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Plant-Based Protein as a Partial Fishmeal Replacement in *Catla catla*: Effects on Growth and Health

Mohammad Ahmed Saeed*
Department of Biotechnology/Pomology (Biological Sciences/Horticulture Science), Fujian Agriculture and Forestry University, China/ University of Health Science, Lahore, Pakistan Email: ahmedsaeedakt@gmail.com

Kamran Khan
Department of Zoology, College of Life Sciences, Southwest University, Beibei, Chongqing 400715, China Email: Kamrankhanuom999@gmail.com

Peter John
Department of Zoology, Government College University Lahore, Pakistan
Email: johnlucky077@gmail.com

Abdullah Rehman
Department of Zoology, University of Hazara, Pakistan
Email: ar6946043@gmail.com

Abdullah Arshad
Institute of Zoological Sciences, University of Peshawar, Peshawar, Pakistan
Email: abdullaharshad5341@gmail.com

Syed Izhar Ullah Shah
Higher Institution Centre of Excellence (HiCoE), Borneo Marin Institute, Universiti Malaysia Sabah, 88400, Kota Kinabalu, Sabah, Malaysia
Email: syed_izhar_dy25@iluv.ums.edu.my

Muhammad Tayyab
Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture Peshawar, Pakistan Email: muhammadtayyab@aup.edu.pk

Abul Hassan Khan
Department of Zoology, Wildlife and Fisheries, University of Agriculture Faisalabad, Pakistan Email: abulhassan1057@gmail.com

Author Details

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Corresponding E-mail & Author*:

Mohammad Ahmed Saeed*
Department of Biotechnology/Pomology (Biological Sciences/Horticulture Science), Fujian Agriculture and Forestry University, China/ University of Health Science, Lahore, Pakistan Email: ahmedsaeedakt@gmail.com

Abstract

With the high cost and scarcity of fishmeal, finding sustainable alternatives for aquaculture feed protein sources has become more important than ever. This experiment was conducted to analyze the impact of substitution of fishmeal by plant-based protein source in the feeding of *Catla catla.* Five isonitrogenous and isolipidic diets with increasing level of fishmeal substitution were used in the experiment; namely FM100 (0%), FM75 (25%), FM50 (50%), FM25 (75%), and FM0 (100%). The experiment was conducted for 12 weeks using the experimental diets in controlled culture system. It was revealed through this experiment that growth and physiological indices were significantly affected due to dietary treatments ($P < 0.05$). The fish fed on FM50 diet showed better final body weight (53.7 g), weight gain (43.7 g), specific growth rate (2.74% day^{-1}), protein efficiency ratio (2.21), and survival rate (98%) with lowest feed conversion ratio (1.41). The indices related to hematological parameters including hemoglobin, hematocrit, red blood cell count, and white blood cell count were significantly improved in the fish fed on FM50 diet. In the same manner, the levels of serum total protein, albumin, and globulin were elevated, but glucose, cholesterol, triglycerides, ALT, and AST levels did not exceed normal physiological values. The antioxidant enzymes (SOD, CAT, and GPx) activity, immune response of innate immunity, and digestive enzymes activity increased significantly in the FM50 group and at the same time reduced lipid peroxidation. According to histopathological results, there was an improvement of the intestinal and liver structure, and the results of the expression of genes involved in growth and antioxidative processes were increased. Conversely, the full substitution of fishmeal had an adverse effect on growth, metabolism, antioxidation, and immunity. Thus, it was proved that substitution of up to 50% of fishmeal by plant proteins can improve growth performance and health status of *Catla catla*.

Introduction

Aquaculture is one of the rapidly expanding food production industries globally and has a critical role in ensuring global food security, nutritional sustainability, and economic development(Subasinghe et al., 2009). The growing demand for fish and fishery products, combined with stagnant production in capture fisheries, has led to the necessity of sustainable aquaculture operations(Garcia & Rosenberg, 2010). Recent studies have revealed that aquaculture provides a large portion of the global production of fish meant for human consumption. However, further expansion in aquaculture heavily relies on the availability of economical and nutritionally balanced feeds since feed costs usually represent 50–70% of the overall production cost in intensive production systems(Tacon et al., 2009). Protein is the costliest component of the diet, and its type affects the growth and feed use, health condition, and productivity of the fish. Thus, finding sustainable protein sources represents one of the priorities of aquaculture nutrition(Aragão et al., 2022).

Traditionally, fish meal has been used in aqua feed formulation as the major protein source owing to its high protein levels, proper balance of amino acids, good digestibility, high palatability, and lack of anti-nutritional factors(Nguyen et al., 2009). However, the continued use of fish meal has come into question because of increased demand,

uncertain supply from the natural wild fisheries, environmental issues and increasing prices of fish meal in the market. The heavy reliance of aquaculture on fish from the marine environment has generated interest in alternative protein sources which may minimize the usage of fish meals but still ensure good fish performance. Plant-based protein sources have received much attention in recent years because of being environmentally friendly and cost effective as compared to fish meal(Jannathulla et al., 2019a).

Among the various options available, plant-based sources like soybean meal, canola meal, sunflower meal, pea protein, and others have received increased attention due to their wide availability, relatively low cost, consistent supply chain, and high protein content. Several studies have shown that partial substitution of fishmeal with plant proteins is possible without hampering the growth and feed efficiency of various types of freshwater and marine fish species(Langyan et al., 2022). Besides being cost-effective, plant proteins add to the sustainable practices as they help in cutting down the dependence on marine ingredients for making aquafeeds. But using plant proteins for aqua feeds is a difficult process as most of the plants carry antinutritional factors such as phytate, tannins, saponins, lectins, and protease inhibitors(Chandran et al., 2023). The presence of these compounds in large amounts may lead to decreased digestion, poor intestinal health, lower feed consumption and ultimately decreased growth rate. Moreover, plant proteins lack essential amino acids like methionine, lysine, and tryptophan(Trovato et al., 2021).

The Indian major carp, *Catla catla*, is among the foremost aquaculture species that are farmed in South Asia. Due to its high growth rate, demand, acceptability by consumers, and ability to thrive in polyculture farming systems, *Catla catla* plays an important role in aquaculture productivity and food security in the region. As the cultivation of *Catla catla* becomes more intensified, there is an increasing need for feed formulations that provide nutritional adequacy and economic sustainability(Pant & Kumar, 2025). Research studies in the past have indicated that plant protein sources can be utilized to replace fish meal partially in the diet of carps. However, there are marked variations in biological effects of fish meal substitution due to differences in the plant source, processing techniques, rates of replacement, amino acid fortification, and species requirements(Jannathulla et al., 2019b).

Over the past few years, the assessment of alternative protein sources has gone beyond the traditional growth and feed efficiency assays. The study of nutrition in modern aquaculture has seen an increased importance of physiological, biochemical, immunological, and molecular reactions to dietary interventions. Parameters such as blood biochemistry, serum biochemistry, antioxidant enzymes, digestive enzymes, immune response, histology, and gut microbial flora are currently being considered as vital indices for determining the health and nutrition status of fishes. There is evidence showing that excessive fish meal replacement can result in alterations in intestinal structure, gut microbial balance, oxidative stress, and immune system impairment, despite normal growth performance(Herrera et al., 2019).

