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EDITED BY
Xueshan Li,
Jimei University, China

REVIEWED BY
Neeraj Kumar,
National Institute of Abiotic Stress Management (ICAR), India
Rakhi Kumari,
Central Institute of Freshwater Aquaculture (ICAR), India

*CORRESPONDENCE
Sarvendra Kumar
sarvo0203@gmail.com

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Dietary licorice (*Glycyrrhiza glabra*) supplementation enhances growth, antioxidant defence, and modulates metabolic enzyme activity in *Labeo rohita*

Ravishekhar Kumar[1], Sarvendra Kumar[1*], Showkat Ahmad Dar[1], Sagarika Swain[1] and Ravi Shankar Kumar[2]

[1]Department of Fish Nutrition, Physiology and Biochemistry, College of Fisheries, Kishanganj, Bihar Animal Sciences University, Patna, India, [2]Department of Fisheries Economics, Extension and Statistics, College of Fisheries, Kishanganj, Bihar Animal Sciences University, Patna, India

This study aims to assess the impact of liquorice (*Glycyrrhiza glabra*) on the growth, immunity, digestive enzyme activity and metabolism of *Labeo rohita*. Five isonitrogenous (30% crude protein) and isocaloric diets were formulated with licorice incorporated at 0%, 1%, 2%, 3%, and 4%, and were designated as C (control), T1, T2, T3, and T4, respectively. A total of 225 *L. rohita* fingerlings (5 $\pm$ 0.5 g) were randomly stocked in triplicate FRP tanks (15 fish per tank). After 60 days of experimental feeding, licorice inclusion significantly ($p<0.05$) enhanced growth performance and feed conversion efficiency in *Labeo rohita* fingerlings compared to the control group. Fish fed the T3 diet (3% licorice) exhibited significantly greater weight gain (%) and specific growth rate, coupled with a lower feed conversion ratio. Lipase activity was significantly affected ($p<0.05$), with the highest activity observed in the T3 group. Muscle aspartate transaminase (AST) and alanine transaminase (ALT) activities were significantly higher ($p<0.05$) in the T4 group compared to the control. The highest superoxide dismutase (SOD) activity was recorded in the T4 group, which was higher ($p<0.05$) than the control and other dietary treatments. Similarly, catalase activity increased with higher licorice inclusion, showing significant elevation ($p<0.05$) in the T3 and T4 groups relative to the control. Serum protein and globulin levels significantly($p<0.05$) vary between controls and treatments. The highest NBT activity was observed in the T1-fed group; however, it did not differ significantly from the other treatment groups, although it was higher compared to the control group. The study revealed that licorice supplementation promotes growth and immunity in fish.

KEYWORDS
growth performance, herbal immunomodulator, metabolic enzymes, oxidative enzyme, transaminases

Introduction

Aquaculture plays a critical role in global food and nutritional security; however, the sustainability of intensive fish farming is increasingly challenged by disease outbreaks, oxidative stress, and suboptimal growth performance. Among cultured species, *Labeo rohita* (rohu) holds a prominent position in the Indian subcontinent due to its excellent nutritional profile, rapid growth, and strong consumer acceptance. However, the intensification of aquaculture systems often exposes fish to environmental and physiological stress, adversely affecting their growth, immunity, and overall well-being. These stressors interfere with metabolic homeostasis, including energy metabolism, nutrient assimilation, and protein synthesis, which are fundamental for optimal growth, physiological performance, and the maintenance of an effective immune system in fish. Therefore, there is increasing interest in identifying natural dietary supplements to enhance fish health, altered metabolic enzyme activities, growth, ultimately affecting productivity and economic returns.

In captive environments, stressors such as overcrowding, chemical usage, and therapeutic interventions can negatively affect fish immunity. In recent years, the use of synthetic growth promoters and antibiotics in aquafeeds has raised serious concerns regarding antimicrobial resistance, environmental safety, and consumer health. At the same time, infectious diseases further compromise their growth performance and survival in aquaculture systems (Zhu, 2020). Use of antibiotics to control pathogens and diseases has been banned due to their adverse effects, including body malformation, immune suppression, microbiota dysfunction, and oxidative stress (Yang et al., 2020; Limbu et al., 2021). Enhancing the nutritional quality of aquafeed is therefore essential for maintaining fish health (Awad and Awaad, 2017). To support fish health, aquaculture diets are often supplemented with functional additives like probiotics, acidifiers and medicinal plants because of their ability to enhance antioxidant defence, inhibit pathogens, and stimulate immune responses (Van, 2015). Consequently, there is growing interest in natural and eco-friendly feed additives that can enhance growth performance, improve antioxidant capacity, and strengthen innate immunity in fish (Shankar and Kiran, 2013). Among these, phytogenic feed additives derived from medicinal plants have gained considerable attention due to their bioactive compounds, including flavonoids, phenolics, saponins, and glycosides, which possess antioxidant, immunomodulatory, and metabolic regulatory properties (Stratev et al., 2018).

Licorice (*Glycyrrhiza glabra*), a well-known medicinal plant, has been widely used in traditional medicine for its anti-inflammatory, antioxidant, antimicrobial, and immunostimulatory effects. *G. glabra* is a medicinal herb belonging to the family Fabaceae. Its roots are the most valuable part of the plant and are extensively used in pharmaceutical, health-related, and food industries (Pastorino et al., 2018). The root contains several key biomolecules, including glycyrrhizinic acid (glycyrrhizin), flavonoids, glabridin, liquiritigenin, and liquiritin, which exhibit notable antimutagenic, antimicrobial, antioxidant, and anti-inflammatory (Alagawany et al., 2019). These beneficial properties enhance its value as a dietary supplement, as reflected in improved non-specific immune responses in Nile tilapia (Abdel-Tawwab et al., 2021) and common carp (Adineh et al., 2021). Because of their improved absorption efficiency, enriched bioactive constituents, and effectiveness at lower inclusion levels, herbal supplements and their extracts are increasingly used in aquaculture nutrition. In particular, licorice root extract in powdered form shows greater potency and uniformity, largely due to its high concentration of bioactive phytochemicals, especially glycyrrhizin (Isbrucker and Burdock, 2006). The present study was designed to evaluate the effects of dietary licorice (*Glycyrrhiza glabra*) fortification on growth performance, feed utilization, antioxidant defence mechanisms, immune response, and metabolic enzyme activities in *L. rohita* fingerlings. The findings of this study aim to contribute to the development of functional and sustainable aquafeeds using herbal additives, thereby supporting environmentally responsible aquaculture practices.

