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Protease activity in *Labeo rohita* fingerlings fed *Lactobacillus acidophilus* and *Spirulina platensis*

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Abstract

In the present 75-day experiment, protease activity was studied in the intestinal tissue of fingerlings of the freshwater fish *Labeo rohita*. The fish were fed diets containing 2%, 4%, and 6% concentrations at the rate of 3% of their body weight at 15-day intervals. The 210 fingerlings were divided into 4 groups; Fish group To (control diet) were fed a diet containing feed ingredients without experimental additives. Fish groups T1L, T2L, and T3L (experimental diets) were fed diets containing 2%, 4%, and 6% *Lactobacillus acidophilus* tablet (SPORALAC-DS) powder, in that order. Fish groups T1S, T2S, and T3S were fed diets containing 2%, 4%, and 6% *Spirulina platensis* powder, respectively. After the 75-day period, total protease was measured in the intestine of the fingerlings. It was observed that intestinal enzyme activity increased over time in both the control and experimental groups. Protease activity was highest in the 6% diet concentration groups, with significant increases seen in the *Spirulina platensis*-fed fingerlings compared to the control group and other groups. The results indicate that protease activity was highest in the group fed 6% *Spirulina platensis* (T3S), followed by the group fed 6% *Lactobacillus acidophilus* (T3L). This suggests that a 6% dietary concentration of either *Spirulina platensis* or *Lactobacillus acidophilus* can help enhance protease activity and growth in *L. rohita* fingerlings, with *S. platensis* showing greater efficacy.

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Introduction

In aquaculture, proper nutrition is critical for maintaining normal growth rate, survival rate, and health of fish. Good nutrition can mitigate stress effects, decrease disease susceptibility, and boost the immune system (Oliva-Teles, 2012). Fish exhibit growth differences from environmental fluctuations Matthias *et al.* (2018). As an important protein source in human diets, fish growth is an increase in overall length and weight under feeding regimes (Albrektsen *et al.*, 2006; Sarder *et al.*, 2011).

Labeo rohita, an Indian major carp, is an importantly herbivorous column feeder preferring algae and submerged vegetation (Javaid Iqbal *et al.*, 2013). In the fingerling stage, there is strong positive selection for zooplankton. In juvenile and adult stages, nutrient requirements increase for biological functions. Thus, studying digestive physiology is critical. Digestive enzymes are responsible for nutrient utilization in *L. rohita* (Portella and Dabrowski, 2008).

Digestion is a key metabolic process determining nutrient uptake and growth. Proteases in the digestive tract largely determine digestion and absorption rates of essential amino acids from dietary protein (Eshel *et al.*, 1993). The fish is a rich protein replacement source. Generally, the pancreas secretes digestive enzymes like proteases through the intestinal lumen. Protease digestion products in the lumen are free amino acids and small peptides. These enter epithelial cells where specific proteases hydrolyze them into absorbable amino acids. Initially, proteins are broken down by intestinal trypsin (pancreatic secretion) (Moraes and de Almeida, 2020).

Probiotics promote microbial balance, inhibit pathogen growth, enhance immunity, and improve digestion through enzymatic activities (Saravanan *et al.*, 2021). Intestinal microflora participates in digestion, supplying nutrients like enzymes, vitamins, and amino acids (Rowland *et al.*, 2018). Probiotics may improve digestibility, absorption, and digestive enzyme activity (Fuller, 1989). This study aimed to determine the effect of different feed concentrations on protease activity in *Labeo rohita* fingerlings, providing insight into protein utilization.