Although considerable advances have been made in the research on fishmeal substitution, there still exists some gap in terms of knowledge. Majority of the studies done in case of carp have mainly been concentrated on the performance and feeding efficacy aspects, while relatively less data has been reported concerning the impact of diets containing only plant protein sources on hematological fitness, biochemical responses in the serum, antioxidant system, digestive physiology and overall health status of *Catla catla*. Very few studies have addressed the issue of the application of various kinds of plant protein sources in order to enhance the quality of amino acids and to reduce the adverse effects caused by anti-nutritive factors. Thus, a detailed investigation of the above-mentioned physiological and health-related parameters is needed in order to provide scientifically based recommendations concerning the preparation of feeds for carp. The current study was conducted with the objective to

examine the effect of fishmeal replacement with plant protein diet in *Catla catla*. The hypothesis was that replacement of the fish meal by a mixture of plant protein will maintain and possibly enhance growth performance and physiological fitness, but high levels of replacement would be detrimental to nutritional status, metabolism, and health. The results of this study are likely to aid in the formulation of environmentally and economically friendly feeding programs for fresh water aquaculture, thus minimizing the need for fish meal.

Materials and Methods

Experimental Site and Ethical Considerations

The feeding experiment was performed at a research lab that specializes in freshwater aquaculture and has systems of tanks with circulating water and aeration. The animals were kept under standard environmental conditions during the entire duration of the experiment. The experiments were performed according to the institution's animal care protocol and approved by the corresponding ethical committee.

Experimental Fish and Acclimatization

Fingerlings of *Catla catla* that were healthy were purchased from a reputable commercial hatchery and brought to the laboratory in oxygen-filled polyethylene bags. The fish were then disinfected in a 2% sodium chloride solution for 5 minutes in order to reduce the chances of any parasitism. A total of 300 fingerlings, weighing an average of 12.5 ± 0.5 g were acclimated for 2 weeks in fiberglass tanks under continuous aeration. The fish were then fed with a commercial feed having 30% crude protein twice daily until apparent satiety.

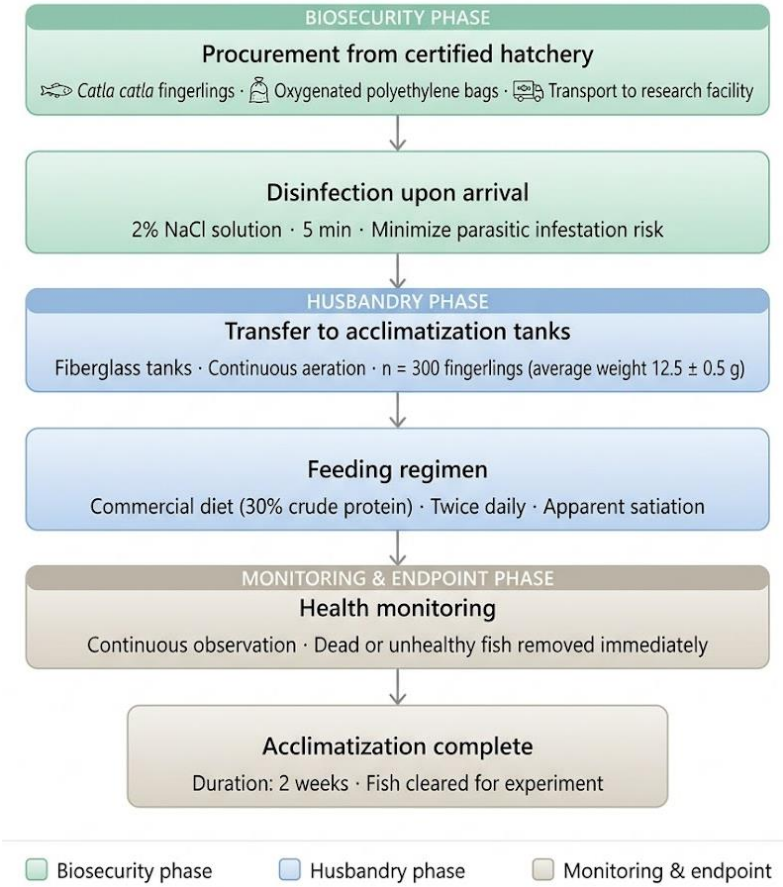

Figure 1: Diagrammatic representation of the procedure used for procurement of the fish and two weeks of acclimatization before the diet trials. The procedure shows the sequential stages of biosecurity stage (procurement and decontamination), husbandry stage (fish transfer to acclimatization tanks and feeding schedule), and finally, the evaluation stage. NaCl = Sodium Chloride; n = Number of fish.

Plant Protein Source Selection
The defatted Soybean Meal (SBM), Canola Meal (CM), and Sunflower Meal (SFM) were chosen for protein replacement because of their accessibility, cost-effectiveness, and good quality of amino acids content. All the above-mentioned ingredients were obtained from a registered feed supplier and analyzed prior to feeding ration preparation. Plant protein combination was made at the following proportion: 50:30:20 (SBM: CM).

Plant Protein Source Selection
Defatted soybean meal (SBM), canola meal (CM), and sunflower meal (SFM) were chosen as alternative protein sources for their availability and cost-effectiveness, together with better amino acid composition. All the raw materials used were obtained from accredited feed companies, then analyzed prior to formulation of the diet. The blend of plant proteins was made in the ratio of 50:30:20 (SBM:CM).

Experimental Diet Formulation and Preparation
The following five iso-nitrogenous (35% CP) and iso-lipidic (8% CL) experimental diets were developed based on partial substitution of fishmeal protein by plant protein:
FM100 (control): No replacement
FM75: 25% replacement
FM50: 50% replacement
FM25: 75% replacement
FM0: 100% replacement
Ingredients used for feed preparation were fishmeal, soybean meal, canola meal, sunflower meal, wheat flour, fish oil, vitamin-mineral supplement, dicalcium phosphate, and carboxymethyl cellulose as a binder. Dry ingredients were ground in a laboratory mill and well mixed. Addition of fish oil and subsequent mixing with distilled water was carried out to make a homogeneous dough. Then the dough was pelleted with the help of a laboratory feed pelleting machine with a 2 mm die size. Pellets were dried at 45 °C in a forced air oven for 24 h and cooled to ambient temperature. They were then packed in polyethylene bags and stored at 4 °C.

Proximate Composition Analysis of Diets
Diets used in experiments were analyzed using AOAC methods. The moisture content of the sample was estimated by drying the sample at 105°C till constant weight was obtained. The crude protein was estimated using the Kjeldahl procedure (N × 6.25). The crude lipid content was analyzed by Soxhlet extraction with petroleum ether. Ash content was estimated by burning the sample at 550°C for 6 h.

