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EVALUATION OF DIETARY L-ASCORBIC ACID, L-LEUCINE, AND THEIR COMBINED SUPPLEMENTATION ON DIGESTIVE ENZYME ACTIVITIES OF *Labeo catla* FINGERLINGS

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ABSTRACT

The present study was conducted to evaluate the effects of dietary supplementation of L-ascorbic acid, L-leucine, and their combination on digestive enzyme activities in *Labeo catla* fingerlings. A 75-day feeding trial was carried out using nine experimental diets containing different levels of L-ascorbic acid and L-leucine. Fingerlings with an average initial weight of 0.85 ± 0.04 g were randomly distributed into nine treatment groups with three replicates each. The dietary treatments consisted of a control diet (T0), L-leucine-supplemented diets (T1 and T2), L-ascorbic acid-supplemented diets (T3 and T4), and combined supplementation diets (T5–T8). At the end of the experimental period, protease, amylase, and lipase activities were analyzed from gut tissue samples. The results revealed significant differences ($p < 0.05$) in digestive enzyme activities among the treatments. The highest protease (2.516 ± 0.037 U mg^{-1} protein), amylase (1.620 ± 0.020 U mg^{-1} protein), and lipase (0.303 ± 0.018 U mg^{-1} protein) activities were recorded in treatment T6, containing 0.4 g kg^{-1} L-ascorbic acid and 10 g kg^{-1} L-leucine. Fish fed diets supplemented with L-leucine alone exhibited higher enzyme activities than the control, whereas L-ascorbic acid alone produced moderate improvements. Combined supplementation treatments consistently showed superior digestive enzyme activities compared to individual supplementation, indicating a synergistic interaction between the two nutrients. However, increasing dietary leucine from 10 to 15 g kg^{-1} did not result in further enhancement of enzyme activities. The findings suggest that dietary supplementation with L-ascorbic acid and L-leucine improves digestive physiology and nutrient utilization in *Labeo catla* fingerlings. Among the tested diets, supplementation with 0.4 g kg^{-1} L-ascorbic acid and 10 g kg^{-1} L-leucine proved to be the most effective in enhancing protease, amylase, and lipase activities.

***Keywords*:** Labeo catla; L-ascorbic acid; L-leucine; digestive enzymes; aquaculture nutrition.

Introduction

Aquaculture is the fastest-growing food-producing sector worldwide and plays a crucial role in ensuring global food security and nutritional sustainability (Bhadarka *et al*., 2026). In India, Indian major carps constitute the backbone of freshwater aquaculture production, among which *Labeo catla* is one of the most commercially important species owing to its rapid growth, high consumer acceptance, and adaptability to diverse culture systems (Jhingran, 1991). The success of intensive aquaculture largely depends on efficient feed utilization and nutrient assimilation, which are directly influenced by digestive physiology and enzyme activity. Digestive enzymes are essential biological catalysts involved in the hydrolysis of proteins, carbohydrates, and lipids into absorbable forms. The activities of protease, amylase, and lipase are considered important indicators of digestive efficiency and nutritional status in fish (Furne *et al*., 2005). Enhanced digestive enzyme activity facilitates improved nutrient absorption, feed conversion

efficiency, and growth performance. Consequently, nutritional strategies that stimulate digestive enzyme secretion and activity have gained increasing attention in aquaculture nutrition research. Vitamin C (L-ascorbic acid) is an indispensable dietary nutrient for most teleost fishes because they lack the enzyme L-gulonolactone oxidase, which is necessary for endogenous synthesis of ascorbic acid (Halver & Hardy, 2002). Ascorbic acid functions as a potent antioxidant and is involved in collagen synthesis, wound healing, stress tolerance, immune modulation, and growth regulation (NRC, 2011). Several studies have reported that dietary vitamin C supplementation enhances growth performance, feed utilization, digestive enzyme activity, and disease resistance in various fish species (Ai *et al*., 2006; Kumar *et al*., 2011). Furthermore, vitamin C contributes to maintaining intestinal integrity and protecting digestive tissues from oxidative damage, thereby improving digestive function and nutrient assimilation.

