Effect of Probiotic (*Lactobacillus rhamnosus*) Supplemented Feed on Growth Performance, Proximate Analysis, and Enzyme Activity of Grass Carp

Safa Imran[1], Muhammad Mubeen Jamal Anwar[2*], Kiran Akram[3], Muhammad Junaid Farooq[4], Kishwar Akram[5], Arshia Mukhtar[6], Muhammad Talha Mukhtar[7], Mariyam Munir[8]

[1,3,5,6,7]Institute of Zoology, University of the Punjab, Lahore, Pakistan.
[2]Department of Food Science and Technology, Faculty of Agriculture and Environment, The Islamia University of Bahawalpur, Pakistan.
[4]National Institute of Food Science and Technology (NIFSAT), University of Agriculture, Faisalabad, Pakistan.
[8]Department of Economics, Management and Territory, University of Foggia, Italy.

Abstract: Aquaculture, a rapidly growing global food-production industry, faces persistent challenges including diseases triggered by high stocking density, overfeeding, and substandard water quality, often necessitating the use of antibiotics and posing risks of immune suppression in fish. Probiotics show a viable alternative for disease mitigation and improved aquaculture practices. **Major Objective**: Thus, the present study evaluated the impact of probiotic (*Lactobacillus rhamnosus*) on the growth dynamics, proximate analysis, and enzymatic activity of grass carp (*Ctenopharyngodon idella*). **Method:**120-grass carp were subjected to two different groups (control and experimental) with triplicate having 20 fish in each. The experimental fish, receiving *L. rhamnosus* at a concentration of 3×10^8 CFU/g, exhibited significant enhancements in weight gain rate of 137%, specific growth rate of 0.91%, survival rate of 77%, and final body weight increased by 50.4 g compared to the control group. The proximate composition demonstrated a significant increase in crude protein (46.32±0.96%) in the experimental group, while moisture content (64.43±0.72%), crude fat (34.30±0.40%) and total ash (9.80±0.80%) were reduced. However, dry matter remained consistent. Furthermore, a significant increase in digestive enzyme activity (amylase, protease, cellulase) was observed. Crucially, key water quality indicators such as dissolved oxygen, pH, and temperature remained unaltered between the experimental and control groups**. Conclusion:** These findings highlight the potential of *L. rhamnosus* to positively influence growth and well-being in aquaculture, emphasizing it as an antibiotic option for disease reduction and sustainable aquaculture.

Keywords: Probiotic, Feed supplementation, Growth performance, Enzyme activity, *Lactobacillus rhamnosus* and *Ctenopharyngodon idella*.

I. INTRODUCTION

Aquaculture plays an essential role in addressing the pressing need for nourishment and food security in our ever-growing global population (Magee, 2018). However, the industry faces a major challenge in the form of bacterial infections, resulting in the widespread utilization of antibiotics as a common approach to managing illnesses (Årdal et al., 2020). The extreme usage of antibiotics has led to negative consequences, such as the build-up of antibiotics in fish tissues, weakened immune systems, and depletion of beneficial microorganisms caused by the rise of antibiotic-resistant bacteria ((Adel et al., 2016; Adel et al., 2017). Probiotics are being considered as a substitute for conventional chemotherapeutic agents in the aquaculture sector, recommended as an entirely novel perspective (Chauhan & Singh, 2019).

Modern aquaculture methods increasingly make use of probiotics. Probiotics have proven time and time again in aquatic environments to increase feed intake, boost cultured species growth and survival, and effectively reduce the spread of disease (Ibrahem, 2015). Probiotics serve as both preventive and curative supplements, and they perform a vital role in enhancing the health of organisms living in water (Hoseinifar et al., 2016; Kiron, 2012; Kühlwein et al., 2014; Nayak, 2010). (LAB), such as *L. rhamnosus*, *L. thermophilus*, *L. casei*, and *L. bulgaricus* have been shown to be valuable biological agents in aquaculture(Ashraf & Shah, 2011). The genus *Lactobacillus* is a gram-positive bacterium. It is unable to move and does not produce spores.