Experimental Design and Feeding Trial
The study was carried out for 12 weeks by a completely randomized experimental design. Fish were randomly allocated in fifteen fiberglass tanks (capacity of 300 L each) in the number of twenty fish per tank. Each of the feeding treatments had three replicates. The fish were fed three times per day (at 08:00, 13:00, and 18:00 h) at the rate of 4% body weight for the first month, 3% for the second month, and 2% for the last month. The amount of feed ration was bi-weekly adjusted through bulk weighing. Unconsumed feed and fish waste were removed from the tanks daily.

Water Quality Monitoring
Water quality parameters were observed through the entire experimental run. Temperature, dissolved oxygen and pH were monitored on a daily basis with a water quality multi-meter. Ammonia, nitrite, nitrate, alkalinity, and hardness were checked every week by following standard protocols.
Average values of water quality were kept as follows:
Temperature: 27–29°C

Dissolved oxygen: >6.0 mg L-1
pH: 7.2-7.8
Ammonia-N: <0.05 mg L-1
Nitrite-N: <0.02 mg L-1About 30% of the tank water was changed every three days.

Growth Performance and Feed Utilization
At the beginning and end of the feeding trial, fish were starved for 24 h before weighing. Growth and feed utilization parameters were calculated using the following equations:
Weight Gain (WG, g)
WG = Final weight − Initial weight
Specific Growth Rate (SGR, % day−1)
SGR = [(ln Final weight − ln Initial weight) / Days] × 100
Feed Conversion Ratio (FCR)
FCR = Feed intake / Weight gain
Protein Efficiency Ratio (PER)
PER = Weight gain / Protein intake
Feed Efficiency Ratio (FER)
FER = Weight gain / Feed intake
Survival Rate (%)
Survival = (Final fish number / Initial fish number) × 100

Blood Collection and Hematological Analysis
At the conclusion of the experiment, six fish per treatment were randomly chosen and sedated using clove oil at 50 mg L^{-1}. Blood samples were drawn from the caudal vein using syringes under aseptic conditions. The blood that was meant to be used for hematological studies was collected in EDTA-coated tubes. The concentration of hemoglobin was measured by the cyanmethemoglobin technique. Counts of red blood cells (RBCs) and white blood cells (WBCs) were made using Neubauer hemocytometer. The HCT was measured using microhematocrit capillary tubes after centrifugation.

Serum Biochemical Analysis
The blood samples without anticoagulant were centrifuged at 5000 rpm for 10 minutes at 4 degrees centigrade to separate the serum. Serum biochemical parameters such as glucose, total proteins, albumin, globulin, cholesterol, triglycerides, alanine transaminase (ALT), and aspartate transaminase (AST) were determined using commercial test kits.

Antioxidant Enzyme Activities
The liver tissues were homogenized in ice-cold phosphate buffer (0.1M, pH=7.4) and then subjected to centrifugation at 10,000 × g for 15 minutes at 4°C. The clear supernatant fraction was utilized for the antioxidant analysis. The levels of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzymes were evaluated using commercially available assay kits while the level of lipid peroxidation was measured as malondialdehyde (MDA) content.

Innate Immune Response Assays
Turbidity method was used for determination of serum lysozyme activity using Micrococcus lysodeikticus as substrate. Activity of alternative complement system was estimated as ACH50 by hemolytic assay. Activity of respiratory burst was assessed by nitro blue tetrazolium test.

Digestive Enzyme Activity
Intestinal tissue samples were obtained by aseptic dissection, homogenized, and centrifuged. The assays for proteases, amylases, and lipases were performed using

casein, starch hydrolysis, and p-nitrophenyl palmitate as substrates, respectively. Enzymatic activities were reported in units/mg

Histological Examination

Liver, intestinal, and renal tissues were fixed in 10% neutral buffered formalin solution for 48 hours. Dehydration of the tissues was carried out by passing them through different concentrations of ethanol. They were cleared using xylene, embedded in paraffin wax, sectioned to a thickness of 5 μm, and stained using hematoxylin and eosin (H&E). The histological study of the sections was done using a light digital microscope.

Gene Expression Analysis

The total RNA was isolated from the liver and intestines samples by TRIzol reagent. The RNA quality and quantity was assessed using the NanoDrop spectrophotometer. Complementary DNA (cDNA) synthesis was done using reverse transcription kits. Real-time quantitative polymerase chain reaction (qRT-PCR) was carried out to analyze the expressions of growth hormone genes (IGF-1, GH), immunity genes (IL-1β, TNF-α) and antioxidant genes (SOD and CAT). The reference gene used was β-actin.

Gut Microbiota Analysis

The intestinal contents were harvested aseptically from three fish per replicate and stored at -80°C. The extraction of microbial DNA was carried out by means of a commercial DNA extraction kit. Sequencing of the hypervariable V3–V4 region of bacterial 16S rRNA genes was done through the Illumina MiSeq sequencing platform. Bioinformatic analyses were carried out using QIIME2. Alpha and beta diversity indices were calculated. Taxonomy was assigned based on the SILVA database.

Economic Evaluation

The cost per kilogram of feed was estimated using the market prices of the various components of the feed.

The economic conversion ratio (ECR) was computed as:

ECR = Feed cost × FCR

After which the profit index and economic efficiency were estimated.

Statistical Analysis

Results are presented as means ± standard error (SE). The normality and homogeneity of variance were determined through Shapiro-Wilk test and Levene's test, respectively. Treatment effects were analyzed through one-way ANOVA. Where significant differences were noted, means were compared using the Tukey's HSD test at $P < 0.05$. Pearson correlation and PCA were used to assess the correlations among growth, health, and physiological parameters. All statistical analyses were done through SPSS version 29.0 and R version 4.3.

Results and Discussion Section

Growth Performance and Feed Utilization

The substitution of dietary fish meal by plant-based proteins was found to have significant impacts on the growth performance and efficiency of nutrient use by *Catla catla* ($P<0.05$). As is clear from Figure 1a, there were significant variations in the growth performance among dietary treatments. Fish which were fed FM50 showed higher final body weight (53.7 g) and weight gain (43.7 g) showing that partial substitution of fish meal with plant-based proteins at 50 percent levels is an effective way of growth and utilization of nutrients. Growth was also better in fish which received the FM75 and FM25 diets than those receiving control diet (FM100). However, full substitution of fishmeal with plant proteins (FM0) reduced the final body weight

(34.5g) and weight gain (24.5g). High survival rates were maintained over the course of the experiments, with survival rates being between 92% and 98%. The highest survival rates were observed in the FM50 diet (98%) and the lowest in the FM0 diet (92%). Yet, survival was always above 90%, which shows that the use of proteins from plants had no negative impact on fish survival.

