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RESEARCH PAPER

Influence of Varied Carbon Sources and Carbon-Nitrogen Ratio on Microbial Community in a Biofloc-Based Culture System and Their Effects on Growth Performance, Proximate Composition, Hematology and Oxidative Stress in Nile Tilapia

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Abstract

This study investigates the influence of varied carbon sources and carbon-nitrogen ratio on microbial community in a biofloc-based culture system and their effects on growth performance, proximate composition, hematology, and oxidative stress in Nile tilapia. The experiment involved three different carbon-nitrogen ratios (10:1, 15:1, 20:1) and two carbohydrate sources (molasses: ML-10, ML-15, ML-20, starch: S-10, S-15, and S-20) with three replicates each. A diet containing 25% crude protein was fed to fish in 18 experimental units for 120 days. Carbohydrate supplementation significantly increased the total heterotrophic bacterial (THB) count from C/N10 to C/N20 while decreasing pH, total ammonia nitrogen, and nitrate nitrogen contents. In addition, growth was considerably greater in the ML-10 and ML-15 groups compared to the C/N20 group. Increasing the C/N ratio increased body protein and ash, decreased lipid contents, and increased the activities of lysozyme, SOD, CAT, and GPx. Finally, the C/N10 and C/N15 groups showed significantly lower GLU and COR concentrations in biofloc compared to the C/N20 group. Furthermore, increasing the C/N ratio up to 15 resulted in improved outcomes in terms of Hb, WBCs, RBCs, and Ht content. Overall, the utilization of the C/N15 group resulted in suitable growth, proximate composition, and immunity in Nile tilapia.

Introduction

Aquaculture is critical to the production of sustainable seafood, but its growth is challenged by land and water shortages, rising feed prices, and environmental concerns (Shourbela et al., 2021). As a result, intensive or super-intensive operations have replaced conventional methods in the aquaculture industry across the world to overcome major difficulties and boost production capacity (Mohammadi et al., 2021). In this aspect, the "biofloc" system functions as a nutrient retention trap, lowering the expense of maintenance as well as providing dietary additives for marketable organisms being raised. This fast expansion of aquaculture has been termed the blue revolution as it contributes to human nutrition and food security (Rind et al*.,* 2023).

The biofloc technique has sparked considerable attention in aquaculture due to its capacity to provide a sustainable and economically viable substitute for traditional aquaculture operations. The key idea behind this technique is to construct a nitrogen cycle by continually aerating the water and adding carbon sources with the purpose of maintaining the carbon-to-nitrogen ratio in an aquaculture system that has stagnant water and fostering the growth of heterotrophic microorganisms (Luo et al., 2020). The *bacillus sp* are responsible for the digestion of nitrogenous waste, which is then utilized as a feed source for the cultivated species. This system can maintain a high concentration of microbial floc in the water while controlling water quality characteristics, which include temperature, pH, and level of dissolved oxygen (Dauda, 2020).

The carbon-nitrogen ratio (C/N) is crucial for optimal biofloc performance, as it minimizes nitrogen buildup and produces microbial communities (Minabi et al., 2020). Increased C/N ratios in the culture system promote heterotrophic bacteria and natural products (Polysaccharides, microbial protein, lipids and enzymes) increasing oxygen demand and reducing pH levels. To control water quality and regulate suspended particles, it is common to use low-cost, locally accessible carbon sources to improve the C/N ratio above 10:1. An external carbon source is required for the effective development of biofloc microorganisms, boosting bacterial biomass, and reducing waste nitrogenous metabolites. To lower settling rates and make carbonated organic matter more accessible, it should be soluble in water or in powdered form (Saha et al., 2022).

To maintain a suitable C/N ratio and facilitate the proliferation of heterotrophic microorganisms, a suitable supply of carbohydrate sources must be provided. Biofloc technology is highly influenced by the availability and composition of this carbon source (Silva et al., 2017). Common carbohydrates in aquaculture, such as glucose, acetate, starch, wheat, glycerol, and molasses, are often used in BF systems to promote floc formation (Ulloa Walker et al., 2020). Biofloc properties, including floc volume, biochemical compounds, and capacity to hold bioactive constituents, are influenced by the type of carbon source and their ratio, which in turn affects the productivity of farmed fish (Dilmi et al., 2021).

Carbon sources and the ratios employed in biofloc technology influence a variety of essential aquaculture variables, including growth rates, feed conversion ratios, and organism's health (Li et al., 2018). The microbial floc composition and quality might vary depending on the carbon sources and C/N ratios employed to form it, affecting its viability as a natural source of food for cultured organisms (Ahmed et al., 2017). According to current research, the kinds of carbohydrate sources and C/N ratios used in biofloc technology may have an effect on the immunity and disease resistance of the fish populations (Ahmad et al., 2019). Microbial communities found in biofloc technology may benefit fish digestive enzyme performance and act as a natural probiotic, increasing the overall immunity of fish (Emerenciano et al., 2013).

Nile tilapia is a valuable freshwater fish species that is grown across various countries worldwide because to its remarkable growth characteristics, ease of adaption to intensive culture, and ability to consume artificial feeds (Khanjani et al., 2021). Furthermore, Nile tilapia has an extensive ability to feed on plankton, detritus, suspended particles in the water column, and microbial masses (Abdel-Tawwab, 2011). As a result, Nile tilapia may grow intensively in a BFT-based system where it has ability to utilize biofloc biomass (Durigon et al., 2020). Carbon sources addition serves as the backbone for establishing the system, with a C/N ratio of 10:1 or above ensuring an activation of the heterotrophic bacteria (De Schryver et al., 2008). Silva et al. (2017) indicate that a C/N ratio of around 15:1 is suitable for bacterial growth. The bacteria have the ability to intake waste nitrogen and recycle it into microbial protein, which is used as a supplemental feed source for species such as tilapia, carp, and shrimp. As a result, water quality and fish culture performance improved (Crab et al., 2010). Moreover, the various carbon sources employed in the BFT-based system have distinct impacts on growth rate, proximate analysis, antioxidant capacity, and immune responses of diverse aquatic species (Khanjani and Sharifinia, 2020). However, the goal of this experiment was to investigate the effects of different C/N ratios and carbon sources on the dominance of heterotrophic bacteria, water quality, biofloc biomass and the health of Nile tilapia fingerlings.

