Fish and Shellfish Immunology 95 (2019) 440–448

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Biofloc technology improves growth, innate immune responses, oxidative status, and resistance to acute stress in common carp (*Cyprinus carpio*) under high stocking density

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ARTICLE INFO

Keywords:
Biofloc
Stocking density
Immunity
Digestive enzyme activities
Common carp

ABSTRACT

This study aimed to evaluate the effects of biofloc technology (BFT) on the performance of common carp under high stocking density. Fish (8.41 ± 0.44 g) were stocked into 12 tanks (70 L) in a 2 × 2 factorial design consisting of clear water (CW) and BFT systems at two stocking densities. Fish were divided into four groups according to culture system and stocking density: CW6 (clear water, 6 kg m^{-3}), CW12 (clear water, 12 kg m^{-3}), BFT6 (biofloc, 6 kg m^{-3}), and BFT12 (biofloc, 12 kg m^{-3}). After 49 days, the fish were exposed to acute crowding stress (40 kg m^{-3}) for 7 days to evaluate the fish performance to additional stressors. Total ammonia nitrogen (TAN) concentration was significantly lower in BFT groups at the end of the trial. The growth of fish was significantly higher and the feed conversion ratio was lower in BFT groups. The final density of fish was highest in BFT12 group (25.41 ± 0.48 kg m^{-3}). The amylase and protease activities in BFT groups were significantly higher than the CW12 group. The highest serum catalase, superoxide dismutase, and glutathione peroxidase activities, and malondialdehyde content (3.07 ± 0.16 nmol mg^{-1} protein) were observed in CW12 group. Serum LDL, cholesterol, triglyceride, total protein, and albumin levels were significantly higher in CW6 and BFT6 groups, followed by BFT12 group, and lowest in CW12 group. Serum globulin level was lowest in CW12 group (0.68 ± 0.06 g dL^{-1}). After 49 days, serum lysozyme, alternative complement (ACH_{50}) and ALP activities were significantly higher and serum cortisol and glucose levels and ALT and AST activities were lower in BFT groups than the CW12 group. After 7 days of acute stress, serum lysozyme activities were significantly lower and ACH_{50} and ALP activities were higher in BFT groups than the CW12 group. Also, fish in CW12 group were more sensitive to the acute stressor, showing higher cortisol and lower glucose levels. Overall, biofloc had the anti-stress effect, and the culture of common carp in the biofloc system at a stocking density of 12 kg m^{-3} could be recommended. BFT can also exert beneficial effects on the welfare of fish subjected to acute stress.

1. Introduction

Common carp (*Cyprinus carpio*) is widely reared in Iran and many other countries. The world aquaculture production of this species reached 4.129 million tons in 2017 [1]. Recently, intensive common carp culture in concrete ponds have been developed in different countries, due to rising population and local market demands. In aquaculture systems, profitability depends on the growth of fish and culture density [2]. Thus, culture density is a key factor for the productivity and profitability of common carp farms. The apparent efficiency of culture systems can be maximized by increasing the stocking densities [3]. However, the development of high-density systems could be the cause of accumulation of organic matter and toxic inorganic nitrogen and has led to an increase in concerns regarding the development of sustainable aquaculture [4,5]. Stress due to the increased stocking density have deleterious effects on growth, feed utilization, antioxidant and immune systems, welfare and health of common carp [6,7] and other farmed fish [8–11]. Therefore, alleviating the adverse effects of stress and/or strengthen immunity are important goals of the aquaculture industry.

One of the aquaculture systems that can be applied in intensive culture of fish is the biofloc system. The basic principle of biofloc technology (BFT) is the microbial conversion of nitrogenous wastes in culture systems into microbial biomass (biofloc), which can *in situ* be used by the cultured fish as an additional food source [12,13].

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https://doi.org/10.1016/j.fsi.2019.10.057
Received 1 September 2019; Received in revised form 11 October 2019; Accepted 28 October 2019
Available online 31 October 2019
1050-4648/

Microbial flocs continuously provide additional protein (essential amino acids), polyunsaturated fatty acids, vitamins and minerals [4,13]. Thus, BFT can improve water quality, feed efficiency and immunity of fish [5,14]. BFT focused on the more efficient use of nutrient input with minimal or zero water exchange during the culture period [15], and thereby can improve sustainability, biosecurity, and production in fish culture system [12]. The biofloc system have beneficial effects on growth, feed utilization, digestive enzyme activities, immune system and robustness against stress and diseases [4,16–18].

In the biofloc system, the fish stocking density can be increased higher than that in the clear water system [16,18]. Thus, under higher stocking density condition compare to the clear water system, the welfare of fish may not be impaired, and they can still grow well [19]. demonstrated that microbial flocs can improve water quality and growth performance of common carp under zero water exchange. BFT has a high potential for disease control and management in the common carp intensive culture [17]. However, very little is known about the effects of BFT on growth, immunity and stress resistance of common carp under high density conditions. Furthermore, it was found that although common carp responded with a mild stress response to increased stocking density and adaptation occurred, the crowded fish were more sensitive to an additional acute stressor [20]. Acute stress tests have been used to evaluate the effects of chronic stressors [10]. Therefore, further experiments are required to study whether high stocking density affects the ability of fish to resist additional stressors, thus being more susceptible to stress. This study aimed to evaluate the effects of BFT system on water quality, growth performance, feed utilization, digestive enzyme activities, antioxidant and immune responses, as well as resistance to acute stress in common carp under intensive culture. Alleviating the detrimental effects of high stocking density stress and improve fish immunity by BFT system are important goals of this research. The main aim of our study was to assess the biofloc *in situ* has anti-stress efficacy. The results of this study could be very useful and applicable to common carp farmers.

2. Materials and methods

2.1. Experimental design and biofloc production

The experiment was conducted at Gonbad Kavous University, Gonbad, Golestan, Iran, and in accordance with the ethics and animal care committee of Gonbad Kavous University. A total of 900 common carp were obtained from a commercial farm, Sari, Mazandaran, Iran. The fish were acclimatized to experiment conditions and fed with a commercial diet (Faradaneh Co., Shahrekord, Iran) three times a day (09:00, 13:00 and 17:00 h) at 3% of biomass for 14 days. The nutritional composition of the commercial diet consisted of 38% crude protein, 7% crude lipid, 5% crude fiber, 9% ash, and 1.25% phosphorus. The experiment was applied in a 2 × 2 factorial design consisting of 2 clear water (CW) groups and 2 BFT groups with 3 replications per group. After acclimatization, all of the tanks were stocked with fish from the stock (acclimatization) tank. The fish were randomly captured (netted) from the stock tank. The fish (initial weigh of 8.41 ± 0.44 g) were randomly distributed into 12 indoor cylinder fiberglass tanks (0.6 m diameter, 0.3 m height, the effective volume of 70 L) at a stocking density of 50 fish per tank (6 kg m^{-3}) and 100 fish per tank (12 kg m^{-3}) following a completely randomized design (CRD). Then, the fish were divided into four experimental groups according to culture system and stocking density: CW6 (clear water, 6 kg m^{-3}), CW12 (clear water, 12 kg m^{-3}), BFT6 (biofloc, 6 kg m^{-3}), and BFT12 (biofloc, 12 kg m^{-3}).