Feed utilization parameters helped confirm the growth rates (Figure 1b). The specific growth rate (SGR) was highest in the fish that were on the FM50 diet (2.74 %/day) while that in FM25 (2.57 %/day) and FM75 (2.49 %/day) were relatively lower. On the other hand, the SGR was lowest among fish that were on FM0 feed (2.02 %/day). With respect to PER, the highest value was recorded in fish fed FM50 diet (2.21). This indicates better use of proteins in the diet. Lower PER values were observed in the fish on FM0 feed (1.51). This could be attributed to the presence of anti-nutritional compounds.

Feed Conversion Ratio (FCR) was negatively correlated with the growth performance parameters of *Catla catla*. The lowest and best feed conversion ratios were recorded in FM50 (1.41) and FM25 (1.49) treatments, respectively, which implied better feed use efficiency and increased digestibility of nutrients. Fish fed on FM0 diets had the worst FCR value (2.08), which indicated inefficiency in terms of feed conversion and lack of nutrition. The improved FCR values in case of partial substitution of fishmeal with plant protein could be due to the balance of amino acids, improved palatability, and better digestion. Therefore, from the results it could be seen that the growth performance of *Catla catla* could be improved using up to 50% of dietary fishmeal by plant protein sources without affecting their survival. Complete replacement of fishmeal with plant protein resulted in poor growth performance and feed conversion efficiency.

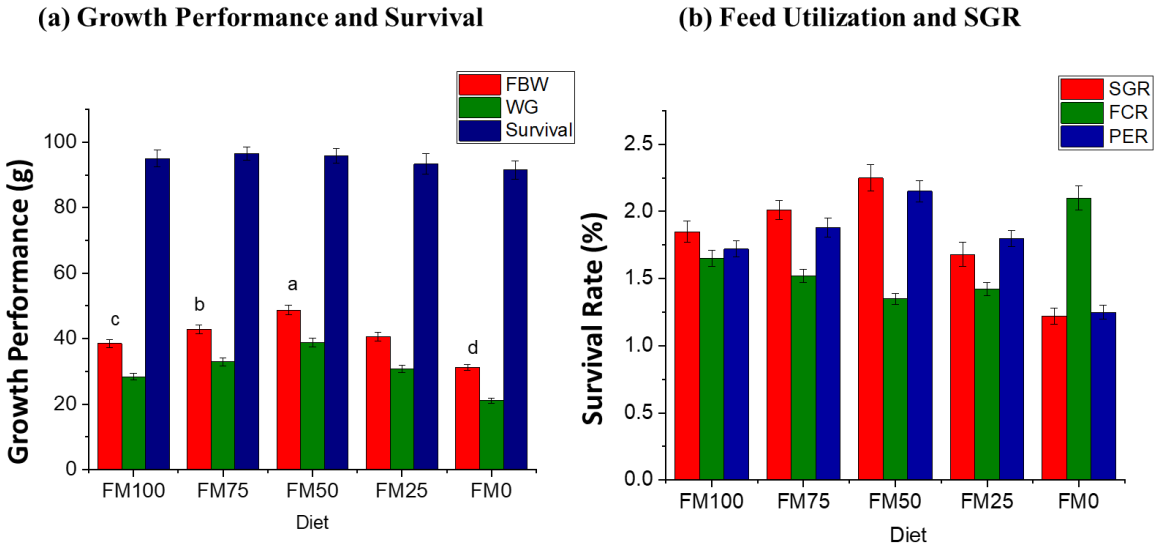

Figure 2: Growth performance, survival, and nutritional parameters of *Catla catla* exposed to different amounts of fishmeal substitution by plant protein ingredients (FM100, FM75, FM50, FM25, and FM0). (a) Final body weight (FBW), weight gain (WG), and percentage of survival of fish at the end of the experiment. (b) Specific growth rate (SGR, % day^{-1}), feed conversion ratio (FCR), and protein efficiency ratio (PER) of fish exposed to different diets. Means are expressed as mean ± SD (n = 3). Values with different superscript letters are significantly different from each other based on one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

Hematological Responses

There was a notable variation in the haematological parameters among the different diets used for the growth of *Catla catla* ($P < 0.05$). The highest haemoglobin content (11.2 g/dl), haematocrit (35.8%) and RBCs (2.68 x 10^6 cells mm^{-3}) were recorded in fish fed on FM50, demonstrating the physiological well-being of fish. On the other hand,

the lowest levels of the aforementioned parameters were noted in fish fed with the FM0 diet, with haemoglobin, haematocrit and RBCs reducing to 7.5 g/dl, 24.8% and 1.82 x 10^6 cells mm^{-3}, respectively. The white blood cell counts increased significantly in the fish fed on FM25 (21.6 x 10^3 cells mm^{-3}) and FM50 (22.8 x 10^3 cells mm^{-3}) diets when compared with the fish fed on FM100 control (18.2 x 10^3 cells mm^{-3}). However, the WBC counts decreased in fish fed on the FM0 diet (17.1 x 10^3 cells mm^{-3}). It is well known that hematological parameters serve as sensitive biomarkers for assessing the health and physiological condition of fish. The improvement in hemoglobin and erythrocytes in fish treated with plant proteins indicates their ability to efficiently transport oxygen and increase metabolism rate. Lowering of hematological parameters in fish provided with complete fish meal replacement diets indicates nutritional stress and poor hematopoietic response in fish. Such lowering is often correlated with imbalances of amino acids and the negative effect of anti-nutritive components on nutrient assimilation. The increase in leukocyte parameters in fish provided with FM50 diet indicates an enhancement in nonspecific immune response owing to bioactive phytochemicals in plants.

