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# Exogenous papain supplementation: impacts on growth, digestibility, digestive enzyme activities and oxidative stress in *Labeo rohita* fingerlings

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## Abstract

This study was conducted to evaluate the effects of varying levels of dietary exogenous papain on the growth performance, survival rate, nutrient utilization, protein digestibility, digestive enzyme activity, and oxidative stress response in *Labeo rohita* fingerlings. A control diet was formulated using rice bran, groundnut oil cake, wheat flour, and a vitamin-mineral mixture. Experimental diets were prepared by supplementing the control diet with different levels of papain enzyme powder: T0 (0% papain, control), T1 (1.0%), T2 (2.0%), T3 (3.0%), T4 (4.0%), and T5 (5.0%). The diets were fed to the fish for 60 days. Results indicated that weight gain, weight gain percentage, daily growth rate (DGR), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and apparent protein digestibility (APD) significantly improved ($P < 0.05$) with the inclusion of up to 2% papain in the diet. Throughout the experiment, a 100% survival rate was observed in all groups except T4. Significant differences ($P < 0.05$) were also noted in PER, protein absorption ratio (PAR), and digestive enzyme activities (lipase and protease) among the treatments. The highest intestinal protease and lipase activities were observed in fish fed the diet supplemented with 2% papain. Additionally, liver antioxidant enzyme activities, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), were significantly enhanced with up to 2% papain inclusion in the diet. Therefore, a 2% inclusion of papain enzyme in the diet is recommended to optimize growth performance, protein digestibility, digestive enzyme activities, and oxidative stress response in *Labeo rohita* fingerlings.

**Keywords** Papain enzyme, Rohu, Growth performance, Digestibility, Digestive enzymes, Oxidative stress

## Introduction

The aquaculture sector is vital for food production, significantly contributing to nutritional security, agricultural exports, and providing livelihoods to around 61.8 million people globally [11]. The increase of aquaculture production is expected to exhibit a positive correlation with the concurrent enhancement of quality feed production, aimed at fulfilling the nutritional needs of cultivated fish [45, 46]. Quality nutritive artificial feed is a determining element for fish growth, thus a good artificial feed supply in aquaculture becomes increasingly crucial to a higher level of feed consumption

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[16]. The criteria guiding the selection of feed supplements include their natural origin, safety, and high concentrations of active compounds. However, concerns regarding low nutrient digestibility in plant protein ingredients has led to an increasing interest in feed enzymes. It is known that specific dietary enzymes and feed additives can improve the digestion and assimilation of complex food items by breaking them down. These additives are products of external sources, and a few of them are more commonly referred to as nutrizymes because of their function in the assimilation and digestion of food. The incorporation of exogenous enzymes into fish feed has been demonstrated to enhance the utilization of feed components, consequently reducing losses. Moreover, the use of exogenous enzymes has been verified to not only improve the nutritional value of the feed but also contribute to a decrease in environmental pollution [31, 43].

Papain, the principal and highly potent protease enzyme found in different parts of green papaya (*Carica papaya*), stands out as a remarkable natural digestive catalyst, surpassing the digestive efficacy of both pepsin and pancreatin [28]. Its enzymatic action significantly improves the digestibility of low-quality feed, leading to reduced feed costs and minimizing the environmental footprint by lowering the output of waste materials. According to Kazerani and shahsavani [18], it is possible to increase the efficiency of feed exploitation by introducing papain enzyme. Adding exogenous enzymes to animal feed improves digestion by breaking down complex feed components into smaller, more digestible molecules. These enzymes, such as papain, act on long-chain proteins and carbohydrates, converting them into simpler forms that animals can more easily absorb and utilize [4]. Exogenous enzymes, such as papain, enhance feed digestibility by breaking down complex feed particles into smaller, more digestible molecules. This process increases nutrient assimilation efficiency and reduces feed waste [42]. Specifically, papain targets long-chain amino acids, crucial for protein digestion, thereby improving protein digestibility and nutrient uptake [1, 27, 33]. As a feed supplement, papain boosts protein digestibility, enhances nutrient assimilation, and positively affects feed utilization and growth performance in livestock [44]. Thus, supplementing with papain may be an effective strategy to improve growth performance and nutrient utilization in *Labeo rohita* fingerlings. Therefore, this study aims to address the gap by investigating the effect of exogenous papain supplementation on improving the digestibility, growth performance, oxidative stress, and digestive enzyme activities in *Labeo rohita* fingerlings, focusing on enhancing protein digestibility and feed utilization through feed additives.

## Materials and methods

### Rearing conditions and experimental design

The 60-day feeding trial was conducted in the wet laboratory of the College of Fisheries, Udaipur, Rajasthan, India (Latitude: 24.5854° N; Longitude: 73.7125° E). Labeo rohita fingerlings, with an average initial body weight of 17 ± 0.5 g, were procured from the Fish Seed Production Unit at Maharana Pratap University of Agriculture and Technology, Udaipur. Prior to the start of the experiment, the fish were acclimated to the experimental conditions for 15 days, during which they were fed the control diet. After acclimation, 10 fish were randomly allocated to each of 18 tanks (250-L capacity per tank), following a completely randomized design (10 fish/replicate, $N = 30$ fish/treatment). The experiment was conducted from September to October 2019, during which the average air temperature was recorded at 29.4 ± 0.24 °C.