For the formation of microbial flocs stock (inoculum), 100 L of the effluent water from common carp culture tank (acclimatization stage) was transferred to a conoid tank, and total ammonia nitrogen (TAN) concentration was measured. To stimulate biofloc growth, sugar was used as the organic carbon source, and after weighting and mixing with water was added to the tank daily. Sugar is rapidly assimilated by bacteria, increasing biofloc production in less time [21]. The carbon: nitrogen (C:N) ratio was maintained at 15:1 using the carbon source (sugar) according to [5]. Tank aeration was stopped when TAN concentration decreased to almost zero and total suspended solids (TSS) reached 300 mg L^{-1}, after which the biofloc tanks were inoculated with 200 mL L^{-1} of microbial flocs [22].

Fish were fed the aforementioned commercial diet three times a day by hand (09:00, 13:00 and 17:00 h) at a feeding rate of 3% of biomass for 49 days. During the experiment period, demand carbon source (sugar) was determined based on the protein content (%) of the diet and the amount of feed supplied in each biofloc group according to [5], assuming that protein contain 16% nitrogen and fish will excrete 75% of protein nitrogen. To maintain the C:N ratio of 15:1, the carbon source was added to the biofloc tanks daily depending on the experimental group after the second feeding at 13:00 h. The C:N ratio of 15:1 was chosen, because the previous studies indicated that the C:N ratio of 15:1 was suitable for the fish [18,23]. All experimental tanks were continuously aerated and agitated using air stones connected to an air pump. In clear water groups, a regular water exchange at a level of 25% of the total volume was performed daily, whereas in BFT groups, clean freshwater was only added to replace loss due to evaporation. The light condition was maintained at 12:12 h (light/dark, artificial luminosity of ~600 lx). No disease was breakout before and during the experiment period.

2.2. Assessment of water quality

Water temperature, salinity, dissolved oxygen, and pH were measured daily between 08:30 and 09:30 using Hach HQ40d portable apparatus (Loveland, Colorado, USA). The alkalinity was measured daily by acid titration according to [24]. Total ammonia nitrogen (TAN), nitrate-nitrogen (NO_3-N), and phosphate (PO_4^{3-}) concentrations were analyzed spectrophotometrically every 15 days (on days 3, 18, 33 and 48 of rearing) following standard methods [25]. Water samples (50 ml) were also collected from each tank every 15 days and filtered under vacuum pressure through pre-dried and pre-weighed filter paper (Whatman). The filter paper containing suspended materials was dried at 105 °C in an oven until a constant weight was reached, and the dried filter paper (sample) was weighed afterward. Then, the weight difference and the amount of total suspended solids (TSS) were calculated [4]. Biofloc volume (BFV) was determined using an Imhoff cone every 15 days, where the biofloc volume was registered after 30 min of 1000 mL water sedimentation of each tank [12].

2.3. Growth performance and sampling

After 49 days, fish were starved for 24 h. Then, all fish from each tank were weighed and the final weights were measured to calculate growth and feed utilization parameters. Also, the blood samples were randomly collected from the caudal vein of 4 fish from each tank (12 fish per group). The fish were anesthetized using 200 mg L^{-1} clove powder before sampling [26]. The time from capture to blood collection was < 4 min to avoid cortisol increase due to handling during sampling. After clotting, the blood samples were centrifuged at 5000 g for 10 min at 4 °C, and serum separated. After being bled, the whole digestive tract was also collected from each fish for the determination of digestive enzyme activities. All the samples were quickly frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.4. Acute crowding stress

After 49 days of rearing, the fish from all clear water and BFT groups were exposed to acute crowding stress by decreasing water volume in tanks as previously described [7]. The fish were reared for acute crowding stress (40 kg m^{-3}) for 7 days in aforementioned

(section 2.1.) indoor cylinder fiberglass tanks (0.6 m diameter, 0.3 m height, the effective volume of 70 L). All tanks were continuously aerated and agitated and dissolved oxygen concentration was $> 5\ mg\ L^{-1}$. Fish were fed three times a day (09:00, 13:00 and 17:00 h) at a feeding rate of 3% of biomass during the acute crowding stress. In clear water groups, water exchange at a level of 50% of the total volume was performed daily, whereas in BFT groups, clean freshwater was only added to replace loss due to evaporation. Before and after 7 days of crowding stress, the blood samples were randomly collected from 4 fish in each tank (12 fish per group). After centrifugation (at 5000 g for 10 min at 4 °C), the serum samples were stored at −80 °C for further analysis.

2.5. Digestive and antioxidant enzymes activities

The whole digestive tract of fish was homogenized as described by [22]. The homogenates were centrifuged at 25000 g for 20 min at 4 °C to eliminate debris and lipids, the resultant supernatants (enzyme extracts) collected, and frozen at −80 °C until the analysis of digestive enzyme activities. Amylase activity was assayed according to [27]; using 0.3% soluble starch as substrate dissolved in NaH_2PO_4 buffer (pH 7.4). Lipase activity was assayed for 15 min at 30 °C using p-nitrophenol myristate as the substrate that is dissolved in 0.25 M Tris-HCl (pH 9.0) [28]. Protease activity was determined at 25 °C using 1% (w/v) casein (Sigma, USA) as a substrate in 0.2 M phosphate buffer at pH 7.0 [29].

Serum catalase (CAT) activity was assayed following the method of [30]. The activity of serum superoxide dismutase (SOD) was determined according to the method of [31]. Serum glutathione peroxidase (GPx) activity was measured using the method described by [32]. Serum malondialdehyde (MDA) content was assayed colorimetrically using the method of [33]. Total protein concentrations in the digestive enzyme extracts and serum were determined according to [34]; using bovine serum albumin (BSA) as the standard. The specific activity of measured enzymes was expressed as unit enzyme activity per mg protein ($U\ mg^{-1}$ protein).

