Effects of *Lactobacillus sporogenes* supplementation on growth performance, survival, immune parameters, and disease resistance in *Labeo rohita* exposed to *Aeromonas hydrophila*

ABSTRACT

The present study ~~was~~ aimed to investigate the ~~influence~~ effect of *Lactobacillus sporogenes* on the growth~~ characteristics~~performance, immune ~~system~~responses, and susceptibility to *Aeromonas hydrophila* infection in *Labeo rohita*. Diet containing different levels of *L. sporogenes* at 10^4 cfu/g (F1), 10^6 cfu/g (F2), 10^8 cfu/g (F3) and without (F0, control) in write as cfu $g_{\text{-}}^{-1}$ throughout the manuscript triplicates for 90 days meaning is not proper rewrite. Results indicated ~~meaningful~~ significant ($p<0.05$) increase ~~in~~d weight performance and decreased feed conversion ratio (FCR) at 10^8cfu/g (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 ~~measures,~~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 ~~of~~ mortality (F1: 23.33±0.5; F2: 26.67±1.15 and F3: 30±1.35) and higher relative percent survival (F1: 77.73±1.51; F2: 79.69±1.26 and F3: 83.69±0.89) authors should mention only cululative mortality when challenge study is performed 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 ~~exposed to~~challenged with *A. hydrophila* infection.

Keywords: *Labeo rohita*, Lactic Acid Bacteria, *L. sporogenes*, non-specific immune parameters, cfu/g Better keywords may be provided

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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 2018 provide recent reference). 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 67% of the total production of freshwater fish (Parthasarathy et al. 2011 provide recent data and reference). Aquatic animal production at large-scale facilities ~~is~~are subjected to stress, disease, and deterioration of the environment, which results in significant economic losses (Subasinghe et al. 2005 provide recent reference). 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 ~~lead~~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 ~~organisms~~ animals have been investigated (Merrifield et al. 2010; 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) include recent studies on the application of probiotics in rohu culture with emphases on lactobacillus species. There is, however, a lack of information on Indian Major Carp, *L. rohita* *hypothesis is not clear*. Hence, a research investigation was undertaken to investigate the effects of *L. sporogenes* on growth ~~efficiency~~ performance ~~and~~ feed consumption, carcass composition, non-specific immunity, and its potential for enhancing ~~resilience~~ resistance ~~in the presence of~~against *A. hydrophila*.infection

2. Materials and Methods

2.1 Research diet ~~and structure~~formulation

The probiotic bacterium *L. sporogenes* was obtained from M/S Altech Biotechnology Pvt. Ltd, Bangaluru (Bangalore, Karnataka, India). The dry ingredients were mixed well to make a smooth dough after cooking with required temperature and water not clear. The pellets were formed using a manual pelletizer with a diameter of 3 mm. Subsequently, the pellets were subjected to air drying in a hot air oven set at a temperature of 60°C until they reached a moisture content of approximately 10%. Each diet was packaged differently in thick polythene bags, marked and maintained at 4 °C in a freezer. Experimental diets were evaluated for the proximate composition in accordance with AOAC (1995). The nutrient composition of dietary components and its protein contribution ~~exhibited~~ is shown in Table 1.

The *L. rohita* spawn was obtained from the Bhadra Reservoir Project fish seed farm in Shivamogga, Karnataka, India. It was then raised for 3 to 4 months in the college's research and instructional fish farm to reach fingerling stage. Twelve ~~(12)~~ outdoor cement tanks with a 1×1×1 m bare bottom each were used for the investigation. *L. rohita* fingerlings of uniform size, measuring approximately 1.17±0.45 cm in length and weighing around 0.76±0.15 g, were released in each tanks with 15 fingerlings. Each diet was replicated in triplicate not clear. Three experimental diets, labelled as F1, F2, and F3, were formulated ~~to include~~incoporating 104

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cfu/g, 106 cfu/g, and 108 cfu/g 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. After cooking the dough, the feed was rapidly down to ambient temperature (29±2 °C) putting it out in an aluminium tray. Following the methodology outlined by Nesara et al. (2018), the feed was supplemented with *L. sporogenes*, the probiotic bacterium. There is no proper flow in sentence formation for diet formulation section and experimental design…both should be separately written.

