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Research Article

Efficacy of an Insect-Based Diet with Addition of Probiotics on Growth, Proximate Composition, Enzymatic Efficiency, and Immune Response of Nile Tilapia

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Insects are potential alternative protein sources to replace fish meal (FM) in aqua feed. The role of insect species as replacements in the aqua industry has been a hot debate in the current era. The present study evaluated the influence of FM replacement with insect-based meals (black soldier fly (BSF) and maggot fly) in the feed of Nile tilapia. Eight diets with graded replacement levels of FM were formulated along with basal diet as T_0 (control group having basal diet), T_1 (25% maggot meal (MM) with 2% probiotics), T_2 (50% MM + 2% probiotics), T_3 (75% MM + 2% probiotics), T_4 (100% MM + 2% probiotics), T_5 (25% BSF meal (BSFM) + 2% probiotics), T_6 (50% BSFM + 2% probiotics), T_7 (75% BSFM + 2% probiotics), and T_8 (100% BSFM + 2% probiotics). Fish having an initial body weight of 7 g were fed on formulated feed for 16 weeks. Growth parameters, including weight, length, feed conversion ratio, specific growth rate, and survival rate, were observed weekly, and significant ($p < 0.05$) results were attained up to 75% replacement level with no adverse effect on growth. However, at 100% replacement of FM, fish growth was retarded. Maximum survival rate was observed in T_1 and T_5 and minimal in T_4 and T_8. Body composition, including crude protein, fat, moisture, and ash, showed significant ($p < 0.05$) results. Similarly, digestive enzyme (protease, lipase, and amylase) activity was measured at the end of the trial. Fish in T_1 and T_5 groups had the highest digestive enzyme activity, which slightly decreased with the inclusion of insect-based meals and probiotics. Statistically significant ($p < 0.05$) results were observed for antioxidant enzyme activity in catalase, glutathione peroxidase, and superoxide dismutase. The most vigorous immune response was shown in T_1 and T_5, with higher white blood cells and high levels of IgM. The present research showed that FM could be replaced with BSF meal and MM with probiotics up to 50% for better fish health performance; however, 75% replaced FM without compromising growth and health status.

1. Introduction

Aquaculture is a part of agriculture industries that has grown steadily in the last few decades, producing aquatic animals and plants [1]. It is an important food production source to fulfill the growing population's demand for food [2]. According to FAO [3], its production attained another record high of 114.5 million tonnes in live weight with a total value of USD 263.6 billion. Fish is globally consumed because it is a good animal protein source and highly nutritious [4]. Fish nutrition is the most critical part of aquaculture, as good feed leads to a healthy and resistant population [5]. Among nutrients, protein is the most essential biomolecule among all energy sources because the main feed ingredient for protein is fish meal (FM) [6]. FM is the most widely used protein source due to its high amino acid profile. Aqua feed primarily relies on FM and fish oil that are formed by capturing wild fish [7]. There has been adequate progress in finding the best alternative protein sources to replace FM in fish feed that lowers the cost of fish nutrition [8].

Various plant-based protein sources (soybean meal, canola meal, cottonseed meal, rapeseed, and maize) and insect-based meal (maggot fly (MF), black soldier fly (BSF) and silkworm, etc.) are considered good alternatives to replace FM [9, 10]. The studies showed that about 98% of the soybean meal feeds fish, livestock, and other animals [11]. However, soybean meal results negatively when used at a higher substitution level because of its antinutritional properties. Some antinutritional factors like trypsin, lectins, saponins, protease, and phytase are present in soybean meal, limiting its use in fish feed [12]. Moreover, plant-based protein sources are poorly digestible and lack two essential amino acids, i.e., methionine and lysine [13]. These amino acids are added as supplements in feed to enhance the efficiency of soybean meal to be used as an alternate [14].

To overcome this problem, research has been conducted to find a better alternative protein source for FM that is not just rich in protein but also digestible. For 20 years, insect meal has been considered an alternative protein source to replace FM [15]. Insects are rich protein sources with balanced amino acids, and they have fair content of lipids [16]. Moreover, insect meal production is cost-effective and environmentally friendly because they are reared on waste substances with less emission of ammonia and other greenhouse gases. However, insect-based meals have a higher nutrient conversion ratio than soybean meals [17]. As the insect meal is ingested, it soon starts providing protein, antimicrobial properties, and other derivatives that enhance fish growth and boost their immunological responses [18].

Insect species used for insect meal production are silkworms, black soldiers fly, maggot flies, crickets, and beetles [19]. Among all insect species, BSF (*Hermetia illucens*) meal is a novel protein source and an essential component in animal feed [20]. The amino acid profile of BSF meal is relatively the same as that of FM, with 42% and 35% fat content [21]. Favorable amino acids and essential fatty acids are abundant in BSF meals [22]. These insect species did not just have high nutritional value and provided multiple advantages, like converting food waste into rich energy biomolecules, i.e., protein [23]. High-fat contents and CP levels make it more suitable for aqua feeds [24]. Housefly maggot (*Musca domesticus*) meal is another alternative protein source used in fish feed to replace FM [25]. Maggot meal (MM) is more nutritious and more accessible to produce than other protein sources like FM or plant-based meals. MM contains dual properties, rich in nutrients and transforming waste [26], and has high activity of digestive enzymes, including protease, lipase, and amylase [27]. Its nutritional value is comparable to FM, where crude protein level lies between 43% and 62%. MF meal has been recorded to have high fatty acid content and a good profile of B-complex vitamins [28]. It generates no oxidative stress and does not influence fish metabolism [29].

Nile tilapia (*Oreochromis niloticus*) is the second most farmed fish, and a 70% increase in its production rate for the past 8 years has influenced its contribution to the aquaculture industry. Tilapia is an important fish in aquaculture because of its omnivorous nature and fast growth [30]. It is tolerant to different environmental conditions and can be easily handled, so it is preferably used in fish farming [31]. The present study aimed to check the effects of an insect-based meal as a replacement for FM in the feed of Nile tilapia. Moreover, the fish's proximate body composition, growth profile, antioxidant enzyme activity, and digestive enzymes assay were measured.

2. Materials and Methods

2.1. Rearing of Maggots. Housefly larvae were reared with fermented rice bran and poultry droppings mixed and used as the substrate to grow flies.

2.1.1. Fermentation of Rice Bran. One kilogram of rice bran was collected from the local grain market and fermented using 1 l of spoiled milk and 20 g of yeast. Water was added to the mixture to moisten it and poured into the airtight medium for about 2 weeks. Then, the rice bran was fermented due to its pungent smell and dark coloration.

2.1.2. Preparation of Culture Medium. After fermenting the rice bran, 5 g of poultry droppings with fruit waste were added to the mixture. The mixture was placed into a $50 \times 50 \times 50\,cm^3$ container and left open. The pungent odor of waste and fermented rice bran was used as a stimulus to attract maggot flies. The maggots laid eggs on the medium, and a large number of maggots were produced on the substrate [32].