2.6. Metabolic and biochemical parameters

Levels of the low-density lipoproteins (LDL), high-density lipoproteins (HDL), cholesterol, and triglyceride in the serum were measured using commercial kits (ZiestChem Diagnostics, Tehran, Iran) following the company protocol. Serum total protein and albumin levels were determined colorimetrically using commercial kits (Pars Azmun, Karaj, Iran) based on the manufacturer's procedure. The serum globulin level was calculated by subtracting serum albumin from total protein.

2.7. Stress and innate immune parameters

The serum cortisol concentration was measured by radioimmunoassay (RIA) as described by [35]. Serum glucose content was determined using a commercial kit (Pars Azmun, Karaj, Iran) following the manufacturer's procedure. Serum lysozyme activity was assayed according to [36]; based on the lysis of the lysozyme sensitive Gram-positive bacterium, *Micrococcus luteus* (Sigma). The dilutions of hen egg white lysozyme (Sigma) ranging from 0 to 20 $\mu L\ ml^{-1}$ (in 0.1 M phosphate citrate buffer, pH 5.8) were taken as the standard. This together with the undiluted serum sample (25 μL) was placed into wells of a 96-well plate in triplicate. Then, one hundred and seventy-five μL of *M. luteus* suspension (75 $mg\ ml^{-1}$) prepared in the same buffer was added to each well. After rapid mixing, the change in turbidity was determined every 30 s for 5 min at 450 nm at approximately 20 °C using a microplate reader. Alternative complement (ACH_{50}) activity was determined using the method of [37] with modifications as described previously [38]. The volume of serum complement producing 50% hemolysis (ACH_{50}) was determined, and the number of ACH_{50} $U\ mL^{-1}$ was calculated for the sample. Serum enzymatic activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate transaminase (AST) were determined using commercial kits (Pars Azmun, Karaj, Iran) with an automated biochemical analyzer (Beckman Coulter, USA).

2.8. Statistical analysis and calculations

Data (mean ± SD) were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to compare the means between groups. Before analyses, data were assessed for normality and homogeneity of variance with the Shapiro-Wilk test and Levene's test, respectively. Significant differences between means obtained before and after acute crowding stress were analyzed by independent samples *t*-test. Two-way ANOVA was also used to test the effects of the culture system, stocking density, and their interactions. Statistical analyses were conducted using SPSS software version 23 and $P < 0.05$ was the accepted significance level. The following parameters were also calculated:

Survival rate (%) = 100 × final number of fish/initial number of fish

Weight gain rate (WGR, %) = 100 × (final weight – initial weight)/initial weight

Specific growth rate (SGR, % day^{-1}) = 100 × (ln final weight – ln initial weight)/days

Feed conversion ratio (FCR, g g^{-1}) = dry feed intake/(final weight – initial weight)

Protein efficiency ratio (PER, g g^{-1}) = (final weight – initial weight)/protein intake

Condition factor (CF, %) = 100 × [final weight (g)/(final length (cm))^3]

Hepatosomatic index (HSI, %) = 100 × (liver weight/body weight)

Gut weight ratio (%) = 100 × (gut weight/body weight)

3. Results

3.1. Biofloc development and water quality

The results of water quality parameters in the experimental groups during 49 days trial are shown in Table 1. The biofloc development in terms of total suspended solids (TSS), and biofloc volume (BFV) is shown in Fig. 1. The TSS and BFV levels increased during the experiment. Also, TSS and BFV levels were increasing with the increase in stocking density. Dynamics of inorganic nitrogen (TAN and NO_3-N) and phosphate (PO_4^{3-}) in different groups during the experiment are shown in Fig. 2. BFT groups had generally lower TAN concentrations than clear water (CW) groups. Higher stocking densities also led to higher inorganic TAN. On day 48, the NO_3-N concentrations in the BFT groups were significantly higher than in the CW groups (Fig. 2). While PO_4^{3-} concentration increased in CW groups during the experiment, it decreased in BFT groups as the trial progressed.

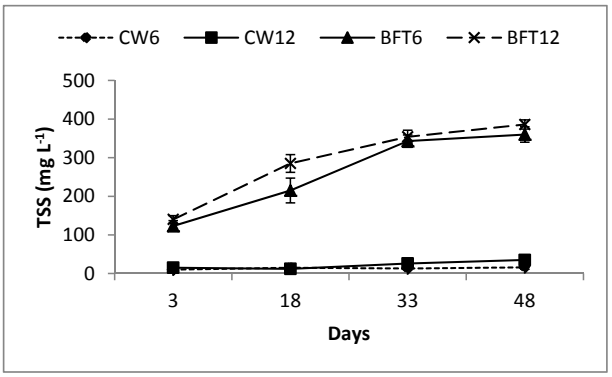
3.2. Growth performance and feed utilization

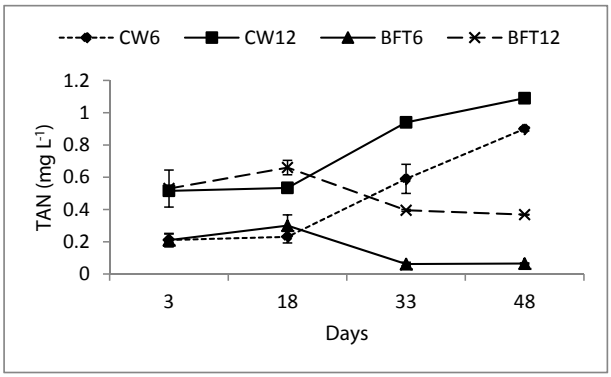
Growth performance and feed utilization of common carp reared in CW and BFT systems at different stocking densities (6 and 12 $kg\ m^{-3}$) for 49 days are shown in Table 2. The final weight, weight gain ratio (WGR), specific growth rate (SGR), and protein efficiency ratio (PER) of fish in BFT groups were significantly higher than those in the clear water (CW) groups. The feed conversion ratio (FCR) was significantly

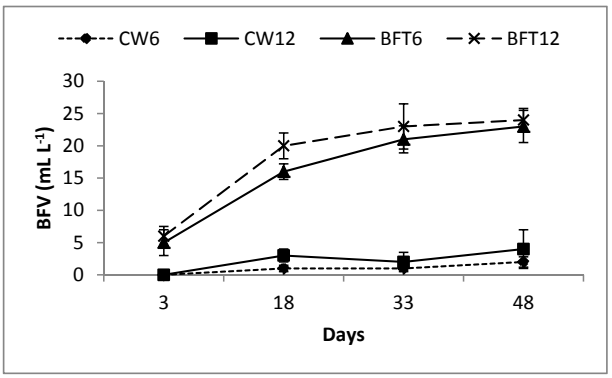
Table 1
The overall means of selected water quality parameters in the CW and BFT systems stocked with common carp at different densities (6 and 12 kg m^{-3}) for 49 days (mean ± SD, n = 12).

