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Biofloc Technology Improves Keeled Mullet (*Liza carinata*) Growth, Antioxidants, Immunological Responses, and Expression of Immune-Growth Related Gene

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ABSTRACT

Biofloc technology represents a complex ecosystem of microorganisms intended to enhance the efficiency and sustainability of aquaculture. The present trial was designated to analyze *Liza carinata* (Keeled mullet) suitability to be cultured in biofloc system. Two treatments were designed including clear water system (CWs) and biofloc technology system (BFTs) in triplicates for each treatment. Keeled mullet fry (weighted 0.49 ± 0.01g) were assigned randomly to six round plastic tanks (120 liters) of the two treatments at 15 fry/tank. After 90 days, the results revealed that water quality were kept at appropriate levels in both treatments. Fish growth in BFTs was significantly better than CWs. Better proximal body composition with higher protein and lipids percentages were found in fish reared in BFTs. BFTs improved intestinal activities of amylase, lipase and protease. Fish raised in BFTs had a significant improved antioxidant response and lower malondialdehyde than CWs. Fish serum showed higher content of total protein, globulin, albumin, lysozyme, phagocytic activity, respiratory burst activity (NBT) and immunoglobulin M in BFTs than CWs (P<0.05). Reduction of both cortisol and stress enzyme were reported in fish in BFTs than fish in CWs. Higher length and width of *villi* along with a higher number of goblet cells in addition to higher total bacterial and *Bacillus* count were recorded in BFTs than CWs. BFTs up-regulated immunity genes, including Tumer Necrosis Factors alpha (*TNF-α*) Interleukin 1β (*IL-1β*), Interleukin 8 (*IL-8*) and Interleukin 10 (*IL-10*). In addition, higher levels of selected growth genes were recorded in BFTs fish. In conclusion, the present results demonstrated that BFTs maintained water quality and improved growth, fish proximate analysis, activity of digestive enzymes and antioxidants enzymes. BFTs also enhanced hematological, serum-immunological parameters and improved gut health.

INTRODUCTION

The aquaculture industry has witnessed a remarkable development since the 1970s, which contributes significantly to food safety for the increased population since it offers a good source of animal protein **(FAO, 2023)**. This rapid development has resulted in increasing traditional aquaculture activities that unfortunately had detrimental effects

on the aquatic environments together with the global limitation in water and land sources **(Long *et al.*, 2015)**. Therefore, there is high need toward adopting responsible and sustainable aquaculture technologies **(Khanjani *et al.*, 2022)**. Biofloc technology (BFTs) is regarded as an environmentally-friendly fish culture system **(Bossier & Ekasari, 2017)**. Systems applying biofloc technology (BFTs) have sustainable features due to the intensive production of fish with efficient use of nutrients and little or no-water exchange that maximizes the use of water, moreover it doesn't have bad impacts on the natural resources **(Avnimelech, 2015)**. Additionally, BFTs contain microbial load that assimilate nitrogenous wastes to produce high quality single-cell protein in their bodies, which is considered as a supplementary food for the reared fish **(Wasielesky *et al.*, 2006)**. In BFTs the carbon-to-nitrogen ratio (C/N) should be kept at about 10 to 20:1 with adopting a vigorous aeration in order to develop the beneficial microbial load.

Floc particles constitute of algae, protozoa, bacteria, feces, remains of dead microorganisms **(Hoang *et al.*, 2020)** which are regarded as a valuable provider of essential nutrients for fish, however the efficiency of using floc particles differ with fish behavior and their feeding habits. In this regard, species with low trophic level had an increased interest to be used in BFTs.

Biofloc system has a natural probiotic effect due to their content of beneficial microbial populations and their biochemical composition that are considered as growth-promoters and immunostimulants. Accordingly, BFTs stimulates growth, enhances non-specific immunity, antioxidative status, and reduces stress **(Liu *et al.*, 2018)**. Also, BFTs can enhance intestinal health by affecting intestinal histomorphometry, microbial composition, and digestive enzymes activity **(Hersi *et al.*, 2023)**. Furthermore, biofloc can boost the expression of genes related to the immune system **(Menaga *et al.*, 2019)**.

The Mugilidae family is among the economic important euryhaline and eurythermal species commonly scattered across tropical, subtropical, and temperate seas, including China **(Chang *et al.*, 2004)** and Egypt **(Saleh, 2008)**. It is also regarded as ecologically significant and serves as a key food source for human communities in various regions of the world **(Durand *et al.*, 2012)**. Some mullets have been studied as suitable species in BFTs in a polyculture system for example, *liza ramada* **(El-Kady *et al.*, 2016)**, *Mugil curema* **(Legarda *et al.*, 2019)**, and *Mugil liza* **(Holanda *et al.*, 2020)**. However, few studies have been performed on the monoculture of mullets such as the *Mugil cephalus* **(Haridas *et al.*, 2021; Garcés & Lara, 2023)**. Among mullets, the keeled mullet is highly valued as a premium food source and an excellent candidate for aquaculture. Its high market demand, superior meat quality, and rich polyunsaturated fatty acid content make it a versatile and profitable species for farming **(Pombo *et al*., 2005; Küçükgülmez *et al*., 2011)**. A study have been conducted by **Küçükgülmez *et al.* (2011)** to understand the keeled mullet feeding habits. Furthermore, **El-Halfawy (2004)** investigated the reproductive performance of this species. However, limited studies have assessed the suitability of culturing the keeled mullet in biofloc technology (BFT) systems under

monoculture conditions. To date, only **Khalil *et al.* (2016)** have investigated the zootechnical performance of the keeled mullet in BFT. To develop an effective culture strategy for this species, a comprehensive understanding of its performance in BFT systems is essential. Therefore, this study aimed to evaluate the effects of BFT on the keeled mullet, focusing on growth performance, proximate body composition, digestive enzyme activity, gut health, innate immunity, antioxidant capacity, intestinal histology, and the expression of key genes related to immunity and growth.

MATERIALS AND METHODS

Location and experimental layout

The trial consisted of two treatments: a clear water system (CWS, control) and a biofloc technology system (BFT), each with three replicates (2 × 3 factorial design). The keeled mullet (Planiliza carinata) fry were wild-caught from the Suez Gulf near El-Cabanoon region (Suez Governorate, Egypt) and were transported to the laboratory in aerated plastic tanks.