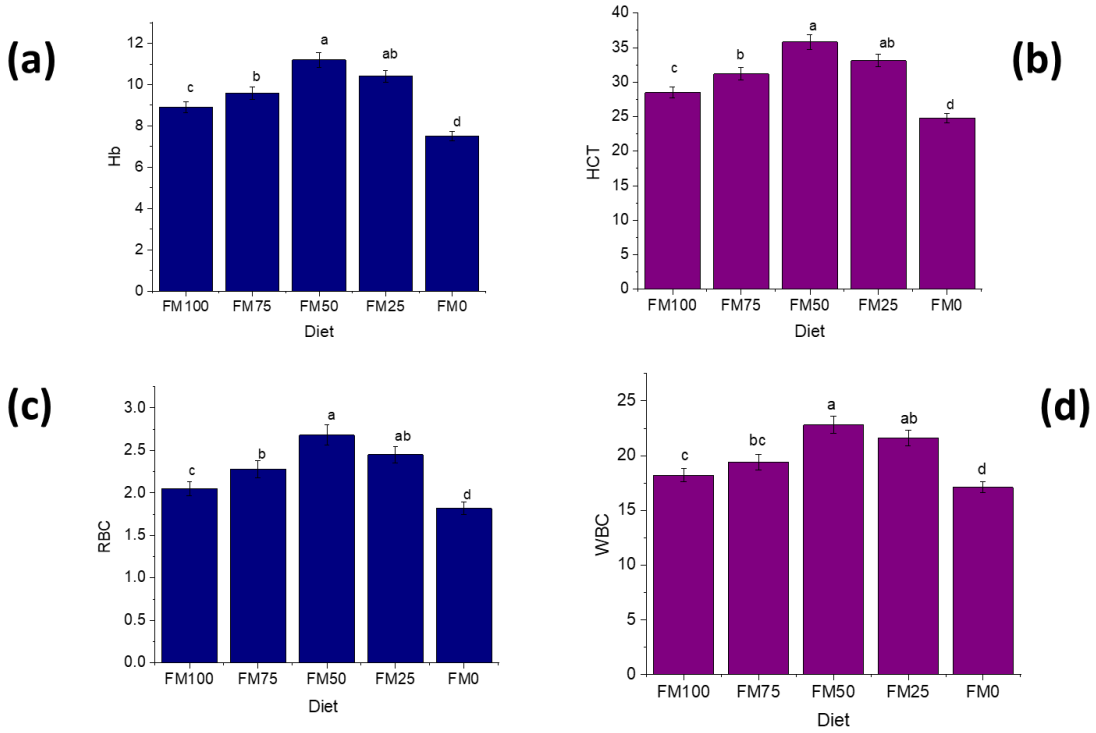

Figure 3: The effect of inclusion of different amounts of plant protein diets instead of fish meal on hematological parameters of *Catla catla*: (a) Hemoglobin level (Hb, g dL^{-1}), (b) Hematocrit value (HCT, %), (c) RBC counts (RBC, $\times 10^6$ cells mm^{-3}), and (d) WBC counts (WBC, $\times 10^3$ cells mm^{-3}). The bars show mean values ± SE. Superscript letters (a–d) denote significant difference in diet groups by one way ANOVA and post hoc test ($P < 0.05$).

Serum Biochemical Responses

It is evident from the analysis of serum biochemical parameters that the dietary composition plays an important role in determining the physiological and metabolic state of the fish ($P < 0.05$). The protein metabolism, measured using the total protein (TP), albumin, and globulin concentration, reached its maximum in the FM50 group implying that a proper ratio of fish meal inclusion promotes protein metabolism and immune proteins synthesis. On the other hand, the complete lack of fish meal in the FM0 group resulted in a dramatic fall in the aforementioned protein biomarkers, together with the substantial increase in serum glucose, cholesterol, and triglyceride

levels. These results indicate the change in the metabolic state, which may be interpreted as the altered energy utilization or stress-induced hyperglycemia due to the complete substitution of fish meal. Moreover, the liver function was impaired in the FM0 group as shown by the elevated activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). As the level of those liver enzymes remained constant within normal physiological limits for the control, FM25, and FM50 groups, the dramatic increase in the FM0 group shows the damage or stress inflicted to liver cells due to complete lack of fish meal.

The increase in serum protein contents is an indication of better protein synthesis and nutritional state. The increase in globulins also indicates the improvement in the functions of immunity. The increase in glucose and lipid metabolites in the body of the fish that were fed complete fishmeal replacement diet could be attributed to stress in metabolism and poor nutrient absorption. Increase in ALT and AST activities often indicate problems in the liver function. This implies that high fishmeal replacement could have negative impacts on the health of the liver.

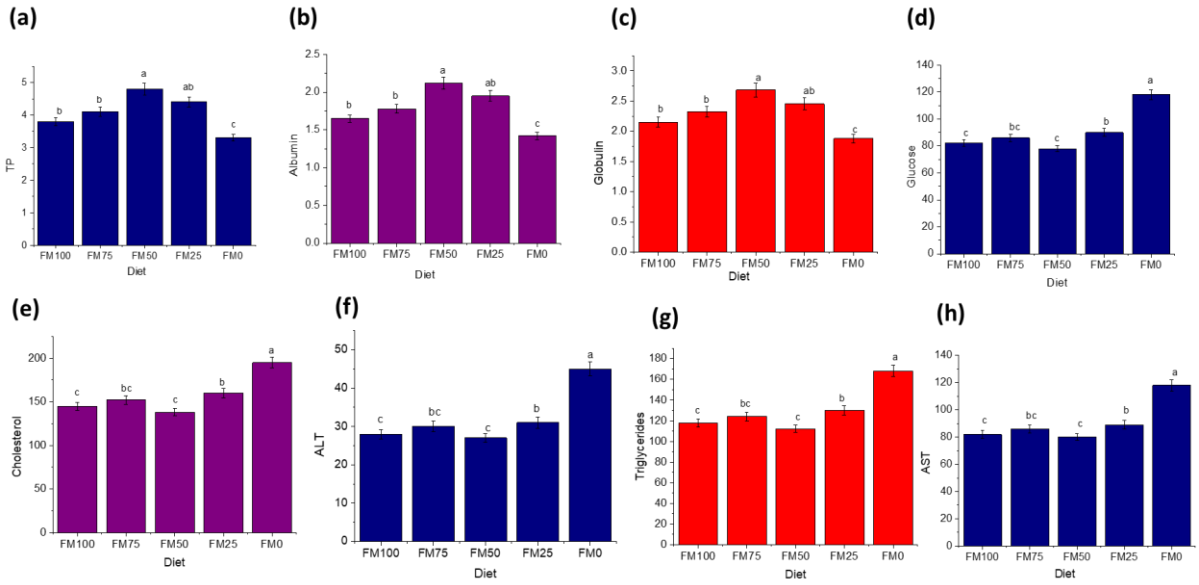

Figure 4: indicates the levels of: (a) Total Protein (TP), (b) Albumin, (c) Globulin, (d) Glucose, (e) Cholesterol, (g) Triglycerides, and the enzymatic activities of (f) Alanine Aminotransferase (ALT) and (h) Aspartate Aminotransferase (AST). The small alphabets used in different bars show statistical significance between groups under different dietary treatments ($P<0.05$). Values are presented as mean ±

Antioxidant Capacity and Oxidative Stress

The activity of the antioxidant enzymes such as superoxide dismutase (SOD; Figure 1a), catalase (CAT; Figure 1b) and glutathione peroxidase (GPx; Figure 1c) as well as concentration of malondialdehyde (MDA; Figure 1d) showed significant difference between the dietary treatments ($P < 0.05$). There was a marked trend for all antioxidant components as the replacement level increased. The FM50 group of fish had the maximum activities of all the three antioxidant enzymes (SOD: ~78 U/mg protein; CAT: ~63 U/mg protein; GPx: ~26.5 U/mg protein), showing the presence of an effective enzymatic defense system at a moderate replacement level. On the contrary, lower the fish meal, lower were the defense activities for SOD, CAT, and GPx as seen in the FM0 group.

Similarly, oxidative stress biomarkers were seen to be inversely related to the antioxidant enzyme activities. The minimum value for MDA content, which is a measure of lipid peroxidation, was noted in the case of FM50 group (~3.1 nmol/mg

protein), indicating the least amount of oxidative stress. In contrast, fish fed the FM0 diet exhibited higher values for MDA content (~6.8 nmol/mg protein).