Materials and Methods

Experimental Design

The experiment was carried out at the Fish Nutrition Laboratory, Department of Zoology, Wildlife, and Fisheries, University of Agriculture, Faisalabad. Fingerlings were taken from the Fisheries Research Farm, University of Agriculture, Faisalabad. The fish were acclimatized in laboratory conditions for 7 days before beginning the experiment feed with commercial diet. Commercially available molasses and starch was utilized in this experiment. There were six treatments with different C/N ratios (10:1, 15:1, 20:1) and two carbohydrate sources (molasses: ML-10, ML-15, ML-20; and starch: S-10, S-15, S-20) each with three replications. One hundred eighty Nile tilapia (*Oreochromis niloticus*) were dispersed in 18 rectangular glass aquariums (length 65cm, width 47cm, height 33cm); each aquarium had a capacity of 100L and was filled with 80L of water. During the experiment, no water was changed; instead, water loss from evaporation in the culture aquarium was replenished to maintain the level of 80L. Each aquarium was supplied with ten fingerlings with an initial weight of 0.8±0.1g. The experiment continued for 16 weeks.

Floc Preparation and Carbohydrate Supplementation

The inoculum was produced in accordance with the methodology outlined by Avnimelech (1999) in a separate aquarium one week before the initiation of the trial. In this research, with 1,440L total water in 18 aquariums (each contain 80L), 14.4L inoculum was required. To prepare this inoculum, a separate clean aquarium was filled with 15 liters of water by adding 288g pond soil, 0.144g ammonium sulfate/urea, and 2.88g carbon sources. The produced inoculum was then introduced to the appropriate experimental aquariums. The water in each aquarium was aerated and agitated continuously using porous stone with length of 7.6cm (3 inches) connected to a 100-W air pump (YAMANO, LP-100).

Molasses and starch, readily accessible carbon sources, were employed to maintain C/N ratios of 10:1, 15:1, and 20:1 in treatments CN10-CN20, respectively. For that purpose, the total utilized feed (TUF) per day in each replicate was determined. Total Feed Protein (TFP) was calculated by multiplying TUF by 0.25 (feed protein %). According to Crab et al. (2012) 16% of protein is nitrogen, Total feed nitrogen (TFN) was determined by multiplying TFP by 0.16. On average, 75 percent of the feed nitrogen is excreted in the water, hence the excreted feed N (EFN) was calculated by multiplying the TFN by 0.75. To achieve the desired C/N ratios in water, EFN was multiplied by 10, 15 and 20 in each treatment. Molasses and starch contain 39% and 89% organic carbon, respectively, therefore divide the carbon amount by 0.39 and 0.89 to find the amount of each. Molasses and starch was mixed into the water in two separate containers for each treatment and added to the water daily, 2 hours after the morning meal. The carbon in these carbs allows heterotrophic bacteria to proliferate and hence preserving water quality.

Feeding

In this investigation, sinking feed was used. The feed's proximate composition was as follows: 89.93% dry matter (DM), 6.11% ether extract (EE), 25.16% crude protein (CP), and 7.12% ash. Feed was provided to the fingerlings of Nile tilapia, containing 25% crude protein at 4% of their body weight. Fish weight gain was considered while adjusting the amount of feed delivered after each sampling and growth monitoring. Fish weight gain was measured fortnightly, although daily inspections were made to check survival rates.

Assessment of Water Quality

Water quality viz. temperature (°C), pH, and DO (mgL^{-1}) using a thermometer (TP-101), pH meter (HANNA HI98107), and DO meter (HANNA HI9146), was monitored regularly. Total ammonia nitrogen (TAN), (NO_3), and (NO_2) were measured twice a week according to standard protocols (APHA, 2005). Floc volume and TSS were determined weekly using the Imhoff cone following 15 minutes of sedimentation and the evaporation process throughout the experiment (Avnimelech, 1999).

Determination of Microbial Biomass

Water samples of biofloc were taken for the quantification of total heterotrophic bacteria at the end of the experiment. Sterile poly-propylene bottles were used to take water samples from the tank's centers. The samples of water were promptly shifted to the lab and stored at 4°C in the refrigerator. After homogenizing 200 milliliters of the sample, it was diluted tenfold in normal saline solution (NSS). To figure out the total number of heterotrophic bacteria, 0.1 milliliters of the corresponding dilutions were plated on Zobell marine agar. Plates were kept in an incubator at room temperature for 24 hours, and colonies were enumerated and quantified as CFU/mL (Dosta et al., 2015).

Growth Performance

Growth parameters, i.e., weight gain (g), FCR, SGR (%), and survival (%), were measured fortnightly and calculated according to the formula described by Manduca et al. (2021). Average daily weight gain (g/day) and Protein efficiency ratio were also determined according to the following formulas:

$$\text{Average daily weight gain (ADWG)} = \frac{\text{FBW-IBW}}{\text{Duration (days)}}$$

where, FBW= Final Body Weight; IBW= Initial Body Weight

$$\text{Body weight gain (WG)} = \text{final fish weight } (W_F) - \text{initial fish weight } (W_I)$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed intake (g)}}{\text{Total weight gain (g)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}}$$

$$\text{Specific growth rate (SGR)} = (\ln W_F - \ln W_I) \times 100 / \text{duration period}$$

$$\text{Survival (\%)} = \frac{\text{Final fish number}}{\text{Initial stock number}} \times 100$$

Proximate Analysis

After the end of the experiment, the proximate analysis was determined using conventional methods given by AOAC (2012), including the Kjeldahl method for crude protein content. To determine the amount of ash, samples were burned for four hours at 600°C and Soxhlet extractor was used to determine the ether extract.