Our viability testing showed that the probiotic bacterium remained viable after incorporation into the diethow the vaiability was checked. The probiotic's survival bacterium, *L~~actobacillus~~ sporogenes*, in the fish feed was evaluated using the plate count method. Nutrient agar plates (Himedia) were prepared, and the probiotic-containing feed was poured onto the agar plates after being serially diluted. The plates were then incubated and estimated the number of live bacterium cells. The fish received feed for the duration of the experiment in a quantity equal to 5% of their body weight. Physico-chemical parameters were recorded during fortnightly sampling and accordingly all the parameters were maintained. There is no proper flow in sentence formation…rewrite this section

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2.2 Proximate composition of formulated diets

While formulating test diets, all the feed items underwent proximate composition analysis using accepted techniques (AOAC 1995, Table 2) . ~~-~~Samples are heated for 30 minutes at 105 °C, allowing them to cool, and then weighing them at a steady weight, the moisture content was calculated. The FOSS Kjeltec system was used to assess crude protein, the Soxtech system (PELICAN) to determine fat content, and the Fibretech system to determine fiber content (PELICAN). The difference approach (Hastings, 1976) was used to calculate the amount of carbohydrates present in nitrogen free extract (NFE) with the standard formula. There is no proper flow in sentence formation…rewrite.

2.3 Growth parameters

To assess the growth's effectiveness and feed utilization, fish samples were taken every 15 days. Each sampling involved the collection of at least 50% of the stocked fish, which were then each individually weighed and measured. After each sampling, the quantity of feed provided was modified in accordance with the recorded growth in fish weight during the assessment. Various growth parameters, such as weight gain, feed conversion ratio (FCR),

survival rate, and specific growth rate (SGR), were calculated using standard formulae formula maybe provided. There is no proper flow in sentence formation…rewrite.

2.4 Sample collection and immunological parameters

2.4.1 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, the sera obtained from three fish from each treatment group were combined or pooled together. The pooled sera were then stored at a temperature of -40 °C check? for next analysis, specifically for measuring parameters such as total serum protein and lysozyme activity. There is no proper flow in sentence formation…rewrite this section

2.4.2 Nitroblue tetrazolium assay (superoxide anion production)

The nitroblue tetrazolium assay was assessed according to Anderson and Siwicki (1995). Three fish were sampled, and blood was collected from their caudal veins and transferred to EDTA-coated tubes (EDTAK2). After centrifugation (5000 rpm for 10 minutes) the leukocyte-containing buffy coat was dispensed into a microtitre plate for cell adhesion. Following an hour of incubation, the plate was treated with 0.3% NBT solution for another hour. Upon removal of the NBT solution, the cells were treated with a fixing agent, washed, and air-dried. To dissolve the formazan, a solution consisting of 60 µl of 2N KOH and 70 µl of dimethyl sulphoxide (DMSO) was added to each well. Following that, the solution with its distinctive turquoise blue color was analyzed using a microplate reader, measuring the optical density at a wavelength of 620 nm. There is no proper flow in sentence formation…rewrite this section

2.4.3 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 subjected to sequential dilutions, gradually decreasing the amount of sample by 50 µl in each consecutive well. Following that, 125 µl of a suspension of *Micrococcus luteus* was evenly introduced into all the wells. The plate was placed in an incubator at ambient temperature, and the decrease in absorbance at 450 nm was assessed during a duration of 0 to 15 minutes using an ELISA reader. Lysozyme function was quantified based on the rate of reduction in absorbance per minute (0.001 min^{-1}), with one lysozyme unit defined as the enzyme activity that resulted in a decrease in absorbance at this rate. There is no proper flow in sentence formation…rewrite this section

2.4.4 Total plasma protein

Serum plasma protein was determined by the Lowry method with the GeNei™ protein analysis kit. In order to complete the technique, 50 µl of distilled water and 50 µl of serum were combined and added to each well of a 96-well plate. The Lowry reagent was then applied in 200 µl well, and placed in an incubator for 10 minutes at ambient temperature. After the initial incubation, the plate was incubated for an additional 30 minutes with 200 µl of the Folin-Ciocalteu reagent inserted into each well. Then, a spectrophotometer had used to measure the absorbance of each well at 650 nm. There is no proper flow in sentence formation…rewrite this section

2.5 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 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.

2.6. 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. 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 1501-2015-2018/01).