Among essential amino acids, L-leucine has received considerable attention due to its dual role as a substrate for protein synthesis and a signaling molecule regulating cellular metabolism. Leucine is one of the branched-chain amino acids (BCAAs) and is known to activate the mammalian target of rapamycin (mTOR) signaling pathway, which promotes protein synthesis, tissue growth, and nutrient metabolism (Li *et al*., 2008; Wu, 2013). Dietary leucine supplementation has been shown to improve growth performance, feed efficiency, and digestive physiology in several aquatic species by stimulating digestive enzyme secretion and enhancing intestinal development (Dai *et al*., 2013). Adequate leucine levels are therefore essential for maximizing nutrient utilization and growth in cultured fish. Recent advances in fish nutrition have emphasized the importance of functional nutrients that not only satisfy basic nutritional requirements but also improve physiological performance and health status. The combined supplementation of vitamins and amino acids may exert synergistic effects on digestive and metabolic processes. While ascorbic acid enhances antioxidant defense mechanisms and intestinal health, leucine promotes protein synthesis and nutrient metabolism through activation of growth-related signaling pathways. Their combined dietary inclusion may therefore provide greater benefits than individual supplementation by improving digestive enzyme activity and nutrient utilization efficiency. Despite the recognized importance of vitamin C and leucine in fish nutrition, limited information is available regarding their individual and combined effects on digestive enzyme activities in *Labeo catla* fingerlings. Understanding these effects is important for developing nutritionally optimized diets that enhance digestive capacity, growth performance, and overall aquaculture productivity. Therefore, the present study was undertaken to evaluate the effects of dietary L-ascorbic acid, L-leucine, and their combined supplementation on digestive enzyme activities in *Labeo catla* fingerlings.

Materials and Methods

The present research work was conducted for 75 days in the wet lab, Department of Aquaculture, College of Fisheries Science, Kamdhenu University, Veraval, Gujarat. Healthy Catla fingerlings (*Labeo catla*, Family *Cyprinidae*, and Order Cypriniformes) were used in an in vivo experiment. The stock of *Labeo catla* fish was obtained from the Aqua fish farm, Ranagadh, Limbdi. The fish had a mean weight of 0.85 ± 0.04 g. Before placement in an acclimatization tank, the fish underwent prophylactic treatment in compliance with the accepted protocol, which included a bath in a 0.05% potassium permanganate solution. Fingerlings of *Labeo catla* were acclimatized in rectangular plastic tanks (250 Liters). Fish were reared for 1 month under laboratory conditions to ensure proper acclimatization under optimal conditions before taking them for the in vivo experiment. Fish feed was formulated with ingredients like fish meal, groundnut cake, wheat flour, tapioca powder and vitamins & minerals mixture. The ingredients for feed were procured from the nearby market of Veraval. Nine different isonitrogenous diets were prepared with L-ascorbic acid and L-leucine respectively, while substituting tapioca powder at different percentages. The Crude protein (CP%) in the formulated diets for experiment was kept 30%. The substitution of different ingredients is depicted in Tables 1.

Table 1: Feed Formulation

Ingredients	Grams of ingredients per 100 grams of feed								
	T0	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**
L-Leucine	0	1	1.5	0	0	1	1	1.5	1.5
L-Ascorbic acid	0	0	0	0.03	0.04	0.03	0.04	0.03	0.04
Fish Meal	37	36	36	37	37	36	36	36	36
GNOC	23	23	23	23	23	23	23	23	23
Wheat Flour	17	17	17	17	17	17	17	17	17

Tapioca Powder	17	17	16.5	16.97	16.96	16.97	16.96	16.47	16.46
Fish Oil	2	2	2	2	2	2	2	2	2
Plant Oil	2	2	2	2	2	2	2	2	2
Vitamins & Minerals	2	2	2	2	2	2	2	2	2

The research was conducted in pretreated (2ppm KmNO4, washed 3-4 times thoroughly) rectangular tanks, and filled with pre-quality tested water. The experimental setup followed CRD design and the tanks were arranged for treatments and control in triplicates of each (Table 2).