### Diet preparation

Feed ingredients were procured from local feed suppliers. Control diet prepared with GNOC, rice bran, wheat flour and vitamin-mineral mix (Agrimin, Kamadhenu Nutrients Pvt. Ltd Gujarat, India). All ingredients, except the vitamin-mineral mix, underwent oven drying (Yona, Indian Instruments Manufacturing Co. Kolkata) at 60 °C for 24 h, followed by fine grinding and thorough mixing with water (70% W/V) to form dough. The resulting mixture was then enclosed in a muslin cloth, and the cloth-bound preparation was subjected to autoclaving for 30 min at 15 lbs pressure. Vitamin-mineral mixture was incorporated into the dough after cooling. Following that the dough was pelleted (using hand pelletizer), air dried and stored in airtight glass container. The test diets were formulated by replacing equal amount of control diet with commercially available papain enzyme powder (HiMedia Laboratories, Mumbai, India) viz. control -T0 (0% papain enzyme), T1 (1.0%,), T2 (2.0%), T3 (3.0%), T4 (4.0%), and T5 (5.0% papain enzyme) using Excel spreadsheet (Table 1). For digestibility chromic oxide ($Cr_2O_3$; digestibility indicator) was incorporated to each diet at 1% at the replacement of wheat flour. Throughout the experimental period of 60 days, the fish were fed experimental diets at a rate equivalent to 3% of their body weight. The feeding regimen consisted of two equal doses administered twice daily, in the morning at 10 AM and in the evening at 5 PM.

**Table 1** Ingredients proportion and proximate composition of experimental diet supplemented with different proportion of papain enzyme during experimental period

| S. No | Ingredients | Quantity (g/Kg) | | | | | |
|---|---|---|---|---|---|---|---|
| **Control diet** | | | | | | | |
| 1 | GNOC [a] | 400 | | | | | |
| 2 | Rice bran [a] | 400 | | | | | |
| 3 | Wheat flour [a] | 190 | | | | | |
| 4 | Vitamin-mineral mixture [b] | 10 | | | | | |
| **Experimental diets** | | | | | | | |
| | | **T0** | **T1** | **T2** | **T3** | **T4** | **T5** |
| 1 | Control diets (g) | 1000 | 990 | 980 | 970 | 960 | 950 |
| 2 | Papain enzyme [c] (g) | 0 | 10 | 20 | 30 | 40 | 50 |
| **Proximate composition of the diet (on dry matter basis; mean of triplicates)** | | | | | | | |
| 1 | **Moisture (%)** | 7.13 ± 0.12 | 7.27 ± 0.09 | 7.37 ± 0.03 | 7.43 ± 0.12 | 7.63 ± 0.03 | 7.70 ± 0.06 |
| 2 | **Crude protein (%)** | 22.71 ± 0.02 | 23.00 ± 0.29 | 23.32 ± 0.21 | 23.10 ± 0.17 | 22.90 ± 0.29 | 22.80 ± 0.29 |
| 3 | **Crude lipid (%)** | 5.09 ± 0.15 | 5.10 ± 0.06 | 5.12 ± 0.09 | 5.14 ± 0.02 | 5.18 ± 0.01 | 5.19 ± 0.01 |
| 4 | **Total ash (%)** | 6.53 ± 0.01 | 6.52 ± 0.03 | 6.52 ± 0.00 | 6.51 ± 0.01 | 6.51 ± 0.00 | 6.51 ± 0.00 |
| 5 | **Carbohydrate (%)** | 60.90 ± 0.12 | 58.11 ± 0.25 | 57.68 ± 0.18 | 57.81 ± 0.22 | 57.77 ± 0.26 | 57.80 ± 0.27 |

[a] Purchased from local animal feed ingredient dealer, Udaipur, Rajasthan, India

[b] Composition of vitamin mineral mix (Agrimin) (quantity $kg^{-1}$): vitamin A- 55,00,000 IU; vitamin D3- 11,00,000 IU; vitamin B2- 2,000 mg; vitamin E- 750 mg; vitamin K- 1,000 mg; vitamin B6- 1,000 mg; vitamin B12- 6 mcg; calcium pantothenate—2,500 mg; nicotinamide—10 g; choline chloride—150 g; Mn—27,000 mg; Cu—2,000 mg; I—1,000 mg; Zn—5,000 mg; Fe—7,500 mg; Co—450 mg; L-lysine—10 g; DL—methionine- 10 g; selenium—125 mg

[c] HiMedia Laboratories, Mumbai, India

### Water quality parameters

Water quality parameters including water temperature, dissolved oxygen (DO), pH, total dissolved solids (TDS), and electrical conductivity were measured at the beginning and at 15-day intervals using a water quality digital probe (HANNA, Romania). Additional parameters such as total alkalinity, total hardness, were recorded following standard methods [3].

### Proximate analysis

The moisture, crude protein, crude lipid, and total ash content of the experimental diets were determined according to AOAC [2] methods. Moisture content was measured by oven-drying the weighed and ground samples at 60 °C for 24 h. Nitrogen content was estimated using a micro Kjeldahl apparatus, with crude protein calculated by multiplying the nitrogen content by 6.25. Crude lipid content was determined using a Soxhlet apparatus (Pelican Instruments, Chennai, India), and total ash was estimated by incinerating the samples in a muffle furnace at 550 °C for 6 h. Carbohydrate content was estimated by the difference method. The proximate composition of the experimental diets is presented in Table 1.

