











Research Article

Biofloc System with Different Carbon Sources Improved Growth, Haematology, Nonspecific Immunity, and Resistivity against the *Aeromonas hydrophila* in Common Carp, *Cyprinus carpio*

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Received 7 December 2023; Accepted 16 January 2024; Published 13 February 2024

Academic Editor: Lee Seong Wei

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Biofloc technology (BFT) is one of the most sustainable aquaculture system, which is based on the principle of nutrient recycling and addition of carbon to enable heterotrophic microorganisms to the system. To evaluate the performance of the biofloc culture system for *Cyprinus carpio* fingerlings, a 60-day growth trial was conducted. The fingerlings ($n = 600$) of average body weight ($4.92 \text{ g} \pm 0.14$) were stocked in 12 circular fiberglass tanks (300 L, volume 10.59 cft) to form three biofloc treatments (T1, T2, and T3) along with one control group. The carbon sources for treatments were sugarcane molasses, tapioca, and wheat. The C/N ratio of 15 was maintained for all treatments. After 60 days of rearing, the fish were challenged with *Aeromonas hydrophila*, and the relative percentage survival (RPS) was observed over 14 days. A haematological, nonspecific immune, and stress parameters were analyzed using blood and serum samples collected at intervals of 20, 40, and 60 days. According to the results, the carbon sources affected the water quality parameters but were still adequate for fish welfare. An increased biofloc volume was observed with tapioca. Growth performance and better feed conversion ratio were recorded in biofloc with the tapioca group. The haematological parameters, including haemoglobin (Hb), hematocrit (HCT), white blood cells and lymphocytes were significantly ($P < 0.05$) higher in biofloc-based tapioca group than in other treatments and control. Further, the serum protein, globulin, albumin, total immunoglobulin, and respiratory burst activity were also found significantly ($P < 0.05$) higher in biofloc with tapioca as carbon source. However, the lysozyme activity was higher in biofloc with the wheat group. The RPS in tapioca was significantly higher, followed by biofloc with wheat. In conclusion, the tapioca-based biofloc can improve *C. carpio* growth, haematology, and nonspecific immune response under zero water exchange.

1. Introduction

The fish utilize approximately 26% of the nitrogen and 30% of the phosphorus in their feed, with the remainder (uneaten feed) increasing nitrogen and ammonia concentrations [1, 2]. To maintain good water quality in intensive fish culture, continuous and partial water exchanges are essential, which may account for approximately 10% of the total cost of production [3]. However, biofloc technology (BFT) offers an alternative approach by creating a controlled environment where beneficial microorganisms form flocs, reducing the need for frequent water changes. Thus, this method minimizes water exchange costs and, therefore, emphasizes the importance of consistently managing water quality within the system to ensure optimal conditions for aquatic organisms [4, 5]. Bioflocs contain a combination of various floc-forming microorganisms, including bacteria, protozoa, organic polymers, phytoplankton, rotifers, nematodes, microalgae, copepods, annelids, dead cells, cations, colloids, and uneaten feed [6]. The entire floc former combines with the others to build a mass that fish can eat, which helps with nutrient recycling and enhances fish growth [7]. In the biofloc rearing system, the protein content of the commercial feed can be reduced from 35% to 25% due to the availability of microbial protein that compensates for the low protein content in the diet [2].

To sustain the culture system's carbon-to-nitrogen ratio is one of the fundamental requirements for biofloc formation [6]. It is recommended that the carbon/nitrogen ratio in the development of biofloc be between 10 : 1 and 20 : 1 with varied carbon sources. A balanced C:N ratio is essential for the success of biofloc systems in aquaculture. It promotes the formation of a robust microbial community, efficient nutrient cycling, and the stability of the system, ultimately benefiting the health and growth of the cultured aquatic organisms [8].

Culture systems can be supplemented with organic carbon sources, such as tapioca, glucose, corn, wheat, acetate, molasses, and glycerol [9]. The kind and quantity of storage polymers and the flocs' overall composition are determined mainly by the organic carbon source [10]. Compared to conventional practices for farming fish species, BFT is more efficient regarding water and feed usage [11]. Various microbes and their cell components have been utilized as immunostimulants or probiotics to enhance fish growth, immunity, disease resistance, and antioxidant status [12]. Bioflocs contain various bioactive compounds such as polysaccharides, carotenoids, chlorophylls, phytosterols, fat-soluble vitamins, and taurine [13]. However, there is a notable gap in research regarding the impact of biofloc on nonspecific immunity and the physiological health of cultured fish.

Aeromonas hydrophila is one of the most commonly encountered and highly damaging bacterial pathogens, causing severe damage to carp cultures worldwide [14]. In aquaculture, including carp farming, *A. hydrophila* can be introduced through contaminated water sources, equipment, or infected fish [15]. Several common symptoms are associated with it, including abdominal distension, ulceration, and exophthalmia in fish [16]. Stressors like poor water quality, overcrowding, and inadequate nutrition can exacerbate the

susceptibility of fish to *A. hydrophila* infections [17]. The study of *A. hydrophila* in aquaculture is essential for the sustainable and responsible management of fish health, economic viability, and environmental impact in the rapidly growing aquaculture industry. Using BFT in *Cyprinus carpio* culture systems may provide other advantages, including determining the best carbon source to use, improving the quality and quantity of biofloc, and enhancing growth and immune-modulating effects. The present study investigated the influence of three distinct carbon sources within bioflocs on the growth, haematological indicators, body composition, nonspecific immunological response, and disease resistance of *C. carpio* fingerlings.