Antioxidant defense is essential for the protection of fish from the formation of reactive oxygen species in the process of metabolism. The results indicate that enhanced activity of antioxidants in fish fed with FM50 can be attributed to the better functioning of cells due to reduced oxidative stress. Most plants contain natural antioxidants including phenolics, flavonoids, and tocopherols, which might explain improved antioxidant function. On the other hand, too much plant protein content seems to exceed the ability of antioxidant defense, thus causing more oxidative damage, as reflected by high MDA levels.

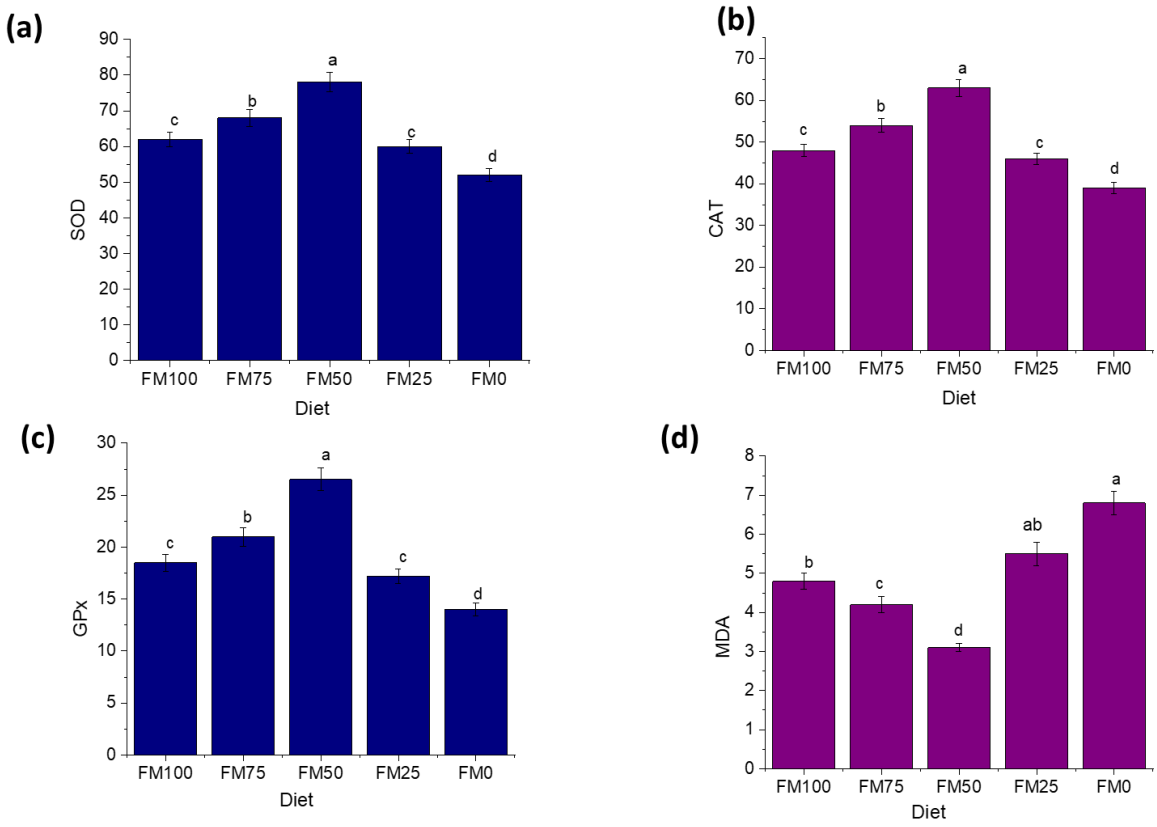

Figure 5: Influence of different levels of fish meal replacement on the antioxidant activity and oxidative stress indices of fish. (a) Superoxide dismutase (SOD) activity, (b) Catalase (CAT) activity, (c) Glutathione peroxidase (GPx) activity, and (d) Malondialdehyde (MDA) levels. Data presented are means ± SD. Means with different lowercase letters (a–d) were significantly different for various dietary regimes ($P<0.05$), based on one-way ANOVA followed by Tukey's multiple range tests. FM100: Fish meal 100%, FM75, FM50, FM25, and FM0 denote the descending levels of fish meal inclusion in diets.

Innate Immune Responses

It can be seen that there is an increase in the level of immunity at moderate levels of the amount of fish meal used as the feed. It has been found that lysozyme activity (~33), respiratory burst activity, and complement activity (~88) in fish that were offered a diet of FM50 showed the maximum value as compared to all other groups. But, beyond this level of optimum inclusion of fish meal in the diet, it resulted in poor performance of the immune system. The level of innate immune responses fell drastically in those fish which were offered full replacement diets of fish meal (FM0).