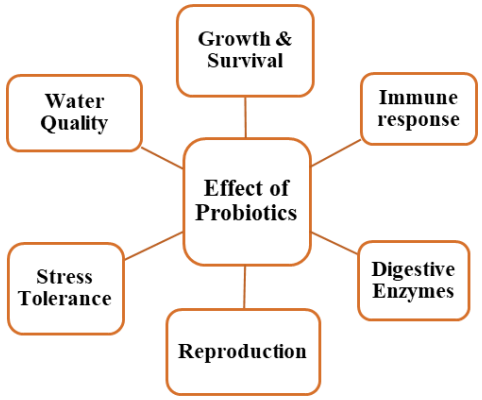

Figure 1: ***Characteristics of probiotics advantageous for aquaculture.***

As depicted in Figure 1, these bacteria play vital physiological functions as beneficial probiotics in the intestines. As part of its role, it aids in ensuring that fish have access to nutrients, produces antimicrobial chemicals, enhances immune responses, and fortifies their resistance to illnesses(Thirumdas et al., 2021). *Lactobacillus* also has a substantial impact on enhancing the

survival, growth, and disease resistance of various aquaculture species (Esteban et al., 2014).

According to Kühlwein et al. (2014), cyprinids are classified under the order *Cypriniforms* based on taxonomic classification. Carp possesses a major role in contemporary aquaculture, accounting for around 72% of the total production. The growing demand for carp species poses a challenge to farmers. They intend to control illnesses while accelerating the growth rates of fish. Due to the expensive aquaculture system and its increased vulnerability to disease outbreaks, extensive methods provided a way to semi-intensive or intense approaches (Chen et al., 2014). Scientists have used novel strategies, innovative technology, and sustainable approaches to address these challenges. **Objective:** Our research aims to optimize grass carp growth characteristics and crude protein content while limiting health risks.

II. MATERIAL AND METHODS

A. Experimental Design

The experimental testing was done at the Microbiology laboratory of the University of Punjab, Lahore. The aquaria were disinfected before stocking with 0.005 g/L of KMnO4 for 15 minutes. After that, the aquariums were thoroughly washed with tap water. The aquarium water was dechlorinated and adequately oxygenated. Tanks were equipped with air pumps that held air stones to provide continuous aeration. 50% of aquarium water was replaced on a daily basis in each tank to maintain the ideal conditions.

One hundred and twenty grass carp were taken from the Manawa Hatchery Research Institute, Tulspura, Lahore. A basic random design was used to divide the fish into control and experimental groups. 120 grass carp were divided into control and experimental groups. Each group consisted of a triplicate of twenty fish each. The control group was fed a commercial feed, while the experimental group was given a feed fortified with Lactobacillus rhamnosus at a concentration of 3×10^8 CFU/g. A further 14-day acclimation period was then mandated for all fish. Pellets equal to five percent of the fish's average body weight were given to them twice daily throughout the acclimation stage as a baseline diet. After acclimation, strong fish were selected and distributed at random into 60×30×30 cm glass aquariums, with 20 grass carp per tank as an initial stocking density. Early on in the experiment, we took the initial measures of the average length and weight of the grass carp.

B. Probiotic Preparation

The experiment used *Lactobacillus rhamnosus*, a probiotic bacterium sourced from Lahore's University of the Punjab's Microbiology and Biotechnology Laboratory. After obtaining the bacterial cells from the strain, they were introduced to the MRS broth using an inoculating loop. Following this, they were incubated at 37˚C for 48 hours and then Centrifuged for 10 minutes at 4000 rpm at a temperature of 25˚C to collect the resulting cells. Using a vortex mixer, the solid cell pellets were combined with distilled water after discarding the liquid part above the cells, called the supernatant. To achieve the desired concentration of live bacteria (3×10^8 CFU/g), the bacterial solution was titrated using spectrophotometry to an optical density (OD) of 600nm after the preparation of the required serial dilutions (Suh et al., 2019).

C. Experimental Feed

The experimental diet contains 30% crude protein. A concentration of 3×10^8 CFU/g of bacterial cells was introduced to the diet of the treatment groups, while the control group received feed devoid of any bacterial cells.