Biochemical Measurement

At the end of experiment, fish were sedated with clove oil (commercially available; 100% pure) by using 50-100mg/L of clove oil (0.5–1.0mL) per 10 liters of water according to Ross and Ross (2009). For fish weighing 56-63g, this dosage was effective for 5-10 fish per 10-liter. For biochemical examination, and samples of blood were obtained through caudal veins and deposited in eppendorf tubes with heparin-anticoagulant. (RBCs) and (WBCs) were counted in accordance with standard procedure (Rusia and Sood, 1992). The concentration of hemoglobin (Hb) was quantified spectrophotometrically using the cyano-methemoglobin technique (Blaxhall and Daisley, 1973). The centrifugation method was used to determine hematocrit (HCT) (Wintrobe, 1967).

Oxidative Stress Parameters

The fish liver samples were taken, washed away, and stored at -60ºC to measure the levels of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) spectrophotometrically (Luck, 1974; Pedrajas et al., 1995). The serum lysozyme activity (LZM) was assessed using a turbidimetric assay in accordance with Ellis's (1990). To measure the levels of the serum cortisol (stress hormone), an immunosorbent test kit that is enzyme-linked was used (ELISA) (Chiu et al., 2003). An automatic biochemical analyzer (Hitachi 902) was used to assess glucose (GLU) (Trinder, 1969).

Statistical Analysis

A completely randomized 2x3 factorial design with two carbohydrate sources (molasses, starch) and three carbon-to-nitrogen ratios (10:1, 15:1, and 20:1) with three replications each. A two-way ANOVA was used to determine significant differences (P<0.05) across treatments. Tukey's test was employed for comparison of treatment means.

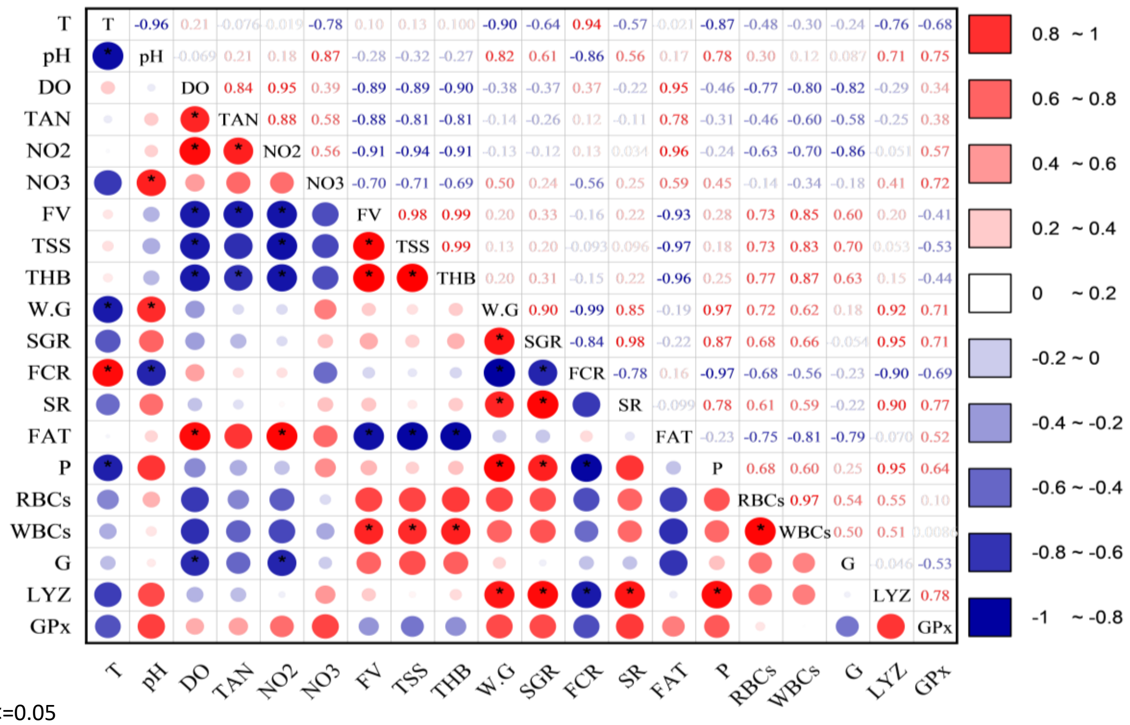
Results

Water Quality Parameters

The temperature remained reliable among treatments (28.23-28.53°C), whereas the pH varied significantly (7.16-7.42). Starch treatments had greater levels of dissolved oxygen (DO), ranging from 5.07 to 5.28 mg/L. Total ammonia nitrogen (TAN) levels dropped with increasing C/N ratios, with values of 0.45, 0.41, and 0.37mg/L, particularly in molasses. Nitrite (NO_2) concentrations decreased with higher C/N ratios (0.17-0.25 mg/L). Nitrate (NO_3) levels were lower in starch treatments (12.5-9.6mg/L) (Figure 1). Floc volume (FV) rose with higher C/N ratios (18.2-23.7mL/L for molasses), and total heterotrophic bacteria (THB) counts were higher in molasses (245-432 CFU/mL) than in starch (222-354 CFU/mL), indicating more microbial activity in molasses as shown in Table 1.

Growth Profile

The growth profile of Nile tilapia (*Oreochromis niloticus*) is shown in Table 2. Molasses treatments, particularly at a 15:1 C/N ratio, showed improved results, including a weight gain (63.6g), specific growth rate (3.71%/day), and reduced feed conversion ratio (1.31). Starch treatments resulted in greater FCR and



* p<=0.05

Figure 1. Spearman correlation between water quality parameters, fish health and biometrics. According to the Pearson correlation analysis, a close relationship was observed between the water quality parameters, total heterotrophic bacteria, growth performance, proximate composition, hematology and oxidative stress of Nile tilapia. A dot plot is used to depict the correlations between these parameters in the biofloc-based raising of *O. niloticus*. The colour variation indicates the level of correlation between them, as determined by Pearson's correlation coefficient. The colour intensity is represented in the right column, ranging from -1.0 to 1.0. The values represented by dots are considered to have insignificant p-values. T: Temperature, DO: Dissolved oxygen, TAN: Total ammonia nitrogen, NO_2: Nitrite, NO_3: Nitrate, FV: Floc volume, TSS: Total suspended solids, THB: Total heterotrophic bacteria, WG: Weight gain, SGR: Specific growth rate, FCR: Feed conversion ratio, SR: Survival, P: Protein, RBC: Red blood Cells, WBC: White blood Cells, G: Glucose, LYZ: Lysozyme, GPx: Glutathione peroxidase.