Parameters	Experimental groups			
	CW6	CW12	BFT6	BFT12
Temperature (°C)	24.32 ± 0.58	24.41 ± 0.54	24.33 ± 0.49	24.52 ± 0.64
Salinity (g L^{-1})	0.45 ± 0.01	0.46 ± 0.02	0.46 ± 0.01	0.47 ± 0.02
Dissolved oxygen (mg L^{-1})	7.06 ± 0.46^{a}	6.63 ± 0.80^{ab}	6.96 ± 0.58^{a}	6.40 ± 0.34^{b}
pH	7.72 ± 0.12^{a}	7.73 ± 0.19^{a}	7.54 ± 0.15^{b}	7.48 ± 0.16^{b}
Alkalinity (mg L^{-1} $CaCO_3$)	298.88 ± 34.03	330.73 ± 77.97	321.56 ± 31.18	341.95 ± 39.58

Means in the same row with different superscripts are significantly different ($P < 0.05$). CW, clear water; BFT, biofloc technology.







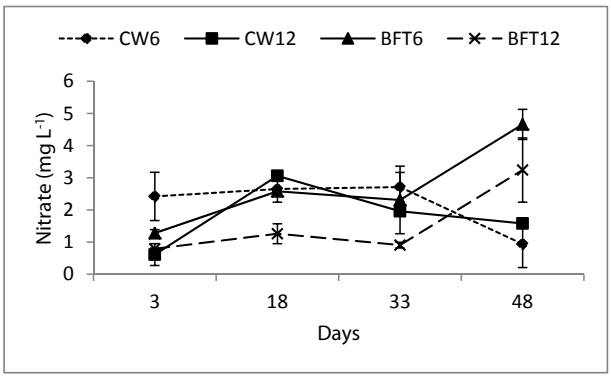


Fig. 1. Changes of total suspended solids (TSS) and biofloc volume (BFV) in the CW and BFT systems stocked with common carp at different densities (6 and 12 kg m^{-3}) for 49 days. Values are means (± SD) of three replicate tanks per sampling time in each group. CW, clear water; BFT, biofloc technology.

affected by the culture system and the interaction of the culture system and stocking density (Table 2). The FCR in BFT groups were lower than those of the CW groups. Interestingly, the final density (25.41 kg m^{-3}) and the final biomass of fish in the BFT12 group were significantly higher than other groups. Hepatosomatic index (HSI) was significantly lower in CW12 than that in other groups.

3.3. Digestive and antioxidant enzymes activities

Digestive enzyme activities and oxidative status of common carp reared in CW and BFT systems at different stocking densities for 49 days are shown in Table 3. The amylase activity was significantly higher in low density (CW6 and BFT6) groups than those in high density (BFT12 and CW12) groups. The amylase and protease activities in fish reared in BFT groups were significantly higher than those of the CW12 group.

According to the results shown in Table 3, the highest serum catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities, and malondialdehyde (MDA) content were observed in CW12

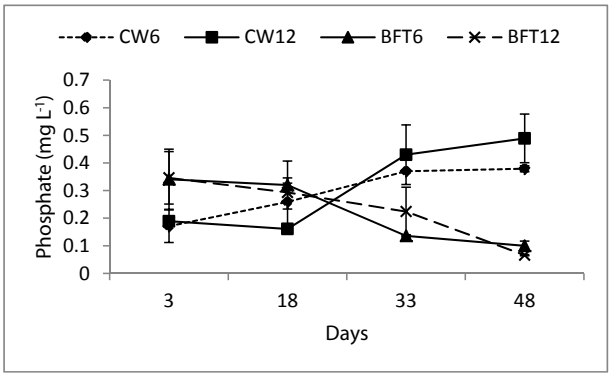


Fig. 2. Total ammonia nitrogen (TAN), nitrate-nitrogen (NO_3-N), and phosphate dynamics in the CW and BFT systems stocked with common carp at different densities (6 and 12 kg m^{-3}) for 49 days. Values are means (± SD) of three replicate tanks per sampling time in each group. CW, clear water; BFT, biofloc technology.

group. The MDA content was significantly affected by stocking density and the interaction of culture system and stocking density (Table 3). The serum MDA contents in fish reared in BFT groups were significantly

Table 2
Growth performance and feed utilization of common carp reared in the CW and BFT systems at different stocking densities (6 and 12 kg m^{-3}) for 49 days (mean ± SD, n = 3).

Parameters	Experimental groups				Two-way ANOVA		
	CW6	CW12	BFT6	BFT12	Culture system	Stocking density	Interaction
Initial weight (g)	8.48 ± 0.45	8.50 ± 0.45	8.36 ± 0.49	8.32 ± 0.42	Ns	Ns	Ns
Final weight (g)	15.92 ± 0.62^{c}	16.15 ± 0.63^{c}	19.51 ± 0.88^{a}	17.79 ± 0.33^{b}	P < 0.001	P < 0.001	P < 0.001
Survival rate (%)	100	100	100	100	Ns	Ns	Ns
Initial density (kg m^{-3})	6.05 ± 0.32^{b}	12.14 ± 0.64^{a}	5.97 ± 0.35^{b}	11.88 ± 0.60^{a}	Ns	P < 0.001	Ns
Final density (kg m^{-3})	11.37 ± 0.44^{d}	23.08 ± 0.91^{b}	13.94 ± 0.62^{c}	25.41 ± 0.48^{a}	P < 0.001	P < 0.001	Ns
Initial biomass (g)	424.16 ± 22.74^{b}	850.00 ± 45.12^{a}	418.33 ± 24.71^{b}	832.08 ± 42.50^{a}	Ns	P < 0.001	Ns
Final biomass (g)	796.41 ± 31.06^{d}	1615.83 ± 63.88^{b}	975.83 ± 44.04^{c}	1779.25 ± 33.83^{a}	P < 0.001	P < 0.001	Ns
WGR (%)	88.21 ± 11.76^{c}	90.50 ± 11.09^{c}	133.95 ± 16.47^{a}	114.34 ± 11.61^{b}	P < 0.001	P = 0.025	P = 0.005
SGR (% day^{-1})	1.28 ± 0.12^{c}	1.31 ± 0.11^{c}	1.72 ± 0.14^{a}	1.55 ± 0.11^{b}	P < 0.001	P = 0.043	P = 0.008
FCR (g g^{-1})	1.99 ± 0.20^{a}	1.93 ± 0.17^{a}	1.32 ± 0.11^{c}	1.55 ± 0.08^{b}	P < 0.001	Ns	P = 0.002
PER (g g^{-1})	1.33 ± 0.12^{c}	1.37 ± 0.12^{c}	1.99 ± 0.17^{a}	1.69 ± 0.09^{b}	P < 0.001	P = 0.001	P < 0.001
CF (%)	1.59 ± 0.26^{b}	1.56 ± 0.20^{b}	1.92 ± 0.20^{a}	1.64 ± 0.20^{b}	P = 0.003	P = 0.016	Ns
HSI (%)	2.51 ± 0.66^{a}	1.71 ± 0.60^{b}	2.09 ± 0.65^{ab}	2.01 ± 0.75^{ab}	Ns	P = 0.029	Ns
Gut weight ratio (%)	4.24 ± 0.99	3.81 ± 0.66	3.71 ± 0.70	3.79 ± 1.04	Ns	Ns	Ns

