EFFICACY OF DIFFERENT PROTEIN LEVELS ON GROWTH, IMMUNITY AND MEAT QUALITY OF NILE TILAPIA (*Oreochromis niloticus*) UNDER ZERO EXCHANGE HETEROTROPHIC CULTURE

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ABSTRACT

Biofloc treatment is an eco-friendly and affordable technology which has been adopted over the last three decades to improve aquaculture sustainability. In this study, fingerlings were randomly distributed into different aquariums and fed diets with varying protein levels (15, 20, 25 and 30%). This study investigated the effect of different crude protein (CP) levels on growth, immunity and meat quality of Nile Tilapia (*Oreochromis niloticus*) under zero exchange heterotrophic culture conditions. Results showed that fish fed 25 and 30% protein diets had the highest growth rate, feed efficiency and protein efficiency ratio. Weigh gain (WG) and specific growth rate (SGR) significantly increase in fish fed 25 and 30 % CP levels ($p<0.05$). Proximate analysis showed that the fish fed 25 and 30% CP had the highest protein content, lowest lipid content and improved fatty acid profile. Additionally, fish fed 30% protein had the lowest mortality rate and highest survival rate. Immune parameters (WBCs, plasma parameters, lysozyme and antioxidants enzymes activity) were significantly enhanced in fish fed 25 and 30% CP ($p<0.05$). The best growth performance was obtained when the fish fed on the 25 and 30% CP in diet and can be used as sustainable production of fish under zero exchange heterotrophic culture system.

Keywords: Biofloc, Dietary protein, Heterotrophic culture, Nile Tilapia, Nutrition

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INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) has emerged as one of the most significant aquaculture species globally, representing approximately 8% of total finfish culture production worldwide. This species has been successfully introduced throughout tropical and subtropical regions due to its remarkable adaptability, fast growth rate, disease resistance, and ability to thrive in diverse farming systems (Abduljabbar *et al*., 2015; Teodósio *et al*., 2020). The global expansion of tilapia aquaculture has provided substantial benefits for livelihoods in developing countries, serving as both a source of household income and family consumption (Mridha *et al*., 2017). As the aquaculture sector continues to grow (accounting for nearly 47% of total fish production globally) the sustainability of Nile tilapia farming depends increasingly on the development of cost-effective and environmentally friendly feeding strategies (Prabu *et al*., 2019; Teodósio *et al*., 2020).

Dietary protein constitutes the most expensive component of formulated fish feeds, typically representing over 50% of operational costs in intensive aquaculture systems (Abdel-Tawwab & Ahmad, 2009; da Silva *et al*., 2018). Consequently, optimizing protein levels in tilapia diets has become a critical research focus, balancing economic viability with environmental sustainability. Extensive research has demonstrated that protein requirements for Nile Tilapia vary significantly across different life stages. Fry weighing less than 1 g requires relatively high protein levels (35-50%), while fish in the 1-5 g range need 30-40% protein, and those in the 5-25 g category perform optimally with 25-30% protein (Hafedh, 2001). Studies have shown that fry (approximately 0.5 g weight) requires 45% crude protein (CP) for optimal growth, whereas juveniles (approximately 40 g weight) achieve maximum performance with 35% CP diets (Abdel-Tawwab *et al*., 2010). These variations reflect the changing metabolic demands as fish develop, with younger fish requiring higher protein for tissue formation and growth, while larger fish can utilize protein more efficiently for maintenance (Hafedh, 2001).

The environmental implications of protein management in aquaculture cannot be overstated. Excess dietary protein that exceeds the fish's metabolic capacity is catabolized and excreted as nitrogenous waste, contributing to water pollution and eutrophication (Teodósio *et al*., 2020; Zablon *et al*., 2022). In traditional aquaculture systems with water exchange, this waste is partially diluted, but in zero-exchange systems (which are increasingly adopted for environmental and biosecurity reasons) the accumulation of nitrogenous compounds becomes a critical concern. Biofloc technology (BFT), a form of zero-exchange heterotrophic culture, has gained prominence as a sustainable aquaculture approach that converts potential pollutants into microbial protein through controlled heterotrophic processes. In such systems, the relationship between dietary protein levels and water quality parameters becomes particularly complex, as the microbial community plays a crucial role in nutrient recycling (Hisano *et al*., 2020; Long *et al*., 2015).

Recent research by Hisano *et al*. (2020) has demonstrated that, in biofloc systems, Nile tilapia fingerlings (6-25g) can maintain growth performance with reduced dietary protein levels (28% CP) compared to conventional recommendations (36% CP), resulting in significant cost savings and reduced environmental impact. These findings challenge traditional protein formulation standards and suggest that the microbial protein generated within BFT systems may partially compensate for reduced dietary protein (Debnath *et al.*, 2025). However, most existing studies have focused primarily on growth performance and feed conversion efficiency, with limited attention to immune function and meat quality parameters.