### Growth performance parameters and survivability

$$\textbf{Daily Growth Rate (DGRg/d)} = (Wf - Wi) \times \mathrm{T}^{-1} \quad (1)$$

(Where: $Wi$ = initial mean body weight (g); $Wf$ = final mean body weight (g); T = rearing time)

$$\textbf{Weight gain}(\%) = \frac{(Final\ Wt - Initial\ Wt)}{Initial\ Wt} x100 \quad (2)$$

$$\textbf{Specific growth rate(SGR\%/day)} = \frac{(Log\ n\prime Wt - Log\ n\ Wo)}{D} x100 \quad (3)$$

(Where: $W_0$ = Initial weight of fish (g); Wt = Final weight of fish (g); D = Time interval)

$$\textbf{Gross conversion efficiency(GCE)} = \frac{Weight\ gain(g)}{Feed\ given(g)} \quad (4)$$

$$\textbf{Feed conversionratio (FCR \%)} = \frac{Feed\ given(g)}{Weight\ gain(g)} \quad (5)$$

$$\textbf{Survival rate (SR\%)} = \frac{Nt}{No} \times 100 \quad (6)$$

(Where, $N_t$ = Final number of fishes $N_o$ = Initial number of fishes)

$$\textbf{Protein efficiency ratio(PER)} = \frac{Gain\ in\ body\ mass(g)}{Protein\ intake(g)} \quad (7)$$

$$\textbf{Protein absorption ratio(PAR)} = \frac{protein\ in\ fish\ diet - protein\ recovered\ in\ faeces}{protein\ in\ diet} \quad (8)$$

#### In vivo digestibility study

During the last 15 days, the fish of each experimental group were fed with 1% chromic oxide ($Cr_2O_3$; an inert marker), containing diet once daily on satiation basis. This assumption is based on the premise that the marker's quantity in both the feed and feces remains consistent throughout the experimental period, and that all ingested markers will be excreted in the feces. Feces were collected by siphoning (filtering through fine meshed nylon cloth; 15 µm) onto a mesh two hours after feeding to avoid leaching of the nutrients and were oven-dried overnight separately in a hot air oven at 60 °C, pooled and then stored at in tubes at − 20 °C until required for analysis. The samples were analysed in triplicate for protein (Nitrogen × 6.25) following the procedure by [2]. Chromic oxide levels in both the diets and feces were analyzed following the method outlined by Furukawa and Tsukahara [12]. The process involved digesting the samples with concentrated nitric acid and subsequently oxidizing the chromic oxide by applying 70% perchloric acid. Following formula was used to calculate chromic oxide.

$$\text{Chromic oxide (\%)} = [\{(\text{absorbance} - 0.0032)/0.2089\}/\text{sample weight}] \times 100 \quad (9)$$

$$\text{Apparent protein digestibility (APD \%)} = 100X\frac{(\%\ \text{Cr2O3 feed X \% protein in faeces})}{(\%\text{Cr2O3 in feaces } X\%\ protein\ in\ feed)} \quad (10)$$

#### Determination of digestive enzyme and oxidative stress response

Liver and intestine tissues were collected from five fish in each treatment group and immediately frozen at − 20 °C. To evaluate protein levels, the samples were homogenized in phosphate buffer (pH 7.0) and then centrifuged at 15,000 × g for 15 min at 4 °C using a Sorvall ST 8R Small Benchtop Centrifuge (ThermoFisher Scientific, Germany). After centrifugation, the supernatants were collected and stored at − 80 °C until further analysis. Total protein content was determined using Lowry's method [22]. Specific enzyme activities were analyzed following established procedures for amylase [32], protease [8], and lipase [7].

#### Oxidative stress parameters

The activities of oxidative stress enzymes were determined in liver samples. Superoxide dismutase (SOD) activity was measured following the method described by Misra and Fridovich [24], which involves monitoring the oxidation of epinephrine to adrenochrome by the enzyme. The absorbance change at 480 nm was recorded over 3 min, and one unit of SOD activity was defined as the amount of protein required to achieve 50% inhibition of epinephrine auto-oxidation. Catalase (CAT) activity was assessed using the method of Takahara et al. [39], in which the reaction was carried out in a phosphate buffer (50 mM, pH 7.0) and initiated by adding hydrogen peroxide ($H_2O_2$). The decrease in absorbance at 240 nm was used to quantify CAT activity, with one unit defined as the amount of protein needed to decompose $H_2O_2$. Glutathione peroxidase (GPx) activity was measured according to the procedure outlined by Faheem and Lone [10].

#### Statistical analysis

The experimental data was sorted by Excel and subjected to a One-way ANOVA utilizing software (IBM SPSS Statistics version 21.0 for Window) to ascertain whether there was any significant difference between the different treatment groups ($P < 0.05$). A posthoc, Duncan's options, descriptive comparisons test was performed after the One-way ANOVA analysis when the substantial differences were discovered. The data has been displayed as means ± SD for each group. Graphs were plotted using Graph Pad Prism 9.5.1 software (GraphPad software, California, USA).