Material and methods

Collection and acclimation of fish

Labeo rohita fingerlings (weight 5±0.4 g, length 5.25±0.2 cm) were collected from Surya Fish Farm, Kallidaikurichi, Tirunelveli District, Tamil Nadu, India (8°40'47"N, 77°28'35"E) using a 12m cast net. Fingerlings were transported in oxygenated plastic bags (density 100-130 kg/m3, thickness 0.1-0.15 mm) to the centre for Aquaculture and Research Extension (CARE) laboratory (8.7180°N, 77.73.88°E) at St. Xavier's College (Autonomous), Palayamkottai, Tamil Nadu, India. In the CARE laboratory, fingerlings were acclimated in a cement tank (9' x 5') with continuous aeration for 14 days prior to experiments. During acclimation, fish were fed a control diet without supplements. The control diet contained 25% fish meal, 25% soybean meal, 25% groundnut oil cake, 12% rice bran, 5% tapioca flour, 3 ml sunflower oil, 4 ml egg albumin, and 1gram multivitamin mix. After acclimation, the control-fed *L. rohita* were used as a reference to evaluate growth and biochemical parameters in fish fed experimental diets containing supplements.

Experimental procedure and feeding regimen

Labeo rohita fingerlings (5±0.4 g weight, 5.25±0.25 cm length) were selected. Feeds were *Spirulina platensis* at 2% (T1S), 4% (T2S), 6% (T3S), 8% (T4L), 10% (T5L) and 12% (T6L). *Lactobacillus acidophilus* was at 2% (T1L), 4% (T2L), 6% (T3L), 8% (T4L), 10% (T5L) and 12% (T6L). Control (TO) have no feed. Triplicate plastic troughs had 10 fish each. Fish were fed 3% body weight twice daily. Aquarium water was siphoned daily. Uneaten feed and feces were collected and sun dried. Experiments lasted 75 days. Effects of bacterial probiotic (*L. acidophilus*) and algal (*S. platensis*) feeds on *L. rohita* fingerling growth were observed. Enhanced growth may be from improved digestion, enzymatic activity and vitamin synthesis. Fish were sampled every 15 days. Total feed increased progressively. Feces and uneaten feeds were recorded. At end, 6% probiotic groups had less feces and uneaten feed than control.

Formulation and preparation of experimental diets

Ingredients were ground and sieved separately. Two groups were made: control diet without microalgae/probiotics, and three experimental diets

with 2%, 4%, 6% *L. acidophilus* replacing fishmeal. Blends were steam-cooked 15 minutes at 95-100°C then cooled. Cooked blends were mixed with *L. acidophilus* coated tablets (≥120 million spores). After 75 days, three fish per trough were sacrificed. Alimentary tracts were dissected, washed externally and rinsed. They were split open and washed thoroughly. Multivitamin tablets, sunflower oil, binders (egg albumen, tapioca flour) were blended. Water was added to make dough. Pellets were made using 3mm die and dried until <10% moisture. Pellets were examined for appearance, uniformity, color and aroma.

Experimental procedure and feeding trait

Labeo rohita fingerlings of uniform size (5±0.4g in weight, 5.25±0.2cm in length) were carefully selected for feeding experiments. Different feeds at varying concentrations were used, including the probiotic bacterium *Lactobacillus acidophilus* at 2% (T1L), 4% (T2L), 6% (T3L), 8% (T4L), 10% (T5L) and 12% (T6L) and the prebiotic alga *Spirulina platensis* at 2% (T1S), 4% (T2S), 6% (T3S), 8% (T4S), 10% (T5S), and 12% (T6S). (0%) To was the control group receiving no supplementary feed. Triplicate groups were maintained, with 10 fishes introduced into each plastic trough. The fishes were fed an amount equivalent to 3% of their body weight twice a day. The water in each aquarium tank was siphoned daily to remove and store the remains of uneaten feed and fecal matter separately for sun drying.