2.1.3. Harvesting and Drying the Housefly Maggots. After the housefly maggots were reared, they were harvested using the floatation method, in which water was added to the mixture, and the larvae started to float. A sieve was used to collect the larvae from manure. The collected maggots were dried using the oven at 60°–80° in laboratory conditions. Then, the desiccated larvae were transformed into fine powder by the user mortar and pestle [33]. The powdered meal was stored in airtight polythene bags to formulate the feed in the future.

2.2. Rearing of Black Soldier Fly. Black soldier flies were reared under laboratory conditions using organic matter and animal waste as substrate. The larvae at the pre-pupae stage were bought from the local market to culture. Rice bran was used as a culture medium in a petri dish over which the larvae were spread. The leftover kitchen waste was added to the mixture. The mixture was kept for 3 weeks, and the pupae turned into developed bees. The reared flies produced the larvae and grew for 8 days. Then, the larvae were harvested by using forceps [17].

2.2.1. Drying and Defatting. The collected larvae were dried in the oven at 70°C for 24 hr to increase their palatability. The larvae were ground into fine powder using the grinder and stored in polythene bags at freezing temperature [34]. For defatting, the frozen larvae were cut into smaller pieces, and pressure was applied to leak intracellular fats that increased the crude protein content in BSF meal [35].

The proximate body composition of feed was determined using the method described by AOAC [36].

2.3. Experimental System. The present study was conducted for 4 months using glass aquaria of size 90 l (40 cm long, 50 cm wide, and 45 cm high) in laboratory conditions. 270 Nile tilapia fingerlings (10 in each aquarium) were collected from Fisheries Research Farm, University of Agriculture, Faisalabad. Fingerlings were fed functional feed in which FM was replaced with an insect-based meal (MM and BSF meal) at different levels, probiotics (*Bacillus subtilis* and *Bacillus lechiniformis*), and supplementation of amino acids. The feeding ratio was 6% of the body weight of fish with 35% crude protein. Feed was given twice a day at 8:00 am and 7:00 pm. Nine groups were made with three replicated; one was control T_0 (control group having basal diet), and eight were experimental. Siphoning was done after 20–30 min of feeding, and all the waste material and feed residuals were removed from the tank. Physico-chemical parameters were monitored weekly at standard protocol; meanwhile, growth parameters, including weight gain, length gain, feed conversion ratio (FCR), and specific growth rate (SGR), were measured weekly. However, antioxidant, immunity, proximate analysis, and digestive enzyme activity were measured at the end of the trial.

2.3.1. Feed Preparation. Feed ingredients, including FM, wheat flour, wheat bran, rice bran, fish oil, vitamins, and probiotics, were bought from the local market, Faisalabad. MM and BSF meal were prepared in the laboratory. Experimental feed was prepared using 35% crude protein, 4% fat contents, and 2% vitamins and minerals. The feed was given to eight experimental groups; meanwhile, the control group was fed a basal diet having 100% FM (Table 1). After collecting the ingredients, they processed them to improve the feed's digestibility. The assessment of amino acid and fatty acid profiles of all diets is also presented in Table S1.

2.3.2. Feed Processing. The feed ingredients were bought from the main market in the required amount, and the feed was prepared with different steps.

(1) *Grinding of Ingredients*. First, all the ingredients were ground into small fine particles using physical methods, including mortar and pestle or hammer mill.

(2) *Weighing of Ingredients*. In the second step, the ground ingredients were weighed using a balance.

(3) *Mixing of Ingredients*. The ingredients were thoroughly mixed using the mixer to increase the palatability of the feed. However, minerals and vitamins were mixed separately, added to the mixture, and blended.

(4) *Addition of Oil.* Then, oil was added to the whole mixture and stirred for 5 min. Preferably, warm oil was used to ensure the proper mixing.

(5) *Addition of Probiotics*, *Vitamins*, *and Minerals*. Vitamins, minerals, and probiotics were added to the feed to improve quality. *B. subtilis* strain was provided for resistance against harmful pathogens, so 2% probiotics were used in fish feed.

2.4. Proximate Body Composition. At the end of the trial, the pithing technique was performed to destroy both experimental and control fish brains and ultimately immobilize them.

Table 1: Feed ingredients and composition of different diets T_0–T_8.

Ingredients	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8
Fish meal	48	36	24	12	–	36	24	12	–
Black soldier fly	–	–	–	–	–	12	24	36	48
Maggot meal	–	12	24	36	48	–	–	–	
Wheat flour	13	13	13	13	14	13	13	13	14
Wheat bran	18	16	16	16	16	16	16	16	16
Rice bran	15	13.5	13.5	13.5	12.5	13.5	13.5	13.5	12.5
Fish oil	4	4	4	4	4	4	4	4	4
Vit. and min.	2	2	2	2	2	2	2	2	2
Probiotics	–	2	2	2	2	2	2	2	2
Total	100	100	100	100	100	100	100	100	100

The meat samples from both groups were collected to analyze the proximate body composition of fish. The samples were homogenized with the help of mortar and pestle, and standard protocols were used to determine the body composition.

The sample of fish and feed were subject to proximate analysis following the methods by AOAC [37] (Table 2).

2.5. Measurement of Growth Parameters

2.5.1. Growth Performance. The growth parameters, including length gain (cm), weight gain (kg), SGR, and FCR, were measured weekly throughout the experimental period.

The following formulas were used to measure these parameters.

2.5.2. Length Gain (cm).

$$\text{Length gain (cm)} = \text{Final length} - \text{Initial length}. \quad (1)$$

2.5.3. Weight Gain (g).

$$\text{Weight gain (g)} = \text{Final weight} - \text{Initial weight}. \quad (2)$$

2.5.4. Specific Growth Rate.

$$\text{SGR} = \frac{\ln(\text{final weight}) - \ln(\text{Initial weight})}{\text{number of days}} \times 100. \quad (3)$$

2.5.5. Feed Conversion Ratio.

$$\text{FCR} = \frac{\text{Total feed consumed}}{\text{Final weight} - \text{Initial weight}} \times 100. \quad (4)$$

2.6. Antioxidant Enzyme Profile. To determine the antioxidant enzyme activity, catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) were checked by using liver and gill samples of experimental fish. First, the liver and gills were dissected, and the sample was homogenized in a cold sulfate buffer solution having 6.5 pH with 1 : 4 weight by volume. The mixture was centrifuged at 1,000 rpm

Table 2: Proximate composition of feed.