D. Feeding Procedure

The fish were provided with feed two times a day at a feeding rate of 5% of their total body weight. The bacterial-cell-augmented feed was administered via syringes and divided uniformly among the experimental group's duplicates according to their body weight. Alterations were made to the daily nutrition allocation every two weeks in response to fluctuations in body weight. Following an hour of feeding, this process is executed to remove any residual feed through syphoning, thereby mitigating contamination in the aquarium.

E. Assessment of Physio-Chemical Parameters

The physicochemical parameters were recorded twice a day during the entire experimental trial. The temperature was assessed using a thermometer. Dissolved oxygen was found using a DO meter. The pH was determined using a pH meter. The measurement of nitrates and phosphates was conducted using the HANNA Nitrate Test Kit HI3874 (Gawankar & Masten, 2023).

F. Growth Performance

The growth performance was assessed by individually weighing all fish in both the treatment and control groups at the beginning and the end of the 8-week study period. The feed ratios were modified after two weeks in response to the fish's weight gain (Aman et al., 2022). The growth parameters were calculated;

$$IAW = W_i / N_0$$
$$FAW = W_f / N_t$$
$$SR\ \% = N_t / N_o \times 100$$
$$WGR\ \% = (W_f - W_i)/W_i \times 100$$
$$SGR\ \% = (LnW_f - LnW_i) / t \times 100$$
$$Survival = (C_f / C_i) \times 100$$

In the following equations, the IAW represents the initial average body weight, FAW is the final average body weight, W_i means the initial body weight of all fish, N_0 means the number of fish in aquaria, N_t represents the number of fish at the end of trial, N_o represents number of fish at start, W_f means final body weight, W_i means initial body weight, t stands for days of feeding trial, C_f means final fish count and C_i means initial fish count.

G. Proximate Composition

Fish were scheduled for proximate analysis after the feeding trial ended. Standard methods are used to evaluate moisture, crude protein, fat, and ash percentages (AOAC, 2002).

H. Digestive Enzyme Investigation

During the harvesting process, a total of 9 fish/aquaria were anaesthetized using clove oil at a con. of 0.20ml. Fish guts were dissected in an ice bag and cleaned with saline solution. For quantitative analysis, 0.5 g of intestine was measured and then crushed in 5 ml of cooled PBS-buffered saline. At 4.0ºC, the homogenate was centrifuged for 10 minutes at a speed of 10,000

rpm. The enzyme solution was transferred to a test tube for investigation of enzyme activity.

i. Cellulase Activity

Denison and Kohen (1977) technique were used to assess the activity of the cellulase enzyme. Concisely, 1 mL volume of 1% carboxymethyl cellulose (CMC) solution was introduced into a 10-mL glass tube combined with 1.0 mL of citrate buffer. The solution was incubated at 50°C for 30 min. After incubation, 3.0 mL of DNS reagent was added and incubated at boiling point for 15 min. Afterwards, 1.0 mL of Na K tartrate (40%) was added, and let it cool. The activity of cellulose-degrading enzymes was analyzed at 540 nm on a UV-visible spectrophotometer.

ii. *Protease Activity*

The proteolytic activity was assessed using the casein-hydrolysis technique by Kothary and Kreger (1985). Concisely, 0.50 mL of casein with 0.50 mL of glycine-sodium hydroxide buffer and 0.2 mL of enzyme extract were added. The mixture was incubated at 37°C/h. This reaction was halted by incorporating 1.20 mL of 8% trichloroacetic acid, and then the sample was cooled at 4°C. Afterwards, it was placed in centrifugation for 10 min at 1800 rpm. The resultant supernatant was observed at a wavelength of 280nm, and L-tyrosine served as a reference compound.

iii. Amylase Activity

The a-amylase was quantified using the starch-hydrolysis technique Robyt (2009). The enzymatic reaction mixture consisted of 0.2 mL digestive extract, 0.1 M citrate-phosphate buffer, and a 2% (w/v) starch solution of 0.50 mL. The combination underwent incubation at 37 °C for 1 hour. Subsequently, adding 1 mL of DNS reagent, the mixture was heated to boiling point for 15 min, then analyzed at a wavelength of 600nm. The activity unit of a-amylase was determined using maltose.