Means in the same row with different superscripts are significantly different ($P < 0.05$). CW, clear water; BFT, biofloc technology; WGR, weight gain ratio; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; CF, condition factor; HSI, hepatosomatic index; Ns, not significant ($P > 0.05$).

lower than those of the CW12 group.

3.4. Metabolic and biochemical responses

Serum metabolites of common carp reared in CW and BFT systems at different stocking densities for 49 days are shown in Table 4. Serum LDL, cholesterol, triglyceride, total protein, and albumin levels were significantly higher in CW6 and BFT6 groups, followed by BFT12 group, and lowest in the CW12 group. The serum globulin level was lowest in the CW12 group.

3.5. Stress and innate immune responses

The results of stress and innate immune parameters of common carp reared in CW and BFT systems at different stocking densities (6 and 12 kg m^{-3}) for 49 days and after additional acute crowding stress (40 kg m^{-3}) for 7 days are shown in Table 5. Before acute crowding stress, the highest serum cortisol concentration was observed in the CW12 group, but it was not significantly different from the cortisol concentration in the CW6 group. After 7 days of crowding stress, the serum cortisol concentrations in all groups were significantly higher than the pre-stress levels. Also, serum cortisol concentrations in the BFT groups were significantly reduced 7 days post-stress compared with the CW groups. Before acute crowding stress, serum glucose content was significantly higher in the CW12 group than that in other groups. After 7 days stress the serum glucose contents in CW groups were significantly lower and in BFT groups were significantly higher than those before stress. Serum glucose contents in the CW groups were significantly lower 7 days post-stress compared to the BFT groups. Also, serum glucose content decreased with the increase in stocking density from 6 to 12 kg m^{-3}.

Before acute stress, the highest serum lysozyme activity was observed in CW6 and BFT6 groups, but it was not significantly different from the lysozyme activity in the BFT12 group. Also, the serum alternative complement (ACH_{50}) and ALP activities were significantly lower in the CW12 group than other groups. After 7 days of stress, the serum lysozyme activity in CW12 group significantly increased whereas the other groups did not show a significant difference. Furthermore, serum ACH_{50} activities in the BFT groups were significantly increased 7 days post-stress compared with the CW groups. After crowding stress, the highest serum lysozyme activity and the lowest ACH_{50} and ALP activities were observed in the CW12 group. Before acute stress, the highest serum ALT and AST activities were observed in the CW12 group. Also, serum ALT and AST activities increased with the increase in stocking density from 6 to 12 kg m^{-3}. After acute stress, the serum ALT and AST activities were highest in the CW12 group, followed by the CW6 group, and lower in the BFT groups.

4. Discussion

4.1. Biofloc development and water quality

A significant decrease in pH associated with the high biomass of bacteria in BFT groups was observed. Bacterial respiration rate probably increased the CO_2 concentration resulting in a corresponding decrease in pH [39]. The dissolved oxygen concentration in the BFT12

Table 3
Digestive and antioxidant enzymes activities (U mg^{-1} protein) and oxidative status (nmol MDA mg^{-1} protein) of common carp reared in the CW and BFT systems at different stocking densities (6 and 12 kg m^{-3}) for 49 days (mean ± SD, n = 3).

Parameters	Experimental groups				Two-way ANOVA		
	CW6	CW12	BFT6	BFT12	Culture system	Stocking density	Interaction
Amylase	2.65 ± 0.01^{a}	2.49 ± 0.04^{c}	2.71 ± 0.05^{a}	2.57 ± 0.04^{b}	P = 0.021	P < 0.001	Ns
Lipase	0.83 ± 0.02	0.79 ± 0.04	0.85 ± 0.02	0.84 ± 0.04	Ns	Ns	Ns
Protease	1.28 ± 0.04^{ab}	1.24 ± 0.02^{b}	1.34 ± 0.04^{a}	1.35 ± 0.03^{a}	P = 0.006	Ns	Ns
Catalase	2.13 ± 0.10^{d}	3.50 ± 0.17^{a}	2.62 ± 0.07^{c}	3.29 ± 0.01^{b}	Ns	P < 0.001	P < 0.001
SOD	1.79 ± 0.04^{b}	2.44 ± 0.38^{a}	2.00 ± 0.01^{b}	2.15 ± 0.14^{ab}	Ns	P = 0.009	Ns
GPx	11.73 ± 0.08^{d}	15.82 ± 0.06^{a}	14.28 ± 0.02^{b}	12.32 ± 0.06^{c}	P < 0.001	P < 0.001	P < 0.001
MDA	2.27 ± 0.11^{c}	3.07 ± 0.16^{a}	2.59 ± 0.03^{b}	2.75 ± 0.03^{b}	Ns	P < 0.001	P = 0.001

Means in the same row with different superscripts are significantly different ($P < 0.05$). CW, clear water; BFT, biofloc technology; SOD, superoxide dismutase; GPx, glutathione peroxidase; MDA, malondialdehyde; Ns, not significant ($P > 0.05$).

Table 4
Metabolic and biochemical responses of common carp reared in the CW and BFT systems at different stocking densities (6 and 12 kg m^{-3}) for 49 days (mean ± SD, n = 3).