Materials and methods

Fish species and research methodology

This study was conducted over 60 days at the College of Fisheries, Kishanganj wet laboratory. Fingerlings of rohu average body weight of 5.0 ± 0.5 g were used as the experimental animals. The fish were obtained from Taniska Farm, Purnia, Bihar, and transported to the wet laboratory in polyethene bags. Before the experiment, the fish were acclimated for 30 days (fed 30% CP diet). The feeding trial was subsequently conducted under controlled conditions in the wet laboratory of COF Kishanganj. A total of 225 *Labeo rohita* fingerlings were distributed randomly into five experimental treatments (three replicates per treatment), following a completely randomized design. During the experimental period, the water level was maintained at 500 L, and uninterrupted aeration was ensured in all tanks.

Experimental diets

For diet were formulation and preparation, commonly used ingredients such as soybean meal, rice bran, maize flour, mustard oil cake, CMC (Carboxymethyl Cellulose), vitamin–mineral premix, Butylated Hydroxytoluene (BHT), were utilized. Five experimental diets were formulated to be isonitrogenous, isocaloric, and isoenergetic, each containing 30% crude protein and 6% lipid. The diets differed only in the level of licorice inclusion: (C) 0%, (T1) 1%, (T2) 2%, (T3) 3%, and (T4) 4% (Table 1). The required ingredients, formulated according to the nutritional needs of rohu, were thoroughly blended with an appropriate amount of water to obtain a uniform dough. The prepared dough was then transferred into autoclavable plastic bags and cooked by steaming in an autoclave for 15 minutes. After steaming, the dough was removed and allowed to cool completely. Once cooled, the predetermined quantities of licorice powder along with the vitamin–mineral premix were incorporated and mixed uniformly. The dough was subsequently processed through a mechanical pelletizer to produce uniform pellets. Pellets were spread on paper to air-dry. Once dried, they were packed in polythene bags, sealed in airtight containers, and labelled according to their respective treatments.

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TABLE 1 Ingredients composition (g/100g dry matter) of the experimental diet.

Ingredients	C	T1	T2	T3	T4
SM	32	32	32	32	32
RB	25	25	25	25	25
MOC	27	27	27	27	27
MF	9.98	9.98	9.98	9.98	9.98
CMC	1	1	1	1	1
BHT	0.02	0.02	0.02	0.02	0.02
Vit. min. mix[a]	1	1	1	1	1
Corn starch	4	3	2	1	0
Licorice	0	1	2	3	4

CMC, Carboxymethyl cellulose; BHT, Butylated hydroxyl toluene; SM-Soyabean meal; MOC, Mustard Oil cake; MF- Maize Flour; RB- Rice bran. [a]Composition of vitamin-mineral mix (quantity/kg): Vitamin A – 55,00,000 IU; Vitamin D_3 – 11,00,000 IU; Vitamin B_2 – 2,000 mg; Vitamin E – 750 mg; Vitamin K – 1,000 mg; Vitamin B_6 – 1,000 mg; Vitamin B_{12} – 6 mcg; Nicotinamide – 10 g; Calcium Pantothenate – 2,500 mg; Choline Chloride – 150 g; Mn – 27,000 mg; I – 1,000 mg; Fe - 7,500 mg; Zn – 5,000 mg; Cu – 2,000 mg; Co – 450 DL-Methionine – 10 g; L- lysine – 10 g; Selenium – 50ppm

Feeding

Feeding was initiated at 3% of body weight, and the feeding rate was adjusted accordingly, with feed rations periodically adjusted based on growth performance. The total daily feed allowance was split into two equal meals and administered in the morning (9:00 am) and afternoon (4:30 pm).

Proximate nutrient composition of the formulated diets

The Proximate composition of the experimental diets was determined using standard methods of AOAC (1995) at the Fish Nutrition and Feed Technology Laboratory, College of Fisheries, Kishanganj. The experimental diets were dried at 102 ± 2 in a hot air oven to determine the moisture content. The CP of the dried experimental diet was estimated by the Kjeltec automated method (4800 Kjeltec, Foss, Sweden). The Soxhlet apparatus (Borosil) was used for the estimation of lipid by using diethyl ether as the solvent. Ash content in various experimental diets was determined by using a muffle furnace at 600 °C for 5–6 hours, and Nitrogen-free extract (NFE) was estimated by using the following formulae-100 – [CP (%) + EE (%) + Ash (%) + CF (%)].

Growth parameters

Growth performance of *Labeo rohita* was determined at the end of the feeding trial by recording final and initial body weights after overnight starvation.