Table 2: Treatment Details

Sr. No.	Treatment	Feed Additive (g/Kg)
1.	T_0	Diet prepared without the addition of L-ascorbic Acid and L-leucine (Control)
2.	T_1	Diet prepared with 10 g L-leucine
3.	T_2	Diet prepared with 15 g L-leucine
4.	T_3	Diet prepared with 0.3 g L-ascorbic Acid
5.	T_4	Diet prepared with 0.4 g L-ascorbic acid
6.	T_5	Diet prepared with 0.3 g L-ascorbic Acid and 10 g L-leucine
7.	T_6	Diet prepared with 0.4 g L-ascorbic Acid and 10 g L-leucine
8.	T_7	Diet prepared with 0.3 g L-ascorbic Acid and 15 g L-leucine
9.	T_8	Diet prepared with 0.4 g L-ascorbic Acid and 15 g L-leucine

The experimental fish *Labeo catla* was stocked @ 1g/L of water. Siphoning was done every day and water exchange kept 10-15% to compensate losses during syphoning and evaporation. The fish were fed at the rate of 8% of average body weight in split dosed twice a day for first 30 days and 6% for subsequent time. The experiment aimed to examine how various feed ingredients influenced the activity of digestive enzymes in *Labeo catla*. This evaluation of enzymes for digestion was conducted at the end of the 75-day experimental period. Upon completion of the 75 days, the *Labeo catla* were collected for enzyme analysis. To evaluate enzymatic activity, the gut from each *Labeo catla* treatment group was carefully dissected and stored in appropriate vials. Subsequently, the collected gut samples were homogenized in an appropriate solvent and then centrifuged. After that, the supernatant was used to estimate enzyme activity. The analysis of protease activity in catla gut content was performed using the casein digestion method outlined by Drapean (1974). This involved an enzymatic reaction using casein as the substrate, with subsequent measurement of the resulting hydrolysis products.1% casein in 0.05 M Tris-phosphate buffer (pH 7.8) was employed as the mixture for the enzyme reaction, and it was incubated for five minutes at 37°C. Then, the tissue homogenate was added and incubated for 10 min to complete the reaction, which was then stopped by adding 10 % TCA, followed by filtration of the entire content. The amount of enzyme required to release soluble acid fragments equal to 0.001 at 280 nm per minute at 37°C and pH 7.8 was established as one unit of enzyme activity. Assessment the activity of amylase in the catla gut utilized the dinitrosalicylic acid (DNS) method developed by Rick and Stegbauer (1974). This method triggered an enzymatic reaction between amylase and starch, and the amount of released reducing sugars was measured using the DNS reagent. Concisely, tissue homogenates and reaction mixtures (1% w/v) of a starch solution and phosphate buffer (pH 6.9) were 30 minutes of incubation at 37 °C. To this, DNS is added and kept in a boiling water bath for 5 min. After cooling and diluting with distilled water, 540 nm was used to measure the absorbance. As a standard, maltose was employed. A mole of maltose emitted from starch per minute at 37°C was used to measure the amylase activity. To determine lipase activity in the catla gut, the assay described by Cherry and Crandall (1932) was used. This process involved the hydrolysis of a lipid substrate by lipase, followed by measurement of the released fatty acids. The fatty acids released were estimated by titrating with sodium hydroxide solution. The assay system consists of 1.5 mL of stabilized lipase substrate and 1.5 mL of 0.1 M Tris-HCl buffer (pH of 8.0); to this, 1.0 mL of the crude enzyme extract was added. This mixture was incubated for 24 h at 27ºC, and 3 mL of 95% ethanol was added to stop the reaction. It was then titrated against 0.01 N NaOH (0.9% w/v) using phenolphthalein as the indicator. All data were subjected to One-way ANOVA using SPSS v30, and mean differences were evaluated by Duncan Multiple Range Test at $p < 0.05$. Results are presented as mean ± standard error.

Result

Effect of L-Ascorbic Acid, L-Leucine and Its Combination on Protease Activity

The mean value of protease activity for *Labeo catla* after the completion of 75 day feeding trials are presented in table 3. In the present study, dietary supplementation significantly improved the protease activity across the different treatments ($p < 0.05$). The highest protease activity was recorded in T6 (2.516±0.037) followed by T5 (2.363±0.029) and T7 (2.316±0.020). These values represent a substantial improvement in digestive enzyme capacity compared to T0, which exhibited a protease activity of 1.923±0.021. Statistical analysis indicate that treatment T6 was significantly different ($p < 0.05$)from all other treatments. The result indicates that T5, T6 and T7 effectively enhanced the proteolytic potential of the species, while treatments T3 and T4 did not yield a statistically significant gain over the basal diets. Moderate improvement was also noted in T8 (2.253±0.018),T2 (2.150±0.017) and T1 (2.093±0.022) though they remained statistically comparable to the mid-range treatments.