The interaction between dietary protein levels and immune function in Nile tilapia remains poorly understood, despite evidence that protein nutrition significantly influences immune competence in fish. Adequate protein intake is essential for the synthesis of immune-related proteins, antibodies, and acute-phase proteins, whereas protein deficiency can compromise immune responses and increase disease susceptibility. Conversely, excessive protein intake may induce metabolic stress that could negatively impact immune function (Gaye-Siessegger *et al*., 2006; Nguyen *et al*., 2021). In zero-exchange systems where water quality parameters fluctuate more dramatically than in flow-through systems, the relationship between protein nutrition and immune status becomes even more critical, as suboptimal water conditions can exacerbate stress responses (Hisano *et al*., 2020; Wang *et al*., 2015).

Meat quality represents another crucial dimension affected by dietary protein levels. The proximate composition of fish muscle (including protein, lipid, moisture, and ash content) is directly influenced by dietary formulation. Studies have shown that variations in dietary protein can alter the fatty acid profile, texture, color, and overall sensory characteristics of fish fillets (Hafedh, 2001). For example, Ogweny *et al*. (2023) investigated the impact of protein sources on water quality, growth and feed utilization of Nile tilapia fingerlings reared under BFT. Eid *et al*. (2020) focused on the impact of various protein levels in improving the growth and utilization of food under BFT system. Similarly, a study conducted by Ghaedi and Khanjani (2025) examined the impact of varying protein levels on water quality and reproductive performance of Nile tilapia brood stock in biofloc system. In another study, the meat quality of Nile tilapia flash was evaluated under BFT system (Kattakdad *et al*., 2026). In zero-exchange heterotrophic systems, where microbial communities contribute to nutrient cycling and potentially to the fish's nutritional status, the relationship between dietary protein and final product quality may differ significantly from conventional culture systems (Hisano *et al*., 2020).

However few studies have investigated how varying dietary protein concentrations influence growth, immunity and meat quality in tilapia cultured under zero-exchange heterotrophic conditions, where microbial biomass contributes significantly to nutrition and health. The current study addresses these critical knowledge gaps by investigating the efficacy of different protein levels on growth performance, immune function, and meat quality of Nile tilapia under zero-exchange heterotrophic culture conditions. This research builds upon previous findings that demonstrated the feasibility of protein reduction in biofloc systems (Hisano *et al*., 2020), while expanding the evaluation to include comprehensive immune parameters and detailed meat quality assessments. By establishing the optimal protein level that maximizes growth, maintains robust immune function, and ensures high-quality fillets while minimizing environmental impact, this research aims to provide practical guidance for sustainable Nile Tilapia production in zero-exchange systems, an increasingly important aquaculture approach in the context of water scarcity and environmental regulations. Therefore, the present study was designed to investigate the efficacy of different protein levels on growth, immunity and meat quality of Nile Tilapia (*Oreochromis niloticus*) under Zero exchange heterotrophic culture system.

MATERIALS AND METHODS

Ethics approval: The experiment was conducted in Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture Faisalabad, in compliance with the ethical committee of University of Agriculture, in the guideline of Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries of the same university at Faisalabad. All the ethical principles and standards have been followed during the dissection and handling of specimens.

Experimental design and ethical considerations: The experiment was carried out under indoor conditions in glass aquaria for 4 months. The volume of each aquarium was calculated to be 120 liters using the dimensions: 80cm (length) × 35cm (width) × 43cm (height). The effect of various protein levels 15, 20, 25 and 30% on growth, immunity and meat quality of Nile tile under zero exchange heterotrophic culture was conducted in Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture Faisalabad, in compliance with the ethical committee of University of Agriculture, in the guideline of Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries of the same university at Faisalabad. All the ethical principles and standards have been followed during the dissection and handling of specimens. Acclimatization of Nile tilapia larvae was done for one week on basal diet before being distributed to respective glass aquarium of experimental units.

The experiment was conducted indoor conditions using glass aquarium over a period of 4-months. Four experimental groups were made with the same stocking density (50 fishes/m^3) and fed with various levels of protein (T1 (15%), T2(20%), T3(25%) and T4(30%) with three replications of each aquarium (n=3),As aquarium were considered as experimental unit for statistical analysis and individual fish were tested as subsamples. Prior to the start of the trial, all aquarium were cleaned thoroughly, dried and filled up with fresh water and inoculated of biofloc was done form a prepared cultured aquarium. Juveniles of Nile tilapia were fed daily at the rate of 6% of their body weight throughout the study period.

Floc preparation: The clean aquarium was taken with 45 liters of water and continue vigorous aeration was done. According to the protocol, for the preparation of 45 liters of fresh water, 5 L of inoculum was required for the floc development, So, 600g of pond soil, 0.3 g of Ammonium sulphate and 6 g of carbon source (starch) were added in water aquarium. Within 24-48h the inoculum was ready and then shifted to the main aquarium. The carbon source added daily in the inoculum for floc development. So, every 1kg of feed given, 600 g of carbon source was added to the system to maintain C: N (10:1). Once the floc volume reaches 15-20ml further addition of carbon source was not required (Avnimelech, 1999).