Percentage weight gain

The WG % was determined using the following formula:.

$$\text{WG \%} = \frac{\text{Fish weight at end of experiment in gm} - \text{Initial fish Weight in gm}}{\text{Initial fish weight in gm}} \times 100$$

SGR

The following formula estimated SGR.

$$\text{SGR} = \frac{\text{Log}_e\text{Final wt .} - \text{Log}_e\text{Initial wt .}}{\text{Duration of experiment (in Days)}} \times 100$$

FCR

FCR calculated by using formulae given below:

$$\text{FCR} = \frac{\text{Feed given (gm)}}{\text{Increase body weight (gm)}}$$

PER

PER estimated by formulae given below:

$$\text{PER} = \frac{\text{Weight gain(gm)}}{\text{Protein fed (gm)}}$$

Preparation of samples for enzymatic assay

At the End of experiment, three fish were collected from each replicate and anaesthetized using clove oil (50 µL/L). Subsequently, the intestine, liver, and muscle tissues were dissected, collected, and pooled for each replicate for further analysis. A 5% homogenate was prepared in chilled sucrose (0.25M), centrifuged at 6000 rpm (−4 °C) for 10 min, and the supernatant was stored (−20 °C) for subsequent enzymatic analyses.

Digestive enzyme estimation

Protease activity

Protease was estimated by the casein digestion method of Drapeau (1974). The reaction mixture containing 1% casein in 0.05 M Tris-phosphate buffer (pH 7.8) was incubated at 37 °C, followed by enzyme addition. The reaction was terminated with 10% trichloroacetic acid, and a non-incubated reagent blank was used as the control. The activity was expressed as units, where one unit corresponded to the quantity of enzyme causing an absorbance change of 0.001 min^{-1} at 280 nm due to the release of acid-soluble fragments at pH 7.8 and 37 °C.

Amylase activity

Intestinal amylase activity was assayed using a starch substrate solution (2% w/v in pH 7 phosphate buffer) according to the method of Rick and Stegbauer (1974). Incubation for 30 minutes at 37 °C, the reaction was terminated by adding 3,5-dinitrosalicylic acid (DNS), and the samples were heated in a boiling water bath for 5 minutes. Following cooling and dilution with distilled water. The absorbance was measured at a wavelength of 540 nm. Activity was determined from a maltose standard curve and expressed as µmol of maltose/min. per mg of protein at 37 °C.

Lipase activity

A titrimetric assay based on Cherry and Crandall (1932) was used to estimate lipase activity. The enzyme hydrolyses triacylglycerols, releasing fatty acids. The reaction mixture (3 mL H_2O, 1 mL homogenate, 0.5 mL pH 7 phosphate buffer, and 2 mL olive oil emulsion) was incubated for 24 h at 37 °C. Following this, the fatty acids titrated with NaOH (0.05 N) after adding 95% ethanol and phenolphthalein. A boiled homogenate served as the control. Enzyme activity (units/mg protein) was derived from the milliequivalents of NaOH used in the titration.

Metabolic enzyme

Aspartate aminotransferase and alanine aminotransferase

AST and ALT activities in both liver and muscle tissues of rohu were estimated using a modified method of Wooten (1964). The reaction mixture contained α-ketoglutarate with DL-aspartate (for AST) or DL-alanine (for ALT) in phosphate buffer (0.05 M, pH 7.4). Reaction was initiated by adding tissue homogenate (incubated at 37 °C, 1hrs) and terminated with 2,4-dinitrophenylhydrazine and take absorbance at 540 nm after alkaline development. Enzyme activities were expressed as nmol of oxaloacetate (AST) or pyruvate (ALT) formed per mg protein/minute at 37 °C.

Lactate dehydrogenase

Lactate dehydrogenase E.C.1.1.1.27

Lactate dehydrogenase (LDH) Activity was measured using the prescribed protocol of Wrobleiuski and Ladue (1955). A 3 mL reaction mixture was prepared: 2.7 ml phosphate buffer (0.1M, pH 7.5), NADH solution (0.1 mL, 2 mg/mL in phosphate buffer), tissue homogenate (0.1 mL), and sodium pyruvate (0.1 mL). Reaction was initiated by adding sodium pyruvate. Record at 340 nm every 30 seconds (3 minutes). Enzyme activity was defined as μmol of NAD^+ generated per mg of protein/minute (25 °C), One unit is equal to Δ0.01 OD per minute.

Malate dehydrogenase

MDH (EC 1.1.1.37) activity in various tissues was measured using the method established by Ochoa (1955). The specific activity was calculated in units per milligram of protein/minute (25 °C). One unit is equal to Δ0.01 OD per minute.

Antioxidant enzyme

Superoxide dismutase

SOD activity was measured following the Misra and Frodovich (1972), which measures the inhibition of epinephrine auto-oxidation to adrenochrome. A reaction mixture was prepared by adding 50 μL of the sample to a cuvette containing 0.1 M carbonate-bicarbonate buffer (1.5 mL, pH 10.2) with 57 mg/dL EDTA and 3 mM epinephrine (0.5 mL). After thorough mixing, the absorbance change at 480 nm (three minutes) by UV spectrophotometer (Thermo Scientific). Enzyme activity, expressed in units, is defined as the protein amount needed to inhibit 50% of the epinephrine auto-oxidation rate.

Catalase

Catalase activity was quantified using the Takahara et al. (1960) protocol. A 20 μL enzyme sample was combined with 50 mM phosphate buffer (2.45 mL, pH 7.0). Reaction Mixture initiated by adding hydrogen peroxide solution (1.0 mL). Absorbance at 240 nm was measured every 30 seconds for three minutes to assess the decrease. A control reaction, where the H_2O_2 was replaced with 1.0 mL of distilled water, was run in parallel. Results were calculated as nanomoles of H_2O_2 broken down per minute/milligram of protein.

Collection of blood and serum

After completing the feeding trial, blood was obtained from nine randomly selected fish from each treatment (three fish from each replicate and pooled). The fish were first anesthetized (50 μL/L clove oil). Whole blood was drawn from the caudal vein using an EDTA-rinsed syringe and placed in an EDTA-coated tube to prevent clotting. This sample was used for the Nitroblue Tetrazolium (NBT) assay. In parallel, blood was collected without an anticoagulant, kept at room temperature (for clotting), and then centrifuged for 10 minutes (10,000 rpm at 4 °C). Collected serum, stored at -20 °C, and subsequently used to measure various biochemical and immunological markers.