Prior to the experiment, fish were acclimated for two weeks to the culture conditions, including a salinity of 10ppt. After acclimatization, healthy fry (initial weight: 0.49 ± 0.01g) were randomly stocked into circular plastic tanks (120L, filled to 100L) at a density of 15 fish per tank. The culture water was maintained at 10ppt salinity using a mixture of dechlorinated tap water and saline water from the Suez Canal.

Each tank was equipped with two air stones connected to a centralized aeration system (two alternating air blowers) to ensure sufficient oxygenation and water movement. A polyethylene screen covered the tanks to prevent fish from escaping. Fish were maintained under a natural photoperiod (12h light:12h dark) throughout the trial.

The experiment was conducted over 90 days (April 15 – July 14, 2023) at the Research Laboratory, Faculty of Fish Resources, Suez University, Egypt.

System management

For units of BFTs treatment, starch was introduced once every day to reach an C/N ratio of 15:1 which can stimulate the proliferation of heterotrophic bacterial **(Khalil *et al.*, 2016)**. Starch was combined with some of tank water in a glass beaker then scattered across the surface of each tank after the last meal. No water exchange was experienced in BFTs tanks except evaporation compensation, while, a 10% daily water exchange was applied for CWs tanks.

Feed proximal composition analysis

Fish were fed with a commercial diet (Skretting®, Egypt) with a chemical compsition represented in Table (1). The fish were fed at a rate of 5% of their total body weight, with two meals daily throughout the experiment. Every two weeks, fish were weighed to modify feeding rates accordingly.

Table 1. Proximate composition analysis of the experimental diet based on a dry matter

Constituent	Diet
Dry matter (%)	92.35 ± 0.45
Protein (%)	36.49 ± 0.19
Lipid (%)	9.10 ± 0.28
Fiber (%)	4.09 ± 0.44
Ash (%)	8.56 ± 0.16
Carbohydrate (%)	41.76 ± 0.26

*Data are mean ± SE.

Fish sampling

At the beginning of the experiment and every two weeks, the performance of the fish was assessed by weighing five fish as a sample from each replicate in the two treatments. Before sampling fish, clove oil (50mg/ L) **(Haridas *et al.*, 2021)** was used to anesthetize fish. After the trial, fish were anesthetized and samples were taken for the different measurments in the study. A sample of six fish were taken from each treatment to assess their growth and proximal body composition. Another sample of fish containing nine fish from each treatment were gathered to collect blood samples for the assessment of the hematology and immune-related factors, liver tissues for the assessment of antioxidants and gut tissues for the assessment of digestive enzymes activity. Additionally, nine fish fom each treatment were used to collect gut tissues for bacterial count and liver tissues that were immediately preserved in liquid nitrogen for further determination of gene expression. A final sample of six fish were dissected to collect gut tissues for the histological examination.

Obtaining blood and serum samples

Blood samples were drawn from tail vein by sterile insulin syringe. A portion of the blood sample was stored at 4°C in a solution containing EDTA at a concentration of 10 % as an anticoagulant and directly applied to hematological measures. The other portion of the blood sample was centrifuged at 5,000 rpm for 8 minutes, then allowed to clot in a test tube at room temperature to obtain serum. Serum was preserved at −20°C for further determination of biochemical properties, immunological parameters and antioxidants enzymes.

Water physicochemical parameters

Several water quality indicators were tested such as temperature, salinity, pH, dissolved oxygen, total ammonia nitrogen (TAN) and floc volume. Temperature and dissolved oxygen were monitored twice every day with DO meter (HANNA, HI9146-04). The salinity was measured daily with salinometer (lovipond, Sens Direct Con200). The pH was verified every day with a pH meter (Adwa® AD8000). TAN was measured daily using HANNA, HI97715 photometer. The volume of floc was determined every 48h with Imhoff cone based on procedure reported by **Haridas *et al.* (2021)**.

Fish growth

Indexes for growth and feed usage effeciency included final body weight, weight gain (the average initial weight was subtracted from the average final weight of fish), specific growth rate percentage (SGR%) (Ln (final weight) – Ln (initial weight) / number of days* 100), feed conversion ratio expressed (the ratio between feed quantity (g) and weight of fish (g) per treatment), and protein efficiency rate (body weight gain/crude protein fed) were calculated based on **Yu *et al.* (2021)**.

Proximal body composition

Fish specimens were dried at 60°C until they reached a fixed weight and grounded and used for proximal body composition (protein, lipid and ash). The proximate composition of overall fish body was assessed based on the standards set by the Association of Official Analytical Chemists **(AOAC, 1995)**. The proximate composition analysis was conducted using standard methods: dry matter content was determined by oven-drying samples at 105°C until constant weight was achieved; crude protein content was analyzed using the micro-Kjeldahl method **(AOAC, 2005)** and calculated as % nitrogen × 6.25; crude lipid content was measured through Soxhlet extraction using petroleum ether (60-80°C boiling point range) as solvent; and ash content was quantified by incineration in a muffle furnace at 550°C for 12 hours.

Floc analysis

At the end of the trial, biofloc samples were taken from BFTs treatment and concentrated with a 100-μm mesh nylon bag as mentioned by **Li *et al.* (2019)**. Chemical proximate composition of biofloc was carried out based on the methodology of **AOAC (1995)**. To achieve the analysis of dry matter, biofloc specimens underwent drying in an oven for five hours at 105°C. For the other chemical analysis biofloc samples were powdered after being dried at 80°C till they maintained fixed weight, and stored at −20 °C) for further determination. Regarding mineral composition, using an atomic absorption spectrophotometer, the ratios of Ca, P, Na, K, Mg, Fe, and Zn in biofloc samples were examined following the method outlined by **Martínez-Valverde *et al.* (2000)**.

Tissue preparation for digestive enzymes and antioxidants analysis

Gut and liver samples has been weighed, and washed in freezed buffer, poured into a glass tube, then homogenized with cooled sucrose (0.25 M) solutions with a ratio of 1:19 (tissue to sucrose) using mechanical tissue homogenizer. A cool centrifuge was utilized for homogenized samples at 5000rpm ad 4°C for 20 minutes. For further analysis, the obtained liquid extract was cooled at −20°C.