## Results

#### Water quality parameters

Water quality parameters such as water temperature, pH, electrical conductivity, dissolved oxygen, total alkalinity and total hardness remained within the prescribed ranges from 26 to 29 °C, 8.24 to 8.65, 180 to 189 µS $cm^{-1}$, 6.8 to 7.4 mg $L^{-1}$, 137.6 to 142.0 mg $L^{-1}$ and 608 to 642.8 mg $L^{-1}$ respectively throughout the experimental period.

**Table 2** Growth performance, feed conversion and survival rate of fingerlings *Labeo rohita* fed diets containing varying levels of papain enzyme for 60 days

| Parameters | Treatments | | | | | | |
|---|---|---|---|---|---|---|---|
| | T0 | T1 | T2 | T3 | T4 | T5 | *P*-value |
| IBW (g fish$^{-1}$) | 17.57 ± 1.57 | 17.65 ± 1.21 | 17.72 ± 0.55 | 17.60 ± 0.62 | 17.57 ± 0.42 | 17.66 ± 0.76 | 0.366 |
| FBW (g fish$^{-1}$) | 27.80 ± 1.13$^{e}$ | 29.19 ± 0.87$^{c}$ | 30.75 ± 2.05$^{a}$ | 29.49 ± 0.35$^{b}$ | 29.04 ± 1.70$^{c}$ | 28.07 ± 0.47$^{d}$ | 0.000 |
| WG (%) | 58.23 ± 0.83$^{d}$ | 65.35 ± 0.78$^{c}$ | 73.48 ± 0.70$^{a}$ | 67.55 ± 0.40$^{b}$ | 65.25 ± 1.15$^{c}$ | 58.96 ± 0.95$^{d}$ | 0.000 |
| DGR (g day$^{-1}$) | 1.71 ± 0.01$^{d}$ | 1.92 ± 0.01$^{c}$ | 2.17 ± 0.03$^{a}$ | 1.98 ± 0.01$^{b}$ | 1.91 ± 0.03$^{c}$ | 1.74 ± 0.02$^{d}$ | 0.000 |
| SGR (% day$^{-1}$) | 0.76 ± 0.005$^{d}$ | 0.84 ± 0.005$^{c}$ | 0.92 ± 0.004$^{a}$ | 0.86 ± 0.002$^{b}$ | 0.84 ± 0.007$^{c}$ | 0.77 ± 0.006$^{d}$ | 0.000 |
| GCE (%) | 0.27 ± 0.007$^{bc}$ | 0.29 ± 0.007$^{a}$ | 0.29 ± 0.003$^{a}$ | 0.28 ± 0.003$^{ab}$ | 0.30 ± 0.005$^{a}$ | 0.26 ± 0.005$^{c}$ | 0.002 |
| FCR | 2.90 ± 0.03$^{a}$ | 2.65 ± 0.01$^{b}$ | 2.44 ± 0.01$^{d}$ | 2.59 ± 0.02$^{c}$ | 2.64 ± 0.03$^{bc}$ | 2.88 ± 0.04$^{a}$ | 0.000 |
| SR (%) | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 | 96.67 ± 5.77 | 100.00 ± 0.00 | 0.458 |

Values are presented as Mean ± SD. The means within the same row carrying different superscripts are significant ($P < 0.05$) ($N = 30$/group). Where: *IBW* initial body weight, *FBW* final body weight, *WG* weight gain, *DGR* daily growth rate, *SGR* specific growth rate, *GCE* gross conversion efficiency, *FCR* Feed conversion ratio, SR; survival rate

### Growth performance, fish survival and nutrient utilization

The growth performance, nutrient utilization, and survival rate parameters of *Labeo rohita* fingerlings fed various levels of papain are summarized in Table 2. After a 60-day feeding trial, the 2% papain enzyme powder treatment (T2) showed significantly ($P < 0.05$) higher values for weight gain (73.48 ± 0.70%), daily growth rate (DGR) (2.17 ± 0.03 g day$^{-1}$), and specific growth rate (SGR) (0.92 ± 0.01% day$^{-1}$). Additionally, the feed conversion ratio (FCR) was significantly lower in T2 (2.44 ± 0.01) compared to the control and other treatments. No significant differences ($P > 0.05$) were observed in gross conversion efficiency (GCE) among all treatments. However, PAR (Fig. 1A) did not show significant differences ($P > 0.05$). All groups exhibited a 100% survival rate, except for T4, which had a survival rate of 96.67 ± 5.77%. Furthermore, protein efficiency ratio (PER) was significantly higher ($P < 0.05$) in group T2 (Fig. 1B).

### In vivo protein digestibility

The apparent protein digestibility (APD) percentages for the treatments and control are illustrated in Fig. 1C. The APD percentage was significantly higher ($P < 0.05$) in group T2 (81.93 ± 1.31) compared to the control group (71.41 ± 0.85) and was also higher in group T3 (76.66 ± 2.09) compared to the control. However, no significant differences were observed between the remaining treatment groups.