Diet formulation: Soybean meals, wheat flour, rice bran, wheat bran, fish meal, soy oil, vitamins, and minerals premix were mixed in to four different diet formula to make feed for fishes. All these ingredients were bought from the local market of Faisalabad. Ingredients were weighed and thoroughly mixed before feed diet formulation, and all the feed ingredients were properly grinded into fine particles by using mortar and pestle and the large sized particles were removed with the help of a sieve. Weight balance was used to weigh the ingredients. A Small quantity of minerals and vitamins were added and mixed well. At the end soya oil was added and mixed well to ensure thorough mixing. The diets were prepared, packed and labeled for ease (Table 1).

Table 1: Formulation and composition of experimental diet

INGREDIENTS	T1 (15%)	T2 (20%)	T3 (25%)	T4 (30%)
Fish meal	20g	50g	80g	105g
Soybean meal	20g	50g	80g	105g
Wheat flour	120g	96.5g	76.5g	65g
Wheat bran	115g	97g	77g	62g
Rice bran	115g	96.5g	76.5g	63g
Maize flour	80g	80g	80g	70g
Soya oil	20g	20g	20g	20g
Vitamin & minerals	10g	10g	10g	10g
Total	**500g**	**500g**	**500g**	**500g**

Water quality parameters: Water quality parameters were monitored daily throughout the experimental time. Temperature in centigrade was measured by using dissolved oxygen meter (TP-101), Dissolved oxygen (DO) was measured by using Dissolved Oxygen Meter (HI-9147). Total dissolved solids (TDS) were measured by using YL-TDS2-A meter, and pH of water was checked by using pH meter (HANNA) pH tester). Total Ammonia, Nitrite and Nitrate were measured by using APA freshwater Ammonia kit.

Proximate analysis of fish meat and diet: The proximate composition (Protein, Lipid and Ash) of the biofloc material and fish meat was analyzed according to standard protocols AOAC (2006). Meat quality parameters include crude protein was calculated by Kjeldehal's method. Crude Protein was calculated by applying this formula (Crude Protein = N2×6.25), and crude fat was calculated by Soxhlet apparatus (Total fat (%) = (Weight of empty extraction cup-weight of extraction cup with fat after) / (weight of sample) and ash content was calculated by using this formula (Total ash (%) = (Weight of ash (g)/ (weight of sample) ×100).

Growth performance: Growth parameters including body weight in grams and length gain in cm were calculated on weekly basis. Survival percentage (%), Specific Growth Rate (SGR), feed conversion ratio (FCR) was also measured. At the end of experimental trial, the following variables were evaluated: Weight gain was assessed by the given formula:

$$\text{Weight gain (g)} = \text{Final weight (g)} - \text{Initial weight (g)} \ldots\ldots\ldots \text{(a)}$$

Length gain was measured by using this formula:

Gain in length (cm) = Final length (cm) - Initial length (cm)…(b)

The formula for the calculation of SGR is given below:

$$\text{Specific growth rate (SGR)} = \frac{\ln WF - \ln W1}{\text{time Duration}} \times 100 \ldots\ldots\ldots\ldots\ldots (c)$$

FCR was assessed by following formula:

$$\text{Food conversion ratio (FCR)} = \frac{\text{Feed consumption (g)}}{\text{Total weight gain (g)}} \ldots\ldots\ldots\ldots\ldots (d)$$

Survival rate assessed by following formula:

$$\text{Survival rate (\%)} = \frac{\text{Number of harvested fish}}{\text{Number of stocked fish}} \times 100 \ldots. (e)$$

Innate immunity and blood profile

Immunoglobulin levels were measured directly by subtracting the values of albumin from those of total protein by using 12% of polyethylene glycol solution.

WBCs (White blood cells) were calculated by using Neubauer hemocytometer.

$$\text{No of WBCs } (10^3/\mu L) = \frac{no\ of\ WBCs \times diluion\ counted}{area\ counted \times depth\ of\ fluid} \ldots\ldots\ldots\ldots\ldots\ldots (f)$$

Lysozyme activity: The turbid metric assay was used for determining lysozyme activity.

Lysozyme activity (µl/ml) = (Transmittance (530 nm))/ (serum in mi used) x 1000. (g)

RBCs The hemocytometer method used to take readings of red blood cells (RBCs) (Rusia [et al.], 1996) No. of RBCs = $\frac{\text{No.of RBCs x dilution count}}{\text{Area counted x depth of fluid}}$

Hemoglobin content, Cyanmethemoglobin methods were employed to evaluate the hemoglobin content of fish blood. Hemoglobin test kit was used (Blaxhall & Daisley, 1973).

$$\text{Hemoglobin concentration } \left(\frac{g}{dl}\right) = \frac{\text{Abs.of test}}{\text{Abs.of standard}}$$

Hematocrit contents, A standard microhematocrit method was needed for the measurement of hematocrit. The capillaries tubes will be used for the collection of Heparinized, non-clot blood. The capillaries tubes will be sealed with wax and will be centrifuged at 12000 rpm for 5 min (Wintrobe, 1967)

$$\text{Hematocrit} = \frac{\text{Column Length of packed red cells}}{\text{Length of whole blood column}} \times 100$$

Antioxidant enzyme activity: Assay of antioxidant enzyme activity were done to assess the antioxidant enzyme activity of Nile Tilapia (Asghar & Masood, 2008). Superoxide dismutase (SOD) was measured by using Tetrazolium salt (Maier & Chan, 2002). Glutathione peroxidase (GPx) activity was measured indirectly by the kinetic colorimetric assay (Paglia and Valentine, 1967). Catalase activity was determined by Catalase assay kit (Weydert & Cullen, 2010).