Digestive enzymes activities

The activities of digestive enzymes were assessed using standardized methods: protease activity was determined through casein digestion **(Liu *et al*., 1991)**, amylase activity was measured using 2% (w/v) starch solution as substrate following **Rick and**

Stegbauer's **(1974)** protocol, and lipase activity was evaluated according to **Zamani *et al*. (2009)** with olive oil emulsion serving as the substrate for the titration method.

Antioxidant responses

Catalase, superoxide dismutase, glutathione peroxidase and malonaldehyde in fish liver and serum were used to gauge antioxidant response. The superoxide dismutase activity was determined using hydroxylamine method **(Xu & Pan, 2014)**. The catalase activity was measured using specific-commercial kits (Bio-diagnostic Co., Egypt) based on the method of **Kim *et al.* (2015)**. The activity of glutathione peroxidase was assessed based on turbidimetric technique **(Feng *et al.*, 2016)**. Malonaldehyde was assessed by the thiobarbituric acid (TBA) procedures of **Yin *et al.* (2018)**.

Hematological and serological parameters

Hematological parameters, including hematocrit, hemoglobin the total count of red blood cells (RBCs) and of white blood cells (WBCs), also the number of differential WBCs and thrombocytes were determined following the methodology of **Savari *et al.* (2011)**.

Regarding stress indicating parameters, the enzyme-linked immunosorbent assay (ELISA) method was used to assess the level of serum cortisol. The serum levels of alanine aminotransferase and serum aspartate aminotransferase were determined by the method of **Adeyemi *et al.* (2015)**.

Total serum protein (mg/ ml) and albumin (mg / ml) were assessed following the procedures of **Patriche *et al.* (2011)** with Standard kits. By deducting total serum albumin from total serum protein, the total serum globulin (mg/ ml) is computed **(Coles, 1974)**.

For the assessement of immunological parameters, lysozyme activity in serum was determined as µg/ ml serum by turbidimetric procedures **(Chen *et al.*, 2018)**. Phagocytic activity was assessed based on the approach of **Kitao and Yoshida (1986)**. The phagocytic index was computed using the subsequent formulas: phagocytic index is the ratio of the number of phagocytised beads to the number of phagocytizin leukocytes multiplied by 100. Respiratory burst activity (the formation of superoxide anion O_2^-) was measured by reducing nitroblue tetrazolium (NBT) to formazan utilizing the technique described by **Cook *et al.* (2001)**, and immunoglobulin levels (immune turbidimetry) were determined with a commercial kit (Fish Immunoglobulin M, ELISA Kit, Cat.No: MBS042385, My-BioSource, Co., Southern California, San Diego, USA) following **Li *et al.* (2019)** description.

Bacterial counts in fish gut

Bacterial count analysis of fish gut was estimated based on the method of **APHA (2005)**. The gut samples were sliced into tiny pieces and aseptically homogenized for 15–30 seconds in 10 millilitres of distilled water at ambient temperature using a tissue

homogenizer. Then, serial dilutions of the homogenate were made using Aliquots of each dilution (0.1ml) of sterilized physiological saline solution up to 10–9 and were plated on Petri dishes with tryptic soy agar media (TSA) for total bacterial count. Other aliquots were spread on Petri dishes containing MRS *Bacillus* Agar for total *Bacillus* count and kept at 37°C throughout the entire night. To prevent fungal from growing, the solidified media were supplemented with nystatin (50mg/ l) **(Haridas *et al.*, 2021)**. For fish gut microbial count, colonies were enumerated in colony-forming units (CFU/g) following incubation.

Intestinal histomorphometry analyses

The intestinal samples (proximal, middle, and distal segments) were taken as mentioned by **Pirarat *et al.* (2011)**. Afterward, the samples were preserved for 24 hours in a solution of 10% formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E) or Periodic-acid Schiff reagent (PAS) and Alcian blue (AB) mix stain to count goblet cells. Then, the light microscope (Olympus CX, Tokyo, Japan) was used to examine the stained sections of intestine. Histomorphometric assessments were carried out to evaluate intestinal histoarchitecture alterations in the two studied treatments. Intestinal villi were measured in height, width, and area (10x), and the goblet cell counts (x10) were count in every villus **(Samanya & Yamauchi, 2002)**.

Gene expression analysis

The total RNA was extracted using TRIzol Kit (Bioline) from the liver based on the guidelines of the manufacturer. A NanoDrop spectrophotometer (Quawell, Uv-Vis spectrophotometer Q5000/USA and 1.5% agarose gel electrophoresis) was used to check the quality and integrity of RNA. Thereafter, cDNA was synthesized from the extracted RNA by a reverse-transcriptase synthesis kit (the SensiFAST cDNA synthesis kit (Bioline)) according to the producer's guidelines. After that, the synthetized cDNA was preserved at - 40°C for further use. The transcriptional expression has been studied for β-actin (reference gene), four cytokines (Tumer Necrosis Factors alpha (*TNF-α*) Interleukin 1β (*IL-1β*), Interleukin 8 (*IL-8*) and Interleukin 10 (*IL-10*)), growth hormone (*GH*) and insulin like growth factor 1 (*IGF-1*) using primers set before by **Byadgi *et al.* (2016)** and **Abdel-Mageid *et al.* (2020)** (Table 2). Quantitative real-time PCR (qRT-PCR) analysis was performed using the SensiFast™ SYBR Lo-Rox kit (Bioline) on an MxPro qPCR system (Agilent Technologies, USA). Each 20µl reaction contained: 10µl of 2X SensiFast™ SYBR Lo-Rox mix, 1µl of cDNA template, and 0.5µl each of forward and reverse primers (10µM). The thermal cycling protocol consisted of: initial denaturation at 95°C for 30sec; followed by 45 cycles of denaturation at 95°C for 15sec and annealing/extension at 60°C for 20sec (with the exception of il10 which was amplified at 58°C). The precision of realtime PCR amplification was verified by assessing the melting curve using agarose gel electrophoresis to confirm that just a single PCR product was

amplified precisely to the required size. By normalizing each gene to β-actin, each gene's relative expression level was determined based on $2^{-\Delta\Delta CT}$ technique **(Livak & Schmittgen, 2001)**.