### Digestive enzyme activities

Changes in intestinal digestive enzyme activity of *Labeo rohita* fingerlings fed with papain enzyme for 60 days are depicted in Fig. 2A, B, C. Supplementing feed with different levels of papain enzyme significantly influenced digestive enzyme activities. Lipase activity was significantly ($P < 0.05$) higher in T2 &T3 than that of T5 and control group. The activity of protease enzyme was also significantly ($P < 0.05$) higher in T2 compared to control

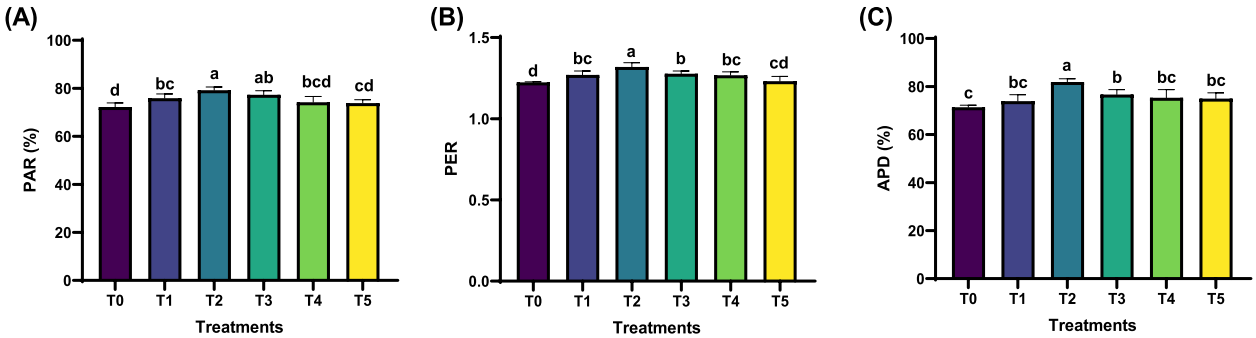


**Fig. 1** Change in PAR, PER and apparent protein digestibility *Labeo rohita* fingerlings fed diets supplemented with papain enzymes for 60 days. Values are expressed as mean ± SD. The bars with different letters differed significantly ($P < 0.05$, one-way ANOVA). **A** PAR; protein absorption ratio; (**B**) PER; protein efficiency ratio; (**C**) APD; Apparent protein digestibility

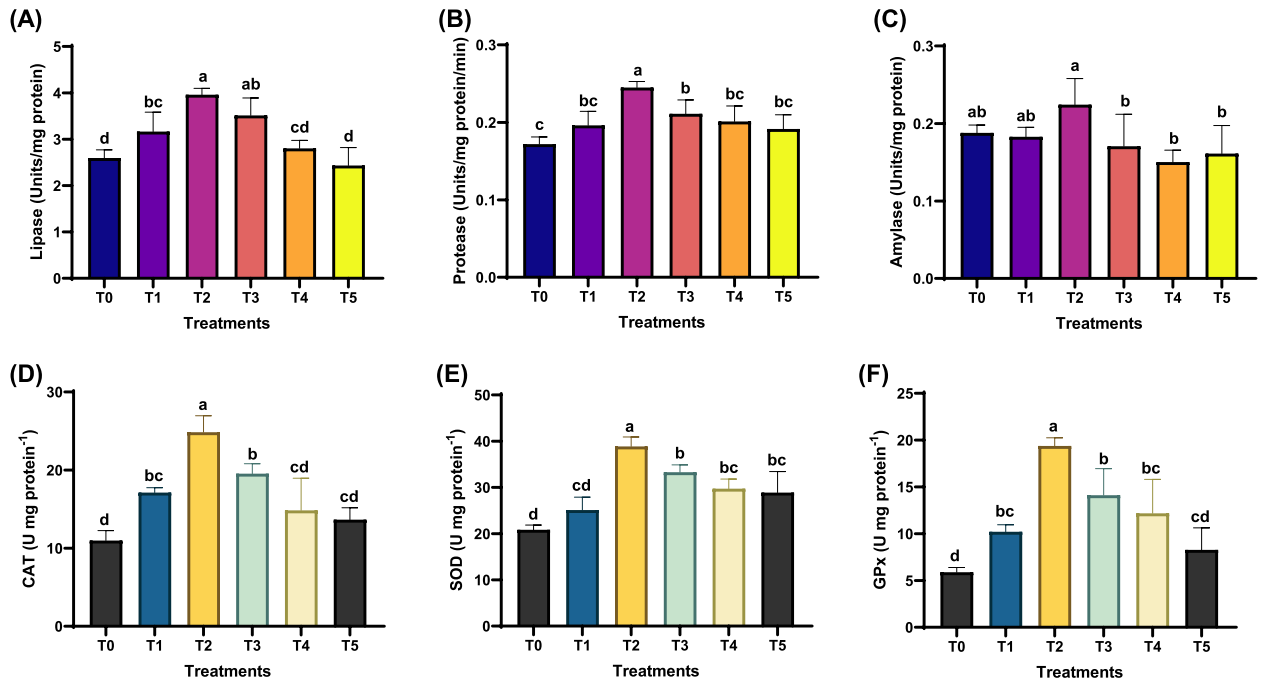


**Fig. 2** Intestinal digestive enzymes and oxidative stress enzymes of *Labeo rohita* fingerlings fed diets supplemented with papain enzymes for 60 days. Values are expressed as mean ± SD. bars with different letters differed significantly ($P < 0.05$, one-way ANOVA). **A** Lipase (Units/mg protein), (**B**) Protease (Units/mg protein/min), (**C**) Amylase (Units/mg protein), (**D**) CAT (U mg protein$^{-1}$), (**E**) SOD (U mg protein$^{-1}$), (**F**) GPx (U mg protein.$^{-1}$)

and rest of other treatments. No significant difference ($P > 0.05$) was evident for amylase activity in all groups. However, group T2 showed significantly higher value with the group T3, T4 and T5.