Statistical Analysis: The data arranged in excel and mean± standard error was calculated. The experiment consisted of 4 different crude protein treatments, each conducted in three replicate aquaria (n=3). The aquarium was considered as experimental unit. The statistical analysis was performed using Statistix version 8.1. The data was subjected to ANOVA under complete randomized design (CRD) and mean were further subject to post hoc test by Tukey HSD (using honest significant difference) ($p<0.05$).

RESULTS

Water quality maintenance and fish survival: During the experimental period, the mean system temperature was 27 °C ± 2 (range 26–30 °C). Dissolved oxygen (DO) concentration averaged 5.2 mg/L (range 4–6 mg/L), and pH averaged 7.06 (range 7.06–8.003), generally within the optimal range for tilapia culture, except for occasional low pH values observed during the trials. Nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_4^+), total dissolved solids (TDS), and total suspended solids (TSS) exhibited fluctuations across tanks with different dietary crude protein (CP) levels (15, 20, 25, and 30%) (Fig. 1). No significant differences ($p < 0.05$) were detected in nitrite, nitrate, or TDS levels among treatments. However, the highest nitrate, nitrite, and TSS concentrations were observed in the 30% CP group (10.06 ± 1.07 mg/L and 694 ± 6.3 mg/L, respectively) (Fig. 1). Floc volume (FV, ml/L) increased significantly throughout the study, with maximum values of 105.9 ± 0.5 ml/L recorded at 20% and 30% CP, though these differences were not statistically significant. Overall, FV development did not differ significantly among treatments ($p < 0.05$) (Fig. 1).