Components	Control	MM 25%	MM 50%	MM 75%	MM 100%	BSFM 25%	BSFM 50%	BSFM 75%	BSFM 100%
Dry matter (%)	91.0	94.31	92.93	93.51	90.32	93.21	91.45	90.35	91.53
Crude protein (%)	70.69	68.23	66.75	65.36	63.56	70.32	69.35	67.53	65.36
Crude fat (%)	7.80	12.10	15.41	15.32	14.32	11.56	13.32	12.34	11.42
Ash (%)	18.30	22.35	23.95	21.23	22.98	20.35	21.56	20.63	20.63
NFE (%)	7.25	9.513	9.32	10.12	9.96	9.37	10.35	9.67	8.69
Calcium (%)	1.35	1.42	1.32	1.24	1.26	1.21	1.20	1.23	1.21
Phosphorus (%)	0.81	0.75	0.66	0.81	0.62	0.80	0.71	0.69	0.72
Gross energy (MJ/kg)	23.1	24.31	22.31	21.75	22.36	23.9	23.2	22.34	21.56

NFE, nitrogen-free extract.

at 4°C for 15–20 min. The supernatant was collected and stored at a high temperature (80°C) to measure the antioxidant enzyme assay.

2.6.1. CAT Activity. Protocol was used to measure the CAT activity. About 2 ml of reaction mixture containing 0.05 ml CAT extract and 1.95 ml buffer was placed in a spectrophotometer, and absorbance was measured at 240 nm.

The following formula was used to measure the CAT activity:

$$\text{Activity (units/ml)} = \frac{\frac{\Delta A}{\text{min}} \times \text{dilution} \times 2\ \text{ml}}{0.04\ \text{mM} - 1\ \text{cm} - 1 \times 0.05\ \text{ml}}. \quad (5)$$

2.6.2. Glutathione Peroxides. GPx activity was calculated by measuring the rate of decrease in NADPH level for 1 min at 340 nm. A reaction mixture containing phosphate buffer, NADPH, GSH GR, and supernatant was formed in 1 ml. Then, 3 mM C.H.P. was added to the solution mixture, and GPx activity was expressed in mmol/mg p/min.

2.6.3. Superoxide Dismutase Assay. Free superoxide radicals were measured by using Tetrazolium salt [38]. About 1 ml of blank buffer was taken in the cuvette and placed in a spectrophotometer, where the reading was set to auto zero to neglect the buffer reading when the solution was added. Riboflavin, an enzyme extracted from the buffer and the buffer solution, was incubated for 12 min. About 0.33 ml N.B.T. and 0.067 ml NaCN/EDTA were added as illuminators and placed in the spectrophotometer. After 20 s, the absorbance was measured at A_{560} nm.

By using the following formula, SOD activity was measured.

$$\text{Percentage inhibition (Abs)} = \frac{\text{Blank} - \text{Sample}}{\text{Blank}} \times 100. \quad (6)$$

2.6.4. Digestive Enzyme Assay. A digestive enzyme assay was measured at the end of the experiment, where the fish from each aquarium were collected at random and anesthetized. Dissection was performed to get an intestine sample. The sample was homogenized with the help of a prechilled sucrose solution. Homogenate was centrifuged by using a refrigerated centrifuge. The supernatant was collected and stored at 20°C. UV visible spectrophotometer was used to check the absorbance to measure the digestive enzyme activity (UV visible Hitachi U-2001).

2.6.5. Amylase Assay. Amylase enzyme activity was determined by using the [39] method. About 0.5 ml starch solution and 0.5 ml enzyme extract were mixed to prepare the solution. The reaction mixture was then incubated at 37°C for half an hour. To stop the reaction, dinitro salicylic acid was added to the reaction mixture. Then, the solution was diluted by adding distilled water, and absorbance was measured at 540 nm. Amylase activity was measured through the maltose standard curve.

2.6.6. Protease Assay. Kunitz's [40] protocol was used to calculate the protease activity. One percent casein substrate, phosphate buffer, and enzyme extract were mixed to prepare the reaction mixture. The prepared mixture was then incubated for 15 min at 37°C. The 5% trichloro acetate was added to the solution to stop the reaction, and the solution was filtered. A blank reagent was formed without incubation through tissue homogenate before stopping the reaction. The spectrophotometer was used to measure the absorbance at 280 nm. To calculate the protease activity, a tyrosine standard curve was used.

2.6.7. Lipase Activity. Lipase activity was calculated by using a P-nitrophenyl palmitate substrate [41]. The reaction mixture was prepared with 0.9 ml P-nitrophenyl palmitate solution, 0.5 M phosphate buffer, and enzyme extract, which was incubated for 30 min at 37°C. Absorbance of P-nitrophenol was measured at 410 nm to measure the lipase activity.

2.7. Physicochemical Parameters. Throughout the experimental period, the physicochemical parameters were measured weekly. Dissolved oxygen (DO), total hardness, total alkalinity pH, and temperature were measured using the methods of APHA [42].

2.8. Statistical Analyses. The statistical design of the experiment was completely randomized design, and the Tucky test was used to analyze the data. The mean values were analyzed by

comparing the growth profile, proximate body composition, antioxidant enzyme activity, and digestive enzyme activity.

3. Results

The highest weight gain was recorded in the T_1 and T_5 groups (25% MM and 25% BSF meal (BSFM) along with 2% probiotics) with 77.1 ± 0.548 and 77.5 ± 0.548, respectively, when compared with the control group weight gain 72.3 ± 0.491. It was followed by T_2 (76 ± 0.548) and T_6 (73.8 ± 0.705) groups, where 50% FM was replaced with maggot and housefly meals. At 75% replacement of FM with MM and housefly meal in T_3 and T_7, the weight gain was 71.1 ± 0.779 and 71 ± 0.808, indicating that 75% replacement level conferred relatively similar results with the control group. However, when 100% FM was replaced with MM and BSFM in T_4 and T_8, a drop in weight gain was noticed and recorded at 65 ± 0.617 and 67 ± 0.519, respectively. The results indicated that FM could be substituted with insect-based meals up to 50% to get effective results regarding weight gain (Table 3).

When FM was replaced with the insect-based meal, the experimental fish showed a significant increase in length ($p < 0.05$). Fish in T_1 and T_5 groups attained 15.8 ± 0.346 and 15.6 ± 0.223 length gain, showing the most effective replacement results compared to the control group (14.8 ± 0.145). At 75% replacement in the T_3 and T_7 groups, the results were comparable to the control group, with recorded values of 14.7 ± 0.057 and 14.5 ± 0.066. Results evaluated that replacement could be done up to 75% without negatively impacting growth performance. T_4 and T_8 groups had the lowest recorded values for length gain, with 14.6 ± 0.635 and 14.3 ± 0.152, indicating that length gain was negatively affected when 100% FM was replaced (Table 3).

The lowest FCR was recorded in the T_1 group (1.4 ± 0.075) along with the T_5 group (1.4 ± 0.057), which were comparably more effective than the control group (1.6 ± 0.057). In T_2 and T_5 groups (1.5 ± 0.057 and 1.6 ± 0.057), the FCR was slightly higher than in T_1, T_4, and the control group. The 1.7 ± 0.057 and 1.76 ± 0.088 FCR were shown by T_3 and T_7 groups at 75% replacement level where the values were constant according to the control group. The 100% replacement of FM had sharply increasing values of FCR in T_4 and T_8 groups, where recorded values were 1.96 ± 0.088 and 2.03 ± 0.066, respectively. Results indicated that replacing FM with a cost-effective insect-based meal could be an alternative in fish feed without compromising fish health if replaced by up to 75% (Table 3).