### Oxidative stress response

The activities of liver oxidative stress markers, including CAT, SOD, and GPx, in *Labeo rohita* fingerlings were significantly affected by the dietary inclusion of papain enzyme, as shown in Fig. 2D, E, F. Fish fed the 2% papain enzyme diet (T2) exhibited the highest activities: CAT (24.84 ± 2.12 U mg protein$^{-1}$), SOD (38.85 ± 2.05 U mg protein$^{-1}$), and GPx (19.37 ± 0.89 U mg protein$^{-1}$). In contrast, the control group displayed the lowest activities, with CAT at 10.98 ± 1.26 U mg protein$^{-1}$, SOD at 20.83 ± 1.26 U mg protein$^{-1}$, and GPx at 5.88 ± 0.50 U mg protein$^{-1}$.

### Pearson correlation analysis between growth, digestive metabolism and oxidative stress

Pearson correlation analysis was conducted to examine the relationships between fish growth (SGR), digestive metabolism (PER, PAR, APD, lipase, protease, and amylase), and oxidative stress enzymes (SOD, CAT, and GPx), as depicted in Fig. 3. A statistically significant positive correlation ($P < 0.05$) was found between SGR and PER, with a correlation coefficient (r) of 0.87. SGR also showed positive correlations with PAR at $r = 0.81$ and APD at $r = 0.75$. Among digestive enzymes, lipase demonstrated a strong positive correlation with SGR ($r = 0.86$), followed by protease ($r = 0.79$) and amylase ($r = 0.42$). Furthermore, oxidative stress enzymes exhibited significant positive correlations with fish growth, with catalase (CAT) at $r = 0.88$, superoxide dismutase (SOD) at $r = 0.75$, and glutathione peroxidase (GPx) at $r = 0.89$.

### Principal component analysis between growth and water quality parameters

Principal Component Analysis (PCA) was conducted to assess the overall correlation between fish growth and water quality parameters. The PCA results, shown in Fig. 4, indicated that the first three principal components collectively accounted for 68.3% of the variability in the dataset. Specifically, PC1 explained 32.5% of the variance, PC2 contributed 20.2%, and PC3 accounted for 15.6% of the variation. In the PCA biplot, acute angles (< 90°) between specific growth rate (SGR) and parameters such as total alkalinity, total hardness, water temperature, conductivity, and dissolved oxygen (DO) demonstrated a strong positive association with fish growth. Conversely, obtuse angles (> 90°) involving total dissolved solids (TDS) and pH indicated a negative relationship with fish growth.

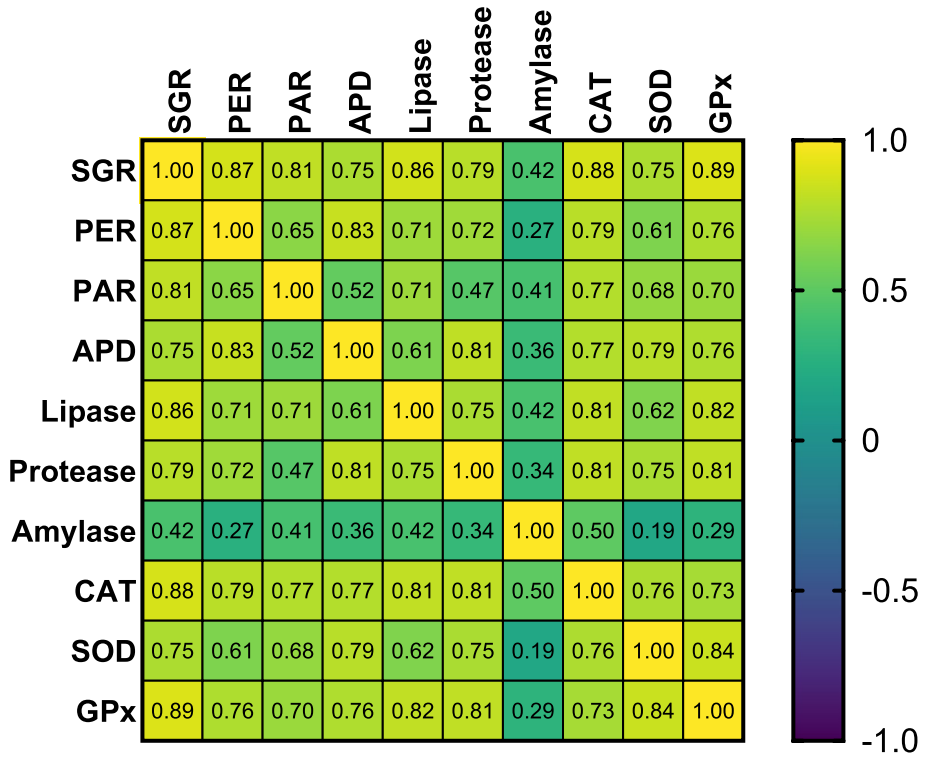


**Fig. 3** The correlation matrix heatmap shows the values of the pearson correlation coefficient for all studied parameters. It ranges from -1 to 1, whereby -1 means a perfect negative linear relationship between variables, 1 indicates a perfect positive linear relationship between variables and 0 indicates that there is no relationship between studied variables