Respiratory burst activity

The Nitroblue Tetrazolium (NBT) assay was conducted following Stasiack and Bauman (1996) methodology. Blood was collected from fish (three fish from each replicate) with an anticoagulant (2.7% EDTA). A 50 μL blood sample was placed in a microplate well and incubated 1hrs (37 °C) to promote cell adhesion. After removing the supernatant, the microplate wells were washed (three times) with PBS. Then, 50 μL of a 0.2% NBT solution was introduced to each well and incubated for another hour. Cells were subsequently fixed with methanol (100% for 2–3 minutes) and then washed three times with methanol (70%). Then air-dried the plate and added 60 μL of 2N KOH and 70 μL of dimethyl sulfoxide per well to solubilize the resulting formazan precipitate. Absorbance of the turquoise solution (blue) was measured at 620nm (Thermo Scientific Multiskan FC ELISA).

Serum protein, albumin, globulin

Serum protein, albumin, and globulin value analyzed using standardized diagnostic assay kits (Erba Diagnostics Mannheim, supplied by TransAsia Bio-Medical Ltd., India). Serum glucose levels were quantified via the Trinder's method using a commercial assay kit (Erba Diagnostic Mannheim, TransAsia Ltd., India).

$$\text{mg/dl Glucose} \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{X N}$$

Where N = 100 mg/dl (Standard).

Statistical analysis

The significance of the experimental results was evaluated by one-way analysis of variance (ANOVA) performed in SPSS version 22.0. Differences among treatment means were further analyzed using Duncan's multiple range test, with statistical significance was accepted at $p< 0.05$. Results are presented as mean ± standard error (SE).

Results

Proximate composition of diets

Post-formulation analysis confirmed the nutrient composition of the diets, with crude protein ranging from 30.1 to 30.6%, crude lipid from 6.0 to 6.6%, ash from 8.3 to 9.6%, and crude fiber from 10.26 to 12.0%. The nitrogen-free extract was calculated to range between 40.83 and 44.93% (Table 2).

Growth performance and feed utilization

Significant differences ($p<0.05$) in growth indices and nutrient utilization efficiency were observed between the treatments and the control at the end of the experiment (Tables 3, 4). Both WG% and SGR showed a significant improvement in licorice fed group. Fish fed the T3 diet exhibited the highest WG% and SGR values, indicating superior growth performance, followed in descending order by the T2 and T4 dietary treatments. This pattern was inversely reflected in the feed conversion ratio (FCR), where T3 showed the lowest value, followed by T2 and T4, all of which were significantly better than T1 and the control. The highest FER and protein PER were observed in fish fed the T3 diet, followed by the T2 and T4 diets, and these values differed significantly ($p<0.05$) from those of T1 and the control (Table 4).

Digestive enzymes

The activity profiles of key digestive enzymes revealed a differential response. Protease and amylase activities were unaltered by dietary treatment ($p>0.05$). Dietary licorice showed a pronounced effect ($p<0.05$), on lipase activity. Lipase activity showed a significant effect ($p < 0.05$), with the highest value observed in the T3 group, which was statistically similar to that in the T4 group (Table 5).

Metabolic enzymes

Aminotransferase enzyme (ALT and AST)

The results show that the activity of AST in liver tissue remained statistically unchanged ($p>0.05$) across all the treatments. The activity of AST in muscle tissue was higher ($p<0.05$) in T4 treatment, followed by T3 and T2 (Table 6).

The ALT activities in Muscle and liver tissue were observed statistically higher ($p<0.05$) in the T4 compared to other treatments.

TABLE 2 Proximate composition of experimental feed (% DM basis).

Treatment	C	T1	T2	T3	T4
DM	88.8± 1.89	89.86 ± 2.19	92.2± 1.15	92 ± 1.15	90.2 ±1.8
CP	30.37±0.28	30.2 ± 0.20	30.1± 0.15	30.63 ± 0.03	30.3 ± 0.28
EE	6.13±0.13	6.83 ±0.60	6.33± 0.44	6.67 ± 0.72	6.17± 0.60
CF	11.13±1.67	12.00 ±0.34	11.27±1.15	12.2±1.40	10.27±1.88
NFE	43.92±1.16	41.86±1.01	42.63±1.26	41.44±0.83	44.93±2.40
Ash	8.45±0.88	9.11±0.77	9.67±0.69	9.06±1.25	8.33±0.57
DE	352.35±4.75	349.72±2.52	347.93±8.90	348.31±7.59	356.43±5.43

DM, Dry matter; CP, Crude protein; EE, Ether extract; CF, Crude fibre; NFE: Nitrogen free Extract. DE: - Digestible Energy. DE is calculated using following formula: (Kcal/100g) = (CL%×9) + (%CP×4) + (%NFE×4) Halver, 1976.

TABLE 3 Growth performance of *Labeo rohita* fingerlings fed with licorice root powder supplemented diets.

Treatment	Initial weight (gm)	Final Weight (gm)	WG (%)	SGR
Control	5.02±0.02	10.8^{a}±0.23	116.40^{a}±5.26	1.28^{a}±.04
T1	5.04±0.02	11.0^{a}±0.21	119.66^{a}±5.30	1.31^{a}±.03
T2	5.03±0.03	12.5^{bc}±0.25	149.93^{bc}±5.70	1.52^{bc}±.03
T3	5.06±0.03	13.1^{c}±0.42	159.60 ^{c}±7.45	1.58^{c}±.04
T4	5.02±0.02	11.9^{b}±0.22	138.77^{b}±5.57	1.44^{b}±.03
p value	0.788	0.001	0.002	0.001

Data is presented as Mean ± S.E. (n=3), values with different superscripts in the same column differ significantly ($p< 0.05$); C-Control; T-Treatments; WG, Weight gain; SGR, Specific growth rate.