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

2.7 Data analysis

One-Way Analysis of Variance (ANOVA) was performed on the experimental and control groups. To assess the significance the Duncan test was employed. These statistical tests were utilized to evaluate and compare the results obtained from the different treatments. Which statistical software was employed for data analysis

3. Results and Discussion

3.1 Experimental ingredients and diets

In the ~~current~~ 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 ~~make~~ prepare various experimental diets and their ~~approximate makeup~~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

Authors justify why there is no vitamin and other supplements were added to diet

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

Check the units

3.2 Growth metrics and feed utilization

The observed data showed dietary supplementation of probiotics has significantly enhanced fish biomass (weight gain) compared to the non-probiotic group (F0). In 90 days of trail the growth found higher in F3 followed by F1, F2 and F0 (Table, 3). However, no notable disparity were noted in the treatment groups. The F3 group exhibited the lowest but optimal feed conversion ratio (FCR), with the F1, F2, and F0 groups following in that order. The results show that group F1 and F3 had the highest specific growth rates, with values of 2.35±0.11 and 2.37±0.10, respectively. Group F2 had an intermediate specific growth rate of 2.23±0.02, which did not differ from the F1 and F3 groups however differed significantly from the non-supplemented (F0). The control fishes (F0) had the lowest specific growth rate of 2.17±0.11. Furthermore, the inclusion of *L. sporogenes* in the diet generated significantly higher SGR in comparison to the control group.level of significance should be included

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]

FCR is very high…which may not be ideal…justify

Evidence of previous works done by few authors the optimal dietary supplement of *L sporogenes* range was decided at levels of 10^4cfu/g, 10^6cfu/g, and 108cfu/g. In our study results found that there was no discernible variation in treatment groups (F1, F2) although F3 group fishes showed increase weight gain compared to the fishes fed with 10^4cfu/g, 10^6cfu/g of *L. sporogenes* and control group. Results suggested that group F3 with 10^8 cfu/g able to meet fish requirement. However, Eid and Mohamed (2008) opinioned that incorporation of commercial blend of probiotics Bifilac® and Pronifer® had a beneficial effect on weight gain, feed utilization on monosex tilapia (*Oreochromis niloticus*). Similarly, Kumar et al. (2006) validated that nutritional feeding of *B. subtilis* orally at 1.5 $\times 10^3$ cfu/ g^{-1} to *L. rohita* for 60 days improved growth with decrease in FCR. Also, Chaudhary and Qazi (2006) found that the incorporation of *B. circulans* into the feed of *L. rohita* fingerlings positively influenced their growth performance and feeding efficiency. In contrast, Noh et al. (1994) discovered that the utilization of lactic acid bacteria as a dietary component in sea bass did not exhibit significant effectiveness when compared to its potential outcomes on the feeding efficiency of juvenile carp. However, their investigation revealed that higher yield and feed utilization were achieved through the dietary supplementation of *Streptococcus facium* in Israeli carp. In another study by Kennedy et al. (1998) noted higher survivability and feed utilization in marine fish with probiotic inclusion diets. A 20 days feeding trial with *A. hydrophila* in *Pangasianodon hypophthalmus* showed improved growth performance and diet efficiency (Mamun et al., 2020). References was very old…rewrite this section quoting with recent literature

3.3 Non-specific immune parameters

Following the 90-day feeding trial, noteworthy discrepancies ($p<0.05$) in total serum protein levels and superoxide anion production in the blood between the treatment and control groups. Although significant higher super oxide anion production was marked in the 10^6 cfu/g inclusion of *L. sporogenes* group (F2) however these values were not remarkably different with inclusion level of 10^8 cfu/g group (F3). Highest lysozyme activity was recorded in 10^8 cfu/g incorporated

diet (F3), followed by 10^6 cfu/g (F2) and 10^4 cfu/g (F1) diets. Conversely, a meaningful decrease in lysozyme function recorded in the fishes that did not received probiotic supplementation. Authors should describe the results presented in table

The notable results showed that blood respiratory burst is considered as one of vital bactericidal mechanisms in fish (Ellis, 2001) and it also indicates the health status of the fish (Anderson, 1992). Successful use of probiotic bacteria like *Bacillus* sp. which provide higher yield of fish production and better health condition by evoking innate and adaptive immune system (Zhao et al., 2012). Previous findings showed elevated respiratory burst level with incorporation of 5% *Saccharomyces cerevisiae* in the diets of rainbow trout (Siwicki et al., 1994), seabream (Ortuno et al., 2002) and turbot (Toranzo et al., 1995). Similarly, EL-Boshy et al. (2010) documented a notable augmentation in the activity of neutrophils and macrophages in *Bacillus* sp., supplemented group in comparison to the control group. In our study we found significantly higher respiratory burst encountered in *L. sporogenes* supplemented group than the control. 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). References was very old…rewrite this section quoting with recent literature

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*. Notably, major improvements in serum protein levels was noticed in the F2 group, which had a probiotic count of 10^6 cfu/g. 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 (*Labeo 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 tailoring 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 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 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).