In the control group, the recorded SGR was 2.4 ± 0.773, used as a reference to compare the experimental groups. The maximum growth rate in the T_1 and T_5 groups was recorded at 2.6 ± 0.057 and 2.63 ± 0.082, respectively. T_2 (2.5 ± 0.057) and T_5 (2.46 ± 0.082) showed the second-highest SGR. The T_3 (2.3 ± 0.033) and T_7 (2.3 ± 0.088) showed similar results when compared with control. At high replacement levels in T_4 (2.2 ± 0.088) and T_8 (2.2 ± 0.057), the SGR was recorded lowest, indicating negative effects of 100% replacement (Table 3). The survival rate of fish was recorded weekly, and results showed that the lowest mortality rate was in the T_1 and T_5 groups, where 96 ± 1.154 and 95 ± 1.527 fish survived, respectively (Table 3).

For protein content, the highest results were in the T_1 (16.6 ± 0.073) and T_5 (16.7 ± 0.115) groups, where the fish were fed with 25% MM and BSFM. Protein content in T_2 (16.5 ± 0.05) and T_6 (16.5 ± 0.033) groups were more significant ($p < 0.05$) than T_0 (16.3 ± 0.0577). However, the ratio decreased when the replacement level increased to 75% in T_3 (16.3 ± 0.120) and T_7 (16.2 ± 0.057). The negative results with the lowest protein content were recorded at the highest substitution level in T_4 (15.9 ± 0.288) and T_8 (15.8 ± 0.145) (Table 4).

Crude fat in insect-based meals significantly affected the fat contents in fish. With increasing replacement levels, the fat content in experimental fish increased, indicating negative results. Fat content was lowest in groups with the lowest substitution levels, including T_1 and T_5, which had equal results (3.76 ± 0.088) compared to T_0 (4.3 ± 0.152). The fatty acid profile was equally better in both T_2 and T_6 groups (3.8 ± 0.057) at 50% replacement. Neutral effects were observed in the T_3 and T_7 groups; the recorded value was the same as in the control diet (4.4 ± 0.12). T_4 and T_8 groups showed the highest noted value (4.53 ± 0.145), which reflected the high fatty acid content in both MM and BSFM (Table 4).

The percentage of moisture content in fish varied according to the replacement rate of FM in fish feed. The moisture content significantly increased with increasing levels of insect-based meals in feed. Moisture content was lowest in the T_1 group (74.82 ± 1.66) and T_5 (75.7 ± 0.89), which slightly increased in T_2 (78.6 ± 0.585) and T_6 (76.53 ± 0.783). Fish in the T_3 and T_7 groups had 78.6 ± 0.58 and 78.5 ± 0.60 moisture content (Table 4). The analyzed ash content in fish fed was recorded between 3.4 and 3.8, and the most significant results ($p < 0.05$) were in T_1, and T_5 had 3.3 ± 0.033 and 3.4 ± 0.088, respectively. The highest ash content was noted in T_4 (3.7 ± 0.057) and T_8 (3.8 ± 0.057), where FM was completely replaced with MM and BSFM in the feed of Nile tilapia (Table 4).

The protease activity in the control group with basal diet was 70.8 in T_0. The highest protease activity was measured in T_1 (71.7 ± 0.365) and T_5 (71.5 ± 0.548), while the activity recorded in T_2 (71.7 ± 0.251) and T_6 (71.4 ± 0.491) was minimal different. The T_3 and T_7 groups had 70.4 ± 0.409 and 70.2 ± 0.351 protease activity. The lowest protease activity was in T_4 (69.7 ± 0.371) and T_8 (69.8 ± 0.333), indicating that 100% replacement of FM with insect-based meal showed negative effects on feed digestibility. The lipase activity in T_0 was 195.5; meanwhile, the increase in the enzymatic index was noted in T_1 and T_5 groups. Fat content was digested poorly in fish fed with T_4 and T_8, where lipase activity was reduced to 193.8 ± 0.848 and 193.4 ± 0.606 (Table 5). The amylase activity was low in T_4 (4.46 ± 0.233) and T_8 (4.3 ± 0.115) with respect to the T_0 group (4.63 ± 0.088). However, the highest recorded amylase activity was in T_1 and T_5 replacement of FM. The T_2 (5.1 ± 0.1) and T_3 (4.6 ± 0.288) showed minimal variation from the most increased

Table 3: Weekly observations of growth parameters in terms of weight gain, length gain, feed conversion ratio, specific growth rate, and survival rate of Nile tilapia fed with different insect meals.

Treatments	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8
Initial weight	7.2 ± 0.057	7.4 ± 0.088	7.6 ± 0.115	7.4 ± 0.120	7.5 ± 0.057	7.7 ± 0.115	7.6 ± 0.115	7.7 ± 0.152	7.8 ± 0.173
Final weight	79.5 ± 0.871	84.5 ± 0.984	83.6 ± 1.074	78.5 ± 1.155	72.5 ± 0.768	85.2 ± 1.162	81.4 ± 0.982	78.7 ± 0.902	75.5 ± 1.115
Weight gain	72.3 ± 0.491^{c}	77.1 ± 0.548^{a}	76 ± 0.548^{ab}	71.1 ± 0.779^{c}	65 ± 0.617^{d}	77.5 ± 0.548^{a}	73.8 ± 0.705^{bc}	71 ± 0.808^{c}	67.7 ± 0.519^{d}
Initial length	5.2 ± 0.057	5.2 ± 0.033	5.3 ± 0.115	5.2 ± 0.088	5.2 ± 0.120	5.3 ± 0.145	5.3 ± 0.176	5.3 ± 0.088	5.4 ± 0.152
Final length	20 ± 0.578	21 ± 0.585	20.9 ± 0.577	19.9 ± 0.548	19.8 ± 0.896	20.9 ± 1.003	20.8 ± 0.721	19.8 ± 0.635	19.7 ± 0.635
Length gain	14.8 ± 0.145^{a}	15.8 ± 0.346^{ab}	15.6 ± 0.260^{ab}	14.7 ± 0.057^{ab}	14.6 ± 0.635^{ab}	15.6 ± 0.233^{ab}	15.5 ± 0.305^{ab}	14.5 ± 0.066^{ab}	14.3 ± 0.152^{a}
FCR	1.6 ± 0.057^{cd}	1.4 ± 0.075^{d}	1.5 ± 0.057^{cd}	1.7 ± 0.057^{bcd}	1.9 ± 0.066^{ab}	1.4 ± 0.057^{d}	1.6 ± 0.057^{cd}	1.7 ± 0.088^{abc}	2.0 ± 0.066^{a}
SGR	2.4 ± 0.773^{ab}	2.6 ± 0.057^{a}	$2.5 \pm 0.057_{ab}$	2.3 ± 0.033^{ab}	2.2 ± 0.088^{b}	2.6 ± 0.082^{a}	2.4 ± 0.033^{ab}	2.3 ± 0.088^{ab}	2.2 ± 0.057^{b}
Survival rate	92 ± 1.145^{abc}	96 ± 1.154^{a}	94 ± 1.101^{ab}	88 ± 1.154^{abc}	86 ± 1.4701^{c}	95 ± 1.527^{ab}	93 ± 1.154^{abc}	$88 \pm 1.527b^{c}$	86 ± 1.527^{c}

The values in the same row with different superscripts differ significantly ($p<0.05$). FCR, feed conversion ratio; SGR, specific growth rate. T_0 (100% fish meal), T_1 (25% maggot meal with 2% probiotics), T_2 (50% maggot meal + 2% probiotics), T_3 (75% maggot meal + 2% probiotics), T_4 (100% maggot meal + 2% probiotics), T_5 (25% BSFM + 2% probiotics), T_6 (50% BSFM + 2% probiotics), T_7 (75% BSFM + 2% probiotics), T_8 (100% BSFM + 2% probiotics).