I. Statistical Analysis

The data is shown as Means ± SEM and was analyzed using a paired t-test in the SPSS 20.0 programme (Valizadeh et al., 2016). A p-value ($p<0.05$) indicates significant differences between the two groups.

III. RESULTS

Probiotics-fed diet enhanced the growth and survival of C. idella. The growth parameters of *C. idella* were monitored by comparing the body weight of the control with the treatment group. The diet, which contained probiotic *Lactobacillus rhamnosus,* was given to the treatment group, and after two months, results were drawn. Final body weight (FBW), final body length (FBL), specific growth rate (SGR) and weight gain were significantly enhanced ($p<0.05$) in the probiotic-supplemented group as compared with control, which was fed with a normal diet (Table 1). SR varied by 73% in the control and 77% in the treatment group, respectively.

Table 1: Effects of *Lactobacillus rhamnosus* on grass carp (*Ctenopharyngodon idella*) growth parameter after dietary supplement.

Parameters	Control Group	Treatment Group
IBW (g)	15.44±0.01	14.94±0.03
FBW (g)	32.53±0.23[a]	50.42±1.42[b]
IBL (cm)	11.01±0.00	11.97±0.07
FBL (cm)	18.31±0.09[a]	27.86±0.59[b]
Wt. gain (g)	17.09±0.23[a]	35.48±1.40[b]
Wt. gain % (g)	10.69±1.50[a]	137.55±9.18[b]
SGR (%BW /day)	0.44±0.01[a]	0.91±0.03[b]
ADG (g)	0.19±0.00[a]	0.39±0.01[b]
IBM (g)	231.86±0.00	224.03±0.63
FBM (g)	357.83±2.55[a]	588.14±36.01[b]
GBM (g)	125.96±2.55[a]	364.11±35.37[b]
Survival %	73±0.00[a]	77.33±4.33[b]

Mean± SEM is the data representation. Significance is indicated by means that have distinct letters ($p<0.05$).

Physio-chemical analysis

The statistical analysis of the basic parameters related to water quality is demonstrated (Table 2). The results on temperature, dissolved oxygen and pH values exhibited no statistical change in both feeds supplemented with probiotic and control groups.

Table 2: In a 60-day culture trial of grass carp, water quality indicators were measured for both the control and experimental groups.

Parameters	Control Group	Treatment Group
T (°C)	24±0.00[a]	24±0.00[a]
DO (mg /L)	5.9±0.03[a]	5.9±0.01[a]
pH	7.7±0.04[a]	7.6±0.02[a]

Values are expressed as Mean ± SE.

Probiotics Increased the Protein Content in the Muscles of C. idella.

A study was done to determine the impact of *L. rhamnosus* supplementation on the proximate composition of *C. idella* muscle tissues. We observed a statistically significant increase in the percentage of protein contents, while moisture content, crude fat, and total ash were reduced in *L. rhamnosus* fortified treatment as compared with the control. However, the dry matter content remains unchanged (Table 3).

Table 3: Effect of probiotic fortification on proximate composition of grass carp (*Ctenopharyngodon idella*) muscles.

Parameters	Control Group	Treatment Group
Moisture	74.79±0.96[a]	64.43±0.72[b]
Crude protein	41.10±0.99[a]	46.32±0.96[b]
Crude Fat	38.17±0.61[b]	34.30±0.4[a]
Total Ash	12.65±0.99[b]	9.80±0.80[a]
Dry Matter	90.45±0.39[a]	90.77±0.28[a]

Values are expressed as Mean ± SE.

Probiotic-fed Supplements increased Enzyme Activity

The relative functions of digestive enzymes in *C. idella* were assessed following a 2-month feeding study, comparing the effects of a probiotic diet and a baseline diet.