Table 2. Primers used sequences, amplicons, and associated data for real-time quantitative PCR

Genes	Primer sequence	Size (bp)
β-actin		
F	TGCAGTCAACATCTGGAATC	191
R	ATTTTTGGCGCTTGACTCAG	
TNF-α		
F	GCGCAGTCTGTCATTGGTT	251
R	ACTGGACACGCTCACTGTAGTG	
IL-1β		
F	GAGGAGCTTGGTGCAGAACA	221
R	CTTTGTTCGTCACCTCCTCCA	
IL-8		
F	CACTGCTGGTCGTCCTCATT	146
R	CAGTCGGAGGTCGGAAG	
IL-10		
F	CTTGTCCTTCTTCGGCACTA	186
R	TTGAAAGAGTCCTCCACGGTC	
GH		
F	TGCTTCAAAAAGGACATGCA	175
R	GATGTTTGCAGGTTGAGAC	
IGF-1		
F	ACCTGATGAGTGGGAAGTGG	215
R	GCATCTCCGGCTCATCTTTG	

*****F**:** Forward; R: Reverse

Statistical analysis

Statistical analyses were carried out with SPSS software (Version 25.0). The measurements were first asserted to be normal using Kolmogorov-Smirnov test and homogeneity of variances using levene's test. Using t-test, there were notable variations between the two treatments with $P< 0.05$ indicating significance. All results were presented as mean ± standard error (SE).

RESULTS

1. Water physicochemical parameters

Differences among CWs and BFTs treatments in water physicochemical parameters in terms of salinity, temperature, dissolved oxygen (DO), pH, ammonia-N levels and the volume of floc volume are displayed in Table (3).

No statistical variations were reported in water temperature between the two systems. Salinity was significantly higher in BFTs than CWs. While, DO and pH levels were statistically reduced ($P< 0.05$) in the BFTs than CWs. Comparing BFTs with CWs, the ammonia-N concentrations were higher in the BFTs, whereas it remained in the range suitable for fish culture. The floc volume in BFTs was 28.33 ± 1.67ml/ L.

Table 3. Water physicochemical parameters values for the keeled mullet reared under CWs and BFTs

Item	Treatments	
	CWs	**BFTs**
Salinity (g/L)	$10.51^{b} \pm 0.128$	$11.19^{a} \pm 0.327$
Temperature (°C)	$29.06^{a} \pm 0.49$	$28.97^{a} \pm 0.474$
pH	$8.24^{a} \pm 0.093$	$7.68^{b} \pm 0.024$
DO (mg/L)	$6.97^{a} \pm 0.111$	$5.92^{b} \pm 0.27$
Ammonia-N (mg/L)	$0.014^{b} \pm 0.002$	$0.11^{a} \pm 0.017$
Floc volume (ml/L)	0.00^{b}	$28.33^{a} \pm 1.67$

* Within a parameter, the means denoted by distinct superscripts are significantly (P<0.05) varied from one another. Data are mean ± SE.

2. Fish growth and proximal body composition

Growth performance of the keeled mullet raised in CWs and BFTs for 90 days are displayed in Table (4). The results revealed greater (P<0.05) final weight, weight gain, ADG, SGR% and survival rate in BFTs compared to CWS. Significantly higher feed utilization efficiency presented with lower FCR and significantly increased PER was recorded in BFTs group.

Table 4. Growth parameters of the keeled mullet reared under CWs and BFTs

Item	Treatments	
	CWs	**BFTs**
Final weight (g)	$5.70^{b} \pm 0.149$	$7.85^{a} \pm 0.097$
Weight gain (g)	$5.20^{b} \pm 0.154$	$7.36^{a} \pm 0.109$
ADG (g / day)	$0.058^{b} \pm 0.002$	$0.082^{a} \pm 0.001$
SGR (%)	$2.53^{b} \pm 0.048$	$2.85^{a} \pm 0.045$
Survival rate (%)	$77.78^{b} \pm 2.22$	$91.12^{a} \pm 2.21$
FCR	$1.42^{a} \pm 0.085$	$1.33^{b} \pm 0.027$
PER (%)	$1.97^{b} \pm 0.118$	$2.10^{a} \pm 0.042$

* Within a parameter, the means denoted by distinct superscripts are significantly (P<0.05) varied from one another. Data are mean ± SE.

3. Proximal body composition

Proximal composition of the keeled mullet raised in CW_S and BFT_S for 90 days are displayed in Table (5). Concerning proximate composition, greater dry weight ($P<0.05$), crude protein and crude lipid content were observed in BFTs group as opposed to CWs group. While, lower ash content was observed in ash content in BFTs compared to CWs.

Table 5. Proximal body composition of the keeled mullet reared under CWs and BFTs

Item	Treatments	
	CWs	**BFTs**
Dry weight (%)	$26.84^{b} \pm 0.087$	$27.81^{a} \pm 0.136$
Crude protein (%)	$51.36^{b} \pm 0.58$	$55.78^{a} \pm 1.03$
Crude lipid (%)	$21.15^{b} \pm 0.40$	$23.56^{a} \pm 0.59$
Ash (%)	$22.16^{a} \pm 0.29$	$15.39^{b} \pm 0.56$

* Within a parameter, the means denoted by distinct superscripts are significantly ($P<0.05$) varied from one another. Data are mean ± SE.

4. Biofloc analysis

The dry matter-based biofloc chemical composition is presented in Table (6). The analysis revealed the following nutritional components: crude protein (27.32%), crude lipid (7.56%), ash (11.73%), and ether extract (48.99%). Mineral analysis demonstrated the presence of essential macro- and micro-minerals including calcium (Ca), phosphorus (P), sodium (Na), potassium (K), magnesium (Mg), iron (Fe), and zinc (Zn).

Table 6. Biofloc chemical and mineral composition

Item	Floc composition
Dry matter (%)	29.52 ± 0.14
Protein (%)	27.32± 1.67
Lipid (%)	7.56 ± 0.42
Fiber (%)	4.40 ± 0.23
Ash (%)	11.73 ± 0.187
Carbohydrate (%)	48.99 ± 1.89
Ca (mg/g)	17.14 ± 0.52
P (mg/g)	15.71 ± 0.50
Na (mg/g)	15.22 ± 0.34
K (mg/g)	4.31233 ± 0.257
Mg (mg/g)	2.76267 ± 0.053
Fe (mg/g)	1.01404 ± 0.051
Zn (mg/kg)	157.24 ± 1.90

*Data are mean ± SE.