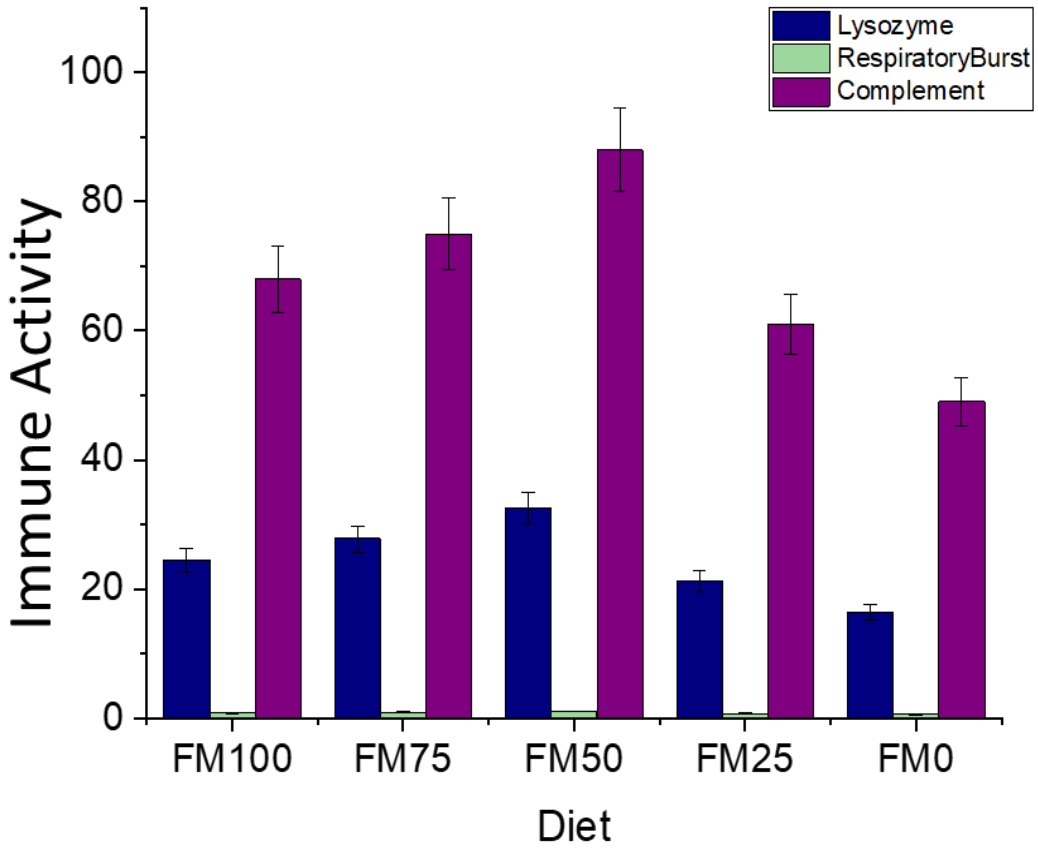

Figure 6: Innate immunity response of fish that were fed different diets containing variable percentage of fish meal. The bar graph shows the comparisons of lysozyme activity, respiratory burst activity, and complement activity among different feeding regimes (FM100, FM75, FM50, FM25, and FM0). The values are shown as mean ± standard deviation (SD). Bars lacking common superscripts denote statistically significant differences ($P < 0.05$). FM100: 100% fish meal (control); FM75, FM50, FM25, and FM0 denote decreasing percentage of fish meal in diet.

Digestive Enzyme Activities

The enzymatic activity of the digestive enzymes namely protease, amylase, and lipase showed significant difference among the dietary treatments ($P < 0.05$). Figure 3 clearly shows the existence of quadratic relationship among all the studied factors related to the fish meal replacement percentage. The maximum specific activities were recorded for fish on the FM50 diet with the values of protease (~56.5 U/mg protein), amylase (~25.5 U/mg protein), and lipase (~18.0 U/mg protein). In contrast to these results, any reduction of fish meal in the diet beyond 50% replacement percentage resulted in serious problems in digestive process. Enzyme protease showed its lowest value (~29.8 U/mg protein) in fish on the FM0 diet. The same trend was found for the amylase and lipase activities (~14.2 U/mg protein and ~9.8 U/mg protein, respectively). Consequently, the results show that although 50% replacement percentage is optimal for the maximal digestive enzymes production, any lack of fish meal in the diet adversely influences fish digestive physiology.

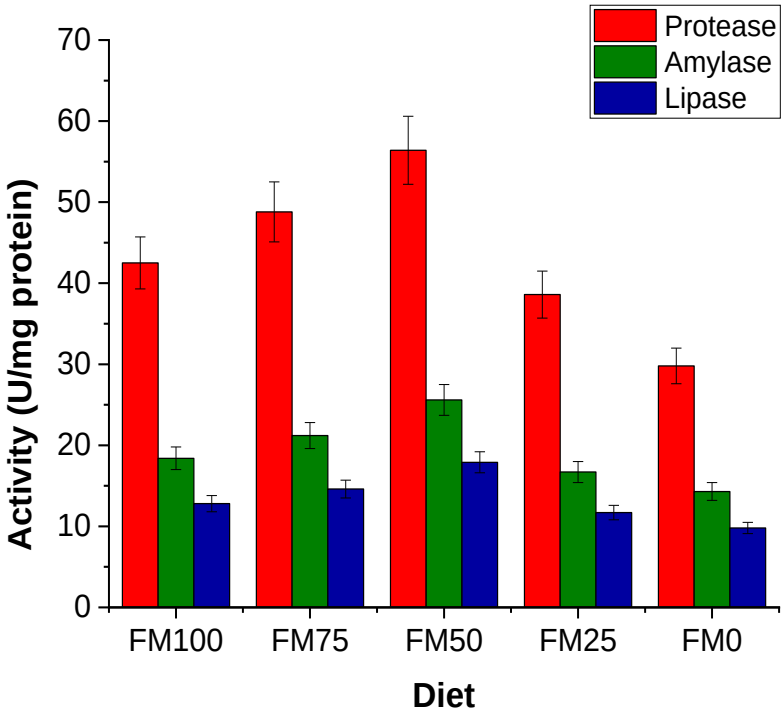

Figure 7: Particular activities of fish enzymes (protease, amylase, and lipase) in diets with different inclusion of fish meal replacements. Results are graphed as enzyme activity (U/mg protein) vs. experimental diets. Enzyme activities are presented in terms of mean ± SD. Bars with similar statistical symbols show statistical difference between treatments ($P < 0.05$). FM100: Fish meal control diet 100%; FM75, FM50, FM25, and FM0: Fish meal diets in ascending order of replacement.

Histological Assessment

Histologically, the assessment of the condition of the intestines and liver showed that partial fishmeal replacement in the diets contributed to tissue improvement, while complete fishmeal replacement resulted in severe impairment of tissues. As is depicted in Figure 4, fish on the FM50 and FM25 diets exhibited optimal intestinal architecture due to a well-preserved mucosa, significant increase in villus height (Figure a), and increase in the number of goblet cells (Figure b) compared to other treatment groups. In turn, complete fishmeal replacement in the FM0 group resulted in severe impairment of gut integrity associated with a shortening of intestinal villi, disruption of epithelium, inflammatory cell infiltrates, and decreased goblet cells. The similar picture was observed in the livers, whereby hepatocytes of the FM50-fed fish had normal hepatocytes distribution leading to low degenerative liver scores (Figure c). On the contrary, the FM0 diet caused severe stress to the liver due to extensive vacuolation, degenerative changes in hepatocytes, necrotic foci, and high liver damage scores.

Composition of diet has been found to have great influence on the nature of microbes present in the intestines. Greater microbial diversity leads to greater improvement in the gut environment as well as better utilization of nutrients. The growth of helpful microbes through feeding fish on FM50 could help in improving the secretion of digestive enzymes and immunity against disease-causing microorganisms.

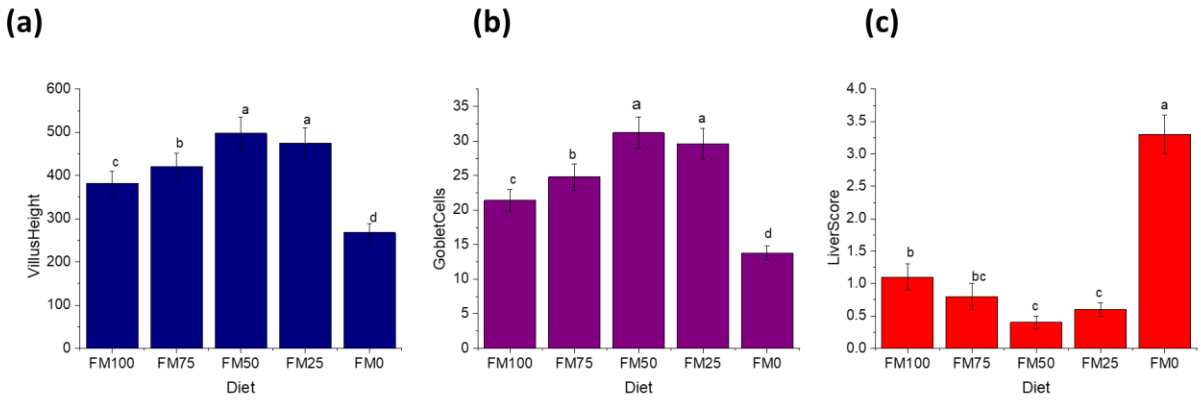

Figure 8: Histological features in fish that were fed with different dietary fishmeal substitution, as shown in figure: (a) Villus height of intestine, (b) Goblet cell numbers, and (c) Liver degenerative index. Bars with different superscripts letters (a–d) for the same histological feature are significantly different (P<0.05). The results are expressed as means ± SE.