2. Materials and Methods

2.1. Experimental Design. A 2-month (60 days) experiment was carried out in 12 fiberglass tanks (circular shape, 300 L, volume 10.59 cft) with one control (C) group and three treatments (T1, T2, and T3). The carbon sources include T1: sugarcane molasses, T2: tapioca, and T3: wheat. Initially, the soil was collected from the bottom of the pond in Punjab (Pakistan) for floc formation. In plastic tanks of 5 L, inocula were prepared by the addition of pond bottom soil (20 g) in 1 L of well-aerated water containing $(\text{NH}_4)_2\text{SO}_4$ (10 mg/L) and different carbon sources of 400 mg/L. The suspension was incubated for 24 hr to develop microbial growth. After 24 hr of floc formation, the prepared inocula was added to each group. Aeration was maintained in the tanks for 10 days to ensure optimal floc production. C/N ratios of 15 were regularly maintained by adding nitrogen and organic carbon sources [18]. The *Cyprinus carpio* fingerlings were collected from the Kallar Kahar fish hatchery, Punjab, Pakistan. The acclimation period of the fish was 28 days in a fiberglass tank of 1,000 L and fed with feed containing 30% protein content. Furthermore, during the acclimatization period, any fish that showed unhealthy signs, apparently undernourished, was separated from the tank. *C. carpio* fingerlings ($n=600$) of average body weight ($4.92 \text{ g} \pm 0.14$) were placed randomly in 12 tanks (300 L) to form three experimental and one control group. The experimental fish were fed at 3% of their body weight twice daily in morning and evening. The detail of the diet is given in Table 1.

2.2. Water Quality Parameters. On a daily basis, the water quality parameters, including water temperature, dissolved oxygen (DO), and pH, were measured on the spot through multi-item meter "YSI-650 Inc., Yellow Spring Instruments, USA." Total suspended solids (TSSs) were measured using a TSS-meter "HM-COM-80." The determination of total ammonia nitrogen "TAN, $\text{NH}_3\text{-N}$," nitrite " $\text{NO}_2\text{-N}$ " and nitrate " $\text{NO}_3\text{-N}$ " was done weekly using a spectrophotometer "Perkin Elmer lambda 25 UV/Vis." The biofloc volume in each biofloc tank was determined using an Imhoff cone. Measurements were recorded after allowing 1 L of water from each tank to settle for 30 min [19]. A total of 30 observations were made for each treatment, consisting of 10 replicates, with three replicates per specific treatment.

TABLE 1: Ingredients and nutritional composition of diet for *C. carpio* fingerlings.

Basic ingredients (%)	Diet (%)
Soybean meal	22
Fish meal	24
Wheat flour	23
Corn flour	25
Sunflower oil	4
Vitamin and mineral premix ^{1,2}	2
Chemical composition (%) dry matter	
Dry matter	83.5
Crude protien	30
Crude fiber	3.5
Crude lipid	7.9
Ash	10.2
NFE ³	31.9
Energy ⁴ (kJ/g)	15.59

¹Vitamin premix: vitamin A (18,000 IU), vitamin B D3 (2500 U), vitamin E (250 mg/kg), vitamin K3 (12 mg/kg), vitamin B1 (25 mg), vitamin B2 (50 mg), vitamin B3 (270 mg), vitamin B6 (20 mg), vitamin B12 (0.06 mg), vitamin C (200 mg), folic acid (10 mg), calcium d-pantothenate (50 mg), biotin (1 mg), inositol (120 mg), and choline chloride (2000 mg). ²Mineral premix: Fe (75.3 mg), Cu (12.2 mg), Mn (206 mg), Zn (85 mg), I (3 mg), Se (0.350 mg), and Co (1 mg). ³Nitrogen-free extracts (NFE) dry matter – (crude lipid + crude ash + crude protein + crude fiber). ⁴Energy calculated according to 23.6 kJ g⁻¹ protein, 39.5 kJ g⁻¹ lipid, and 17.0 kJ g⁻¹ NFE.

2.3. Fish Growth Parameters. Fish growth parameters ($n = 10$) were assessed biweekly in different treatment groups. These parameters, including weight gain, specific growth rate (SGR), feed conversion ratio (FCR), and feed efficiency ratio (FER) [20], were determined using the following equations:

$$\text{Weight gain (\%)} = \frac{FW - IW}{IW} \times 100, \quad (1)$$

$$\text{SGR (\%)} = \frac{\ln(FW) - \ln(IW)}{N} \times 100, \quad (2)$$

$$\text{FCR} = \frac{\text{Feed given DW}}{\text{body weight gain WW}}, \quad (3)$$

$$\text{FER} = \frac{1}{\text{FCR}} \times \text{SGR (\%)} = \frac{\ln(FW) - \ln(IW)}{N} \times 100, \quad (4)$$

where FW is the final weight, IW is the initial weight, DW is the dry weight; WW is the wet weight, In is the natural log, and N is the number of culture days.