Table 4: Body proximate analysis of Nile tilapia fed with different substitution levels of fish meal with insect meals.

Treatments	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8
Protein	16.3 ± 0.0577^{abc}	16.6 ± 0.073^{a}	16.5 ± 0.057^{ab}	16.3 ± 0.1^{abc}	15.9 ± 0.2885^{bc}	16.7 ± 0.115^{a}	16.5 ± 0.057^{ab}	16.2 ± 0.057^{abc}	15.8 ± 0.145^{c}
Fat	4.3 ± 0.152^{ac}	3.76 ± 0.088^{c}	3.8 ± 0.057^{c}	4.43 ± 0.120^{ab}	4.5 ± 0.115^{a}	3.73 ± 0.120^{c}	3.86 ± 0.033^{bc}	4.4 ± 0.152^{ab}	4.53 ± 0.145^{a}
Moisture	76.96 ± 1.065^{ab}	74.83 ± 1.666^{b}	75.76 ± 0.899^{ab}	78.6 ± 0.585^{ab}	78.83 ± 0.338^{ab}	75.23 ± 0.783^{b}	76.53 ± 0.548^{ab}	78.5 ± 0.608^{ab}	79.86 ± 0.856^{a}
Ash	3.53 ± 0.088^{abc}	3.366 ± 0.033^{c}	3.5 ± 0.057^{abc}	3.633 ± 0.088^{abc}	3.7 ± 0.057^{abc}	3.433 ± 0.088^{bc}	3.66 ± 0.033^{abc}	3.766 ± 0.088^{ab}	3.8 ± 0.057^{a}

The values in the same row with different superscripts differ significantly ($p < 0.05$).

Table 5: Digestive activity of Nile tilapia under the influence of insect-based meals.

Treatments	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8
Protease	70.83 ± 0.284^{ab}	71.7 ± 0.365^{a}	71.7 ± 0.2511^{a}	70.43 ± 0.409^{ab}	69.76 ± 0.371^{b}	71.56 ± 0.548^{ab}	71.46 ± 0.491^{ab}	70.2 ± 0.351^{ab}	69.83 ± 0.333^{ab}
Lipase	195.56 ± 0.548^{bc}	198.2 ± 0.351^{ab}	197.5 ± 0.577^{ab}	195.83 ± 0.28^{abc}	193.83 ± 0.848^{c}	198.5 ± 0.577^{a}	198.16 ± 0.352^{ab}	194.5 ± 0.577^{c}	193.46 ± 0.606^{c}
Amylase	4.63 ± 0.088^{a}	5.26 ± 0.120^{ab}	5.1 ± 0.1^{ab}	4.6 ± 0.288^{ab}	4.46 ± 0.233^{ab}	5.23 ± 0.066^{a}	5.03 ± 0.120^{ab}	4.56 ± 0.185^{ab}	4.3 ± 0.115^{b}

The values in the same row with various superscripts differ significantly ($p < 0.05$).

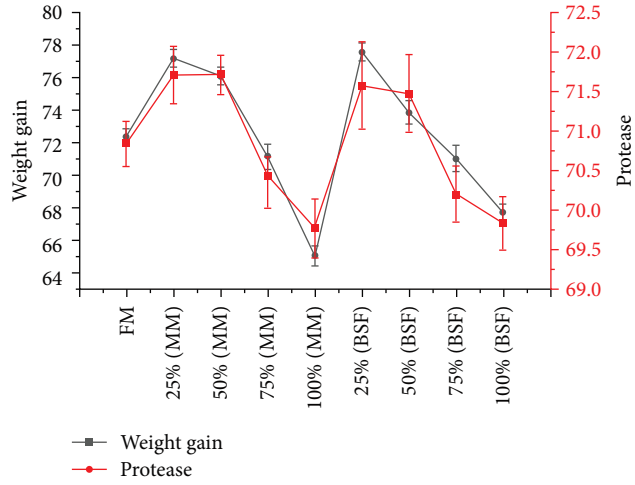

Figure 1: Interaction between protease activity and weight gain in Nile tilapia fed by insect-based feeds (FM, fish meal; MM, maggot meal; BSF, black soldier fly).

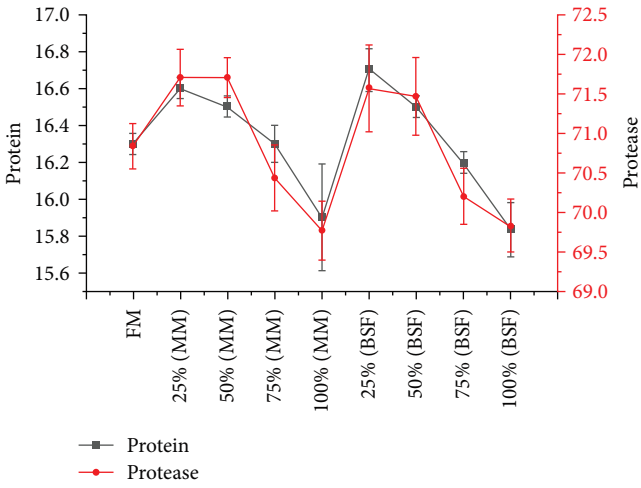

Figure 2: Interaction between protease activity and protein contents of Nile tilapia fed with insect-based feed (FM, fish meal; MM, maggot meal; BSF, black soldier fly).

measured activities. With more insect meal inclusion, the enzymatic activities showed a decreasing trend in T_3 (4.6 ± 0.288) and T_7 (4.56 ± 0.185) (Table 5).

The correlation between protease activity and weight gain in tilapia showed a direct relation among them. It was noted that adding probiotics improves digestive activity and fish health. In T_1 and T_5, the highest protease enzyme activity was measured, resulting in the highest weight gain. The results showed that significant results ($p < 0.05$) regarding protease activity and protein content were achieved in T_1 and T_5, followed by T_2 and T_6. However, in T_3 and T_7, the noticeable similarities were monitored with T_0. The weight gain and protein contents decrease by decreasing the protease activity. The interaction between protease activity and weight is shown in Figure 1, and the between protease activity and protein contents is shown in Figure 2.