5. Digestive enzymes

The gut digestive enzyme activity of the keeled mullet raised in CWs and BFTs for 90 days are displayed in Fig. (1). The results revealed significantly higher levels of amylase, lipase and protease enzymes in fish raised in BFTs compared to CWs group ($P< 0.05$).

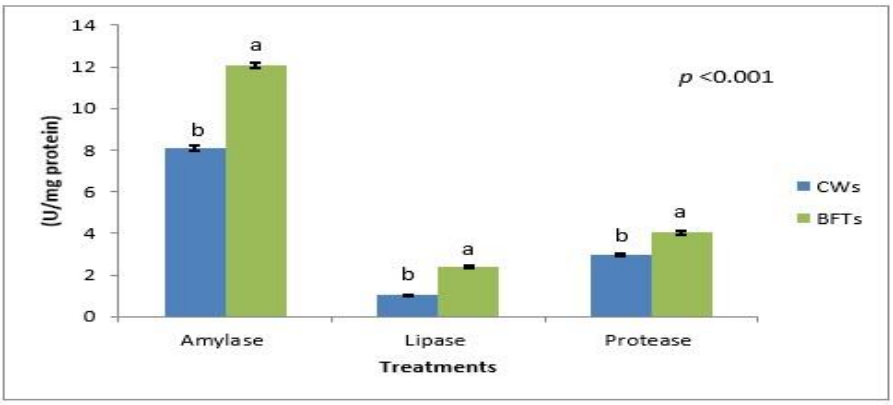

Fig. 1. Activity of digestive enzymes in the keeled mullet's gut from CWs and BFTs. Within a parameter, the means denoted by distinct superscripts are significantly ($P<0.05$) varied from one another. Data are mean ± SE

6. Antioxidant responses

The antioxidants response of the keeled mullet reared under BFTs and CWs for 90 days are presented in Fig. (2). Superoxide dismutase, catalase, glutathione peroxidase and malonaldehyde antioxidants were measured in liver and serum of the fish. BFTs positively affected the antioxidants status of fish, as it increased the activities of superoxide dismutase, catalase and glutathione peroxidase while decreasing malonaldehyde level in the liver and serum of fish compared to CWs ($P< 0.05$).

7. Hematological and serological parameters

The hematological and serological parameters of the keeled mullet were estimated at the completion of the study (Table 7). The hematological results revealed that BFTs group had a significantly higher hematocrit, hemoglobin, RBCs, WBCs and thrombocytes than CWs group ($P< 0.05$). The higher number of WBCs is presented with significantly higher percentages of lymphocyte %, monocyte %, and eosinophil % in the blood of fish group reared in BFTs. While, there were no notable variations found in basophil % regarding BFTs or CWs group. However, a significantly increased count of heterophil % was found in BFTs reared fish compared to CWs group.

The stress indicators in terms of cortisol, alanine aminotransferase and aspartate aminotransferase were reduced significantly in BFTs in comparison with CWs.

The serum biochemical and innate immunological parameters are presented as total protein, globulin, phagocytic activity %, phagocytic index, lysozyme, nitro blue

tetrazolium and immunoglobulin that were significantly higher in the fish stocked in BFTs than CWs, except for albumin and immunoglobulin which showed no significant variations between the two groups.