2.4. Body Proximate Composition. After the experiment completion, the fish sample proximate composition ($n = 10$) was determined according to the standard methods [21]. To determine the moisture content of the samples, they were dried at temperature 105°C until they reached a constant weight and measured by the difference in weight of the sample. Kjeldahl method was used to estimate nitrogen content. The Kjeldahl procedure involves the sequential steps of digestion, distillation, and titration to analyze the nitrogen content in a sample, providing a comprehensive measurement that includes both

organic and inorganic nitrogen forms. The crude protein content was determined by multiplying the nitrogen percentage by 6.25. Soxtec's solvent extraction method was used to determine crude lipid using diethyl ether at boiling point (40–60°C). The ash content of the samples was determined by burning them at 600°C for 8 hr in a muffle furnace.

2.5. Haematological Parameters. Fish from each group ($n = 5$) were anesthetized using clove oil (60 mg/L) [22] after every 20-day interval, and blood was taken from the caudle vein (with and without an anticoagulant) for plasma and serum. To assess the haematological parameters, 50% of each blood sample was stored at 4°C in an aqueous solution containing heparin as an anticoagulant. After dilution with phosphate-buffered saline, white blood cells (WBCs) and red blood cells (RBCs) were counted using a Neubauer hemocytometer. Complete blood was centrifuged in heparinized microhematocrit capillary tubes for 10 min at 3,500 g to determine the hematocrit (HCT). The concentration of haemoglobin (HB) was determined using the cyanohemoglobin technique [23]. Differential WBCs were calculated using Giemsa-stained smears [24].

2.6. Stress and Nonspecific Immune Parameters. For the serum, the collected blood was allowed to clot for 4 hr. Serum and plasma were then collected after the blood had been centrifuged at 5,000 rpm for 5 min and stored at –80°C until further analysis.

A total protein kit (Merck, Germany) was used to estimate serum protein using bromocresol green and biuret dye-binding methods [25]. An albumin kit was used to estimate albumin using the bromocresol green binding technique [26]. Total serum protein values were subtracted from albumin values to calculate globulin. Using polyethylene glycol, total immunoglobulin (TIG) was precipitated from plasma [27]. Using the method described by Secombes [28], the respiratory burst activity was determined using the nitroblue tetrazolium (NBT) assay. An assay for lysozyme activity was conducted using the turbidimetric method developed by Sankaran and Gurnani [29], in which the white of hen egg lysozyme was used as a reference. Using the Cayman's cortisol EIA kit (USA chemical), serum cortisol was estimated. A standard kit was used in order to determine serum glucose levels as instructed by the manufacturer (Merck, China).

2.7. Challenge Test. After 60 days of the experimental period, the fish ($n = 12$) from each experimental group were challenged with the pathogenic bacterial strain *Aeromonas hydrophila* (ATC-7966) obtained from the Microbiology Department, University of Punjab, Pakistan. The bacteria were cultured in nutrient broth at 35°C for 24 hr. At 3,000 rpm for 10 min, the cultures were centrifuged. After this, the supernatants were discarded, and the pellets were suspended in phosphate-buffered saline of pH 7.4, with an OD of 0.48 at 450 nm corresponding to the final bacterial concentration of 1.8×10^7 cfu/mL. A bacterial suspension (20 µL) was injected intraperitoneally into four experimental fish per replicate in each treatment using a tuberculin syringe (1 mL) [30], and mortality was observed for 14 days.

TABLE 2: Water quality parameters of control and experimental groups during 60 days study.

Parameters	Control	Sugarcane molasses	Tapioca	Wheat
Temperature (°C)	23.75 ± 0.84	23.67 ± 0.79	24.98 ± 0.92	24.86 ± 0.96
DO (mg/L)	7.18 ± 0.39	6.91 ± 0.24	6.89 ± 0.34	6.87 ± 0.26
pH	8.49 ± 0.14 ^a	8.03 ± 0.21 ^a	8.08 ± 0.16 ^a	7.68 ± 0.14 ^b
TAN (mg/L)	2.36 ± 0.11 ^c	4.48 ± 0.07 ^b	4.81 ± 0.04 ^b	5.72 ± 0.09 ^a
TSS (mg/L)	31.3 ± 5.27 ^b	354.5 ± 65.32 ^a	313.7 ± 71.54 ^a	363.5 ± 73.16 ^a
Nitrite (mg/L)	0.33 ± 0.17 ^b	0.89 ± 0.15 ^a	0.92 ± 0.12 ^a	0.93 ± 0.14 ^a
Nitrate (mg/L)	1.02 ± 0.18 ^b	7.87 ± 1.34 ^a	7.69 ± 1.38 ^a	9.76 ± 2.43 ^a
Biofloc volume (mL)	0.82 ± 0.12 ^d	17.59 ± 3.38 ^c	30.74 ± 4.21 ^a	26.24 ± 3.64 ^b

Note: Within the same row, the mean shows different superscripts are different significantly.

TABLE 3: Growth performance of *C. carpio* fingerlings cultured in BFT with different groups.