TABLE 4 Feed utilisation of *Labeo rohita* fingerlings fed with licorice root powder supplemented diets.

Treatment	FCR	FER	PER
Control	2.24^{b}±.07	0.44^{a}±.01	1.47^{a}±.05
T1	2.16^{b}±.07	0.46^{a}±.01	1.53^{a}±.05
T2	1.76^{a}±.02	0.56^{b}±.00	1.87^{b}±.03
T3	1.70^{a}±.08	0.59^{b}±.02	1.93^{b}±.09
T4	1.87^{a}±.02	0.53^{b}±.00	1.75^{b}±.02
p value	0.000	0.001	0.001

Data is presented as Mean ± S.E. (n=3), values with different superscripts in the same column differ significantly (p< 0.05); FCR-Feed conversion Ratio; FER-Feed efficiency Ratio; PER-Protein efficiency ratio.

Lower ALT activities in muscle tissue are shown in the control group, which is significantly similar to T1, T2 and T3 treatments. Similarly, hepatic ALT activity was low and did not differ between the control and T1 group (Table 6).

Lactate dehydrogenase and malate dehydrogenase activities

Liver LDH activity was significantly higher (p<0.05) in the T4 group compared to all other treatment groups. Malate dehydrogenase (MDH) activity exhibited a contrasting response, remaining unchanged in the liver while showing a significant increase (p<0.05) in muscle tissue, with the highest levels observed in T4 and the lowest in the control group. The control's muscle MDH activity was statistically similar to that of the T1 group (Table 7).

Oxidative enzyme

The analysis of key antioxidant enzymes revealed a clear enhancement in the licorice- supplemented groups. While superoxide dismutase (SOD) activity was significantly greater in the T4 treatment (Figure 1) , catalase activity was markedly increased in both the T3 and T4 groups (Figure 2). In contrast, the control group demonstrated the lowest activities for both SOD and catalase, underscoring the positive effect of the treatments on the antioxidant status.

TABLE 5 Digestive enzyme activity of *Labeo rohita* fingerlings fed with licorice root powder supplemented diets.

Treatments	Protease	Lipase	Amylase
C	23.39±1.38	1.99^{a}±0.22	5.06±0.47
T1	26.59±0.59	1.88^{a}±0.20	6.18±0.61
T2	27.44±0.66	2.88^{b}±0.24	6.02±0.59
T3	27.98±1.74	3.83^{c}±0.17	6.79±0.39
T4	25.94±1.78	3.67^{c}±0.21	4.63±0.48
p value	0.208	0.000	0.082

Different superscripts in the same column differ significantly (p<0.05). Data expressed as Mean ± S.E., n=3. activities are expressed as follows: protease as nanomoles of tyrosine released/min/mg protein; amylase as micromoles of maltose released/min/mg protein; lipase as units/mg protein.

Serum biochemical parameters

The health status of *L. rohita* fingerlings, as indicated by serum parameters such as total protein, albumin, globulin, A/G ratio, and glucose, varied across dietary treatments (Table 8). A significant increase in serum protein levels was observed in the licorice-treated group compared to the control. Serum globulin levels were higher (p<0.05) in the T2 group, followed by T3, T1, and T4. Conversely, the A/G ratio was significantly elevated in the T1 and control groups compared to the other treatments. Blood glucose levels were highest in the control group and significantly reduced (p<0.05) in T4, with no significant difference observed between T4 and T3.

Respiratory burst activity

The evaluation of innate immunity in *L. rohita* fingerlings, as measured by respiratory burst activity, revealed a significant (p<0.05) enhancement in all treatments (licorice-supplemented diets). This immunostimulatory effect, quantified via nitroblue tetrazolium (NBT) reduction, was most pronounced in the T1 group, which exhibited the highest NBT value. However, the NBT value was higher in T1, which did not differ significantly from treatment groups (T2, T3, and T4), all of which were similarly elevated above the control (Figure 3).

Discussion

In aquaculture, the feed input costs account for the largest portion of recurring expenses, depending on the culture system intensity (Riaz, 1997). An intensive aquaculture system requires specialized techniques to sustain high production levels, with dietary modulation emerging as a key strategy. Due to bans on growth-promoting antibiotics over safety and environmental concerns, natural feed additives are now prioritized in aquaculture (Hojati et al., 2014). Herbal immunostimulants, in particular, offer a promising research avenue. This shift has spurred significant interest in plant-derived compounds that can act as immunostimulants and growth promoters. Herbal additives contain bioactive compounds (alkaloids, flavonoids, saponins, phenolics) that strengthen the immune system, increasing resistance against pathogens and reducing disease outbreaks. Unlike synthetic additives, herbal products are biodegradable, non-toxic, and environmentally safe, supporting sustainable aquaculture practices (Ferraz et al., 2022). Among these, *Glycyrrhiza glabra* is a promising candidate. Licorice, with its long history of food and medicinal use, represents one such candidate for enhancing fish health and performance (Tamer et al., 2019; Alagawany et al., 2019).

In this research, WG % and SGR of *Labeo rohita* fingerlings were significantly higher in the 3% licorice root powder-supplemented groups. The licorice root powder significantly increased (p<0.05) the growth of *Labeo rohita* fingerlings fed with a licorice-enriched diet compared to the control groups. FCR was highest in the control group (0% of licorice), and it decreased subsequently with an increase in the concentration of licorice in the diet. FCR was

TABLE 6 AST and ALT activities in liver and muscle tissues of *Labeo rohita* fed with licorice root powder-supplemented diets.