Table 1. Water quality parameters of Nile Tilapia culture in biofloc tanks with varying carbon to nitrogen ratios and carbohydrate sources for 16 weeks.

Variables	C/N Ratios	Carbon sources	
		Molasses	Starch
[1]Temp (^{0}C)	10:1	28.23±0.35	28.36±0.35
	15:1	28.26±0.31	28.43±0.43
	20:1	28.33±0.57	28.53±0.53
pH	10:1	7.42±0.09	7.31±0.06
	15:1	7.39±0.07	7.22±0.07
	20:1	7.27±0.06	7.16±0.07
[2]DO (mg L^{-1})	10:1	5.18±0.12	5.28±0.15
	15:1	5.10±0.10	5.24±0.13
	20:1	5.07±0.12	5.15±0.10
[3]TAN (mg L^{-1})	10:1	0.45±0.10	0.63±0.13
	15:1	0.41±0.10	0.44±0.10
	20:1	0.37±0.0	0.39±0.10
[4]NO_2 (mg L^{-1})	10:1	0.25±0.09	0.31±0.07
	15:1	0.22±0.06	0.27±0.07
	20:1	0.17±0.05	0.21±0.05
[5]NO_3 (mg L^{-1})	10:1	13.3±0.18	12.5±0.23
	15:1	11.5±0.24	10.6±0.20
	20:1	10.5±0.17	9.6±0.25
[S6]FV (ml L^{-1})	10:1	18.2±0.32	16.3±0.31
	15:1	22.4±0.20	20.4±0.23
	20:1	23.7±0.37	21.5±0.23
[7]TSS (mg/L)	10:1	245±1.45	222±1.15
	15:1	366±1.15	298±1.15
	20:1	432±1.15	354±1.51
[8]THB (CFU/ml)	10:1	19.05±0.10	18.05±0.11
	15:1	24.60±0.12	21.32±0.11
	20:1	25.82±0.10	23.25±0.10

There were no notable variations found in the mean values. Temp = [1]Temperature, [2]DO =Dissolved oxygen, [3]TAN = Total ammonia nitrogen, [4]NO_2 = Nitrite, [5]NO_3 = Nitrate, [6]FV = Floc volume, [7]TSS = Total suspended solids, [8]THB = Total heterotrophic bacteria

reduced growth. Molasses treatments had the highest survival rates (up to 98.06%), while starch yielded reduced survival at higher C/N ratios. Overall, molasses was more successful at increasing fish growth and feed efficiency. The positive relationship between weight gain and body protein content is represented in Figure 2.

Proximate Composition of fish

The proximate body composition of fish was noted after the end of the experiment. Table 3 shows that fish fed molasses had higher crude protein (CP) levels, maximum at 59.5% at a C/N ratio of 15:1, compared to lower values for starch. Ether extract (EE) was also higher in molasses (11.6%) and starch (12.3%) at a C/N ratio of 10:1, but ash levels increased with higher C/N ratios, reaching 24.6% for molasses at 20:1. Molasses boost protein and ether extract levels in Nile Tilapia.

Proximate Analysis of Biofloc

The maximum crude protein (CP) was 32.3±0.16 at a 15:1 C/N ratio with molasses, and the highest ether extract (EE) was 3.66±0.14 with starch at a 10:1 ratio, as indicated in Table 4. The ash level in the molasses biofloc reached 33.7±0.25 at a 20:1 ratio. There were no significant differences seen across values with the same letter ($P<0.05$).

Hemato-biochemical Indices

Fish fed with molasses at a 15:1 C/N ratio had the highest RBC count ($3.39±0.10×10^6$ μL^{-1}) and hemoglobin levels (12.5±0.10 g/dL). In contrast, with a C/N ratio of 10:1, the starch group had the lowest RBC and hemoglobin levels. Overall, the results show that carbohydrate supply and C/N ratio have a substantial impact on the fish's hemato-biochemical health (Table 5).

Oxidative Status and Non-Specific Immunity

At a 15:1 C/N ratio with molasses, the highest levels of catalase (CAT) and superoxide dismutase (SOD) were observed, with respective values of 36.1±0.11 U/mg and 56.5±0.11 U/mg (Table 6). Furthermore, at 15:1 C/N ratio (48.3±0.10 U/mg), glutathione peroxidase (GPx) activity increased (Figure 3). In comparison to other treatments, the 15:1 C/N ratio had the highest lysozyme activity, which is a measure of immunological function (Figure 3D).

Serum Glucose and Cortisol Activity

Serum glucose and cortisol activity were noticed after the experiment (Table 7). There was a significant difference ($P<0.05$) in the mean values of GLU among different treatments. A 20:1 C/N ratio with molasses

Table 2. Growth profile of Nile Tilapia raised in biofloc tanks with varying carbon nitrogen ratios and carbon sources for 16 weeks