Parameters	Control	Sugarcane molasses	Tapioca	Wheat
Weight gain (%)	29.2 ± 1.2 ^d	58.32 ± 0.7 ^c	71.63 ± 1.9 ^a	64.18 ± 1.3 ^b
Specific growth rate (SGR) (%/day)	2.47 ± 0.03 ^b	2.49 ± 0.06 ^b	2.58 ± 0.02 ^a	2.55 ± 0.04 ^a
Feed conversion ratio (FCR)	2.56 ± 0.05 ^a	1.47 ± 0.09 ^b	1.08 ± 0.03 ^d	1.28 ± 0.05 ^c
Feed efficiency ratio (FER)	0.32 ± 0.02 ^d	0.46 ± 0.04 ^c	0.62 ± 0.04 ^a	0.53 ± 0.07 ^b

Note: Within the same row, the mean shows different superscripts are different significantly.

TABLE 4: Proximate composition of *C. carpio* fingerlings of different groups.

Parameters	Control	Sugarcane molasses	Tapioca	Wheat
Moisture	74.68 ± 0.46 ^a	73.03 ± 0.16 ^b	71.83 ± 0.61 ^c	74.72 ± 0.33 ^a
Crude protein (DW%)	48.65 ± 0.06 ^b	50.12 ± 0.16 ^a	50.21 ± 0.03 ^a	50.38 ± 0.26 ^a
Crude lipid (DW%)	1.72 ± 0.02 ^c	4.19 ± 0.05 ^a	3.62 ± 0.07 ^b	4.28 ± 0.03 ^a
Ash (DW%)	12.01 ± 0.06 ^c	14.32 ± 0.02 ^a	13.22 ± 0.02 ^b	13.73 ± 0.05 ^a

Note: Within the same row, the mean shows different superscripts are different significantly. DW, dry weight.

2.8. *Relative Percentage Survival*. Based on the formula shown below, the relative percentage survival (RPS) at 14 days post-infection was calculated [31]:

$$\text{RPS (\%)} = \frac{\text{Numbe of live fish after challange test}}{\text{Number of fish injected with bacteria}} \times 100. \quad (5)$$

2.9. *Statistical Analysis*. The analysis of data was carried out through the SPSS 27 version. To separate the means at a significance level ($P < 0.05$), one-way ANOVA was used. Further two-way ANOVA was used to statistically analyze the difference between serum parameters. A Duncan multiple range test was used to determine whether there were significant differences between the treatments.

3. Results

The results of the water quality parameters of the control and experimental groups are illustrated in Table 2. The parameters, including temperature, DO, and pH values, did not differ notably ($P > 0.05$) among the groups. The total ammonia nitrogen (TAN) value in the biofloc group with wheat is significantly ($P < 0.05$) higher compared to other groups. However, no significant difference was observed in TAN level

between the two experimental groups (sugarcane molasses and tapioca). The level of TSSs, nitrite, and nitrate in all biofloc-based experimental groups did not change significantly but exhibited significantly ($P < 0.05$) higher results than the control group. The biofloc volume was influenced by all carbon sources, with a notably higher volume observed in the tapioca-based unit.

The outcomes of the growth performance of *C. carpio* fingerlings in different groups are depicted in Table 3. The percentage of weight gain of fish in the tapioca-based group exhibited ($P < 0.05$) higher results compared to control and other treatments. A significantly higher SGR was recorded in the tapioca-based unit and lower in the control group. The best FCR was observed in the tapioca-based biofloc group, followed by a wheat-based unit, while in the control group, a higher FCR was found. FER was notably greater ($P < 0.05$) in the tapioca-based group compared to others.

The analysis of body proximate composition of *C. carpio* cultured in different groups is presented in Table 4. The fish of wheat-based groups exhibited higher ($P < 0.05$) moisture content than the control and other experimental groups. The crude protein level revealed no significant ($P < 0.05$) change in all treatment groups, whereas the lower crude protein level was recorded in the control group. A significantly higher level of crude lipid was found in the wheat-based group

TABLE 5: Analysis of haematological parameters of *C. carpio* fingerlings of different groups.

Parameters	Control	Sugarcane molasses	Tapioca	Wheat
WBCs ($10^3/\mu\text{L}$)	$26.34 \pm 1.16^{\text{d}}$	$31.26 \pm 1.22^{\text{c}}$	$39.54 \pm 1.17^{\text{a}}$	$35.34 \pm 1.31^{\text{b}}$
RBCs ($10^6/\mu\text{L}$)	$1.33 \pm 0.13^{\text{a}}$	$1.34 \pm 0.15^{\text{a}}$	$1.35 \pm 0.11^{\text{a}}$	$1.35 \pm 1.19^{\text{a}}$
Hematocrit (HCT; %)	$26.42 \pm 0.47^{\text{c}}$	$27.34 \pm 0.28^{\text{c}}$	$36.23 \pm 0.53^{\text{a}}$	$32.65 \pm 1.17^{\text{b}}$
Haemoglobin (HB; %)	$7.95 \pm 0.25^{\text{b}}$	$8.56 \pm 0.14^{\text{b}}$	$11.78 \pm 0.36^{\text{a}}$	$9.05 \pm 0.42^{\text{b}}$
Neutrophils (%)	$60.45 \pm 0.87^{\text{c}}$	$63.32 \pm 0.47^{\text{b}}$	$65.04 \pm 0.52^{\text{a}}$	$65.06 \pm 0.47^{\text{a}}$
Monocytes (%)	$4.04 \pm 0.13^{\text{a}}$	$4.43 \pm 0.11^{\text{a}}$	$4.25 \pm 0.15^{\text{a}}$	$4.32 \pm 0.13^{\text{a}}$
Lymphocytes (%)	$30.24 \pm 0.52^{\text{c}}$	$34.53 \pm 0.77^{\text{b}}$	$38.67 \pm 0.74^{\text{a}}$	$35.34 \pm 0.68^{\text{b}}$

Note: Within the same row, the mean shows different superscripts are different significantly.

than in the tapioca and control. The ash content exhibited higher ($P < 0.05$) results in the wheat-based group, while lower was obtained in the control.