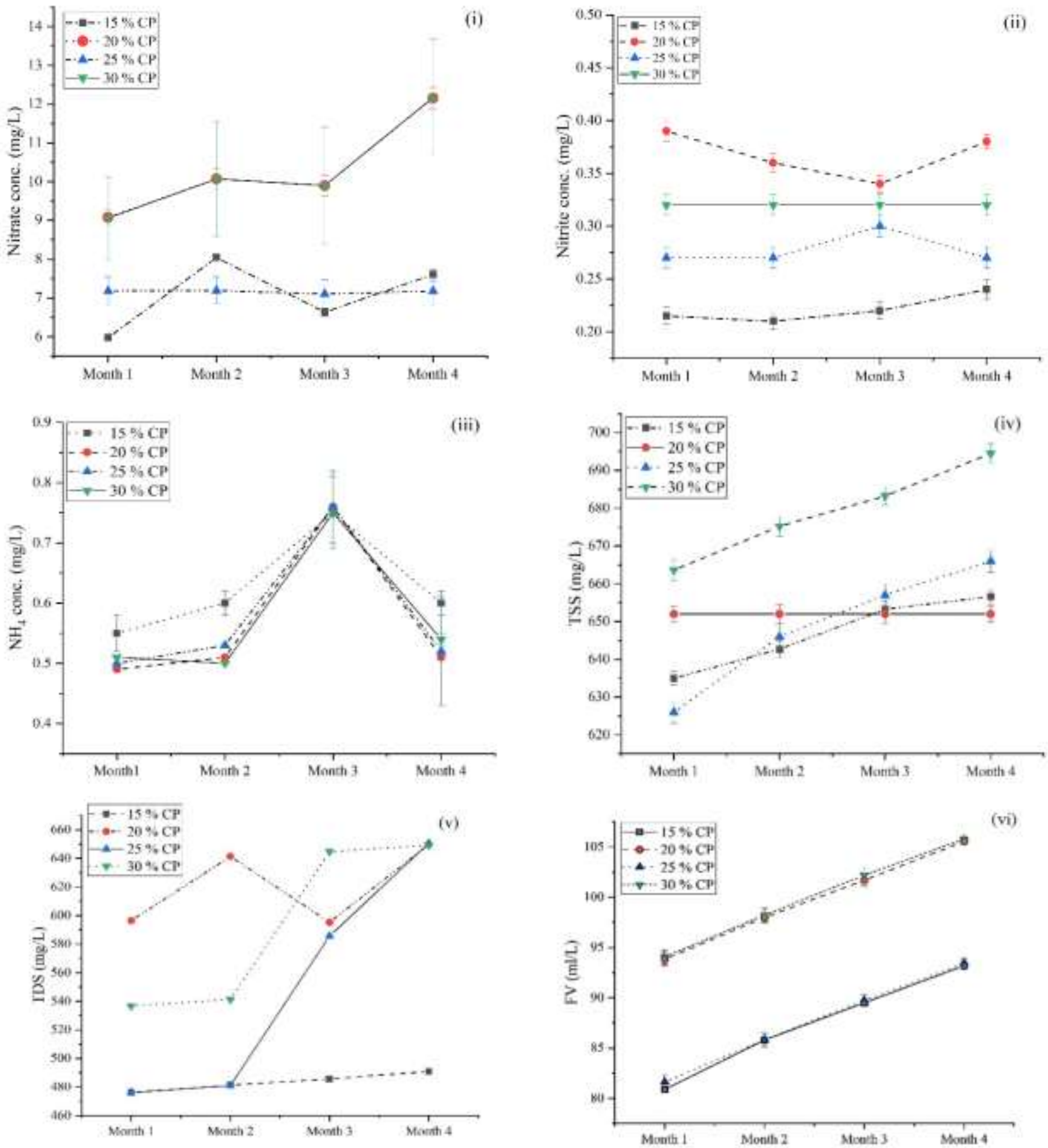

Figure 1: Effect of different crude protein (CP) levels on dissolved inorganic nitrogen concentrations, TSS, TDS and FV of rearing cages throughout the experimental period. Values showed the mean ±S.E of four different CP levels with replication. (i) Nitrate, (ii) Nitrite and (iii) Ammonia, and (iv) TSS= Total soluble solids, (v) TDS= Total dissolve solids and (vi) FV= floc volume.

Proximate composition of experimental diets (%) and Nile tilapia (after trial period): Proximate composition of diet and meat of Nile tilapia (*O. niloticus*) fed with different CP-levels under zero exchange heterotrophic culture system showed (Tab. 2). The nutritional values in the experimental diet (crude protein (CP), crude lipid (CL), ash (%) and dry matter (DM) content percentage) were calculated. The results indicated that highest CP, and DM values, and the lowest CL and ash values were recorded in the diet and fish meat fed with 30 % CP, followed by 25, 20 and 15 % CP under zero exchange heterotrophic culture (Tukey HSD; $p<0.05$).

The results showed that supplementation of CP in diet of Nile tilapia significantly enhanced the proximate composition of fish body (p<0.05). The addition of 30 % CP in diet had more significant impact on Nile tilapia body as compared to other treatments (lower levels of CP). It also improves crude lipid, Ash and DM content of Nile tilapia body followed by 25, 20 and 15 % supplementation of crude protein in diet under zero exchange heterotrophic culture (Tukey HSD: p<0.05) (Tab. 2). The proximate attributes of Nile tilapia body were recorded; CP (47.033±0.46 to 52.633±0.26 %), CL (20.900±0.34 to 26.800±0.40 %), ash (10.433±0.18 to 15.500±0.15%) and DM content (91.877±0.04 to 92.123±0.03 %), after the treatment of four different level of crude proteins in diet (p<0.05) (Tab. 2).

Table 2: Proximate composition of diet and meat of Nile tilapia (*O. niloticus*) fed with different CP-levels under zero exchange heterotrophic culture system.

Proximate composition of diet					
Variables (%)	**15 % CP**	**20 % CP**	**25 % CP**	**30 % CP**	**p-value**
Crude Protein	18.033±0.23^{d}	22.167±0.20^{c}	25.767±0.17^{b}	32.367±0.35^{a}	0.0000
Crude Lipides	20.133±0.29^{a}	19.100±0.23^{ab}	18.267±0.31^{b}	15.867±0.48^{c}	0.0001
Ash	21.100±0.32^{a}	18.133±0.26^{b}	17.400±0.32^{b}	15.067±0.37^{c}	0.0000
Dry Matter	92.123±0.03^{a}	92.410±0.03^{b}	91.430±0.02^{c}	91.877±0.04^{d}	0.0000
Proximate composition of fish body					
Crude Protein	47.033±0.46c	48.833±0.34b	50.047±0.01b	52.633±0.26a	0.0000
Crude Lipides	26.800±0.40a	24.267±0.12b	22.467±0.17c	20.900±0.34d	0.0000
Ash	15.500±0.15a	14.200±0.40b	11.800±0.05c	10.433±0.18d	0.0000
Dry Matter	78.700±0.11a	77.700±0.11b	76.500±0.17c	75.500±0.17d	0.0000

Mean ± S.E with lower case letter showed significance difference among levels of crude proteins on proximate composition of diet and fish (Tukey HSD, p<0.05).

Growth response: Throughout the trial period, the results showed that the survival rate (%) of Nile tilapia (*O. niloticus*) was above 90% in aquaria fed with four different levels of crude protein under zero-exchange heterotrophic culture, and all treatments differed significantly from each other (p<0.05) (Tab. 3). Growth parameters such as final body weight (FBW), weight gain (WG), and fork length (FL) were significantly higher in fish fed with 30% CP compared to the other treatments under zero-exchange heterotrophic culture (Tukey HSD; p<0.05) (Tab. 3), followed by 25%, 20%, and 15% CP levels. The highest weight gain (101.1 ± 1.01 g) was recorded in this group compared to the other treatments (Tab. 3). The specific growth rate (SGR) increased significantly with increasing CP levels from 15% to 30%, with each treatment differing significantly from the others (Tukey HSD; p<0.05). Conversely, a significant decrease in feed conversion ratio (FCR) was observed with increasing protein levels from 15% to 30% (Tukey HSD; p<0.05) under zero-exchange heterotrophic culture. There was no significant difference in FCR between the 15% and 20% CP levels, or between the 25% and 30% CP levels (Tab. 3).