Preparation of enzyme source

After the experimental periods 75 days 4 test species (*L. rohita*) from each trough (Triplicate) were removed and starved for 24 hours and sacrificed. The whole alimentary tract was dissected out in ice cold fish ringer solution and washed thoroughly externally. The tissue was then rinsed with cold distilled water, spilt open and washed thoroughly in fish ringer. The tissues were homogenized separately with distilled water using mechanical dispenser. The tissue concentration was made to 10mg/ml with 0.09% chilled Nacl solution. Then the homogenate was centrifuged at 3000 rpm for 10 min. (Remi model K-11). The clear Aliquot of supernatant was used as the enzyme source for subsequent assay. Then the homogenize matter was stored in freezer until used (FDA, 1993).

Protease activity

Total protease activity was determined by the casein hydrolysis method described by Walter (1984) and adapted by Hidalgo *et al.* (1999) Buffers for each pH assays were: 0.1 M glycine-NaoH, pH-10.0.A reaction mixture contained casein at 1% (W/V) (0.25ml) buffer (0.25ml) and supernatant from the homogenates (0.1ml) was incubated for 1h at 37°C. The reaction was stopped by addition of 0.6 ml 8% (W/V) trichloroacetic acid solution; kept for 1hr at 2°C; centrifuged at 1800g for 10 min and the absorbance of supernatant was measured at 280 nm against blank For the blank preparation, the supernatant from the homogenates was added at the end of the incubation period, just before addition of trichloroacetic acid. Tyrosine solution was used as standard. One unit of enzyme was defined as the amount of enzyme required to catalyze the formation of 1.0 µmol of tyrosine per min. Castro *et al.* (2013)

Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) using IBM SPSS Statistics 22 software. Values are expressed as mean plus or minus standard deviation and $p < 0.05$.

Ethics statement

The fish were treated humanely according to SXC/CARE/EC/2018 (consultation 30 November 2018).

Results and discussion

Table 1 represents protease activity in the intestine of fish *Labeo rohita* fed on *Lactobacillus acidophilus* (2%, 4% and 6%) as well as *Spirulina platensis* (2%, 4% and 6%) supplementary feeds. At 75 days of feeding time, higher protease activity was seen in fishes fed on 6% concentration of *S. platensis* (584.00±0.57 µmol/mg/day) and 6% concentration of *L. acidophilus* (577.66±0.3 µmol/mg/day).

Table 1. Protease Activity from intestine of fingerlings of *Labeo rohita* fed on control and formulated experimental feeds after 75 days.

Supplementary feed	Control	Experimental formulated diets		
Lactobacillus acidophillus	To (0%)	T_1L (2%)	T_2L (4%)	T_3L (6%)
	503.66±0.33	508.00±0.57	521.33±0.33	577.66±0.3
Spiulina platensis	To (0%)	T_1S (2%)	T_2S (4%)	T_3S (6%)
	503.33±0.3	557.6±0.3	578.00±0.00	584.00±0.57

The lowest activity was recorded in rohu fingerlings fed on the control feed (0%) (503.66±0.33 µmol/mg/day). At 75 days, protease activity was significantly increased ($P<0.05$) in rohu fed 2% and 4% concentrations of *L. acidophilus* (508.00±0.57, 521.33±0.33 µmol/mg/day) and 2% and 4% concentrations of *S. platensis* (557.6±0.3, 578.00±0.00 µmol/mg/day). The 2% and 4% concentrations of supplementary feeds were similar among themselves. The highest activity was recorded in rohu fishes fed on 6% concentration of *Spirulina platensis* (584.00±0.57 µmol/mg/day) followed by 6% concentration of *L. acidophilus* (577.66±0.3 µmol/mg/day).

Supplementing feed with 6% *Lactobacillus acidophilus* and *Spirulina platensis* significantly increased intestinal protease activity and growth in *Labeo rohita* fingerlings. Digestive enzymes like proteases are sensitive to diet and affect fish health.