Table 5 shows the haematological parameters of the control and treatment groups of *C. carpio* fingerlings. The value of HCT and HB was notably ($P < 0.05$) higher in tapioca-based unit; however, the lower was recorded in the control group. No significant ($P < 0.05$) difference was found in the value of RBCs among the control and experimental groups. The WBCs of the tapioca-based treatment group revealed significantly ($P < 0.05$) higher values than other groups. The value of neutrophils did not alter significantly ($P < 0.05$) between the two biofloc-based groups, such as wheat and tapioca, but higher results than the control and sugarcane molasses-based unit. Monocytes revealed no significant difference among all the groups. Significantly ($P < 0.05$) higher results of lymphocytes were observed in the tapioca-based group compared to all other groups.

The independent effect of the carbon source on glucose and cortisol is significant, with lower values observed in the biofloc-based tapioca group, while the control group recorded higher values. Furthermore, the interaction between time and carbon source for these parameters was found to be less significant. After 60 days of the study period, total serum protein, total serum globulin (TSG), and total serum albumin (TSA) exhibited significantly higher results ($P < 0.05$) in the tapioca-based group compared to other treatments and the control group. However, no significant difference was found in the TIG values between tapioca and wheat-based units. The independent effect of the carbon source and time on these two parameters was the least significant; however, the interaction between the carbon source and time was insignificant. Moreover, the independent effect and interaction of the carbon source and time on serum albumin were found to be nonsignificant. Details are depicted in Table 6.

The NBT activity at an optical density (OD) of 620 nm revealed that biofloc-based experimental groups compared to control showed significantly ($P < 0.05$) higher results. However, a significant increase in NBT activity was observed when tapioca was used as a carbon source for biofloc culture, followed by wheat at the end of the experimental period (Figure 1).

As the experimental period progressed, there was an increasing trend in lysozyme activity in the biofloc treatment groups. The higher activity was recorded in biofloc with the wheat-based group followed by tapioca, while the lowest activity was revealed in the control group (Figure 2).

The RPS of *C. carpio* fingerlings is depicted in Figure 3. The biofloc-based treatments demonstrated significantly ($P < 0.05$) higher RPS against *A. hydrophila* after the challenge compared to the control group. However, the biofloc with tapioca-based unit showed higher RPS than treatments.

4. Discussion

The use of an effective carbon source can influence both the aquatic microbial community and the nutritional composition of biofloc [32]. Various studies have demonstrated that biofloc in culture medium enhances fish growth and immunity [33, 34]. In the current study, we evaluated the growth performance, body composition, haematological, and serum biochemical parameters of *C. carpio* fingerlings grown in three different biofloc systems with different carbon sources, as well as the degree of resistance to bacteria (*Aeromonas hydrophila*) they exhibit.

The carbon-to-nitrogen (C/N) ratio is crucial in biofloc systems as it significantly influences microbial processes and overall system dynamics. Maintaining an appropriate C/N ratio is essential for optimizing microbial activity, nutrient cycling, and the quality of the biofloc environment [8]. In this study, the C/N ratio was maintained at 15 to maximize the efficiency of the conversion of the inorganic nitrogen to protein by the microbes. Aquatic species' health depends heavily on water quality characteristics, which can be a limiting factor [4]. The water quality parameters of our study are according to the study of Ahmad.H et al. [35] on *Labeo rohita* in a biofloc system with different carbon sources, and Rind et al. [6] on Nile tilapia, *Oreochromis niloticus* reared in a biofloc system. Other authors also reported the water quality parameters adequate for fish survival and normal physiological activities, such as Khanjani et al. [36] cultured Nile tilapia with different carbon sources in BFT. Biofloc composition and formation are strongly influenced by temperature [37] and are recorded as suitable for floc formation in this study. The presence of stable microbial flocs can be achieved at a temperature of (25–28°C), as reported by Krishna and Van Loosdrecht [38]. DO strongly relates to the metabolic activity of cells and plays a vital role in the structure of aerobic flocs [39]. The floc volume index increases when the DO level exceeds 3.5 mg/L [40]. According to Nasir et al. [41], the pH range (7–8) is suitable for the average growth and survival of Juvenile carp (*C. carpio*), which is consistent with the present study. In the current

TABLE 6: Stress and nonspecific immune parameters of *C. carpio* fingerlings in different duration reared under different carbon sources in BFT system.