Parameters	Experimental groups				Two-way ANOVA		
	CW6	CW12	BFT6	BFT12	Culture system	Stocking density	Interaction
LDL (mg dL^{-1})	93.24 ± 3.41^{a}	65.25 ± 0.93^{d}	78.86 ± 0.56^{b}	70.51 ± 1.26^{c}	P = 0.003	P < 0.001	P < 0.001
HDL (mg dL^{-1})	30.50 ± 3.41	27.47 ± 2.39	28.92 ± 1.00	28.57 ± 1.43	Ns	Ns	Ns
Cholesterol (mg dL^{-1})	145.17 ± 3.50^{a}	125.49 ± 2.24^{c}	146.26 ± 1.55^{a}	131.80 ± 0.92^{b}	P = 0.023	P < 0.001	Ns
Triglyceride (mg dL^{-1})	205.09 ± 4.13^{ab}	166.73 ± 9.11^{c}	211.88 ± 1.63^{a}	199.96 ± 1.32^{b}	P < 0.001	P < 0.001	P = 0.002
Total protein (g dL^{-1})	2.41 ± 0.05^{a}	1.73 ± 0.04^{c}	2.33 ± 0.12^{a}	2.10 ± 0.06^{b}	P = 0.012	P < 0.001	P = 0.001
Albumin (g dL^{-1})	1.42 ± 0.03^{a}	1.05 ± 0.03^{d}	1.30 ± 0.01^{b}	1.15 ± 0.01^{c}	Ns	P < 0.001	P < 0.001
Globulin (g dL^{-1})	0.98 ± 0.08^{a}	0.68 ± 0.06^{b}	1.02 ± 0.14^{a}	0.94 ± 0.06^{a}	P = 0.022	P = 0.007	Ns
A:G ratio	1.45 ± 0.15	1.55 ± 0.18	1.29 ± 0.19	1.22 ± 0.10	P = 0.031	Ns	Ns

Means in the same row with different superscripts are significantly different ($P < 0.05$). CW, clear water; BFT, biofloc technology; LDL, low density lipoproteins; HDL, high density lipoproteins; A:G ratio, albumin: globulin ratio; Ns, not significant ($P > 0.05$).

group was lower than the other groups. However, continuous intense aeration ensured that dissolved oxygen was not limiting. Significant increases in total suspended solids (TSS) and biofloc volume (BFV) levels were found in the BFT groups during the experiment, reaching the highest levels at the end of the trial. But the biofloc in the high density group (BFT12) increased faster than the low density group (BFT6) due to the higher input of feed, more fish excretion and nitrogen compounds formation [39,40]. The BFV level suggests the aggregation of the biofloc [18]. Similar to our results, it is reported that BFV in biofloc groups were increasing with the increase in fish density and the culture period [16]. In our study, the biofloc development in terms of TSS and BFV is attributed to continued feed input and organic carbon supplementation in the form of sugar [41,42]. The formation and development of the biofloc were linked with the assimilation of nitrogenous compounds by heterotrophic bacteria or autotrophic nitrification [40,42], and thereby concentrations of TAN, NO_2-N, and NO_3-N were controlled. BFT is a technique of enhancing water quality by a C:N balance in the system [5]. In our study, TAN concentrations in the biofloc groups were effectively reduced when the C:N ratio was 15:1. Therefore, heterotrophic bacteria formed due to the increase of the C:N ratio can absorb the ammonia nitrogen from the water which in turn further increases the formation of biofloc [12,13]. In our study, TAN concentrations increased with the increase in stocking density from 6 to 12 kg m^{-3}, because the fish were more than the low density groups, and the excretion of fish was higher. However, TAN concentrations remained at low levels in all experimental groups during the trial, not exceeding 1.09 mg L^{-1}.

The NO_3-N concentrations increased gradually in the BFT groups during the experiment. On day 48, the NO_3-N concentrations were significantly higher in the BFT groups than in the CW groups, because the nitrification was working in the BFT groups [4]. So, the present study indicated that TAN concentration can be effectively controlled in BFT groups by either ammonia assimilation into heterotrophic bacteria or autotrophic nitrification. The PO_4^{3-} concentrations decreased in BFT groups as the trial progressed because the growth and reproduction of the biofloc microorganisms assimilated the phosphorus [43]. Similarly [44], demonstrated that the decrease of phosphate concentration in the heterotrophic groups may be due to the assimilation of phosphate by heterotrophic bacteria.

4.2. Growth performance and feed utilization

In the current study, the growth of fish in BFT groups was significantly higher than those in the CW groups. Interestingly, the FCR in BFT groups was lower than those of the CW groups. This suggests that BFT could be applied to enhance the intensive production of common carp. The positive effects of biofloc system on growth performance and feed utilization of common carp might be explained by different factors, biofloc provides good and stable water quality [5,45], biofloc could be continuously harvested by the fish as a good quality of food source

Table 5
Stress and innate immune responses of common carp reared in the CW and BFT systems at different stocking densities (6 and 12 kg m^{-3}) for 49 days and after additional acute crowding stress (40 kg m^{-3}) for 7 days (mean ± SD, n = 3).

Parameters	Experimental groups				Two-way ANOVA		
	CW6	CW12	BFT6	BFT12	Culture system	Stocking density	Interaction
Before acute stress							
Cortisol (ng mL^{-1})	36.78 ± 2.39^{ab}	43.41 ± 7.83^{a}	31.00 ± 1.03^{b}	33.07 ± 1.09^{b}	P = 0.010	P = 0.108	Ns
Glucose (mg dL^{-1})	135.18 ± 3.68^{b}	140.01 ± 2.00^{a}	124.35 ± 1.18^{d}	128.94 ± 0.92^{c}	P < 0.001	P = 0.006	Ns
Lysozyme (U mL^{-1})	34.70 ± 3.85^{a}	28.10 ± 1.68^{b}	33.13 ± 0.93^{a}	32.04 ± 1.03^{ab}	Ns	P = 0.017	Ns
ACH50 (U mL^{-1})	139.39 ± 3.04^{a}	126.59 ± 2.85^{b}	136.19 ± 1.05^{a}	135.20 ± 0.84^{a}	Ns	P = 0.001	P = 0.002
ALP (U L^{-1})	151.94 ± 3.43^{a}	131.83 ± 2.07^{b}	148.44 ± 1.19^{a}	150.98 ± 2.25^{a}	P < 0.001	P < 0.001	P < 0.001
ALT (U L^{-1})	19.75 ± 1.14^{b}	27.72 ± 0.59^{a}	14.63 ± 1.25^{c}	19.49 ± 0.62^{b}	P < 0.001	P < 0.001	P = 0.022
AST (U L^{-1})	530.28 ± 4.44^{b}	602.73 ± 1.61^{a}	463.38 ± 2.48^{d}	495.51 ± 1.83^{c}	P < 0.001	P < 0.001	P < 0.001
After acute stress							
Cortisol (ng mL^{-1})	137.73 ± 2.04^{b}*	147.55 ± 1.38^{a}*	131.94 ± 1.20^{c}*	132.28 ± 4.90^{c}*	P < 0.001	P < 0.001	P < 0.001
Glucose (mg dL^{-1})	124.35 ± 1.04^{c}*	117.17 ± 0.80^{d}*	136.46 ± 0.69^{a}*	132.33 ± 1.79^{b}*	P < 0.001	P < 0.001	Ns
Lysozyme (U mL^{-1})	31.84 ± 1.19^{b}	36.65 ± 1.30^{a}*	32.86 ± 1.78^{b}	30.99 ± 1.13^{b}	P = 0.019	Ns	P = 0.003
ACH50 (U mL^{-1})	134.60 ± 1.31^{b}	129.35 ± 1.52^{c}	140.74 ± 1.27^{a}*	141.15 ± 1.16^{a}*	P < 0.001	P = 0.014	P = 0.006
ALP (U L^{-1})	239.30 ± 3.01^{b}*	204.66 ± 2.27^{d}*	291.83 ± 2.36^{a}*	209.58 ± 2.29^{c}*	P < 0.001	P < 0.001	P < 0.001
ALT (U L^{-1})	33.22 ± 1.09^{b}*	43.52 ± 1.37^{a}*	28.29 ± 1.19^{c}*	30.20 ± 1.07^{c}*	P < 0.001	P < 0.001	P < 0.001
AST (U L^{-1})	714.36 ± 3.84^{b}*	769.13 ± 3.50^{a}*	562.90 ± 3.69^{d}*	649.81 ± 3.82^{c}*	P < 0.001	P < 0.001	P < 0.001