Treatments	AST (Liver)	AST (Muscle)	ALT (Liver)	ALT (Muscle)
C	14.80±0.87	3.57^{a}±0.40	3.50^{a}±0.11	1.21^{a}±0.10
T1	15.09±0.71	3.59^{a}±0.30	3.38^{a}±0.20	1.25^{a}±0.10
T2	16.75±0.43	4.80^{ab}±0.39	4.10^{ab}±0.31	1.41^{a}±0.06
T3	16.45±0.78	5.23^{b}±0.51	4.81^{b}±0.37	1.38^{a}±0.13
T4	17.25±0.27	5.59^{b}±0.29	4.85^{b}±0.36	1.75^{b}±0.07
p value	0.095	0.012	0.012	0.029

Different superscripts in the same column differ significantly ($p<0.05$), data expressed as Mean ± S.E., n=3. Aspartate aminotransaminase (AST) - Nanomoles oxaloacetate released/min/mg protein at 37^{0}C. Alanine aminotransferase (ALT)-Nano moles Na pyruvate released /min/mg protein at 37^{0}C.

lowest in treatment fed with 3% of licorice supplemented diet. Similarly, the PER was also higher in the 3% licorice supplemented group, which is significantly similar to the 4% and 2% licorice fed group. Our findings were similar to Kumar et al. (2021), who also observed that glycyrrhizin fortification in the form of licorice improved the WG, SGR, and FCR of *C. mrigala* fingerlings feeding at 0.12% dietary level. Further, Adineh et al. (2021) also revealed that growth (SGR, PER, and WG) was significantly higher in common carp fed (10–30 g/kg) *G. glabra* root powder for 60 days as compared to the control group. Treatments with dietary supplementation of glycyrrhizin at 200 mg/kg significantly improved the specific growth rate of sea cucumbers. Jiang et al. (2012) observed that 3% glycyrrhizin in the diet had enhanced the deposition of lipid in channel catfish without a detrimental impact on growth. According to Kumar et al. (2007), licorice acts as an effective growth promoter in fish, as it contains compounds that may activate the thyroid gland, thereby stimulating the secretion of growth hormones. The growth-promoting effects of licorice are likely attributable to its diverse bioactive constituents. These include flavonoids, pentacyclic triterpene saponins, isoflavonoids, liquiritin apioside, liquiritin, chalcones, glycyrrhizic acid, and glycyrrhizin, isoliquiritigenin (Nassiri and Hosseinzadeh, 2008).

The growth-enhancing effects of licorice may be due to its capacity to improve nutrient digestibility and stimulate growth. Dietary supplementation with licorice at 3% and 4% inclusion levels resulted in a significant increase in lipase activity, followed by the 2% licorice fed group. Earlier studies have demonstrated that dietary fortification with herbal substances can enhance digestibility by stimulating the digestive enzymes (Rashidian et al., 2020). Overall, herbal substances have been shown to enhance the activity of digestive enzymes, facilitating digestion and absorption, and consequently improving growth performance and feed utilization (Ahmadifar et al., 2019). Similarly, lipase activity was enhanced ($p<0.05$) in *Cyprinus carpio* fed diets supplemented with *Glycyrrhiza glabra* (licorice) (Adineh et al., 2021). The increased growth in the present study could be due to enhanced digestion and nutrient absorption in the gut, facilitated by dietary supplementation with glycyrrhizin.

Specific hepatic metabolic parameters can serve as indicators of an animal's physiological response to dietary formulations. AST and ALT activities are strongly associated with protein metabolism and overall nutrient utilisation in fish (Lin and Luo, 2011). In this study, AST activity in the liver did not change significantly across treatments ($p>0.05$). However, muscle AST was higher ($p<0.05$) in fish fed 3% and 4% licorice. Alanine aminotransferase (ALT) activity was also significantly elevated ($p<0.05$) in both liver and muscle at these higher inclusion levels (3% and 4%). AST and ALT are key enzymes involved in protein metabolism, and their elevated activity in muscle indicates that licorice supplementation may induce a metabolic shift favoring enhanced fish growth. Herbal compounds are reported to increase the AST and ALT activity, which are the main enzymes involved in protein metabolism and liver function. The increase in these enzyme activities may refer to improved metabolic processes and nutrient utilisation in animals. In *Cirrhinus mrigala*, ALT and AST are lower ($p<0.05$) in both liver and muscle in the 0.12% and 0.24% *G. glabra* fed group (Kumar et al., 2021). Gora et al. (2018) observed that ALT activity of fucoidan-fed groups was increased significantly in the muscle tissue. During stress, increased transaminase activity generates keto acids like pyruvate, which serve as substrates for the TCA

TABLE 7 LDH and MDH activity in liver and muscle of *Labeo rohita* fingerlings fed with licorice root powder-supplemented diet.

Treatments	LDH (Liver)	LDH (Muscle)	MDH (Liver)	MDH (Muscle)
C	2.46^{a}±0.53	2.13±0.07	0.23±0.03	0.14^{a}±0.01
T1	2.54^{a}±0.11	2.10±0.07	0.21±0.19	0.15^{a}±0.01
T2	2.57^{a}±0.10	1.99±0.05	0.24±0.04	0.21^{ab}±0.01
T3	2.80^{a}±0.25	1.95±0.04	0.25±0.02	0.20^{ab}±0.02
T4	3.45^{b}±0.18	2.42±0.18	0.28±0.02	0.22^{b}±0.01
P value	0.010	0.053	0.569	0.046

Different superscripts in the same column differ significantly ($p<0.05$). Data expressed as Mean ± S.E., n=3, LDH, Lactate dehydrogenase; MDH, Malate dehydrogenase. LDH Activities are expressed as: Units/min/mg protein at 37°C; MDH: specific activity expressed as Units /min/ mg protein at 37 °C.