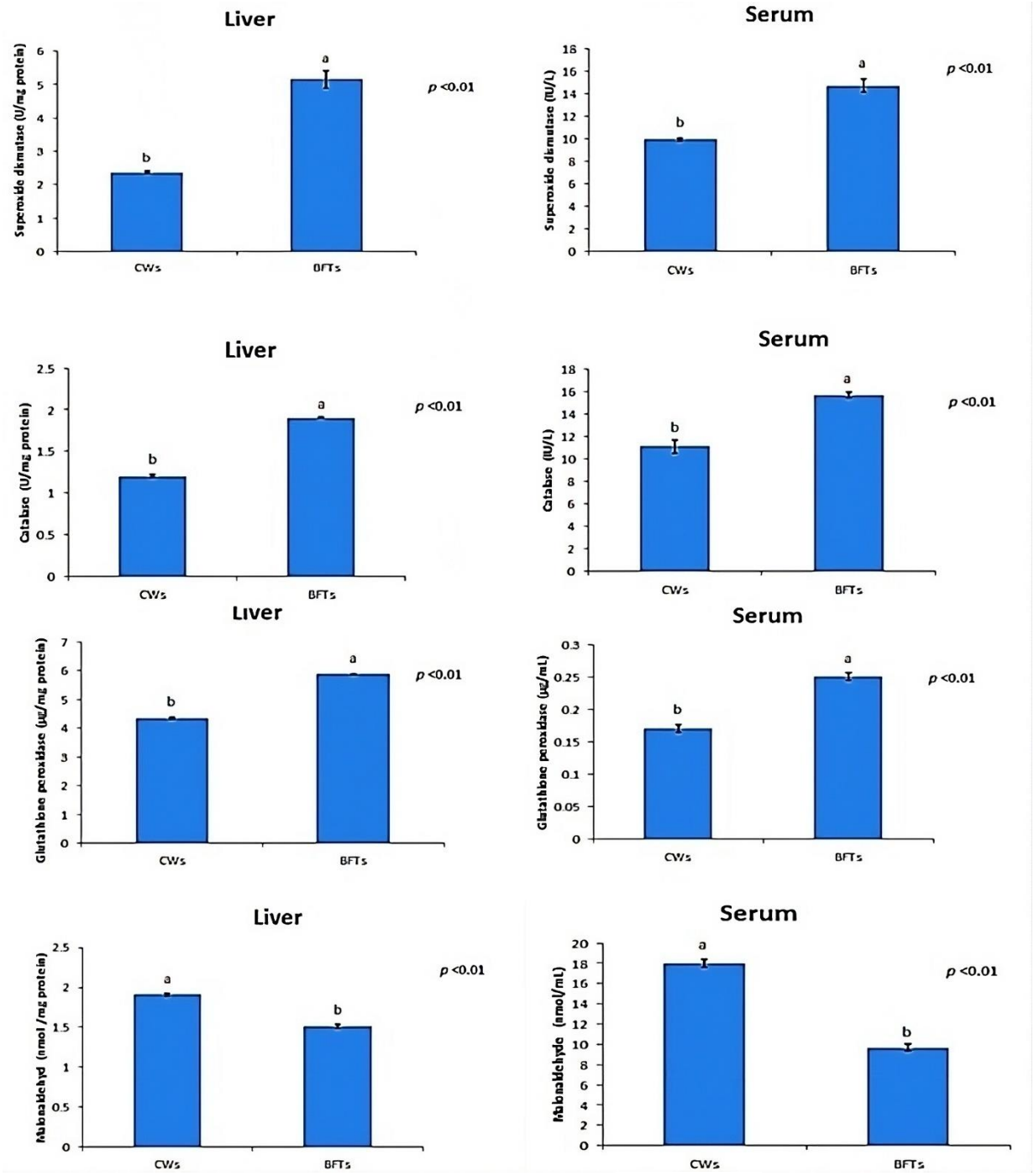

Fig. 2. Antioxidant parameters in liver and serum of the keeled mullet reared in CWs and BFTs. Within a parameter, the means denoted by distinct superscripts are significantly (P<0.05) varied from one another. Data are expressed as means ± SE

Table 7. The hematological and serological parameters of the keeled mullet reared under CWs and BFTs

Item	Treatments	
	CWs	**BFTs**
Hematocrit (%)	$27.51^{b} \pm 0.017$	$31.01^{a} \pm 0.344$
Hemoglobin (g/100 ml)	$9.21^{b} \pm 0.009$	$9.70^{a} \pm 0.0498$
RBCs ($\times 10^6$ cells/ml)	$3.08^{b} \pm 0.009$	$3.22^{a} \pm 0.02$
Thrombocytes ($\times 10^3$ cells/ml)	$95.33^{b} \pm 0.88$	$141.67^{a} \pm 6.01$
WBCs ($\times 10^3$ cells/ml)	$40.7^{b} \pm 0.306$	$50.83^{a} \pm 0.54$
Heterophil %	$18.33^{a} \pm 0.33$	$14.0^{b} \pm 0.577$
Lymphocyte %	$59.33^{b} \pm 0.88$	$70.33^{a} \pm 0.88$
Monocyte %	$2.67^{b} \pm 0.33$	$5.00^{a} \pm 0.58$
Eosinophil %	$11.33^{a} \pm 0.33$	$5.67^{b} \pm 0.88$
Basophil %	$8.00^{a} \pm 0.58$	$5.33^{a} \pm 1.20$
Cortisol (ng/ml)	$35.21^{b} \pm 0.25$	$28.12^{a} \pm 0.31$
Alanine aminotransferase (U/L)	$40.02^{b} \pm 0.12$	$35.95^{a} \pm 0.30$
Aspartate aminotransferase (U/L)	$38.06^{b} \pm 0.61$	$29.82^{a} \pm 0.15$
Total protein (g/dl)	$3.79^{b} \pm 0.02$	$4.00^{a} \pm 0.03$
Albumin (g/dl)	$2.25^{a} \pm 0.06$	$1.98^{a} \pm 0.09$
Globulin (g/dl)	$1.54^{b} \pm 0.04$	$2.03^{a} \pm 0.06$
Phagocytic activity %	$8.89^{b} \pm 0.14$	$9.89^{a} \pm 0.21$
Phagocytic Index	$1.12^{b} \pm 0.01$	$1.34^{a} \pm 0.05$
Lysozyme (µg/ml)	$9.01^{b} \pm 0.04$	$9.71^{a} \pm 0.13$
Nitroblue tetrazolium (OD at 540 nm)	$0.29^{b} \pm 0.02$	$0.41^{a} \pm 0.02$
Immunoglobulin M (µg/ml)	$4.42^{a} \pm 0.32$	$5.19^{a} \pm 0.09$

* Within a parameter, the means denoted by distinct superscripts are significantly ($P<0.05$) varied from one another. Data are mean ± SE.

8. Intestinal histomorphometric observations

Histomorphometric observation results of *Liza cainata* reared under CWs and BFTs for 90 days are shown in Table (8) and Fig. (3). Light microscopy analysis of the intestinal segments of the keeled mullet fish reared in either CWs or BFTs revealed normal gut wall shape, without histopathological alterations. The intestines of fish in both treatments included extensive mucosal folds called intestinal *villi* that reached into the lumen and an undamaged epithelial barrier. Each fold was composed of goblet cells, GC scattered across a basic columnar epithelium (stained PAS) and intra-epithelial lymphocytes, IEL. The morphometric analysis of intestinal sections (Table 6 & Fig. 3). All-intestinal parameters (*villi* length (VL), *villi* width (VW), crypt depth (CD), intraepithelial lymphocytes (IEL) and goblet cells (GC) were increased in the intestine of BFTs groups than CWs.

Table 8. Histomorphometric parameters of *Liza cainata* reared under CWs and BFTs

Item	**Treatments**	
	CWs	**BFTs**
VL (µm)	$151.00^{b} \pm 2.55$	$237.25^{a} \pm 1.93$
VW (µm)	$28.25^{b} \pm 1.55$	$56.25^{a} \pm 3.4$
CD (µm)	$12.00^{b} \pm 0.913$	$27.00^{a} \pm 0.41$
IEL (per 100 µm)	$2.50^{b} \pm 0.289$	$6.50^{a} \pm 0.289$
GC (per 100 µm)	$12.75^{b} \pm 0.48$	$22.5^{a} \pm 0.65$

* Within a parameter, the means denoted by distinct superscripts are significantly (P<0.05) varied from one another. Data are mean ± SE.