## Discussion

Mean values of physico-chemical water properties during the culture period remained within the expected range for carp culture, as outlined by Boyd [6]. The dissolved oxygen levels in this study's treatments ranged from 6.8 to 7.4 mg $L^{-1}$, which is in accordance with the ideal level of 5–7 mg/l [17]. Carp culture requires water with a pH range of 6.5 to 9.0 [38]. In the present study, alkalinity levels were measured in all treatment groups, ranging from 137.6 to 142.0 mg $L^{-1}$, a range considered to be within the ideal parameters for freshwater aquaculture, as outlined by Tiwari et al. [41]. The growth of fish is influenced by environmental parameters [5],therefore, the identification of key abiotic factors affecting fish growth is essential. Notably, fish growth, as measured by SGR, was found to exhibit a significant positive correlation with water temperature, total alkalinity, total hardness, conductivity, and dissolved oxygen (DO. Water temperature plays a crucial role in influencing the feeding rate and body metabolism of fish, ultimately regulating the process of weight gain [25].

WG%, SGR, and DGR are key indicators for evaluating fish growth performance [36]. In this study, *Labeo rohita* fingerlings fed a diet with 2% papain enzyme exhibited significantly higher percent weight gain, DGR, and SGR ($P<0.05$). This improvement is attributed to the enhanced nutrient availability resulting from the inclusion of papain in the diet, which boosts protein digestibility and overall nutrient utilization. Papain, a proteolytic enzyme, enhances protein digestibility by breaking down proteins into shorter peptides and amino acids. This increased digestibility leads to more efficient nutrient absorption and utilization. By improving protein availability, papain supports accelerated growth and better feed conversion rates in fish. This effect is crucial for optimizing growth performance, as it promotes more rapid metabolism and nutrient uptake [20, 33]. The incorporation of papain in fish diets can thus play a significant role in improving overall growth factors and feed efficiency. Saifulloh et al. [34] demonstrated that a 3% inclusion of papain enzyme significantly enhanced growth in Nile tilapia. Similarly, Liono et al. [21] found that adding 1.5% papain to eel (*Anguilla bicolor*) diets improved protein retention and feed efficiency. Singh et al. [37] reported that a 2% papain level was optimal for growth in *Cyprinus carpio*. These results align closely with the present study, which also observed 100% survival across most groups, except T4. Additionally, Rachmawati et al. [29] found no significant differences in growth for *Clarias sp*. when supplemented with papain, respectively.

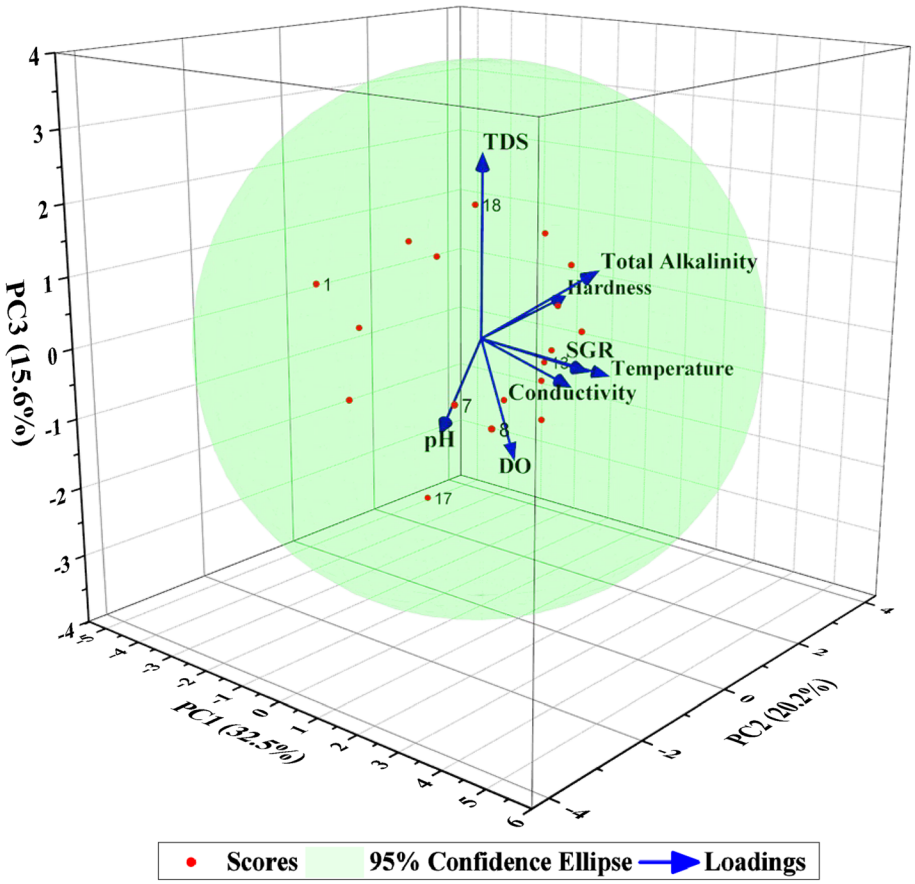


**Fig. 4** Principal component analysis (PCA) based on fish growth and water quality parameters during the experimntal period. The samples were divided into two groups along with three principal components (PCs). PC1, PC2, and PC3 explained 32.5, 20.2, and 15.6 percent of the total variation respectively. Blue arrows specify the increasing values of each variable