Table 3: Growth parameters of Nile tilapia (*O. niloticus*) fed with different crude protein (CP) levels in zero exchange heterotrophic culture.

Parameters	**15%**	**20%**	**25%**	**30%**	**p-value**
IBW (g)	12.16±0.03^{a}	11.89±0.02^{a}	11.50±0.02^{a}	12.01±0.01^{a}	0.1740
WG (g)	67.70±0.7^{d}	77.30±0.57^{c}	87.60±0.65^{b}	101.1±1.01^{a}	0.0000
FL (cm)	10.33±0.21^{c}	11.00±0.24^{c}	12.10±0.48^{b}	14.50±0.22^{a}	0.0000
FCR	2.28±0.02^{a}	2.25±0.02^{a}	2.04±0.01^{b}	1.98±0.003^{b}	0.0000
SGR	1.75±0.02^{d}	2.07±0.03^{c}	2.14±0.03^{b}	2.27± 0.02^{a}	0.0000
Survival (%)	92.58±0.26^{d}	94.40±0.38^{c}	96.72±0.32^{b}	99.12±0.58^{a}	0.0000

Mean ± S.E with lower case letter showed significance of the treatments on growth parameters (Tukey HSD, p<0.05). IBW= Initial body weight (g), FBW= (Final body weight (g), FL= Final length (cm), FCR=Feed conversion ratio, SGR= Specific growth rate

Innate immunity, blood profile and antioxidants activity: Supplementation with varying levels of crude protein (15, 20, 25, and 30%) under a zero-exchange heterotrophic culture system significantly enhanced innate immunity, hematological parameters, and antioxidant activity in Nile tilapia (O. niloticus) (Tukey HSD; p < 0.05) (Tab. 4). Fish fed 30% crude protein exhibited the highest levels of immunoglobulin (IG), erythrocyte content (EC), hemoglobin (Hb), and hematocrit (Hct), followed by those fed 25%, 20%, and 15% crude protein, respectively (Tukey HSD; p < 0.05) (Tab. 4). No significant differences were observed in WBC counts between fish fed 20% and 25% crude protein (p < 0.05); however, fish fed 30% crude protein showed a lower WBC count (28.900 ± 0.17c %) (Tab. 4).

The supplementation of a 30% crude protein level in the diet significantly enhanced the antioxidant activity of Nile tilapia under zero-exchange heterotrophic culture ($p<0.05$), followed by the 25%, 20%, and 15% crude protein treatments (Tab. 4). Significantly higher lysozyme (LZ, mL^{-1}) and catalase (CAT, µmol/mg) activities were recorded in Nile tilapia fed a diet containing 30% crude protein under zero-exchange heterotrophic culture (Tab. 4). The SOD activity did not differ significantly between the 25% and 30% crude protein treatments, with values of 55.33±0.11 and 57.77±0.76 µmol/mg, respectively (Tukey HSD; $p<0.05$). Similarly, GPx activity after supplementation with 25% and 30% crude protein was not significantly different under the zero-exchange heterotrophic culture system (Tab. 4). The lowest antioxidant activities were recorded in fish fed the 15% crude protein diet: LZ (25.10±0.11 µmol/mg), CAT (27.37 ± 0.53 µmol/mg), SOD (43.13±1.01 µmol/mg), and GPx (3.96±0.13 U/g), which were significantly different from all other treatments under the zero-exchange heterotrophic culture system.

Table. 4: Immunological parameters and antioxidant enzyme activity of Nile tilapia (*O. niloticus*) fed with different crude protein (CP) sources under zero exchange heterotrophic culture system.

Variables	15%	20%	25%	30%	p-value
IG (mg/ml)	11.600±0.11^{d}	12.380±0.16^{c}	13.867±0.14^{b}	14.900±0.17^{a}	0.0000
WBCs (×10^{3} cell/ul)	31.40±0.15^{b}	31.96±0.24^{b}	32.23±0.08^{b}	33.73±0.16^{a}	0.0000
EC (×10^{6} cell/ul)	1.733±0.008^{d}	2.516±0.02^{c}	2.883±0.02^{b}	3.140±0.01^{a}	0.0000
Hb (g/dL)	6.333±0.08^{d}	7.400±0.05^{c}	9.167±0.14^{b}	10.800±0.17^{a}	0.0000
Hct (%)	24.067±0.14^{d}	29.033±0.26^{c}	33.000±0.17^{b}	38.033±0.20^{a}	0.0000
LA (mL^{-1})	25.100±0.11^{d}	28.400±0.17^{c}	31.033±0.14^{b}	36.200±0.17^{a}	0.0000
CAT (umol/mg)	27.367±0.53^{c}	29.533±0.34^{b}	30.633±0.12^{b}	33.567±0.53^{a}	0.0000
SOD (umol/mg)	43.133±1.01^{c}	48.133±0.84^{b}	55.33±0.11^{a}	57.766±0.76^{a}	0.0000
GPx (U/g)	3.956±0.13^{b}	4.263±0.18^{b}	5.160±0.02^{a}	5.586±0.19^{a}	0.0000

Mean ± S.E with lower case letter showed significance of the treatments on immunological parameters and antioxidants ability of Nile Tilapia (Tukey HSD, $p<0.05$). IG (mg/ml) = Immunoglobulin, WBCs= White blood cells, LA= Lysozyme activity, EC= Erythrocyte content, Hb= Hemoglobin content, Hct= Hematocrit.