Discussion may be written in elaborated manner for this section

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

Feeding for around 90 days with the diet which contained *L sporogenes* challenged with *A. hydrophila* showed significant increase in relative percentage survival (RPS). The RPS value in F3, F2 and F1 group were 83.65%, 79.69% and 77.73% respectively (Figure 1). Significant incremental ($P<0.05$) cumulative mortality pattern were observed during 10 days of post injection in control group (73.33%) compared to the probiotic groups (26.67±3.95%) shoud be written in reverse manner low mortality data should be in first. Challenged fish in the control group showed severe clinical signs including red spots, haemorrhagic lesions, tail and fin rots and scale loss.

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 fish, first line of protection against invading pathogens is predominantly attributed to the innate immune system (Magnadottir 2006). On that note Balcazar et al. (2007) found that *Cyprinus carpio* supplemented with probiotic diet showed survival rates increased up to 97.8 to 100% when exposed to *A. hydrophila*. According to Newaj-Fyzul et al. (2007), probiotic-fed fishes had higher protection level of 65 to 100% when challenged with *Aeromonas* spp., compared to 5 to 15% for control fish. Furthermore, the application of probiotic *B. subtilis* in *L. rohita* has been demonstrated to be effective in controlling *A. hydrophila* infection (Kumar et al. 2006). Aly et al. (2008) observed similar results with the probiotics *B. subtilis* and *L. acidophilus*. It is also noted that *O. niloticus* fed with probiotics exhibited better protection against *A. hydrophila*. References was very old…rewrite this section quoting with recent literature

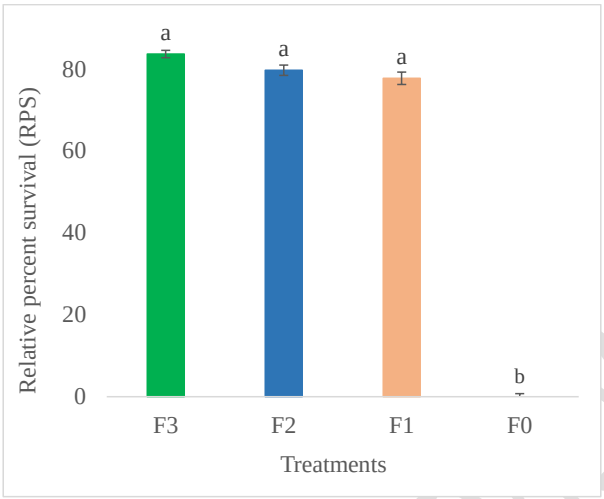

Figure 1: The determination of the relative percentage of survival (RPS) in *L. rohita* was conducted across a range of treatments and the control group following a challenge with A. *hydrophila*.

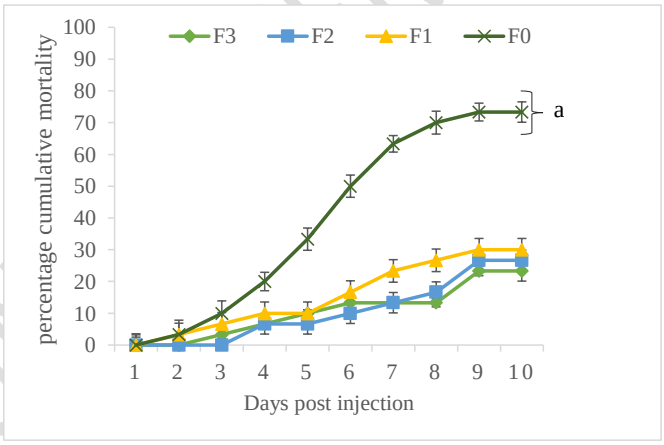

Figure 2: The percentage cumulative mortality (CPM) of *L. rohita* was assessed in the different treatments and control group against *A. hydrophila*.

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. Same view was noted by Tilwani et al. (2022) observed growth improvement and significant higher immunological indices such as lysozyme, phagocytic, immunoglobulin and superoxide dismutase level when Indian Major carp, *Cirrhinus mrigala* fed a probiotic supplemented diet. 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*.

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