Variables	C/N Ratios	Carbon sources	
		Molasses	Starch
[1]IBW (g)	10:1	0.81±0.17	0.84±0.14
	15:1	0.78±0.22	0.75±0.13
	20:1	0.77±0.11	0.74±0.14
[2] ADWG	10:1	0.54 ± 0.11	0.52 ± 0.13
	15:1	0.57 ± 0.12	0.52 ± 0.12
	20:1	0.54 ± 0.14	0.48 ± 0.11
[3]FBW(g)	10:1	62.2±0.11	59.6±0.14
	15:1	64.4±0.11	60.1±0.11
	20:1	61.4±0.14	56.8±0.11
[4]WG (g)	10:1	61.4±0.17^{b}	58.7±0.11^{d}
	15:1	63.6±0.13^{a}	59.3±0.12^{d}
	20:1	60.6±0.14^{c}	56.05±0.16^{e}
[5]SGR (%/day)	10:1	3.62 ±0.01^{ab}	3.57±0.01^{b}
	15:1	3.71 ±0.02^{a}	3.63±0.02^{ab}
	20:1	3.60 ±0.03^{ab}	3.55±0.03^{b}
[6]FCR	10:1	1.36±0.05^{b}	1.47±0.02^{ab}
	15:1	1.31±0.01^{b}	1.45±0.02^{ab}
	20:1	1.39±0.04^{ab}	1.57±0.03^{a}
[7]PER	10:1	2.93 ± 0.01	2.83 ± 0.03
	15:1	2.94 ± 0.03	2.88 ± 0.02
	20:1	2.89 ± 0.01	2.78 ± 0.02
Survival (%)	10:1	89.2±0.60	87.2±0.55
	15:1	98.06±0.69	91.5±0.58
	20:1	87.5±0.41	83.93±0.56

There were no notable variations found in the mean values that shared the same letter in the same row ($P < 0.05$). [1]IBW = Initial body weight, [2]ADWG= Average daily weight gain, [3]FBW = Final body weight, [4]WG = Weight gain, [5]SGR = Specific growth rate, [6]FCR = Feed conversion ratio, [7]PER= Protein efficiency ratio

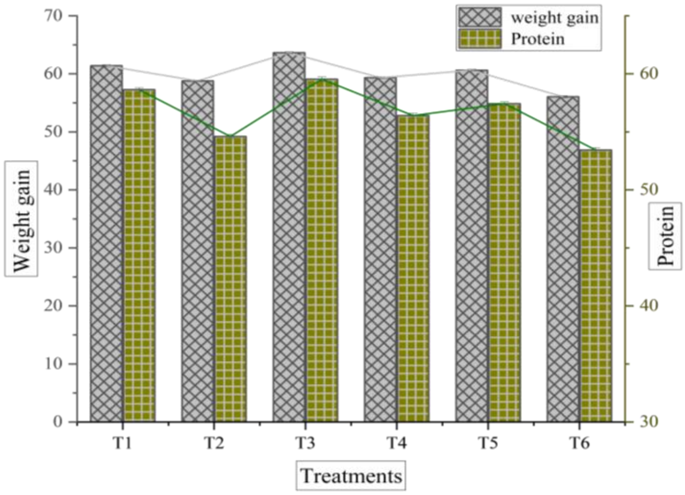


Figure 2. There is a general positive interaction between weight gain and body protein content. As the body protein content increases, the weight of the body also increases. There are discrepancies in the pattern, particularly during the period of maximum weight gain, where the rise in protein is not proportional. The overall graph demonstrates an interaction, but it is not strictly linear. It is likely that other factors have also an influence on the body weight.

Table 3. Proximate body composition of Nile Tilapia raised in biofloc tanks with varying carbon nitrogen ratios and carbohydrate sources for 16 weeks

Variables	C/N Ratios	Carbon sources	
		Molasses	Starch
[1]CP (%)	20:1	58.6±0.17^{b}	54.6±0.17^{e}
		59.5±0.20^{a}	56.4±0.20^{d}
		57.4±0.14^{c}	53.4±0.20^{f}
[2] EE (%)	20:1	11.6±0.17^{a}	12.3±0.20^{a}
		9.2±0.14^{b}	11.6±0.17^{a}
		8.3±0.14^{c}	9.63±0.17^{b}
Ash (%)	20:1	18.6±0.14^{d}	17.5±0.17^{e}
		21.6±0.15^{b}	18.3±0.17^{d}
		24.6±0.14^{a}	20.4±0.14^{c}

There were no notable variations found in the mean values that shared the same letter in the same row ($P < 0.05$). [1]CP = Crude protein, [2] EE = Ether extract

Table 4. Proximate analysis of biofloc using varied carbon nitrogen ratios and carbohydrate sources for 16 weeks.

Variables	C/N Ratios	Carbon sources	
		Molasses	Starch
[1]CP (%)	10:1	28.5±0.14[b]	23.2±0.15[e]
	15:1	32.3±0.16[a]	26.3±0.17[d]
	20:1	27.3±0.17[c]	22.2±0.16[f]
[2]EE (%)	10:1	3.34±0.13[a]	3.66±0.14[a]
	15:1	3.25±0.11[ab]	3.45±0.14[a]
	20:1	3.18±0.10[ab]	2.67±0.11[b]
Ash (%)	10:1	27.6±0.22[c]	25.5±0.24[d]
	15:1	30.6±0.21[b]	28.6±0.25[c]
	20:1	33.7±0.25[a]	30.5±0.23[b]

There were no notable variations found in the mean values that shared the same letter in the same row (P <0.05). [1]CP = Crude protein, [2] EE = Ether extract

Table 5. Hemato-biochemical indices of Nile Tilapia raised in biofloc tanks with varied carbon nitrogen ratios and carbohydrate sources for 16 weeks.

Variables	C/N Ratios	Carbon sources	
		Molasses	Starch
[1]RBC (10^6 µL^{-1})	10:1	2.33±0.10[b]	2.27±0.10[b]
	15:1	3.39±0.10[a]	2.42±0.11[b]
	20:1	3.29±0.10[a]	2.20±0.10[b]
[2]WBC (10^4 µL^{-1})	10:1	39.1±0.12[e]	38.1±0.12[f]
	15:1	49.5±0.12[a]	42.4±0.12[c]
	20:1	48.6±0.12[b]	40.2±0.12[d]
[3]Hb (g/dL)	10:1	10.2±0.10[b]	8.6±0.10[c]
	15:1	12.5±0.10[a]	10.3±0.10[b]
	20:1	12.5±0.10[a]	10.3±0.10[b]
[4]Ht (g/dL)	10:1	18.4±0.11[d]	16.5±0.11[e]
	15:1	24.1±0.11[a]	21.4±0.11[b]
	20:1	23.6±0.11[a]	19.5±0.11[c]

There were no notable variations found in the mean values that shared the same letter in the same row (P <0.05). RBC = [1]Red blood cell, [2]WBC = White blood cell, [3]Hb = Hemoglobin, [4]Ht = Hematocrit

produced the highest glucose level (6.25±0.12 mmol/g), while a 15:1 ratio with starch produced the lowest glucose level (4.19±0.15 mmol/g). The cortisol levels maximum at 4.48±0.12 ng/mg at a 20:1 C/N ratio with molasses, whereas the lowest cortisol levels (3.67±0.12 ng/mg) were found at a 15:1 ratio with starch (Figure 4).