CAT, GPx, and SOD were measured at the end of the experiment. T_0 had a basal diet comprising 100% FM and had 55.5 ± 0.56 CAT activity. The T_1 (61.4 ± 0.606) and T_5 (61.5 ± 0.497) showed the highest CAT activity, which decreased in T_2 (60.5 ± 0.523) and T_5 (61.5 ± 0.497). The T_3 (54.5 ± 0.881) and T_7 (54.0 ± 0.898) had similar ratios with the control group. The lowest activity was found in T_4 (53.0 ± 1.241) and T_8 (52.2 ± 0.497) (Table 6).

The concentration of toxic hydrogen peroxide was high in experimental fish with the lowest GPx activity, such as T_4 (14.2 ± 0.05) and T_8 (14.3 ± 0.057), while in T_0, the observed value was 14.5 ± 0.057. The GPx activity was maximum in T_1 (14.7 ± 0.087) and T_5 (14.7 ± 0.066). T_2 was observed to have 14.6 ± 0.057 reduced to 14.4 ± 0.11 in T_3 (Table 6). With the highest inclusion of insect-based meals, the level of ROS increased in tilapia, increasing the oxidative stress in fish. As in T_1 (17.0 ± 0.328) and T_5 (16.9 ± 0.0384), the maximum SOD activity indicated reduced oxidative stress. Meanwhile, there was a decrease in T_3 (14.3 ± 0.115) and T_7 (14.2 ± 0.497). The lowest results were calculated in T_4 (13.6 ± 0.285) and T_8 (13.8 ± 0.176) (Table 6).

Antibodies production against the pathogen and foreign particles made the fish resistant to survive in different environmental conditions. When IgM levels in treatment groups were observed and compared with the control group, the most effective results were obtained in T_1 and T_5 with the highest IgM. However, at 75% replacement in T_3 and T_7, the IgM level was quite similar to the control group, showing no negative effect of feed (Figure 3).

Immunity parameters, including white blood cell (WBC) count and immunoglobins in experimental fish affected by the formulated feed. The blood profile indicated that the WBC count was highest in T_1 and T_5, with 25% of MM and BSFM replaced with FM. It represented that the effective immune response in the above treatments confirmed the nutritional quality of formulated feed. In T_2 and T_6, relatively similar WBCs were present but higher than T_0. Meanwhile, T_4 and T_8 showed less WBCs and IgM, indicating the harmful effects of replacing 100% FM.

The water quality parameters, including pH, temperature, DO, alkalinity, and hardness, were measured weekly to ensure a suitable environment for fish to grow. The most preferable pH (7) was measured in T_1, T_2, T_5, and T_6 groups, while the more was present in T_4 (7.3 ± 0.145) and T_8 (7.4 ± 0.115). The optimum temperature for fish growth (31.0 ± 0.328) was observed in T_1, while T_4 and T_8 had the least suitable temperature, which was 30.2 ± 0.240. DO level in T_1 was 4.0 ± 0.125, recommended for better fish growth, and the highest recorded DO was observed in T_8. Alkalinity in T_1 was 193.1 ± 193.16, where fish showed maximum growth and body composition; however, T_7 had the maximum alkalinity in water (203.5 ± 1.126). Total hardness in T_1 (168.1 ± 1.449) made it better adaptable for fish to show maximum significant results ($p < 0.05$), while in T_4, the hardness was 166.7 ± 0.938 (Table 7). Figure 4 shows the relationship between water quality parameters, fish growth, and health indices. A positive correlation was found between weight, survival rate, IgM, CAT, protease, and protein.

Table 6: Antioxidant activity of Nile tilapia fed with insect-based diets with different substitution levels.

Treatments	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8
Catalase	55.56 ± 0.560^{b}	61.46 ± 0.606^{a}	60.53 ± 0.523^{a}	54.53 ± 0.881^{b}	53.03 ± 1.241^{b}	61.56 ± 0.497^{a}	61.13 ± 0.548^{a}	54.03 ± 0.898^{b}	52.26 ± 0.497^{b}
GPx	14.5 ± 0.057^{abcd}	14.77 ± 0.088^{a}	14.6 ± 0.057^{abc}	14.4 ± 0.115^{bcd}	14.2 ± 0.0575^{d}	14.73 ± 0.066^{ab}	14.63 ± 0.033^{abc}	14.46 ± 0.088^{abcd}	14.3 ± 0.057^{cd}
SOD	14.6 ± 0.305^{b}	17.03 ± 0.328^{a}	16.83 ± 0.375^{a}	14.36 ± 0.491^{b}	13.6 ± 0.285^{b}	16.93 ± 0.384^{a}	16.86 ± 0.333^{a}	14.26 ± 0.497^{b}	13.86 ± 0.176^{b}

The values in the same row with different superscripts differ significantly ($p < 0.05$). GPx, glutathione peroxidase; SOD, superoxide dismutase.

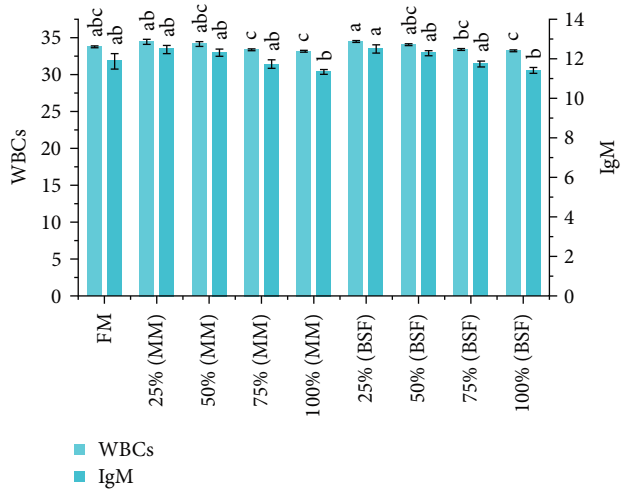

FIGURE 3: Immune response under different replacement levels of fish meal with insect meals in the feed of Nile tilapia (WBCs, white blood cells; IgM, immunoglobulin M; FM, fish meal; MM, maggot meal; BSF, black soldier fly).

Results were negative correlates with pH and DO levels of water. However, alkalinity, temperature, and hardness moderately correlate with fish indices.

4. Discussion

The scarcity of FM and fish oil prompts a quest for other sources of feed components. Certain efforts have been made to reduce the use of FM and fishery products in aqua feed, increasing the inclusion of plant-based products to fulfill the protein demand [43]. However, these products exert stress on the environment with water and land resources and have antinutritional properties restricting their use [44]. Different protein sources have been studied, but insect meal has been considered a better alternative to meet the protein requirements [45]. While considering the suitable insect species, housefly maggots and BSF have been reviewed due to their easy availability, feasible supply, good amino acid profile, and cost-effectiveness [19]. Live microbial strains known as probiotics are used as nutritional supplements and have been demonstrated to promote fish growth and health [46].