Table 4: Results of enzyme activity on grass carp (*Ctenopharyngodon idella*) samples taken at the end of a 60-day culture trial

Parameters	Control Group	Treatment Group
Cellulase activity	0.07±0.00[a]	0.14±0.15[b]
Amylase activity	0.05±0.00[a]	0.14±0.01[b]
Protease activity	0.07±0.00[a]	0.25±0.02[b]

Values are expressed as Mean ± SE.

A notable increase in the activity levels of all three enzymes was detected in the treatment group in comparison to the control group (Table 4).

IV. DISCUSSION

Probiotic species that are distinct from each other have demonstrated beneficial impacts on both feed efficiency and growth. This research aimed to determine the impact of feed supplementation with *Lactobacillus rhamnosus* on *Ctenopharyngodon idella* growth performance, proximate composition, and enzyme activities. Significant results were achieved over 2 months of the experimental trial, which was similar to an earlier investigation (Chaudhary & Qazi, 2007).

According to Dawood et al. (2017), the efficacy of probiotic therapy using a single species is established in terms of improved growth performance and feed utilization. Nevertheless, only a few studies have specifically examined the potential synergistic impacts that may arise from the concurrent introduction of several bacterial species to fish (Giri et al., 2014). The data obtained from our research clearly indicated that the administration of probiotics had a considerable positive effect on the growth parameters of *C. idella* after a period of 60 days, which was similar to an earlier study (Aman et al., 2022). The results of our work are also corroborated by prior research done on several fish species using different probiotics. Our research showed that the *C. idella* SGR had much higher and enzymatic activity in the probiotic (*L. rhamnosus*) group than in the control group, which was found in an investigation on mori fish (Ullah et al., 2018).

A study done on *Labeo rohita* in which feed supplement mixed with probiotics *Bacillus* NL110 and *Vibrio* NE17 has also significantly increased the fish growth performance (Mujeeb Rahiman et al., 2010). These findings have also supported our findings. It is predicted that alterations in body components such as fat and protein levels may be associated with their production, rate of accumulation in muscle and overall development rate. The current study showed a considerable rise in protein levels in the muscles of *C. idella* that were given probiotics-supplemented feed. Similar findings have been found in other investigations on fish species such as *Labeo rohita* and *Cirrhinus mrigala* (Mohapatra et al., 2012; Ullah et al., 2018).

In this study, the *C. idella* probiotic-supplemented group had a significant increase in protease, amylase, and cellulase activity. The previous research on *C. mrigala* using a different probiotic aligns with this study (Ullah et al., 2018). Our experimental result on digestive enzyme activity also aligns with this study's findings that fed dietary probiotic supplementation mix diet to *Oreochromis niloticus*, which exhibited increased amylase activity (Garg, 2015). We also found that probiotic supplements increased cellulase and protease activity.

V. CONCLUSION

The present research concluded that L. rhamnosus is a growth enhancive feed supplement for C. idella. Our findings suggest that strategically formulated probiotics might improve the survival, development, and growth rate of fish, leading to a potential rise in world fish output. Hence, probiotics would be added as a supplement in feed in order to assess their optimal utilization in fish. It is recommended that to comprehensively assess the extensive utilization of probiotics, it is needed to consider factors like species, source, quality, and application techniques.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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AUTHORS

First Author – **Safa Imran**
Institute of Zoology, University of the Punjab, Lahore, Pakistan:

Correspondence Author – **Muhammad Mubeen Jamal Anwar***
Department of Food Science and Technology, Faculty of Agriculture and Environment, The Islamia University of Bahawalpur, Pakistan.
https://orcid.org/0009-0002-7155-6500

Kiran Akram
Institute of Zoology, University of the Punjab, Lahore, Pakistan

Muhammad Junaid Farooq
National Institute of Food science and technology (NIFSAT), University of Agriculture, Faisalabad.

Kishwar Akram
Institute of Zoology, University of the Punjab, Lahore, Pakistan

Arshia Mukhtar
Institute of Zoology, University of the Punjab, Lahore, Pakistan.

Muhammad Talha Mukhtar
Institute of Zoology, University of the Punjab, Lahore, Pakistan.

Mariyam Munir
Department of Economics, Management and Territory, University of Foggia, Italy.