Means in the same row with different superscripts are significantly different ($P < 0.05$). Significant differences ($P < 0.05$) between means obtained before and after acute stress are marked by asterisks. CW, clear water; BFT, biofloc technology; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate transaminase; Ns, not significant ($P > 0.05$).

[15–17] and biofloc environment and consumption improved the fish resistance against stress [16,18]. In our study, the lower FCR and the higher PER in BFT groups might indicate the possibility of biofloc consumption by the fish [46]. reported that the FCR was significantly lower in the biofloc groups than in the control. It is also reported that despite a 25% decrease in daily feeding rate in BFT groups, carbon sources significantly improved FCR and growth performance of common carp [19]. Moreover, the biofloc has been reported to enhance feed utilization of fish by supplementing essential amino acids, lipids, vitamins, and minerals [4,13,47], and by stimulating digestive enzyme activities, resulting in improved digestion of nutrients in fish gut [18,19,22].

Increasing the stocking density is an important strategy to increase the productivity and profitability of fish production [2,16]. However, chronic stress due to high density have adverse effects on growth, feed utilization, immune and antioxidant responses, and welfare in farmed fish [8,11,18]. In our study, the growth of common carp in the CW12 group was depressed, indicating that the high stocking density impaired the growth of fish. But in the BFT12 group, the growth of fish was significantly higher than the CW groups, because the biofloc can be as a supplement food source, or a kind of anti-stress probiotic [18]. Also, the final density of fish and the final biomass produced in the BFT12 group were significantly higher than the CW12 group. In the CW6 group, though the stocking density was equal to the BFT6 group, there was no biofloc, so the growth was still depressed. Hepatosomatic index (HSI) was significantly lower in the CW12 group than that in other groups. An increased mobilization of liver reserves caused by higher energy demands under high density conditions can be responsible for lower HSI in CW12 group [48].

4.3. Digestive and antioxidant enzymes activities

Previous studies have indicated that biofloc contain exogenous digestive enzymes, such as protease and amylase, which may act as supplement to endogenous digestive enzymes, and could facilitate the digestion, absorption and utilization of the feed [18,22,43,49]. In our study, the amylase and protease activities in the fish reared in BFT groups were significantly higher than those of the CW12 group. These results indicated that common carp can adapt well to new nutritional conditions and biofloc stimulated digestive enzyme activities in fish, resulting in improved digestion and utilization of feed, and fish growth performance [19,49]. In our study, the amylase activity decreased significantly with increasing stocking density. Therefore, the high stocking density can impair the digestion and utilization of the feed.

The stress response can increase reactive oxygen species (ROS), resulting in higher lipid peroxidation content and more lipid peroxidation damage [50]. Microbial flocs are rich in various bioactive products that contain fat-soluble vitamins, carotenoids, phytosterols, and taurine [51]. Therefore, we expected BFT to improve the oxidative status in fish. In the present study, the serum MDA contents in the fish reared in BFT groups (BFT6 and BFT12) were significantly lower than the CW12 group, suggesting that BFT can reduce lipid peroxidation and improve the oxidative status in common carp under high density conditions. In our study, the highest serum catalase, SOD and GPx activities were observed in the CW12 group. Also, the highest serum lipid peroxidation (MDA content) was observed in the CW12 group. The highest antioxidant enzyme activities could suggest that the fish reared in the CW12 group did not experience any kind of oxidative stress. However, when these results are integrated with the oxidative status data (MDA content) this conclusion cannot be drawn. On the other hand, lipid peroxidation is thought to be inefficiently inhibited by antioxidant enzymes in the CW12 group. Similar to our results [52], reported that chronic stress can increase serum and muscle MDA contents in fish under crowding conditions.

4.4. Metabolic and biochemical responses

In our study, serum LDL, cholesterol, and triglyceride levels were significantly higher in CW6 and BFT6 groups, followed by BFT12 group, and lowest in the CW12 group. On the other hand, serum metabolite levels decreased significantly with increasing stocking density, indicating that lipid stores were depleted as a result of chronic stress in response to the increased energy demand by high density conditions [8,53]. Also, serum LDL, cholesterol, and triglyceride levels were even lower in fish reared in the CW12 group than in those reared in the BFT12 group, results that coincide with the higher cortisol concentration, indicating a more severe depletion of the lipid reserves in these fish. A strong innate immune system is associated with increasing levels of proteins, such as albumin and globulin, which represent the major proteins in serum [54]. In the present study, serum total protein and albumin levels were significantly higher in CW6 and BFT6 groups, followed by BFT12 group, and lowest in CW12 group, and serum globulin level was lowest in CW12 group, indicating that microbial flocs may act as an immunostimulant [14,23].