In the present study, MM and BSFM, along with 2% probiotics, were used to replace FM at different dietary levels in Nile tilapia, and fish health status was observed. The highest growth performance was observed in groups with the highest weight gain and lowest FCR, where 25% of FM was replaced with either maggot or BSF meal. However, 50% replacement showed significant results, but at 75% replacement, fish showed the same response as the control group. Negative effects were found at complete replacement, indicating that excessive protein intake could not be used for better growth because most energy is wasted during deamination and excretion of undesirable and extra amino acids [33]. The present results were in accordance with the study of Cho et al. [47], in which *Onchorynchus mykiss* showed similar growth performance when house fly pre-pupae meal was used in their feed to replace FM. Moreover, similar results were observed in the study conducted by Khieokhajonkhet et al. [48], in which *Carassius auratus* showed an efficient growth rate and survival ratio when fed on BSF meal.

Substitution of FM with insect-based meal affected whole-body composition in tilapia. Results showed the highest protein and lowest fat, moisture, and ash content in experimental groups fed 25% and 50% of MM and BSFM. There was no significant difference in results between control and 75% replacement. However, the trend went the opposite when the whole FM was replaced. Higher fat content was reported due to an imbalance of omega *n*-3 and *n*-6 ratio in insect-based meals, which leads to fat deposition when added at a high replacement level [49]. The results were in accordance with the study of Caimi et al. [50], where the body composition in Siberian sturgeon juveniles was studied, including ash, moisture, lipid, and protein content. These findings were similar to present research when juveniles were fed on 25% and 50% BSF meal. Moreover, current results showed similarity with the study of Mastoraki et al. [51], in which replacing 30% FM with housefly meal in European sea bass conferred significant results regarding fish body composition. When BSFM was used as a feed supplement replacement agent, considerable growth and health improvement were seen in rainbow trout [52].

Feed type, feeding habits, and antinutritional factors in feed directly influence the digestive activity in fish and affect the enzymatic profile that helps feed absorption and assimilation [53]. Due to its role in immunological defense, digestion, and absorption, the gut is a strong indicator of fish performance and health [54]. In the current study, FM replacement with both MM and BSFM at 25% conferred better results in feed digestibility. Protease, lipase, and amylase activities were recorded highest in T_1 and T_5, followed by T_2 and T_6. However, the effectivity decreased when insect-based meals completely replaced FM. The reduction in digestive enzyme activities was expected due to the presence of chitin in insects [55] because chitin is known to inhibit the absorption of lipids and other nutrients in the gut, which increases the rate of excretion and ultimately affects digestion efficiency [56]. Chitinolytic enzymes are widely mentioned in relation to the effectiveness of chitin consumption by monogastric animals. However, other investigations have verified the presence and activity of chitinolytic enzymes in various fish organs, including the stomach mucosa, intestinal mucosa, pyloric ceca, and pancreas [56, 57]. Present results were in accordance with the study of Li et al. [58], in which replacing FM with 25% and 50% BSF meal in Jian carps resulted in similar digestive enzyme activity (amylase, lipase, and protease). Guerreiro et al. [59] also reported the same results in *Argyrosomus regius* replacing FM with BSF meal. Moreover, Agbohessou et al. [60] observed relatively similar results regarding digestive enzyme activity (protease, amylase, and lipase) in *O. niloticus* after replacing FM with two dipteran species (BSF and housefly).

In T_1 and T_5, the highest protease enzyme activity resulted in the highest weight gain because studies have shown that protease enzymes directly influence the growth parameters, including weight, FCR, and SGR [61, 62].

Table 7: Physiochemical parameters of Nile tilapia.

Parameters	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8
pH	7.2 ± 0.057	7.0 ± 0.145	7 ± 0.057	7.3 ± 0.033	7.3 ± 0.145	7.0 ± 0.120	7.0 ± 0.088	7.1 ± 0.120	7.4 ± 0.115
Temperature	30.3 ± 0.493	31.0 ± 0.328	30.0 ± 0.260	30.1 ± 0.384	30.2 ± 0.240	30.0 ± 0.233	30.0 ± 0.290	30.1 ± 0.202	30.2 ± 0.260
DO	4.13 ± 0.147	4.03 ± 0.125	4.1 ± 0.055	4.1 ± 0.115	4.13 ± 0.120	4 ± 0.057	4.2 ± 0.057	4.1 ± 0.057	4.2 ± 0.0575
Alkalinity	191.33 ± 0.600	193.16 ± 0.881	195.13 ± 1.976	196.13 ± 1.483	198.8 ± 1.479	198.86 ± 1.449	197.16 ± 0.920	203.5 ± 1.1269	202.2 ± 2.307
Hardness	163.8 ± 0.850	168.13 ± 1.449	164.4 ± 1.571	164.1 ± 0.871	166.73 ± 0.938	164.7 ± 1.193	167.76 ± 1.266	166.46 ± 0.606	166.4 ± 0.635

DO, dissolved oxygen.