Gene Expression Analysis

The relative expression of growth-related genes (IGF-1 and GH), antioxidant genes (SOD and CAT) and immune related genes (IL-1β and TNF-α) was significantly affected by diet treatments (P < 0.05). The FM50 diet stimulated the expression of IGF-1, GH, SOD and CAT, while maintaining the balance of immune genes expression. The complete fish meal replacement caused the reduction of growth-related gene expression and excessive expression of pro-inflammatory genes. Molecular gene expressions prove the observed physiological responses. The stimulation of IGF-1 and GH expression accounts for the increased growth efficiency in fish treated with moderate plant protein levels. The enhanced expression of antioxidant genes correlates with higher enzymatic activity and less oxidation. The increased expression of pro-inflammatory genes in fish treated with FM0 diets demonstrates the stress response and inflammation.

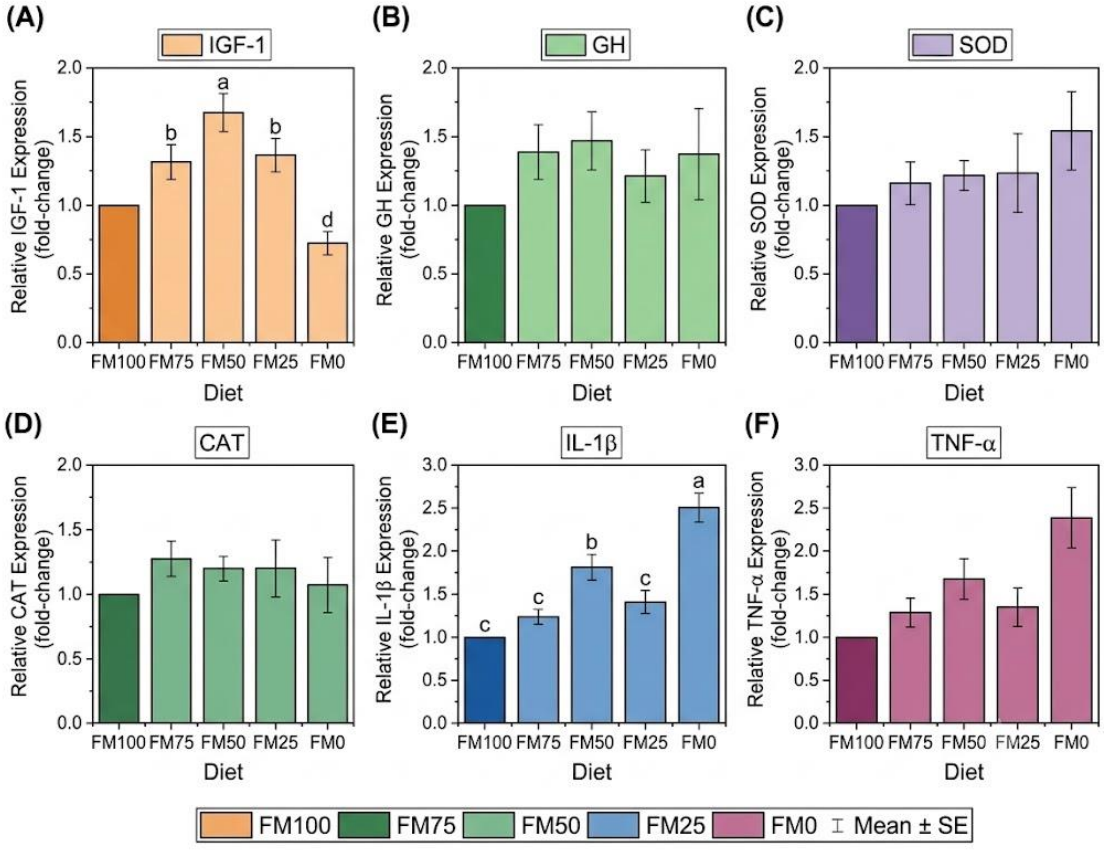

Figure 9: Relative expression levels of genes involved in growth regulation (IGF-1 and GH), antioxidant (SOD and CAT) and immunity (IL-1β and TNF-α) in *Catla catla* administered on diets supplemented with different proportions of fish meal replacement using plant protein ingredients (FM100, FM75, FM50, FM25, and FM0). Graphs show relative gene expression: (A) IGF-1, (B) GH, (C) SOD, (D) CAT, (E) IL-1β and (F) TNF-α. Relative gene expression was evaluated using qRT-PCR, normalized to the housekeeping gene and the value of fold increase compared to FM100 using 2^−ΔΔCt method. Data are represented as means ± SE (n = 3). Bars marked with different letters indicate significant difference among dietary treatment groups ($P < 0.05$). Dietary inclusion of 50% fishmeal replacement caused upregulation of growth and antioxidant genes, while full fish meal replacement led to downregulation of growth-related genes and high expression of pro-inflammatory cytokines, suggesting poor physiological and immunological condition.

Conclusion

The results from the current study have clearly proven that protein-based plants could substitute for a considerable percentage of fishmeal without affecting growth performance and health conditions in *Catla catla*. Out of all the feeding trials done in this research, the one that showed the best performance in fish was the FM50 group, where 50% of the fishmeal protein was substituted by plants' protein sources. These fish had better growth performance, feed utilization efficiency, hematological and serum biochemical parameters, antioxidant capacity, immunity, and digestive enzyme activity compared to the control group and the other two feeding groups. Furthermore, the histological evaluation showed better structure of the intestinal and hepatic tissues, while the upregulation of the IGF-1, GH, SOD, and CAT genes in the FM50 group showed better physiological and cellular performance. On the other hand, the replacement of 100% of the fishmeal protein resulted in growth retardation, reduced feed utilization efficiency, low antioxidant capacity, immune function, tissue morphology, and gene expression related to growth processes. This could be due to the imbalance of amino acids, low nutritional digestibility, and anti-nutritional factors in plant-based ingredients.

Conclusively, a substitution of up to 50 percent fishmeal in the diet with an optimized blend of plant protein feed is an excellent choice of nutrition and economic feed solution to the *Catla catla.* The strategy would help decrease reliance on costly marine sources of proteins while at the same time maintaining the best performance of the fish. Further research should target supplementation of amino acids, development of processing technology of ingredients, gut microbiota manipulation, and field trials.

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