Parameters	Groups	20 days	40 days	60 days	Carbon source	Time	Interaction
Glucose (mg/dL)	Control	142.21 ± 1.43 ^a	129.72 ± 1.23 ^a	98.42 ± 1.02 ^a	**	**	*
	Sugarcane molasses	98.92 ± 1.22 ^b	93.72 ± 2.68 ^b	74.62 ± 1.13 ^b			
	Tapioca	72.44 ± 1.42 ^c	92.63 ± 1.73 ^b	46.38 ± 2.56 ^d			
	Wheat	99.87 ± 1.04 ^b	74.76 ± 1.01 ^c	55.24 ± 1.47 ^c			
Cortisol (ng/mL)	Control	97.12 ± 0.72 ^a	68.52 ± 0.61 ^a	52.31 ± 0.56 ^a	***	**	*
	Sugarcane molasses	67.44 ± 1.32 ^c	66.13 ± 0.82 ^a	38.63 ± 0.72 ^b			
	Tapioca	39.71 ± 0.41 ^d	67.34 ± 0.62 ^a	16.52 ± 0.56 ^d			
	Wheat	73.52 ± 0.81 ^b	38.36 ± 0.61 ^b	21.61 ± 0.56 ^c			
Total serum protein (mg/dL)	Control	2.39 ± 0.03 ^a	3.37 ± 0.52 ^b	3.69 ± 0.42 ^{cb}	*	*	NS
	Sugarcane molasses	1.73 ± 0.03 ^b	3.19 ± 0.34 ^b	3.47 ± 0.17 ^{cb}			
	Tapioca	2.41 ± 0.02 ^a	4.63 ± 0.13 ^a	6.48 ± 0.44 ^a			
	Wheat	1.88 ± 0.04 ^b	3.68 ± 0.03 ^{ba}	4.67 ± 0.53 ^b			
Total serum globulin (mg/dL)	Control	0.39 ± 0.09 ^b	1.77 ± 0.21 ^a	1.86 ± 0.16 ^{cb}	*	*	NS
	Sugarcane molasses	0.47 ± 0.09 ^b	1.66 ± 0.32 ^a	1.75 ± 0.22 ^c			
	Tapioca	1.05 ± 0.02 ^a	2.53 ± 0.03 ^a	4.47 ± 0.34 ^a			
	Wheat	1.19 ± 0.07 ^a	2.09 ± 0.01 ^a	2.82 ± 0.58 ^b			
Total serum albumin (mg/dL)	Control	1.89 ± 0.06 ^a	1.47 ± 0.03 ^b	1.78 ± 0.02 ^b	NS	NS	NS
	Sugarcane molasses	1.35 ± 0.09 ^c	1.48 ± 0.06 ^b	1.69 ± 0.04 ^b			
	Tapioca	1.38 ± 0.02 ^{cb}	1.79 ± 0.11 ^a	2.02 ± 0.12 ^a			
	Wheat	0.69 ± 0.05 ^d	1.48 ± 0.05 ^b	1.68 ± 0.05 ^b			
Total immunoglobulin (mg/dL)	Control	1.41 ± 0.03 ^a	1.55 ± 0.13 ^b	2.04 ± 0.31 ^b	**	*	*
	Sugarcane molasses	1.13 ± 0.12 ^b	2.57 ± 0.13 ^a	1.98 ± 0.04 ^b			
	Tapioca	1.53 ± 0.12 ^a	2.77 ± 0.17 ^a	4.81 ± 0.21 ^a			
	Wheat	1.24 ± 0.03 ^c	2.88 ± 0.16 ^a	4.35 ± 0.71 ^a			

Note: Within the same column, the mean shows different superscripts are different significantly. NS, nonsignificant; *, least significant; **, significant; ***, highly significant.

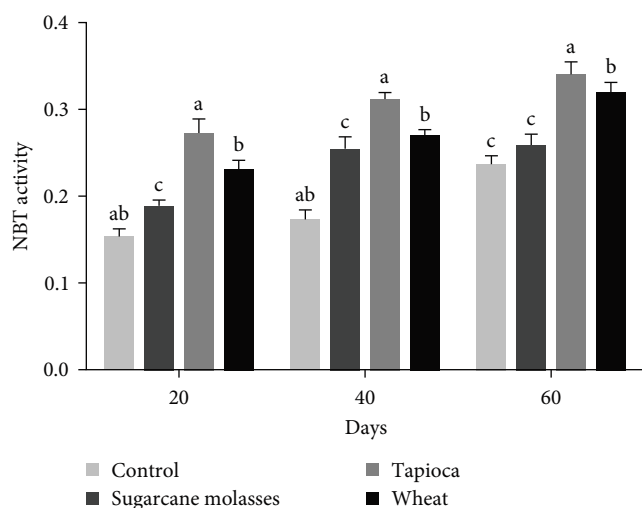


FIGURE 1: NBT activity of *C. carpio* fingerlings reared in biofloc system with different carbon sources. Significant difference ($P < 0.05$) are indicated by mean values that do not show common superscript letter.

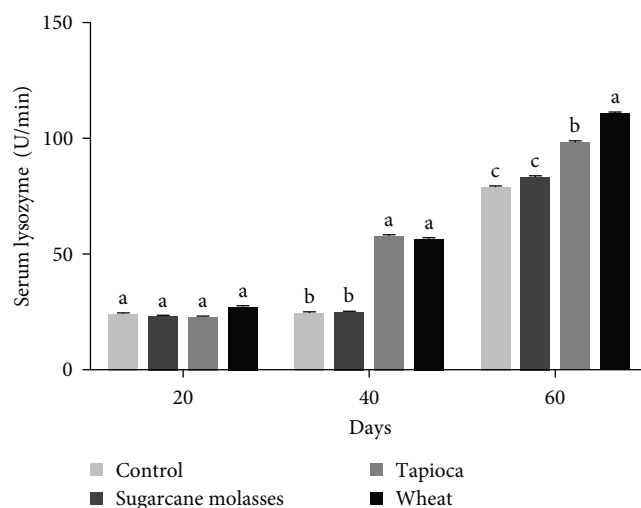


FIGURE 2: Serum lysozyme of *C. carpio* fingerlings reared in biofloc system with different carbon sources. Significant difference ($P < 0.05$) are indicated by mean values that do not show common superscript letter.