Table 3: Protease activity (U/mg protein) of *Labeo catla* after the completion of the experiment

Treatment	Protease(U/mg protein)
T0	1.923±0.021^{a}
T1	2.093±0.022^{b}
T2	2.150±0.017^{b}
T3	1.980±0.050^{a}
T4	2.003±0.012^{a}
T5	2.363±0.029^{d}
T6	2.516±0.037^{e}
T7	2.316±0.020^{cd}
T8	2.253±0.018^{c}

Note: All the values are presented in (Mean±SE) n = 3. Mean values with various superscripts in the same column are significantly different ($p < 0.05$).

Effect of L-Ascorbic Acid, L-Leucine and Its Combination on Amylase Activity

The mean value of amylase activity for *Labeo catla* after the completion of 75 day feeding trials are summarized in table 4. In the present study, dietary supplementation significantly improved the activity of amylase across the different treatments ($p < 0.05$). The highest amylase activity was observed in T6 (1.620±0.020) followed by T5 (1.510±0.017) and T7 (1.483±0.014). These values represent a substantial improvement in carbohydrate digestion capacity compared to T0, which exhibited an activity of amylase 1.103±0.027. Statistical analysis indicate that T6 was significantly different ($p < 0.05$) from all other treatments. The result indicates that T5, T6 and T7 effectively enhanced the amylolytic potential of the species, Furthermore, treatments T3 (1.176±0.020) and T4 (1.213±0.015) also yield a statistically significant gain over the basal diets ($p < 0.05$). Moderate improvement was also noted in T8 (1.446±0.008),T2 (1.350±0.017) and T1 (1.303±0.018) though they remained statistically comparable to the mid-range treatments.

Table 4: Amylase activity (U/mg protein) of *Labeo catla* after the completion of the experiment

Treatment	Amylase (U/mg protein)
T0	1.103±0.027^{a}
T1	1.303±0.018^{c}
T2	1.350±0.017^{c}
T3	1.176±0.020^{b}
T4	1.213±0.015^{b}
T5	1.510±0.017^{e}
T6	1.620±0.020^{f}
T7	1.483±0.014^{de}
T8	1.446±0.008^{d}

Note: All the values are presented in (Mean±SE) n = 3. Mean values with various superscripts in the same column are significantly different ($p < 0.05$).

Effect of L-Ascorbic Acid, L-Leucine and Its Combination on Lipase Activity

The mean value of lipase activity for *Labeo catla* after the completion of 75day feeding trials are presented in table 5. In the current study, dietary supplementation significantly improved the lipase activity across the different treatments ($p < 0.05$). The highest lipase activity was observed in T6 (0.303±0.018) followed by T5 (0.280±0.011) and T7 (0.243±0.015). These values represent a substantial improvement in lipid digestion capacity compared to T0, which exhibited a lipase activity of 0.150±0.006. Statistical analysis showed that treatment T5 and T6 was significantly different ($p < 0.05$) from all other treatments. The result indicates that T5, T6 and T7 effectively enhanced the lipolytic potential of the species, Furthermore, treatments T2 (0.200±0.010) and T4 (0.196±0.012) also yield a statistically significant gain over the basal diets, whereas treatments T1 (0.173±0.012) and T3 (0.183±0.007) remained statistically comparable to the control. Moderate improvement was also noted in T8 (0.220±0.012) which shows a distinct increase in enzymatic activity compared to control.

Table 5: Lipase activity (U/mg protein) of *Labeo catla* after the completion of the experiment

Treatment	Lipase(U/mg protein)
T0	0.150±0.006^{a}
T1	0.173±0.012^{ab}
T2	0.200±0.010^{bc}

T3	0.183 ± 0.007^{abc}
T4	0.196 ± 0.012^{bc}
T5	0.280 ± 0.011^{e}
T6	0.303 ± 0.018^{e}
T7	0.243 ± 0.015^{d}
T8	0.220 ± 0.012^{cd}

Note: All the values are presented in (Mean±SE) n = 3. Mean values with various superscripts in the same column are significantly different ($p < 0.05$).