Nutrient digestibility is a critical factor that influences the overall quality and utilization potential of feed or ingredients [13]. In this study, *Labeo rohita* fingerlings fed a diet supplemented with 2% papain enzyme exhibited a significant increase in protein digestibility compared to the control group. Papain, a protease enzyme, effectively hydrolyzes complex protein compounds into simpler components, such as amino acids [19]. This enzymatic action enhances feed digestibility [23], leading to improved growth performance in fish. The benefits of papain supplementation are further supported by findings from Munguti et al. [26], who observed that the addition of papain to Nile tilapia diets, which included feather meal, significantly improved digestibility. This enhancement in digestibility translates into better nutrient absorption and utilization, which ultimately contributes to improved growth performance and feed efficiency. Thus, incorporating papain into fish diets can be an effective strategy to optimize feed quality and promote better growth outcomes in aquaculture.

This study assessed the activities of key digestive enzymes viz. lipase, protease, and amylase to evaluate their functionality and impact on feed nutrition. Digestive enzyme activity is crucial for determining ingredient availability and nutritional value of feed [15]. Factors such as age, diet quality, and enzyme interactions can influence enzyme activity, as noted by Shabana et al. [35]. In this study, amylase activity remained unchanged with the addition of papain enzyme, indicating that papain does not significantly affect carbohydrate digestion. However, the activities of lipase and protease were notably altered by the papain treatments. Wiszniewski et al. [43] reported that supplementing the diet of *Acipenser ruthenus* with papain at 10 g/kg and 20 g/kg significantly increased lipase activity, suggesting that papain enhances the breakdown of lipids. Similarly, Rachmawati et al. [30] found that adding papain to the feed of *Clarias gariepinus* resulted in significantly higher lipase and pepsin activities, indicating an improvement in the digestion of fats and proteins. These findings highlight the effectiveness of papain in boosting lipase and protease activities,

which can enhance overall feed utilization and growth performance. The unchanged amylase activity suggests that papain's impact is more pronounced on protein and lipid digestion rather than carbohydrate digestion.

Antioxidant enzymes are essential for protecting cells from oxidative damage by catalyzing the conversion of superoxide anions into less harmful molecules such as oxygen and hydrogen peroxide. These enzymes are vital in regulating the production and clearance of reactive oxygen species (ROS), thereby mitigating cellular damage caused by free radicals [47]. Key antioxidant enzymes, such as SOD and CAT, are crucial markers for evaluating the effects of dietary supplements like papain in fish [14]. SOD and CAT work in tandem with other antioxidants, such as GPx, to effectively neutralize oxidative stress [9]. Research has shown that dietary factors, including protein levels and plant-based ingredients, can influence the activities of endogenous antioxidant enzymes in fish [40]. In the current study, *Labeo rohita* fingerlings fed a diet supplemented with 2% papain enzyme exhibited significantly higher activities of SOD, CAT, and GPx compared to those fed the control diet. This suggests that papain enhances the fish's enzymatic antioxidant defense, providing better protection against oxidative stress. However, it was observed that increasing the papain enzyme level beyond 2% led to a decrease in oxidative enzyme activities, indicating potential oxidative stress at higher concentrations. This phenomenon underscores the need for optimal papain levels to balance antioxidant protection without inducing stress. These findings align with those of Wiszniewski et al. [43], who reported that starlet sturgeon fed a papain-enriched diet demonstrated increased SOD and GPx activities compared to the control. This highlights the role of papain in bolstering antioxidant defenses in fish, suggesting its beneficial impact on enhancing oxidative stress management when used at appropriate levels.

## Conclusion

The study found that incorporating 2% papain enzyme in the diet of Labeo rohita fingerlings for 60 days enhanced growth performance, conversion efficiencies, apparent protein digestibility, digestive enzyme activities, and antioxidant status under aquaculture conditions. Future research should explore the impact of papain on fish health, focusing on immune response, non-specific cellular immunity, and gastrointestinal histology. These investigations could provide valuable insights for optimizing the use of papain in aquafeed formulations, contributing to the sustainability and efficiency of aquaculture practices for this species.

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**Authors' contributions**
NKY: Methodology, data duration, software, writing – original draft. SK S: Conceptualization, methodology, validation, investigation, writing – review & editing. DKM: Methodology, data duration, writing – review & editing.

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**Availability of data and materials**
No datasets were generated or analysed during the current study.

## Declarations

**Ethics approval and consent to participate**
All applicable international, national, and/or institutional guidelines for the care and use of fish were followed by the authors.

**Competing interests**
The authors declare no competing interests.

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## Publisher's Note