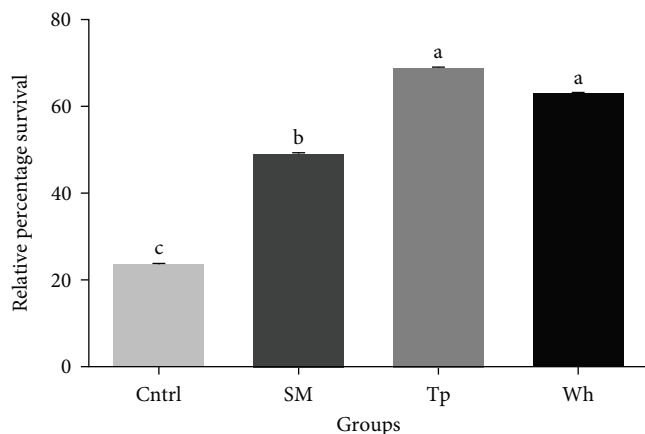


FIGURE 3: Relative percentage survival of *C. carpio* fingerlings reared in biofloc system with different carbon sources. Significant difference ($P < 0.05$) are indicated by mean values that do not show common superscript letter. Codes: cntrl, control; SM, sugarcane molasses; Tp, tapioca; and Wh, wheat.

study, the biofloc with sugarcane molasses as a carbon source among the treatment groups revealed a lower TAN and nitrite value and a higher nitrate level. This showed maximum bacterial population (ammonia and nitrite-oxidizing bacteria) [42]. The nitrogen compounds and TAN level in our study are according to the study of Khanjani et al. [36]. For aquaculture, the toxic levels of nitrite and nitrate are often more than 5 and 60 mg/L, respectively [43]. In this study, the TSS level in biofloc-based units was higher compared to the control. This is due to the addition of organic carbon sources, which produces a favorable environment for biofloc production. Several studies also reported similar results [2, 6]. Kim et al. [44] reported a higher TSS level (760 mg/L). As TSS levels increase, it is necessary to increase aeration and manage the water physics–chemical parameters in the biofloc system with greater precision [45]. Increasing biofloc volume can enhance nutrient recycling and improve system sustainability [18]. In our investigation, the biofloc volume was higher in the tapioca-based carbon source than in other sources, aligning with the study reported by Rind et al. [6].

The current study observed better *C. carpio* growth performance and FCR in biofloc with the tapioca-based group compared to others. This demonstrates that biofloc with a tapioca-based group extensively increases the microbial floc, which is an additional protein source for fish. Also, tapioca has a higher carbohydrate content (90%) than wheat and sugarcane molasses; it may promote microbial growth more successfully [46]. The current study is consistent with the study reported by Rind et al. [6], in which tapioca is a carbon source that enhances the growth performance of Nile tilapia. Another study by Ahmad et al. [47] revealed a higher growth performance of *Labeo rohita* with tapioca as a carbon source in the biofloc system. Other studies on fish species cultured in biofloc with different carbon sources also revealed increased growth performance [34, 35]. According to Khanjani et al. [36], the FCR in the biofloc system should be close to 1, which

is consistent with the current study. Several studies have found that using the biofloc system increases fish growth performance and FCR due to its probiotic properties and comprises different essential nutrients [48, 49]. Biofloc has been found to improve the feed efficiency and protein efficiency ratio of cultivation systems [50].

In this study, no significant difference was observed in the crude protein level of *C. carpio* among the treatment groups. The crude lipid, moisture, and ash content were higher in biofloc with wheat-based treatment. Similar findings were reported by Ahmad.H et al. [35], who reared *L. rohita* in biofloc with different carbon sources. These increased nutrients in fish bodies may be possible due to artificial diet and bioflocs availability in treatment groups compared to control. According to Ju et al. [51], the biofloc contains different essential amino acids, fatty acids, and many nutritional elements. Several studies also revealed that biofloc with different carbon source affect the body composition of different species, such as *C. carpio* [52] and Nile tilapia [36].

To understand the fish's health status, the analysis of haematological parameters is essential [53, 54]. In this study, the biofloc with tapioca significantly enhanced the *C. carpio* WBCs, HCT, HB, and lymphocytes more than other treatments and the control group. There is an essential relationship between fish health and the number of leukocytes, which are critical components of innate immunity during inflammatory events [55]. Similarly, in other studies, it has been found that different carbon sources in biofloc affect the haematology of different aquatic species, such as *C. carpio* [52], *L. rohita* [47], and *Pangasianodon hypophthalmus* [56]. Several studies revealed that the culture system, carbon source, microbial diversity, environment, fish species, age, sex, water parameters, seasons, diet, and disease influence the blood profile of aquatic species [4, 6, 57–59].