4.5. Stress and innate immune responses

Chronic stress such as long-term crowding suppresses the innate and adaptive immune responses through neuroendocrine mechanisms [55,56]. The results of the present study demonstrated that long-term high stocking density stimulated stress responses in common carp. In our study, fish reared in clear water system at high density (CW12 group) showed the highest serum cortisol concentration. After 7 days of crowding stress, the serum cortisol concentrations in all groups were significantly higher than the pre-stress levels. Therefore, fish in all groups were able to show a stress response to an acute stressor, however, fish at the highest chronic stress level (CW12 group) were more sensitive to the acute stressor, showing higher cortisol concentrations than the other groups. Similar to our results [10], demonstrated that after the acute stress, fish at the highest stocking density had significantly higher concentrations of plasma cortisol. After acute crowding stress, the elevated cortisol concentrations in the CW12 group may be due to an additive effect of the acute crowding plus the high stocking density. In our study, serum cortisol concentrations in the BFT groups were significantly reduced compared with the CW groups. These findings suggest that BFT can prevent the increase in serum cortisol concentration induced by acute crowding. Biofloc had an anti-stress effect [18]. Before acute crowding stress, serum glucose content was significantly higher in the CW12 group than that in other groups. It has been shown that one of the primary roles of increased cortisol during stress process is the mobilization of energy stores to help provide energy, usually in the form of glucose, to overcome a stress situation [57]. After 7 days stress the serum glucose contents in the CW groups were significantly lower and in the BFT groups were significantly higher than pre-stress levels. Furthermore, serum glucose content decreased with the increase in stocking density from 6 to 12 $kg\,m^{-3}$. Significant reductions of glucose contents in CW12 group probably occurred due to the chronic high density stress. Long-term stress would eventually exhaust glycogen stores and the content of glucose should decrease [58]. However, in the CW system, high stocking density disrupts the fish's ability to respond to the increase in energy demand associated with an additional acute stressor.

Stress due to high stocking density that induces increased serum cortisol concentrations may also lead to immunosuppression in farmed fish [11,59], which in turn affects immunity-associated parameters. Long-term high density negatively affected the immune responses of fish as demonstrated by the suppression in the transcription of immune-related genes and a significant reduction in some immune factors in fish [9,60]. The results of the present study showed that before acute stress, the serum lysozyme, alternative complement (ACH_{50}) and ALP activities were significantly lower in the high density (CW12) group than

other groups. Similar to our results, decreased lysozyme activity was observed in fancy carp during crowding stress [6]. Determination of serum alternative complement activity is a suitable indicator of fish immunocompetence in studies involving stress [61]. [60] also indicated that long-term crowding stress decreased alternative complement activity. Elevation of plasma cortisol seems to produce depletion of serum hemolytic activity in some fish species [3,60]. Therefore, a significant decrease of serum hemolytic activity in CW12 group during the long-term high density stress could be the result of increased cortisol concentration. It is reported that changes in serum proteins and ALP activity are physiological responses which are associated with immune response and can serve as indicators of fish health [62,63]. According to [9]; the serum ALP activity was significantly reduced with increasing stocking density. In our study, long-term high density through the induction of stress hormones, especially cortisol, impairs the serum lysozyme, hemolytic and ALP activities in the high density (CW12) group. Therefore, high stocking density displays a considerable threat to the immune system of these fish. In those fish reared in biofloc systems (BFT6 and BFT12 groups), the serum lysozyme, hemolytic and ALP activities were higher and similar to fish reared at low density (CW6 group), suggesting the role of BFT in the fish immune mechanism. Thus, the biofloc system seemed to act on the stress response of common carp by preventing stress-related immunosuppression [14]. also reported a significant improvement in lysozyme and hemolytic activities in BFT groups. Similarly [23], reported significant improvements in humoral and cellular immune parameters in all BFT fish. The improvement recorded in the immune status of fish may be due to microbial flocs that contain bioactive products, fat-soluble vitamins, carotenoids, phytosterols, and taurine [51] and some probiotic microorganisms present in the biofloc [18,64]. The complementary protein source rich in essential amino acids provided by the biofloc system might also contribute to the immune function of fish [65].

After 7 days of stress, the serum lysozyme activity in CW12 group significantly increased compared with pre-stress, and this elevated activity was associated with increased serum cortisol concentration observed in this group. The fish in the CW12 group may have increased lysozyme activity to promote stress resistance [36]. Similar results have been observed in the common carp [7]. However, in our study, the serum lysozyme activity of fish in the BFT groups was unaffected 7 days post-stress compared with pre-stress. This finding suggests that BFT can help alleviate the increase in serum lysozyme activity induced by acute crowding stress [63]. Furthermore, serum ACH_{50} activities in the BFT groups were significantly increased 7 days post-stress compared with the CW groups. After acute crowding stress, the lowest serum ACH_{50} and ALP activities were observed in the CW12 group. Similarly, exposure to short-term crowding stress at a density of 100 $kg\,m^{-3}$ decreased serum alternative complement activity in gilthead sea bream [66]. The results of our study revealed that high stocking density stress could influence fish innate immune responses negatively, which would make fish more susceptible to acute stress and ultimately impair fish welfare. Overall, our findings confirmed that while chronic stress suppressed the immune responses, BFT can have beneficial effects on the immune system of common carp.

Serum ALT and AST are important parameters for the diagnosis of liver function and damage, as the increased permeability of injured hepatocytes causes a release of these enzymes into the blood [8]. Before acute crowding stress, the highest serum ALT and AST activities were observed in the CW12 group. Also, ALT and AST activities increased with the increase in stocking density from 6 to 12 $kg\,m^{-3}$, indicated that the liver function was affected by the high density stress [18,26]. After acute stress, the ALT and AST activities were highest in the CW12 group, followed by the CW6 group, and lower in the BFT groups. Our results suggest that BFT may decrease liver cellular destruction of common carp exposed to an additional acute stressor.

5. Conclusions

In conclusion, the present study indicated that TAN concentration can be effectively controlled in BFT groups by either heterotrophic assimilation or autotrophic nitrification. It was concluded that the high stocking density stress can impair the welfare and depress the growth of common carp in clear water system (CW12 group). At the same time, our results demonstrate that biofloc technology could improve the growth and feed utilization, digestive enzyme activities, oxidative status, innate immune responses, and resistance to acute crowding stress in fish. Increasing stocking density from 6 $kg\,m^{-3}$ to 12 $kg\,m^{-3}$ in the biofloc system (BFT12 group) resulted in higher final density (25.41 $kg\,m^{-3}$) and final biomass produced. Therefore, the biofloc *in situ* had the anti-stress effect, and the intensive culture of common carp in the biofloc system at a stocking density of 12 $kg\,m^{-3}$ could be recommended.

Acknowledgments

This work was supported by Gonbad Kavous University, Gonbad, Golestan, Iran (grant number 6/192).

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