According to Bhilave (2019) reported, Proteases can hydrolyse almost any protein, provided the proteins are not part of living cells that are protected by inhibitor mechanisms that characterizing and quantifying protease activities can provide better understanding of fish digestive physiology, enable improvements to feeding After a 75-day time interval, the fish fed with 50% formulated feed exhibited the highest protease activity (0.693±0.226 mg tyrosine per gram of protein-specific activity per hour). This was followed by the fish fed with 100%-formulated feed (0.553±0.181 mg tyrosine per gram of protein-specific activity per hour) and the fish fed with 75% formulated feed (0.513±0.145 mg tyrosine per gram of protein-specific activity per hour). Regimens, and assist development of optimized feeds for fish farming.

Debnath and Saikia (2020) investigated two teleosts, *Labeo rohita* and *Anabas testudineus*, that have divergent feeding habits (herbivorous and carnivorous, respectively), to assess the amylase and protease activity in various segments of their digestive tracts. The research revealed significant variations in enzymatic activity across different regions of the digestive tracts. Specifically, in Rohu, which has a stomachless gut divided into three equal regions, the highest amylolytic activity was observed in the posterior digestive tract, whereas the highest proteolytic activity was primarily localized to the mid-region.

Kumar *et al.* (2007) studied, that rohu had significantly higher total protease activity (1.219 ± 0.059 U mg protein−1 min−1) than silver carp (1.084 ± 0.061 U mg protein−1 min−1) and catla (0.193 ± 0.006 U mg protein−1 min−1). Protease activity in rohu and silver carp showed peak activity at pH 9, while *C. catla* displayed maximum protease activity between pH 8-11. Inhibition studies using soybean trypsin inhibitor (SBTI) and phenylmethylsulfonyl fluoride (PMSF) indicated the proteases were serine proteases. Furthermore, inhibition by N-α-p-tosyl-L-lysine-chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanychloromethane (TPCK) suggested the presence of trypsin-like and chymotrypsin-like enzymes in all three carp species.

de Medeiros *et al.* (2022) investigated that *S. platensis* biomass stimulated greater growth and metabolic activity of *L. acidophilus* compared to the prebiotic fructooligosaccharide (FOS), as evidenced by higher proliferation rates, lower pH, and increased production of acetic, lactic, and propionic acids. Goswami *et al.* (2020) evaluated that protease activity was significantly higher in rohu fed diets containing

fermented duckweed (FTC) and fermented feed (F), while lipase activity was significantly higher in rohu fed the FTC diet compared to other diets. Replacing 300 g/kg of fishmeal with raw duckweed in the feed did not affect Rohu growth.

Recommendation

Aquaculture provides protein, income, jobs, poverty reduction, community growth, and food security while accommodating various fish sizes and feeding habits. Modifying protease activity through probiotics can improve fish growth. The present study found the highest protease activity in *Labeo rohita* fed 6% *Spirulina platensis* and 6% *Lactobacillus acidophilus* supplements. Probiotics may enhance protease activity and protein digestion, benefiting growth in this economically important fish. Further research can optimize probiotic use for sustainable Indian carp aquaculture.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Albrektsen S, Mundheim H, Aksnes A. 2006. Growth, feed efficiency, digestibility and nutrient distribution in Atlantic cod (*Gadus morhua*) fed two different fish meal qualities at three dietary levels of vegetable protein sources. Aquaculture **261**(2), 626-640.

Castro C, Pérez-Jiménez A, Coutinho F, Pousão-Ferreira P, Brandão TM, Oliva-Teles A, Peres H. 2013. Digestive enzymes of meagre (*Argyrosomus regius*) and white seabream (*Diplodus sargus*). Effects of dietary brewer's spent yeast supplementation. Aquaculture **416**, 322-327.

De Medeiros VPB, Salgaço MK, Pimentel TC, da Silva TCR, Sartoratto A, Lima MDS, Sassi CFDC, Mesa V, Magnani M, Sivieri K. 2022. *Spirulina platensis* biomass enhances the proliferation rate of *Lactobacillus acidophilus* 5 (La-5) and combined with La-5 impact the gut microbiota of medium-age healthy individuals through an in vitro gut microbiome model. Food Research International **154**, 110880.