Discussion

In the present study, the activities of protease, amylase, and lipase varied significantly among dietary treatments, demonstrating that dietary supplementation of L-ascorbic acid and L-leucine positively influenced the digestive physiology of *Labeo catla* fingerlings. The highest activities of protease (2.516 ± 0.037 U mg^{-1} protein), amylase (1.620 ± 0.020 U mg^{-1} protein), and lipase (0.303 ± 0.018 U mg^{-1} protein) were recorded in treatment T6, whereas the lowest values were observed in the control group (T0). The increased activities of protease, amylase, and lipase observed in leucine-supplemented treatments (T1 and T2) suggest that L-leucine plays a crucial role in improving nutrient digestion and metabolism. Leucine is a branched-chain amino acid known to regulate protein synthesis and cellular growth through activation of the mammalian target of rapamycin (mTOR) signaling pathway, which controls nutrient sensing and metabolic processes (Kimball & Jefferson, 2006). Zhou *et al*. (2020) reported that dietary leucine supplementation significantly enhanced intestinal digestive enzyme activities and nutrient utilization in rainbow trout (*Oncorhynchus mykiss*) through activation of the mTOR pathway. Therefore, the elevated digestive enzyme activities observed in the present study may be attributed to leucine-mediated stimulation of digestive enzyme synthesis, secretion, and nutrient assimilation. Fish fed diets supplemented solely with L-ascorbic acid (T3 and T4) exhibited moderate improvements in protease, amylase, and lipase activities compared with the control group. This finding suggests that vitamin C exerts an indirect influence on digestive function rather than directly stimulating enzyme production. Ascorbic acid is an essential micronutrient and potent antioxidant that protects intestinal tissues from oxidative damage, maintains epithelial integrity, and supports normal physiological functions (Halver & Hardy, 2002). Zehra and Khan (2021) demonstrated that dietary vitamin C supplementation improved growth performance, antioxidant status, and physiological health in *Channa punctatus*, thereby indirectly enhancing digestive efficiency. Similar mechanisms may have contributed to the improved digestive enzyme activities observed in the present investigation. The combined supplementation treatments (T5–T8) consistently produced significantly higher activities of all three digestive enzymes than treatments receiving either nutrient alone, with T6 exhibiting the maximum response. These findings indicate a synergistic interaction between L-ascorbic acid and L-leucine in enhancing digestive capacity. Leucine promotes protein synthesis and digestive enzyme production through metabolic signaling pathways, whereas ascorbic acid protects digestive tissues and enzymes from oxidative stress, thereby maintaining their stability and functionality (Kimball & Jefferson, 2006; Halver & Hardy, 2002). The complementary actions of these nutrients appear to improve the digestion of proteins, carbohydrates, and lipids more effectively than individual supplementation. Interestingly, increasing dietary leucine supplementation from 10 g kg^{-1} diet (T6) to 15 g kg^{-1} diet (T7 and T8) did not result in further enhancement of digestive enzyme activities. This observation suggests that the beneficial effects of leucine are dose-dependent and that excessive supplementation may lead to amino acid imbalance, reduced nutrient utilization efficiency, or metabolic disturbances. Similar findings have been reported in fish nutrition studies, where optimal amino acid supplementation improved digestive performance and growth, whereas excessive dietary levels failed to provide additional benefits (Wilson, 2002; Zhou *et al*., 2020). The synergistic effects observed in the present study are consistent with previous reports demonstrating the beneficial influence of combined nutrient supplementation on digestive physiology. Liu *et al*. (2018) reported that dietary supplementation with vitamins C and E significantly enhanced digestive enzyme activities, including protease, amylase, and lipase, in juvenile discus fish (*Symphysodon haraldi*). Likewise, the present findings indicate that the combined supplementation of L-ascorbic acid and L-leucine improves digestive enzyme activities and nutrient utilization in *Labeo catla* fingerlings.

Conclusion

The present study demonstrated that dietary supplementation of L-ascorbic acid and L-leucine significantly influenced the digestive enzyme activities of *Labeo catla* fingerlings. Supplemented diets enhanced the activities of protease, amylase, and lipase compared with the control, indicating improved digestive capacity and nutrient utilization. The findings suggest that L-leucine primarily promotes digestive enzyme synthesis and nutrient metabolism, while L-ascorbic acid supports digestive function through its antioxidant properties and maintenance of intestinal

health. The synergistic interaction between these nutrients produced greater improvements in digestive efficiency than their individual supplementation. However, increasing leucine supplementation beyond 10 g kg^{-1} did not provide additional benefits, indicating that excessive amino acid inclusion may reduce nutrient utilization efficiency. Overall, the results indicate that dietary supplementation with 0.4 g kg^{-1} L-ascorbic acid and 10 g kg^{-1} L-leucine is an effective nutritional strategy for enhancing digestive enzyme activities and growth performance in *Labeo catla* fingerlings.

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