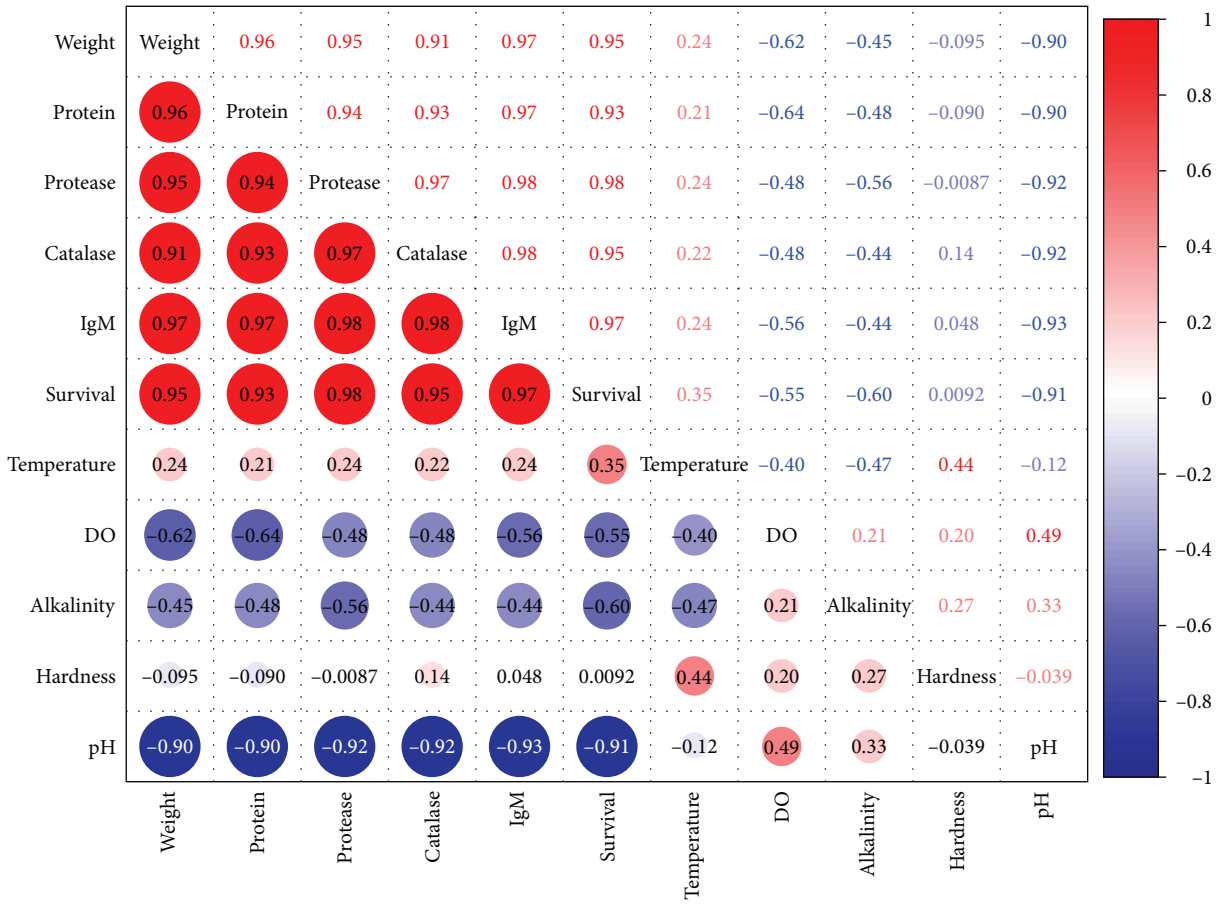

FIGURE 4: Correlation between water quality parameters and fish indices, red and blue, respectively, shows the positive and negative correlation (IgM, immunoglobulin M; DO, dissolved oxygen).

Protease catalyzes the larger protein molecules into smaller subunits, i.e., amino acids, which ultimately help to utilize the available feed [63]. In the T_2 and T_6 groups, both protease enzyme activity and weight gain were also significant compared to the control group, indicating the excellent health profile in fish. However, in T_4 and T_8, the lowest weight gain was observed because the lack of protease enzyme produced by the digestive tract influences the amount of available digestible protein molecules, which retarded growth [64]. The probiotics enhance fish performance, growth, and health. The gut microbiota helps digest otherwise indigestible feed ingredients, generating short-chain fatty acids, the primary energy source for intestinal epithelial cells [65]. In the probiotic-rich diet, gut bacteria diversity was lowest despite reaching the highest growth rate [66]. Probiotics boost feed digestibility and nutrient absorption in cultured fish, leading to better fish growth and conversion rates. They also maintain gut microbiota balance, especially at larval stages [67, 68].

SOD, GPx, and CAT play a key role in maintaining the oxy-radical balance in fish, and they help for better immune response in fish [69]. Furthermore, to sustain cellular homeostasis, CAT is well known to play an essential role in the reduction of toxic hydrogen peroxide into neutral substances like water and oxygen to inhibit oxidative stress [70]. Results of the present study determined that T_1, T_2, T_5, and T_6 improved antioxidant enzymatic activities, indicating the lowest oxidative stress in fish. However, when FM was replaced entirely with maggot and BSF meals, negative enzymatic indices were noticed. In low antioxidant activity, fish are not resistant to oxidative stress, which can decrease [71]. The changes in results of serum antioxidant enzymes in Jian carp juveniles were observed when 25% and 50% FM was replaced with BSF meal [58]. SOD, GPx, and CAT enzymes in *O. mykiss* had similar effects BSFM was replaced with soy and fish oil [72]. By adding probiotics, antioxidant activity increased, and oxidative stress decreased in the experimental groups with insect-based diets. It has been demonstrated that bacillus probiotics like (*B. subtilis*) can assist in controlling the antioxidant enzyme activity of Nile tilapia. The antioxidant enzymes in fish aid in breaking down potentially harmful chemicals in the water and defend against the damaging effects of free radicals [73, 74]. *B. subtilis*, *Bacillus coagulans*, and *Bacillus cereus* boosted SOD activity in Nile tilapia rearing systems. SOD, CAT, and GPx levels are elevated in the fish's liver and intestines, indicating an improved ability to combat disease. Probiotics for fish, such as *Bacillus* sp., have been associated with increased antioxidant enzymes in various fish, including tilapia [75, 76].

A crucial factor in determining how well a fish's immune system functions is its WBC count, which varies depending

on its species, age, sex, diet, and season [77, 78]. The innate immune system is the first line of defense against infection, and Bacillus probiotics (*Bacillus amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, *and B. pumilus*) have been demonstrated to improve phagocytosis in Nile tilapia [79, 80]. WBC count, IgM, and lysozyme activity are essential parameters to assess the immune profile in fish. In the present study, the highest level of WBC and IgM antibodies were observed at 25% replacement, proving it a recommended graded level for replacement. An increase in WBC count and IgM antibodies in fish showed that MM and BSF feed contain immunological properties, thus supporting the idea of replacing FM with insect-based meals up to a certain level. Because with increasing levels of substitution, the immune response started to reduce [77]. Wang et al. [81] observed similar results for IgM expression in *O. niloticus* given the house fly and MM-based diet. It was noted that probiotics improved fish growth, digestion, and immune competency much better than the control group.

5. Conclusion

Insect-based meal, including maggot and BSF meal, has been considered potential alternative protein sources to replace FM in fish feed. Significant results have been reported with 25% and 50% replacement levels with increased growth performance and other health profile parameters. The health status of fish at 75% replacement level was not affected with respect to the control diet. However, the high inclusion of insect-based meal (100%) negatively affected growth profile, body composition, and digestive enzyme activity. Moreover, the complete replacement of FM adversely affected antioxidant enzyme activity and immunity. Therefore, further consideration is needed to understand insect-based meals' nutritional efficiency and optimum inclusion level for more significant results.

Data Availability

Data can be available from the corresponding author upon reasonable request.

Ethical Approval

The work has been approved by the Institutional Biosafety and Bioethics Committee (IBC) of the University of Agriculture Faisalabad. The work has followed all the limitations for the fish trial.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Conceptualization was done by M.N. and A.M.; methodology was done by A.M. and M.N.; software-related task was done by W.M., D.S., M.N., M.N., A.H., and Y.K.; validation was performed by M.N., A.M., F.A., and D.S.; formal analysis was performed by Y.K., A.H., M.B.A., and M.N.; writing—original draft preparation was done by M.N., A.M., D.S., W.M., M.N., Y.K., A.H., F.A., and M.B.A.; writing—review and editing was done by W.M., A.M., M.N., and M.B.A.; supervision was done by A.M. All authors have read and agreed to the published version of the manuscript.

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Supplementary Materials

Table S1: Amino acid and fatty acid profile of experimental diets with different levels of insect meal. (*Supplementary Materials*)

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