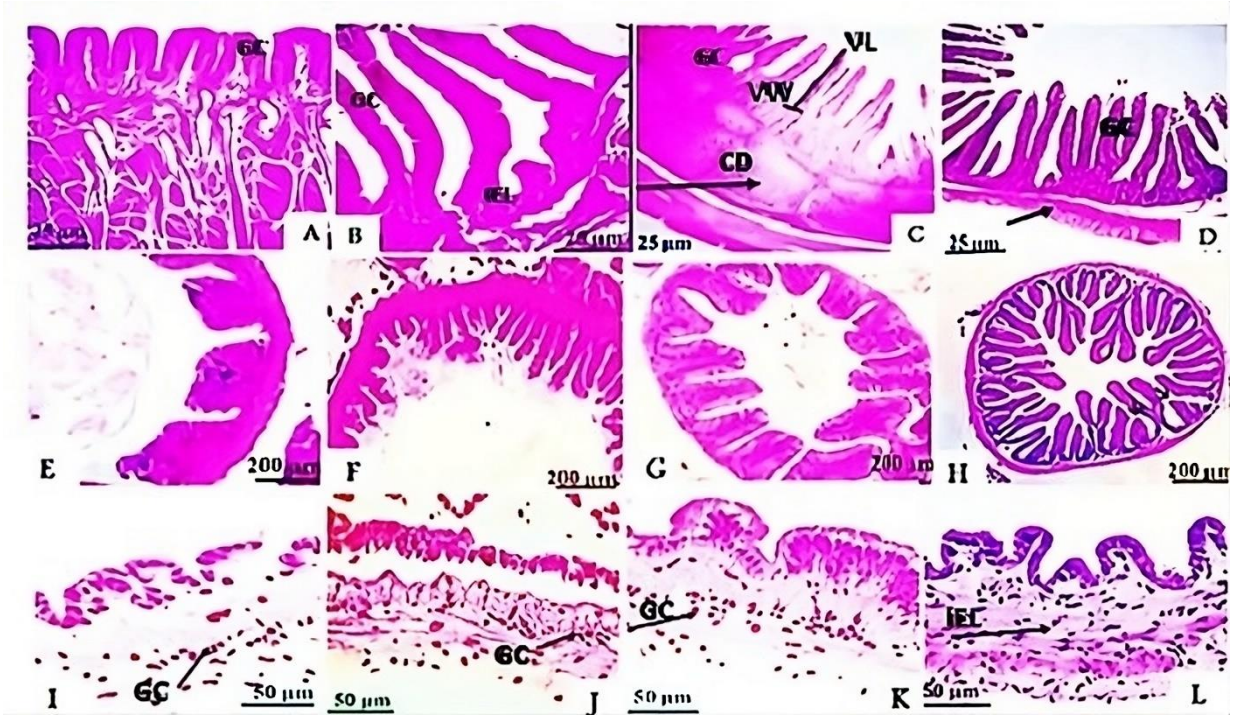

Fig. 3. Photomicrograph of transverse sections of foregut (A & B: CWs, C&D: BFTs) H&E 25µm; midgut (E & F: CWs, G&H: BFTs) H&E 200µm; and hindgut (I & J: CWs, K&L: BFTs) PAS 50µm, from the different groups of the keeled mullet reared in CWs and BFTs. VL: *villi* length; VW: *villi* width CD: crypt depth IEL: intraepithelial lymphocytes; and GC: goblet cells

9. Bacterial analysis of the intestine

Overall plate count and *Bacillus* number of the keeled mullet's gut are displayed in Table (9). Fish guts were shown to have a noticeably higher (P< 0.05) overall bacterial and *Bacillus* number when raised in BFTs compared to CWs.

Table 9. Total plate count (TPC) and *Bacillus* number in the keeled mullet gut

Item	Treatments	
	CWs	**BFTs**
TPC (log CFU / g)	$6.08^{b} \pm 0.004$	$7.37^{a} \pm 0.009$
Bacillus (log CFU / g)	$3.14^{b} \pm 0.039$	$5.39^{a} \pm 0.021$

* Within a parameter, the means denoted by distinct superscripts are significantly (P<0.05) varied from one another. Data are mean ± SE.

10. The expression of immune and growth genes

Gene expression linked to immunological responses and growth in liver tissues of the keeled mullet reared in CWs and BFTs for 90 days is presented in Table (9). Four genes expressions involved in immune responses (*TNF-α*, *IL-1β*, *IL-8* and *IL-10*) and two growth genes (GH and IGF-1) expressions are displayed in Table (9). The findings showed higher mRNA transcript levels of *TNF-α*, *IL-1β*, *IL-8*, *IL-10*, *GH*, and *IGF-1* in BFTs than control.

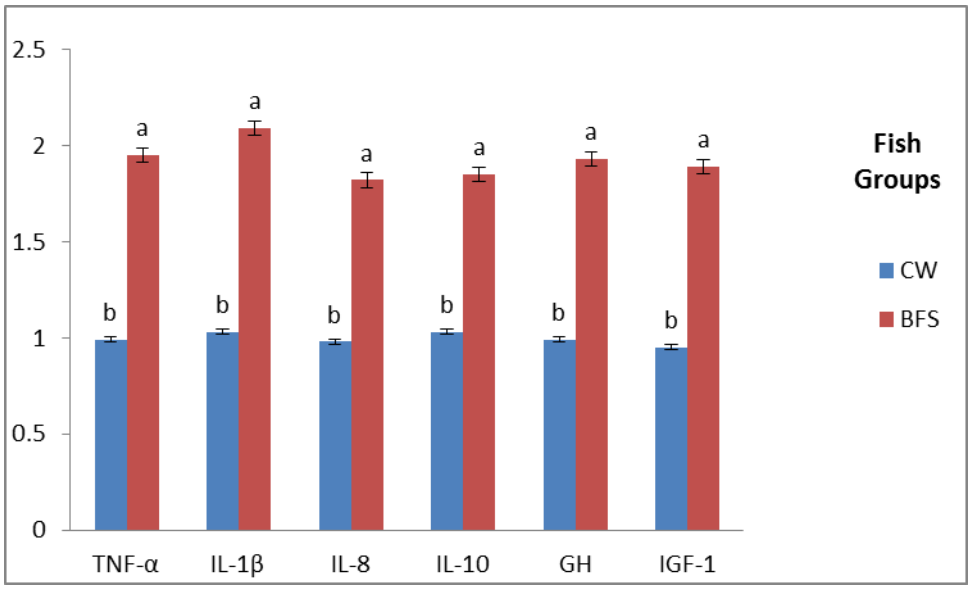

Fig. 4. The fold change in the expression of some selected genes related to immunity and growth in the liver of the keeled mullet. Within a parameter, the means denoted by distinct superscripts are significantly (P<0.05) varied from one another. Data are expressed as means ± SE.

