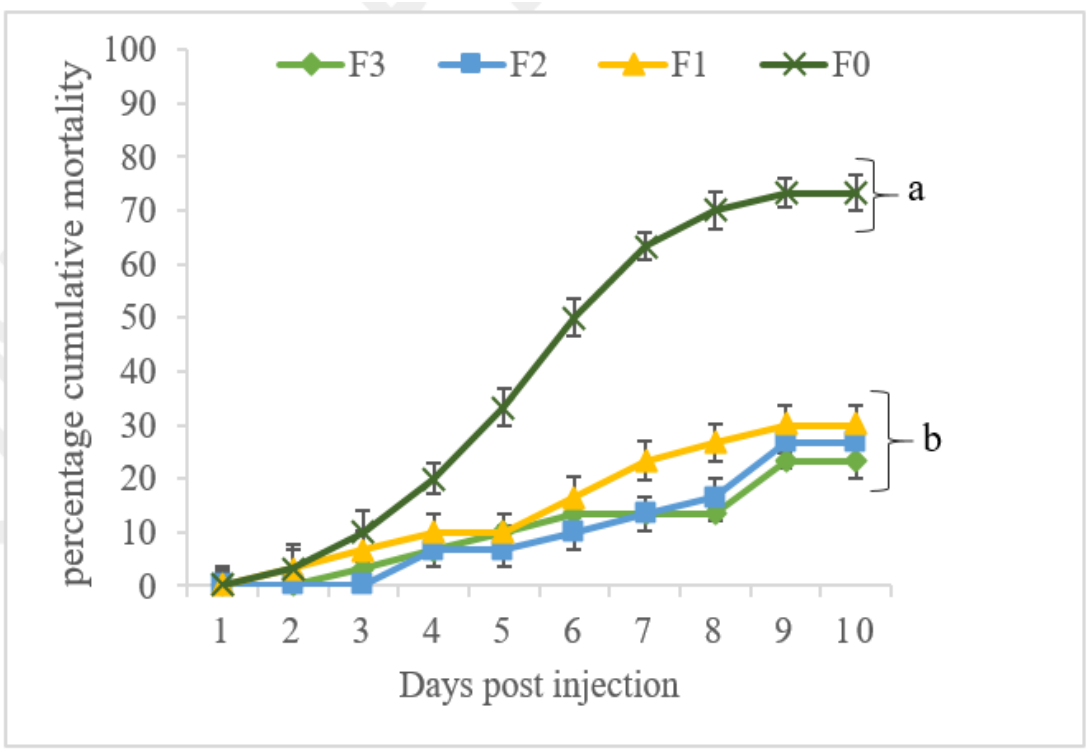

Fig. 2. The percentage cumulative mortality (CPM) of *L. rohita* was assessed in the different treatments and control group against *A. hydrophila*

3.5 Disease Resistance of *L. rohita* to *Aeromonas hydrophila*

Feeding *L. rohita* with a diet containing *L. sporogenes* for 90 days significantly improved resistance to *A. hydrophila* infection, as indicated by lower cumulative mortality and higher relative percentage survival (RPS) values in the probiotic-fed groups. The probiotic-treated groups exhibited significantly lower cumulative mortality during the 10-day post-injection period (26.67±3.95%) compared to the control group, which showed the highest mortality rate (73.33%) $p<0.05$). The RPS values were highest in the F3 group (83.65%), followed by the F2 (79.69%) and F1 (77.73%) groups, whereas the control group showed the lowest survival rates (Fig. 1). Fish in the control group also displayed severe clinical signs, including red spots, hemorrhagic lesions, tail and fin rot, and scale loss, highlighting the protective role of *L. sporogenes* in mitigating the impacts of *A. hydrophila* infection. This demonstrates that dietary supplementation with *L. sporogenes* can enhance disease resistance in *L. rohita*, reducing mortality and improving overall health during pathogenic challenges.

In the present study fishes were challenged with *A. hydrophila* as these *Aeromonas* spp. are Gram-negative aquatic bacteria and ubiquitous in nature which causes fatal haemorrhagic septicaemia in catfishes (Mamun et al. 2022b) and caused severe economic lass in freshwater cultured cyprinid fishes (Nithin et al. 2021). In agreement with these findings, Singh et al. (2024) reported that feeding *L. rohita* with *B. subtilis* (10^9 cfu g^{-1}) for 20 days significantly improved resistance to *A. hydrophila*, with a relative percent survival (RPS) of 64%. Similarly, Barui et al. (2024) demonstrated that dietary inclusion of formalin-inactivated *B. amyloliquefaciens* (10^8 cfu g^{-1}) enhanced survival and innate immune responses in *L. rohita* challenged with *A. hydrophila*. The highest RPS was observed in fish fed with the formalin-inactivated paraprobiotic diet, further supporting the potential of both viable and inactivated probiotics in mitigating the impacts of pathogenic infections. Baisakhi et al. (2024) also highlighted the efficacy of probiotics in disease resistance, showing that *B. subtilis* and *S. cerevisiae* supplementation in *L. rohita* achieved an RPS of 85.7% against *A. veronii* when administered in combination through oral and intraperitoneal routes. These results align closely with the present study, where *L. sporogenes*

supplementation at provided the highest level of protection, as indicated by reduced clinical signs and improved survival rates.

4. CONCLUSION

Dietary inclusion of *L. sporogenes* had a significant impact on growth efficiency, immunity, and exerted a defensive action against *A. hydrophila* pathogen. Different levels of probiotics in the present study acted in one or the other parameters and promoted the higher yield and immunity. Considering all these findings, it has been strongly recommend that including *L. sporogenes* in the diet can effectively improve growth efficiency, enhance immunity, and offer protection against *A. hydrophila*. Nevertheless, it is crucial to emphasize the need for additional research to understand the intricate mechanisms responsible for the observed benefits of *L. sporogenes*. Furthermore, such research will facilitate the optimization of *L. sporogenes* application in aquaculture practices, ensuring its maximum efficacy and potential for enhancing feed utilization, growth improvements, innate immunity, and strategies for combating *A. hydrophila*.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

The author(s) hereby declare that generative AI technology, specifically ChatGPT (Version/Model: GPT-4) developed by OpenAI, was utilized during the writing and editing of this manuscript.

ETHICAL APPROVAL

The approval for fish maintenance, handling and challenged study were performed with the permission of Animal Ethics Committee of the College of Fisheries, Mangalore, Karnataka, India. All experimental procedures were conducted in compliance with the national and international standard guidelines.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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