DISCUSSION

The present study aims to check the effect of different protein levels on the growth, immunity and meat quality of Nile Tilapia under zero exchange heterotrophic culture. The experiment consists of four different experimental groups (CP at 15, 20, 25 and 30% level) each replicated three times (n=3) each with stocking density of 50 fish/m^3. The juvenile of Nile tilapia fed daily basis and physicochemical parameters chemical parameters were checked regularly and maintained during the study period. The pH, temperature and DO were not statistically significant from each other among experimental treatments. The current study showed that floc volume (FV) (ml/L) was significantly higher in treatment T4 (30% CP) and recorded as 105.9±0.5 ml/L. This may be due to degradation of carbon and nitrogen components of floc, which provide large surface area for bacterial and other microorganisms growth, hence enhanced biofloc volume. The present study is in agreement with Zahra and Mateen (2025), who reported that the addition of 25% crude protein with a higher carbon-to-nitrogen ratio significantly improved bacterial biomass in biofloc technology (BFT) systems. Moreover, higher CP may contribute to increased nitrogenous substrate in the culture water, which, in the presence of carbon sources, can enhanced heterotrophic microbial growth and subsequent floc formation. These biofloc not serve as biological filters but also act as supplemental nutrients sources for the culture of Nile tilapia (Li *et al*., 2025; Yadav *et al*., 2025). The current study agreement with Mansour and Esteban (2017) stated that higher carbon and different protein level supplementation enhanced biofloc particles as well as water quality parameters. The study also agreement with Ferreira *et al*. (2015) who started that increase in surface area and substrate for microbial action enhanced the water quality as well as the availability of food, which also impact the fish growth.

The results showed that a higher level of NH_4 was recorded during 3rd month and a decline in the last month of the experimental period, while values of ammonia were not statistically significant among the treatments (15, 20, 25 and 30 % CP levels). The nitrate values increased steadily throughout the study period and higher NO^{-3} level were recorded in aquarium fed with 30 % crude protein (Fig 1). The raising of dissolve inorganic nitrogen by bacterial activity during recycling defecate a substantial amount of nitrogenous ammonia by fish (Burford *et al*., 2004; Crab *et al*., 2010) and bacteria are usually associated with the suspended biofloc particles, providing extra nutrients and exogenous digestive enzymes (Moreno-Arias *et al*., 2017; Wang *et al*., 2015) and stimulating growth and survival (Abduljabbar *et al*., 2015; Ekasari *et al*., 2015).

The TSS and TDS were significantly enhanced in all the aquarium fed with different crude proteins and all the treatments were significantly different from each other's except 20 % CP level whereas the TSS value remained constant throughout experimental period (Fig 1). In the current study the TDS value remains between 626 ±6.8 to 694.5± 2.16 mg/L. The increase in TSS and TDS consistent with recent findings Abd-El Azeem *et al*. (2025) who proposed that diet composition directly influences water quality parameters. Elevated protein diets have been associated with greater dissolved nitrogenous compounds and solids in culture system due to increased waste production and minerals released from feed breakdown (Wani *et al*., 2025). The study agreement with Long et al. (2015) who proposed that total dissolve solid amount reached to 1000 mg/L during the study period. The da Silva *et al*. (2018) reported all treatments of proteins significantly impact the development and formation of biofloc in water aquariums, and gradual increase in TSS and BFV were recorded during the study periods. Furthermore, he stated that the amount of BFV in the treatment T4 was not statistically different from the amount of TSS after 15 days of experiment. The constant aeration and varied conditions keep the level of biofloc constant and stable in the water column (Xu & Pan, 2012).

The current study showed that the survival rate (%) of *O. niloticus* was above 90% in aquariums fed with four different levels of crude protein under zero exchange heterotrophic culture, and all the treatments were significantly different ($p<0.05$) from each other. The highest survival % Nile tilapia was recorded (99.123±3.74a) in an aquarium fed with 30 % crude protein (CP). Similar to the current study Mansour and Esteban (2017) stated that the survival percentage of Nile tilapia was recorded 90 to 100 % throughout study period, fed with 20 and 30 % crude protein or carbon sources (Zahra & Mateen, 2025). Azim and Little (2008) also reported that the Nile tilapia survival rate was recorded 100 % when BFT fed a diet of 24 and 35% crude protein (CP). In an aquarium fed with 30 % CP showed a higher growth rate and lower FCR than the other treatments under zero exchange heterotrophic system (Table 3). Similar findings were reported in recent studies on biofloc-based and heterotrophic systems, where moderate protein-based diets supplementation results in superior growth, survival and lower FCR ration due to improved nutrient recycling and enhanced microbial activity. For example, Ediwarman *et al*. (2025) who proposed that the feed containing 30 % protein yielded the best production performance, including higher growth rate and the lowest Feed Conversion Ratio (FCR) in a zero-water exchange culture system (Adineh *et al*., 2025; Yılmaz *et al*., 2024).