Discussion

Effective management of water quality is vital in aquaculture, with factors like stocking density, environmental conditions, species combinations, and nutritional input playing a significant role (Xu et al., 2016). The present investigation discovered that treatments raised the values of total suspended solids (TSS), possibly as a result of feed and organic carbohydrate supplementation, as in the study of Xu et al. (2016). Treatments with starch had lower TSS values, indicating low settleable solids and biofloc formation. In all C/N ratio treatments, a decrease in total ammonia nitrogen (TAN) and NO_3-N were observed. The microbial assimilation by microbes in the biofloc or nutrient cycling may have contributed to the lower TAN values observed in the molasses treatment. The study suggests that promoting heterotrophic bacteria through floc formation can effectively control water quality. In the current study, all physico-chemical parameters were within the acceptable range of tilapia, as in the study of Cardona et al. (2016).

Understanding the complexity of microbial communities in soils and water is a challenging yet fascinating aspect of scientific exploration that plays a crucial role in aquatic plant and animal growth. It provides crucial services like disease prevention, nutrient cycling, bioremediation, and the sequestration of iron and other metals required for the growth of aquatic plants and animals (Charles and Marco, 2010). According to Xu et al. (2016), carbohydrate supplementation, especially molasses, boosted the total heterotrophic bacterial (THB) population considerably from C/N10 to C/N20. Our results also correlate with Xu's. Bacterial communities such as *Bacillus sp* in biofloc systems thrive by using organic matter and nitrogen molecules, both of which are abundant in biofloc systems (Crab et al., 2007). The progression of culture resulted in a notable impact on the total microbial load due to increasing biomass; higher levels were recorded in C/N15 and C/N20. Our findings align with Panigrahi et al. (2018), revealing a harmony in the observed results.

Table 6. Anti-oxidant enzymes activity and non-specific immune response of Nile Tilapia cultured in biofloc tanks with varied carbon nitrogen ratios and carbohydrate sources for 16 weeks.

Variables	C/N Ratios	Carbon sources	
		Molasses	Starch
[1]SOD U/mg	10:1	55.6±0.11^{b}	52.5±0.11^{d}
	15:1	56.5±0.11^{a}	53.6±0.10^{c}
	20:1	34.0±0.11^{e}	31.4±0.11^{f}
[2]CAT U/mg	10:1	35.1±0.10^{b}	31.0±0.10^{d}
	15:1	36.1±0.11^{a}	32.2±0.10^{c}
	20:1	32.1±0.11^{c}	30.1±0.10^{e}
[3]GPx U/mg	10:1	46.8±0.10^{b}	43.5±0.11^{d}
	15:1	48.3±0.10^{a}	45.2±0.11^{c}
	20:1	34.6±0.11^{e}	31.3±0.10^{f}
Lysozyme activity µg/mL	10:1	15.4±0.10^{b}	12.2±0.11^{e}
	15:1	16.7±0.11^{a}	14.6±0.10^{c}
	20:1	13.6±0.11^{d}	11.4±0.10^{f}