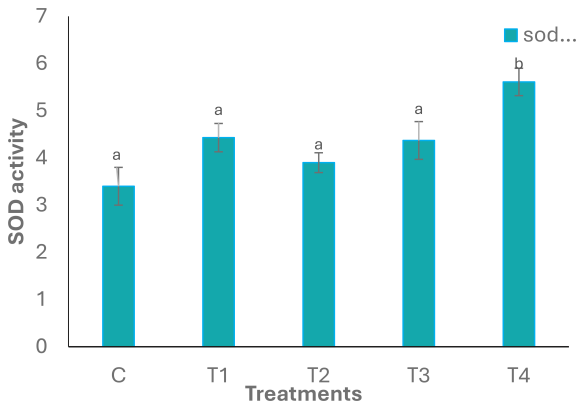


FIGURE 1
Superoxide dismutase (SOD) activities in *Labeo rohita* fingerlings fed with licorice root powder-supplemented diet.

cycle, thereby modulating oxidative metabolism (Chatterjee et al., 2006).

Lactate dehydrogenase serves a vital function in sustaining the glycolysis cycle by regenerating NAD +. Under stress, the anaerobic pathway activates, increasing intracellular conversion of pyruvate to lactate and consequently elevating LDH activity (Kumar et al., 2010). Conversely, MDH catalyzes the reversible conversion of malate to oxaloacetate, releasing NADH. Diets fortified with herbal supplements influence the MDH activity, an enzyme associated with the citric acid cycle. An increase in MDH activity can help maintain cellular energy balance and support overall health and immune response in aquaculture species. Dietary licorice supplementation had no significant effect on LDH activity in muscle. However, LDH activity (hepatic) was higher ($p < 0.05$) in the (T4) 4% licorice-fed group. MDH activity increased in the muscle of licorice fed group, indicating that it increased the metabolic rate of the fish. A reduction in MDH activity in fucoidan-fed groups indicates a decrease in overall metabolic activity (Yengkokpam et al., 2013). This aligns with findings in *Cirrhinus mrigala*, where dietary glycyrrhizin lowered hepatic and muscular activities of both LDH and MDH compared to controls

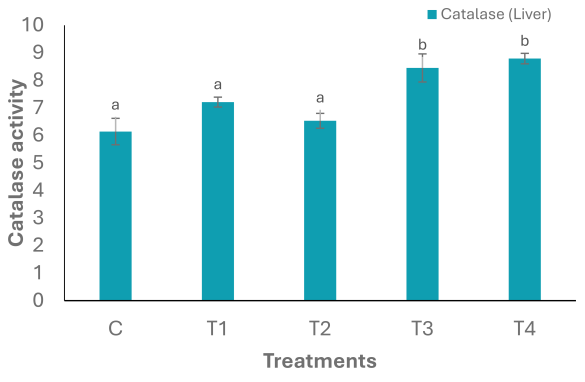


FIGURE 2
Catalase activity in *Labeo rohita* fingerlings fed with licorice root powder-supplemented diet.

(Kumar et al., 2021). However, the response is context-dependent: while *Labeo rohita* showed elevated LDH under thermal stress (Das et al., 2006), supplementation with *Mucuna pruriens* (*L. rohita*) and microbial levan (for *Cyprinus carpio*) reduced LDH and MDH levels in these species (Ojha et al., 2014; Gupta et al., 2008).

The antioxidant enzyme system, which includes superoxide dismutase and catalase, plays a key role in protecting against oxidative damage. SOD is an antioxidant enzyme produced in response to oxidative stress, converting toxic superoxide anions into hydrogen peroxide (Fridovich, 1995). Catalase enzyme activity indicates the ability to break down H_2O_2 into water and oxygen, thereby reducing oxidative stress and protecting cells from damage. Our study showed that the Catalase activity in liver tissue was significantly different ($p<0.05$) in *Labeo rohita* fingerlings fed 3 and 4% licorice than in the control group. Licorice is reported to have hepatoprotective and antioxidant effects in fish cultured in captivity (Yin et al., 2011; Elabd et al., 2016, Elabd et al., 2017). The administration of 200 mg/kg glycyrrhizic acid to silver carp significantly enhances catalase activity levels as compared to the control group (Harikrishnan et al., 2021). Similarly, superoxide dismutase (SOD) activity in the liver of *L. rohita* was significantly elevated in the 4% licorice-fed group. The present study indicates that dietary supplementation with licorice root powder enhances antioxidant capacity in *L. rohita*, highlighting its potential as a natural antioxidant feed additive. This effect may be attributed to bioactive compounds present in *G. glabra*, particularly isoflavonoids such as glabridin (Nassiri and Hosseinzadeh, 2008). Elabd et al. (2016) observed enhanced antioxidant enzyme (SOD, CAT, GPx) activities significantly ($p<0.05$) in yellow perch supplemented with *G. glabra* and *A. membranaceus* after exposure to one week of starvation. An increase in SOD and catalase activities was observed in Cirrhinus mrigala fingerlings fed a diet supplemented with 0.12% glycyrrhizin (Kumar et al., 2021). Dietary supplementation with *Glycyrrhiza glabra* at 0.5%, 1.5%, 2.5%, and 4.0% inclusion levels significantly increased the gene expression of superoxide dismutase (SOD) in *Cyprinus carpio* (Meng et al., 2022). These findings suggest that dietary supplementation with licorice root powder may enhance phagocytic activity in *L. rohita*, leading to increased superoxide anion release and elevated SOD activity within phagocytes.