For optimal growth of fish, high quality of feed has been utilized in recent aquaculture approaches which is not just impact growth but also maintain antioxidant status, physiology and immunological parameters of cultured fish (Kiron, 2012). The current study showed that higher value of immunoglobulin (IG), erythrocyte content (EC), hemoglobin (Hb) and hematocrit (Hct) content were significantly enhanced by the supplementation of 30% crude protein level, followed by 25, 20, and 15 % of crude protein levels, respectively, under zero heterotrophic culture system (Tukey HSD, $p<0.05$). Like current study Wang *et al*. (2024) proposed that increasing dietary protein supplementation evaluated the key immune function (i.e. immunoglobulin concentrations, lysozyme activity and innate immune defense) in Juvenile Hybrid Sturgeon. Long *et al*. (2015) stated that Nile tilapia showed improvement in immune health after the supplement of carbon in form of glucose. However, he also stated that there was no significant difference in WBCs, red blood cell, hematocrit or hemoglobin levels as compared to fish reared in clear water. While Mansour and Esteban (2017) stated that fish reared under BFT showed higher WBC counts than fish maintained in clear water. Furthermore, the increased number of WBCs was due to the increase in both neutrophils and lymphocytes, while monocyte numbers remained unchanged. The number of leucocytes in blood indicates the health status of fish and they play a vital role in innate immunity during inflammation and stress (Klak *et al*., 2024; Philominal *et al*., 2024; Secombes, 1996; Soliman & Barreda, 2023).

The increase in lysozyme activity (LA), CAT, SOD and GPx activity were recorded after the supplementation of different CP levels under zero exchange heterotrophic culture system and the maximum activity of CAT, SOD and GPx was recorded in Nile tilapia fed with 30 % CP. This showed that higher CP level promotes antioxidant defense in Nile Tilapia. Abd-El Azeem *et al*. (2025) also reported that 30 and 35% protein in diet significantly enhanced the antioxidant activity and immune parameters (lysozyme activity and IgM) in Nile tilapia (Sherif *et al*., 2024). Similar to the current study, the Rind *et al*. (2023) stated that different protein levels and carbon sources together significantly improved the SOD and CAT activity in *O. niloticus* ((Mansour & Esteban, 2017).The maximum activity of both enzymes was noticed in fish fed with 30% protein. At protein levels 20 and 30% in integration with carbon sources showed compensating effect of biofloc was manifest in case of catalase activity. He further stated that maximum performance of antioxidant enzymes was noticed in fishes fed with 30 % of protein as compared to control, only contradiction is that the supplementation sources and condition were different from present study (Table 4). CAT and SOD are enzymes linked with the lipid peroxidation inhibition (Gulcin, 2025). The superoxide dismutase involves in the catalysis of superoxide anion to produce H_2O_2 (Tao *et al*., 2013), furthermore the catalyze enzyme acted and converted it into oxygen and water and inhibit the initiation of lipid peroxidation (Tavares-Sánchez *et al*., 2004). The dietary protein level (greater than 26%) significantly enhanced the hepatic SOD and CAT activity and gene expression of other enzymes (Kou *et al*., 2025). Faisal *et al*. (2025) also proposed that dietary purslane extract enhanced the CAT and SOD performance in *Labeo catla* fingerlings. These studies showed that high CP levels influence CAT and SOD activities in fish, reduce oxidative stress, and may improve fish welfare. These studies showed that high CP levels influence CAT and SOD activities in fish, reduce oxidative stress, and may improve fish welfare (Parrilla-Taylor *et al*., 2013; Salamanca *et al*., 2025). Verma *et al*. (2016) proposed that BFT treatment had a strong impact on fish stress as it decreases physiological stress (glucose and cortisol level) in *Labeo rohita*. Similarly, another study lined with current results the shrimps feed on low amount of protein exhibited minimum antioxidant capacity as compared to those reared under zero exchange heterotrophic culture system (Xu & Pan, 2014).

Conclusion: In conclusion study proposed that Nile Tilapia fed with 30% Crude protein had excellent growth performance, antioxidant abilities and efficient immune system. This optimum protein level was essential for maximizing biofloc volume and development of micro fauna on which Nile tilapia feed. The lower level of FCR at 30 % CP level means that feed is efficiently converted into fish weight. Specifically, diets with 25 and 30% crude protein content yielded the best growth, immune response and antioxidant activity, demonstrating the importance of crude protein supplementation in zero heterotrophic exchange system. Furthermore, it is suggested that a cost-effective product can be formulated through industrial linkages, and stakeholders should play their role in this regard.

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