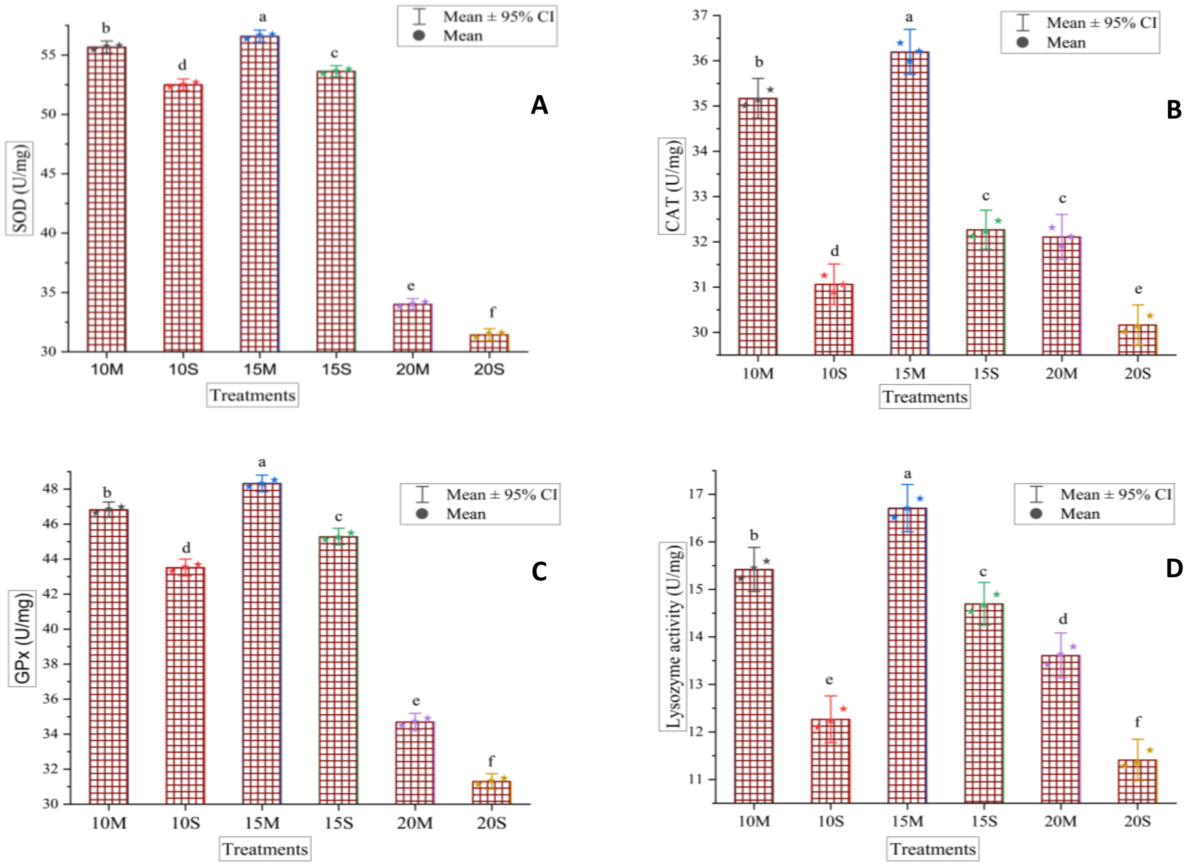


Figure 3. Oxidative stress of Nile Tilapia in biofloc for a duration of 16 weeks, utilizing different C/N ratios and carbon sources **(A)** SOD (Superoxide dismutase activity) **(B)** CAT (Catalase activity) **(C)** GPx (Glutathione peroxidase) **(D)** LYZ (Lysozyme activity).

Table 7. The levels of cortisol (COR) and glucose (GLU) in the liver of Nile Tilapia grown for 16 weeks in biofloc tanks with varying carbon to nitrogen ratios and carbon sources.

Variables	C/N Ratios	Carbon sources	
		Molasses	Starch
[1]GLU (mmol/g)	10:1	5.26±0.11^{b}	4.57±0.12^{cd}
	15:1	5.12±0.18^{bc}	4.19±0.15^{d}
	20:1	6.25±0.12^{a}	5.27±0.14^{b}
[2]COR (ng/ mg)	10:1	4.07±0.12^{abc}	3.93±0.11^{abc}
	15:1	3.89±0.11^{bc}	3.67±0.12^{c}
	20:1	4.48±0.12^{a}	4.27±0.12^{ab}

There were no notable variations found in the mean values that shared the same letter in the same row (P <0.05). [1]GLU = Glucose, [2]COR = Cortisol

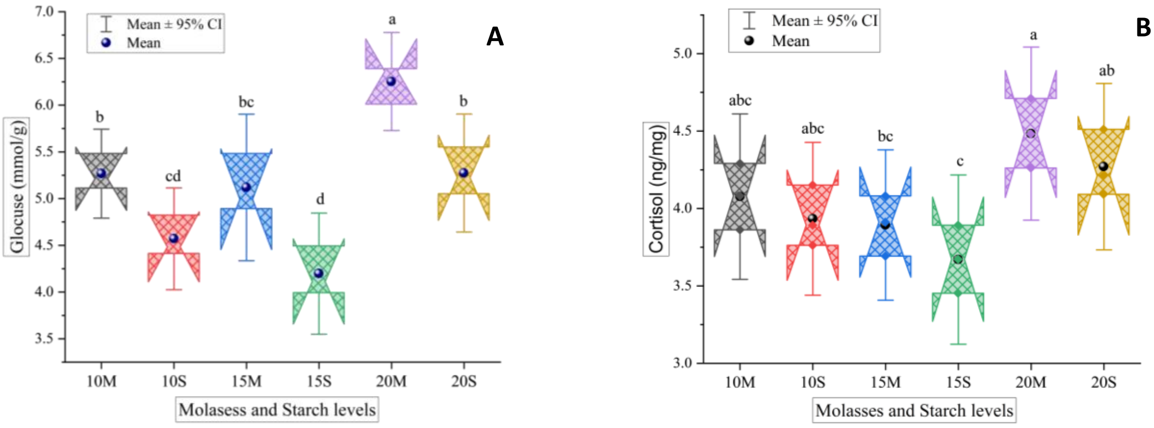


Figure 4. Stress markers of Nile Tilapia in biofloc for a duration of 16 weeks, utilizing different C/N ratios and carbon sources (A) GLU (Glucose activity) (B) COR (Cortisol activity).

In our experiment, Nile tilapia showed better growth performance in C/N10 and C/N15 as compared to the CN-20 group. Normally in traditional aquaculture feed contain 35-40% crude protein but Nile tilapia (*Oreochromis niloticus*) in biofloc systems require 15% less feed than standard quantities, but feed consumption is better (Avnimelech, 2007). The study discovered that in biofloc treatments, the FCR values were lower in the C/N10 and C/N15 groups as compared to the C/N20 group, but FBW, WG, and SGR were greater in the C/N10 and C/N15 groups. However, FCR in the C/N20 group was greater, implying decreased feed consumption. Xu et al. (2016) represent a well-represented tilapia culture at a lower C/N ratio, suggesting that a high C/N ratio can reduce the growth of aquatic species, such as the more heterotrophic bacterial community, which may quickly deplete oxygen levels, stunt fish growth in this system, and have a detrimental impact on water quality. Our results closely correspond with Xu's, showing strong alignment in observed outcomes.

The study examines the proximate analysis of Nile tilapia, focusing Avnimelech, 2007 on protein, fat, and ash content. According to Yu et al. (2020), the research found that in Crucian carp, increasing the carbon-nitrogen ratio improves the carcass composition; however, Mirzakhani et al. (2019) discovered that the C/N ratio of Nile tilapia were raised, the fat and protein content decreases, and ash contents rise. In biofloc treatments, the effects of the carbon-nitrogen ratio, the kind of carbon source, and their interaction on these parameters were significant. Our findings coincide with Mirzakhani, highlighting the correspondence in the observed results. According to the study, the carbon-nitrogen ratio (C/N) and carbon sources affect the total body protein content of biofloc fish. These factors affect the biochemical composition of biofloc and the proximate composition of the cultured species (Khanjani et al., 2017). The C/N15 group had a higher whole-body protein content; both the C/N ratio and carbohydrate sources significantly affected the crude lipid of the fish body (Luo et al., 2014). Long et al. (2015) reported that variations in carbon sources, culture conditions, and rearing temperature may have an impact on the nutrient composition and microbial community of biofloc, leading to discrepancies in the results. In general, when the C/N ratio increased, the whole-body lipid content of biofloc was comparable to that found in the studies by Azim and Little (2008) and Lopez-Elias et al. (2015), where body lipid levels decreased. The group with the highest total body ash content was C/N20 (Serra et al., 2015). The experiment's protein content was similar to that of earlier research with regard to proximate biofloc composition (Azim and Little, 2008; Becerra-Dorame et al., 2012; Ekasari et al., 2014). The lipid content of biofloc was comparable to that found in the studies by Azim and Little (2008) and Lopez-Elias et al. (2015).