In aquaculture, herbal additives serve as immunostimulants to reduce stress and boost disease resistance (Harikrishnan et al., 2011). Consistent with this role, the present study found that dietary licorice significantly elevated ($p<0.05$) serum protein, globulin and albumin levels. This enhancement in key plasma proteins likely contributed to a stronger non-specific immune defence, with the increased albumin potentially facilitating the improved transport of critical humoral compounds. The total protein was higher in the 2% licorice-fed group, which is significantly similar to the other licorice-fed group and higher serum albumin was found in the 1% licorice-fed group, which is also significantly similar to other groups. In the current study, the 2% licorice diet elicited the highest serum globulin level. As elevated serum proteins like albumin and globulin are robust indicators of enhanced non-specific immunity in fish, this result points to an immunostimulatory effect. Supporting this, multiple studies report that dietary licorice and

TABLE 8 Serum total protein, albumin (A), globulin (G), A/G Ratio and glucose activity of *Labeo rohita* fed with licorice root powder supplemented diet.

Treatments	Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G Ratio	Glucose (mg/dl)
C	3.45^{a}±.13	2.80^{a}±.14	0.64^{a}±0.02	4.37^{b}±0.33	98.56^{c}±3.1
T1	7.58^{b}±0.26	6.14^{b}±.13	1.44^{b}±0.18	4.30^{b}±0.39	81.63^{b}±6.05
T2	7.76^{b}±0.46	5.76^{b}±.37	2.00^{c}±0.32	2.91^{a}±0.28	74.44^{ab}±1.68
T3	7.14^{b}±0.29	5.52^{b}±.34	1.61^{b}±0.12	3.44^{a}±0.33	68.49^{a}±2.26
T4	7.01^{b}±0.23	5.67^{b}±.16	1.34^{b}±0.12	4.24^{b}±0.10	64.05^{a}±2.39
p value	0.001	0.001	0.001	0.036	0.000

Mean values with different superscripts in the same column differ significantly ($p<0.05$). Data is presented as Mean ±S.E., n=3.

glycyrrhizin elevate total serum protein, globulin and albumin levels in species such as *Cirrhinus mrigala*, Nile tilapia, and common carp (Kumar et al., 2021; Mohammed et al., 2020). The gamma globulin fraction, in particular, serves as the primary source of most immunologically active proteins in the blood (Jha et al., 2007). Similarly, Adineh et al. (2021) suggested that the serum protein level was higher in common carp fed 20 and 30 g/kg licorice compared to the control. Licorice has been widely utilized as an immunostimulant in fish (Mohammed et al., 2020). Incorporating *Camellia sinensis* significantly elevated serum protein, globulin and albumin in *Oreochromis niloticus* (Abdel et al., 2010).

Serum glucose levels decreased significantly ($p<0.05$) with dietary licorice supplementation. The control group maintained the highest glucose concentration, while the T3 group showed the lowest. This hypoglycemic effect aligns with previous findings where licorice supplementation reduced glucose levels in yellow perch under starvation stress (Elabd et al., 2016), blunt snout bream (Abasubong et al., 2021), and juvenile channel catfish (Jiang et al., 2012). Serum glucose levels reflect energy metabolism and stress in fish. Elevated glucose indicates stress, while lower levels suggest better stress tolerance and metabolic regulation. Licorice root powder (20–30 g/kg) has been shown to reduce stress responses and improve stress resistance in juvenile common carp (Adineh et al., 2021).

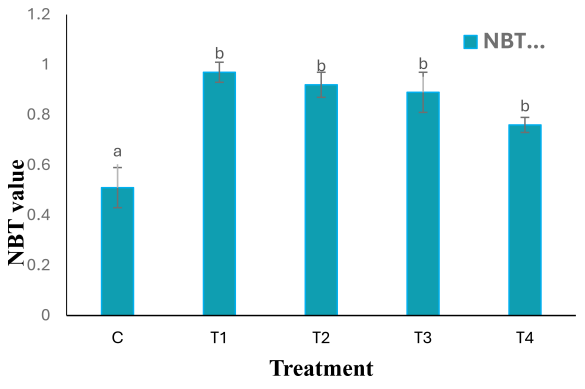


FIGURE 3
Respiratory burst activity in *Labeo rohita* fingerlings fed with Licorice root powder supplemented diets.

Respiratory burst activity refers to the rapid generation of reactive oxygen species (ROS) by immune cells, a key antimicrobial mechanism triggered during pathogen invasion. This process is an essential component of the innate immune system, helping to kill invading microorganisms. Elevated respiratory burst activity indicates an activated immune system, playing a crucial role in defending against infections and enhancing immune responses. In this study, all licorice-supplemented groups exhibited significantly higher ($p<0.05$) respiratory burst activity compared to the unsupplemented control. This finding aligns with previous research where dietary supplements, including alginic acid in *C. mrigala* (Kumar et al., 2018) and licorice in other species (Kumar et al., 2021), similarly enhanced this immune parameter. As the respiratory burst directly correlates with the phagocytes' capacity to eliminate bacterial pathogens (Sharp and Secombes, 1993), the observed increase indicates that licorice supplementation successfully boosted non-specific immunity in *L. rohita*. This immunostimulatory effect contributes to the broader role of functional feed additives, which are employed to enhance feed efficiency, growth performance, and overall animal health.

Conclusion

The findings of the present study demonstrate that dietary licorice functions as an effective growth promoter with antioxidant, immunomodulatory, anti-stress, and metabolic regulatory properties in *Labeo rohita*. Among the tested inclusion levels, supplementation with 3% licorice significantly enhanced growth performance, feed utilization efficiency, digestive enzyme activity, antioxidant status, immune response, and metabolic parameters in *L. rohita* fingerlings. Based on these results, it can be concluded that the incorporation of 3% licorice in the diet is optimal and can be recommended as a functional feed additive to improve growth performance and immunity in *L. rohita*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animal Ethics committee of College of Fisheries, Kishanganj with application no. CoF/BASU/07 dated 11.01.2024. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RK: Formal Analysis, Writing – original draft. SK: Conceptualization, Supervision, Writing – review & editing. SD: Data curation, Writing – review & editing. SS: Data curation, Writing – review & editing. RSK: Formal Analysis, Writing – review & editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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