Debnath S, Saikia SK. 2020. Characterization of amylase and protease activity in the digestive tract of two teleosts (*Labeo rohita* and *Anabas testudineus*) with different feeding habits. Acta Biologica Szegediensis **64**(2), 173-179.
DOI:10.14232/abs.2020.2.173-179173

FDA U. 1993. Enzyme preparations: recommendations for submission of chemical and technological data for food additive petitions and GRAS Notices.

Fuller R. 1989. A review: probiotics in man and animals. Journal of Applied Bacteriology **66**(5), 365-378.

Hidalgo MC, Urea E, Sanz A. 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture **170**, 267-283.

Kumar S, Garcia-Carreño FL, Chakrabarti R, Toro AN, Córdova-Murueta JH. 2007. Digestive proteases of three carps *Catla catla*, *Labeo rohita* and *Hypophthalmichthys molitrix*: Partial characterization and protein hydrolysis efficiency. Aquaculture Nutrition **13**(5), 381-388.
https://doi.org/10.1111/j.1365-2095.2007.00488.x

Madhav Bhilave. 2019. Protease activity profile in freshwater fish *Labeo rohita* fed on formulated fish feed. International Journal of Research and Analytical Reviews **6**(2), 347-355.

Matthias BG, Ahrens RN, Allen MS, Tuten T, Siders ZA, Wilson KL. 2018. Understanding the effects of density and environmental variability on the process of fish growth. Fisheries Research **198**, 209-219.

Mir JI, Sarkar UK, Gusain OP, Dwivedi AK, Joukrushna J. 2013. Age and growth in the indian major carp *Labeo rohita* (Cypriniformes: Cyprinidae) from tropical rivers of Ganga basin, India. Rev Biol Trop **61**(4), 1955-66.

Moraes G, de Almeida LC. 2020. Nutrition and functional aspects of digestion in fish. In Biology and physiology of freshwater neotropical fish (pp. 251-271) Academic Press.

Oliva-Teles A. 2012. Nutrition and health of aquaculture fish. Journal of Fish Diseases **35**(2), 83-108.

Portella MC, Dabrowski K. 2008. Diets, physiology, biochemistry and digestive tract development of freshwater fish larvae. Feeding and digestive functions of fishes. Enfield: Science Publishers, 227-279.

Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, Tuohy K. 2018. Gut microbiota functions: metabolism of nutrients and other food components. European Journal of Nutrition **57**, 1-24.

Saravanan K, Sivaramakrishnan T, Praveenraj J, Kiruba-Sankar R, Haridas H, Kumar S, Varghese B. 2021. Effects of single and multi-strain probiotics on the growth, hemato-immunological, enzymatic activity, gut morphology and disease resistance in Rohu, *Labeo rohita*. Aquaculture **540**, 736749.

Sarder MRI, Yeasin M, Jewel MZH, Khan MMR, Simonsen V. 2011. Identification of Indian major carps (*Catla catla*, *Labeo rohita* and *Cirrhinus cirrhosus*) and their hybrids by phenotypic traits, allozymes and food habits. Asian Fisheries Science **24**(1), 49-61.

Sreekanth GB, Mayekar TS, Kumar S, Rivonkar P, Varghese T, Kumar S, Chakurkar EB. 2021. Fresh Water Ornamental Fish Culture and Management (Scheduled Caste Sub Plan) Govt. of India. ICAR-Central Coastal Agricultural Research Institute, ICAR-Central Institute of Fisheries Education, Mumbai, Maharashtra. Technical Bulletin No. **69**.

Walter HE. 1984. Proteinases: methods with hemoglobin, casein and azocoll as substrates. In: Bergmeyer, H.J. (Ed.), Methods of Enzymatic Analysis, Vol. V. Verlag Chemie, *Weinham*, 270-277 p.