Fish hematological parameters reflect their general health and stress resilience (Harikrishnan et al., 2011). The blood properties of aquatic animals may be adversely influenced by pollutants and environmental stressors (Fazio, 2019). Increased hemoglobin, red blood cell (RBC), and HCT in fish indicate good health, protecting fish from environmental stress (Zafar et al., 2022). This study aligns with previous research by Dauda et al. (2018) on African catfish reared in biofloc-based systems, where improved hematological status was observed. A higher WBC count in C/N15 treatment indicates an improved immune response to infections or stressors, as in the study of Kori-Siakpere et al. (2006). Though Hwihy et al. (2021) found that WBC counts in *Oreochromis niloticus* were higher in the C/N15 groups, these findings are inconsistent with our results.

Liu et al. (2018) collected scientific evidence which suggests that biofloc can boost the immune system of fish, have antiviral properties, and prevent the development of opportunistic pathogens, hence preventing disease (Ekasari et al., 2014). Lysozyme activity is a crucial indicator of innate immunity in fish (Saha et al., 2022). Improved growth performance and water quality have been related to higher lysozyme activity in Nile tilapia growth in biofloc culture systems with a rising C/N ratio (Mirzakhani et al., 2019). In contrast, Dauda et al. (2018) noted that various C/N ratios in glycerol-based biofloc systems showed no impact on serum lysozyme activity in African catfish. Higher serum lysozyme activity in fish raised in biofloc systems with C/N15 might be attributed to heterophilic bacteria and their products enhancing non-specific immunological traits. Azim and Little (2008) demonstrated a rise in non-specific immune responses when *Oreochromis niloticus* is reared as an experimental species (Xu and Pan, 2012). The immunological responses of Tilapia fingerlings may be affected by the C/N ratio. The C/N10 and C/N15 groups had stronger responses, indicating an improved immune condition.

Multiple types of antioxidants are crucial for maintaining the fish immune system and contain both enzymatic and non-enzymatic antioxidant properties (Jia et al., 2012). Antioxidants such as SOD provide the first line of defense against free radicals in organisms by converting reactive oxygen molecules into non-toxic chemicals. A low level of these enzymes can lead to injury and impaired cell function. The results of Kim et al. (2015) are consistent with the observation that fish in the C/N10 and C/N15 groups had elevated levels of SOD, representing a defensive mechanism against lipid peroxidation. According to the study's overall findings, a biofloc environment with C/N15 can improve both the rate of lipid peroxidation reactions and the overall antioxidant response.

Glucose content is a key indication of fish digestion and metabolism, and it is a common response to stress (Li et al., 2016). In this research, the lowest value of glucose level in biofloc groups was detected in the C/N10 and C/N15 groups. This is in contrast to the earlier research conducted by De las Heras et al. (2015), which found that a greater extent of GLU promotes the development of grey mullet. The concentration forms of GLU appear to vary depending on size and/or age (Laiz-Carrion et al., 2012). Haridas et al. (2017) also noted an elevated glucose level in the group exhibiting reduced growth. Our findings demonstrate concordance with these outcomes.

Due to an increase in energy consumption, high plasma cortisol levels over time can have a detrimental effect on physiological procedures such as development, osmoregulation, reproduction, immunological response, and metabolism (Laiz-Carrion et al., 2012). Salas-Leiton et al. (2008) noted that organism development is enhanced when high plasma COR levels down-regulate the production of liver insulin-like growth factor mRNA. Our results are consistent with their results, showing that the groups with optimum COR levels had greater fish development than the others, as the C/N10 and C/N15 groups showed better growth. Similarly, the group that had less growth had a higher cortisol level in the research conducted by Haridas et al. (2017). A high cortisol level is also an effective stress indicator in aquaculture; the optimum range of cortisol in the C/N10 and C/N15 groups showed the best growth. Once again, our findings indicated that tilapia health and growth rates can be increased in biofloc with a suitable C/N ratio and carbon source.

Conclusion

One relatively new kind of aquaculture farming is biofloc technology, which has several potential benefits, such as providing a direct food supply, boosting immunity, and detoxifying the system for cultivated organisms. In conclusion, our research confirmed that C/N15 groups utilizing molasses as organic carbon source was an appropriate strategy for cultivating Nile tilapia fingerlings. The fish in these groups showed improved growth performance, immune system function, antioxidant status, and ultimately a higher survival rate in contrast to the C/N20 treatments. This suggests that the biofloc conditions provided a stress-free environment for the fish. The use of a lower C/N ratio allows for lower production costs by limiting molasses supply and minimizing water renewal, which is related to TSS accumulation. Fertilizing the BFT system with molasses can lower output costs. In conclusion, this research demonstrates that adjusting the carbon-to-nitrogen (C/N) ratio has substantial effects on the advancement of biofloc culture. It was determined that a C/N ratio of 15:1 was sufficient for the rearing of *Oreochromis niloticus* using biofloc. The C/N ratio affects fish development and health through a complex mechanism that may not completely be explained by growth effects or physiological factors. In the future, further research and molecular biology techniques should be applied to clarify this process.

Ethical Statement

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 (ORIC).

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Author Contribution

Andleeb Zahra: performed the research experiment; Abdul Mateen: devised idea and supervised the whole experiment; Andleeb Zahra: sampling and analysis; Abdul Mateen: review and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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