In general, bioflocs stimulate the immune system, although their degree of stimulation depends on the carbon source. In our study, the fish grown in biofloc with tapioca treatment showed higher nonspecific immune parameters such as NBT activity and total serum protein. In many studies, fish immunity was enhanced by using several substances such as *Castanea sativa* [60], *Lactobacillus plantarum* and pineapple peel [61], and *Passiflora edulis* [62]. Bioflocs are a rich source of several antibacterial compounds and immunostimulatory substances [6]. The lower serum protein and globulin levels in biofloc with sugarcane molasses were recorded, which may be probable signs of the lower immune status of fish than other treatments. TIG levels in our study were observed higher in biofloc with tapioca as a carbon source. This is consistent with the results of Kumar et al. [63] in fish "*Carassius auratus*" fed on fortified feed with azadirachtin.

The current study showed that the biofloc treatment groups enhance the nonspecific immune parameters of fish, such as NBT and lysozyme activity. The formation of free radicals takes place due to the destruction of bacterial invaders. It has been reported in different studies that NBT value can be increased by using different extracts, such as microbial levan in *C. carpio* [64] and azadirachtin in *Carassius auratus* [63].

Based on the NBT assay, tapioca-based biofloc treatment was found to have the highest respiratory burst activity.

A high level of lysozyme in fish blood has been recognized as one of their natural protection mechanisms against bacteria [65]. In this study, the higher lysozyme level was observed in biofloc with wheat followed by tapioca-based carbon source than sugarcane molasses and control. This suggests that the floc composition and quality generated by various carbon sources and subsequently consumed by the cultured organisms may impact the nonspecific immunological parameters.

Apart from immunostimulatory action, the results of fish stress parameters such as glucose and cortisol levels were higher in the control group compared to the experimental groups. However, the lower level was found in fish reared in biofloc with the tapioca-based unit. The change in glucose and cortisol levels can be explained by the phenomenon that acute stressors can lead to a swift elevation in both cortisol and glucose, reflecting an immediate response [66, 67]. In biofloc systems within aquaculture, the practice of cofeeding, involving the supplementation of feed alongside naturally occurring microorganisms (biofeeding) and organic components, plays a crucial role in shaping the physiological responses of cultured organisms. The provision of a balanced and nutritious diet, facilitated by cofeeding, is intricately linked to the biochemical parameters of the cultivated species. For instance, glucose levels in the organisms can be influenced by the quality and composition of the cofed diet, reflecting the metabolic processes within the system [68]. Similarly, the levels of TSG, TSA, and TIG are indicative of the immune and physiological status of the aquatic organisms. These parameters are intricately connected to the organic components within the biofloc system. By promoting microbial communities that break down organic matter and improve nutrient cycling, biofloc systems contribute to maintaining optimal water quality. This, in turn, helps suppress environmental stress factors, including temperature and water quality variations, which can impact cortisol levels—a key stress hormone in aquatic organisms [69], as indicated by the current study. Therefore, the cofeeding strategy in biofloc systems directly influences nutritional intake and indirectly regulates the biochemical markers associated with stress and immune responses, highlighting the comprehensive impact of BFT on the health and well-being of cultured aquatic species.

Catecholamine hormones, fight or flight hormones produced from chromaffin tissue, and cortisol during stressful (internal or external) situations mobilize and raise glucose synthesis in fish through glycogenesis and glycogenolysis pathways. According to Fryer and Lederis [70], the corticotrophin-releasing factor stimulates cortisol production in fishes by secreting adrenocorticotropin-releasing hormone (ACTH). The findings of glucose and cortisol levels in this study are, according to Mansour and Esteban [71], using different carbon sources in biofloc for Nile tilapia. Other authors, including Ahmad.H et al. [35], also revealed similar findings.

It is evident from the results of the challenge experiment with different treatments of *A. hydrophila* that biofloc plays

an essential role in boosting the immune response of fish. The application of BFT was found to improve the immunity of cultured fish [6, 72]. Compared to other treatments and the control, the biofloc with tapioca unit had the highest RPS, indicating a stronger immune response and improved resistance to *A. hydrophila* infection.

The results of this study suggest that in situ bioflocs play a part in stimulating the immune system and decreasing the vulnerability of *C. carpio* fingerlings to bacterial infection. This study reveals significant impacts on the growth, haematology, body composition, and nonspecific immunity of *C. carpio* fingerlings in response to biofloc systems using different carbon sources. Notably, tapioca emerged as a particularly influential carbon source, demonstrating a more favorable effect than others.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

The study was approved and conducted after ethical committee approval of Institute of Molecular Biology and Biotechnology, University of Lahore, under the trial registration number (UOL/ZOO/029), Date: 15-Feb-2022, and the study was conducted according to the ethical deliberation of the European Legislation (Animal rights).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

S.S. Habib and S. Tasleem conceived the idea and designed the experiment. The first draft of the manuscript was written by S.S. Habib, S. Tasleem, S. Masud, and Ü. Acar. Samples collection and analysis were performed by B.S. Alotaibi, M. Ullah, and K. Khan. Manuscript was reviewed and edited by F. Fazio, Ü. Acar, and K. Khayyam. All authors have read and agreed to the published version of the manuscript.

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