DISCUSSION

Water quality has been proved to significantly affect aquatic animals performance, health and survival **(Gomes *et al.*, 2023)**. The findings of the current experiment indicated significantly decreased pH values in BFTs compared to CWs which agrees with **Dilmi *et al.* (2021)** and **Garcés and Lara (2023)**. This reduction may be linked to the activity of two types of bacteria (autotrophic and heterotrophic) that utilize alkalinity in order to convert nitrogen wastes either to microbial protein with heterotrophic bacteria or nitrate by nitrification process **(Gomes *et al.*, 2023)**. Besides, lower DO in BFTs than

CWs was observed in current research. **Haridas *et al.* (2021)** mentioned lower DO levels exhibited in biofloc system than the control. This condition could be because of the respiration of diverse living organisms in BFTs. During the present study, ammonia concentrations remained within safe limits for fish farming **(Gomes *et al.*, 2023)**. Meanwhile, higher ammonia levels were recorded in BFTs compared to CWs. **Dilmi *et al.* (2021)** noted a comparable results. However, **Dauda *et al.* (2018)** demonstrated that all BFT units resulted in a decrease in ammonia-N than CWs. Maintaining ammonia levels in safe limits in indoor BFTs is attributed to their uptake by bacteria that are autotrophic and heterotrophic (**Kamilya *et al.*, 2017)**. During this experiment, floc volume was kept in the recommended levels for fish culture (25–50ml/ L) **(Hargreaves, 2013)**. The BFTs treatment resulted in better growth parameters together with a significant enhancement in feed utilization efficiency presented with greater PER and reduced FCR in comparison with CWs. Similar pattern has been recorded by **Holanda *et al.* (2020)** for *M. cephalus* and by **Haraz *et al.* (2023**) for *O. niloticus*. Moreover, **Borges *et al.* (2020)** suggested the possibility of completely dispensing with artificial feed for mullet and the possibility of relying entirely on floc particles. The highest survival was found in BFTs group than CWs group. Close findings were published by **Haridas *et al.* (2021)** for early growth stages of the grey mullet, **Dilmi *et al.* (2021)** for juvenile *O. niloticus* and **Said *et al.* (2024)** for the white-leg shrimp. BFTs treatment resulted in better chemical body composition of fish presented with notably greater lipid, crude protein, and dry matter contents ($P< 0.05$) together with a significantly lower ash contents in BFTs group as compared to CWs. In line with these findings, **Khanjani *et al.* (2021)** found lower ash and highest protein and lipid in biofloc treatment. This enhancement of growth, survival and body proximate analysis could be because of the constant availability of amino acids, fatty acids, numerous minerals as well as additional vital nutrients in biofloc **(Holanda *et al.*, 2020; Khanjani *et al.*, 2021)**.

Regarding biofloc chemical composition, the findings of this trial revealed that the content of protein was 27.32% which is consistent with the range reported in different biofloc systems (25%–50%) **(Pérez-Fuentes *et al.*, 2018)**. Biofloc protein percentage can contribute with a high percentage of *Mugilidae* species protein requirements. The crude protein content of biofloc (27.32%) falls below the 35–40% dietary protein requirement established for Mugilidae species **(Yones *et al.*, 2019).** However, the lipid content (7.56%) aligns with the optimal range (7–9%) reported for mullet nutrition by **Elhetawy *et al.* (2021)**. The presence of zooplankton and different microorganisms (high in protein and lipids) increases protein and lipid level in bioflocs **(Fernandes *et al.*, 2008)**. Biofloc mineral analysis showed that Ca represented the highest concentration of minerals, while Zn was the least abundant mineral, which is consistent with **Binalshikh-Abubkr *et al.* (2021)**. The Na and K contents of biofloc were 15.22 ± 0.34 and 4.31± 0.257, respectively, which concurs the findings of **Kuhn *et al.* (2016)** elucidating that biofloc mineral contents ranged from 12.7 to 15.5mg/ g for Na and from 3.6 to 7.5 for K.

However, they reported higher Mg levels (4.1 to 18.1mg/ g) than our study. The concentration of P and Ca was 12.9 to 13.7 and 10.7–12.8 which was less than what was found in this study (15.71 ± 0.50 P and 17.14 ± 0.52 Ca). However, **Binalshikh-Abubkr *et al.* (2021)** found a higher calcium concentration than of our findings (21.85–69.74mg/ g). The Zn concentration in our findings is close to the levels determined in the study of **Binalshikh-Abubkr *et al.* (2021)**. Minerals as Ca, P, K, Mg, Fe and Zn are regarded as essential for physiological process in fish **(Kumaran *et al.*, 2012)**.

Digestive enzymes have a high importance in the process of food digestion and absorbance in fish body **(Durigona *et al.*, 2019)**. Our results revealed the significant influence of biofloc on the activity of these enzymes as the greatest activities of amylase, lipase and protease were recorded in BFTs rather than CWs, and this aligns with the findings of **Yu *et al.* (2020)** and **Adeih *et al.* (2022)**. Since biofloc is regarded as a nutrients supplement for fish that alters fish intestinal flora, the microorganisms in biofloc can secrete extracellular microbial enzymes and can stimulate the secretion of endogenous enzymes, which cause nutritional compounds degradation in food **(Xu *et al.*, 2013; Luo *et al.*, 2014)**.

Reactive oxygen species (ROS) cause alterations to fat, protein in addition to DNA molecules leading to deterioration in fish health **(Rotilio *et al.*, 1995)**. Antioxidants have major contribution as ROS antagonist (**Yılmaz *et al.*, 2019)**. Malonaldehyde indicates non inefficient antioxidant response of fish **(Storey, 1996**). Elevated levels of glutathione peroxidase, catalase, and superoxide dismutase activities have been found during the current experiment, along with reduced malonaldehyde levels reported in fish liver and serum samples from BFTs group opposed to CWs. This positive impact exhibited by biofloc on antioxidants response agrees with **Yu *et al.* (2020)**, who found higher antioxidants in different body organs and serum of *R. lagowskii* fish treated with biofloc meal compared to a clear water treatment. Opposite to our findings, **Dilmi *et al.* (2021)** recorded no statistical variation in catalase and superoxide dismutase between fish raised in BFTs or CWs.

This experiments' findings revealed enhanced hematological indices with significantly higher values of hematocrit, hemoglobin, RBCs, WBCs and thrombocytes in BFTs fish than CWs fish. These results align with those of **Hoang *et al.* (2020)**, who argued that the fish in biofloc system had a significantly higher hemoglobin and WBC than CWs group. Additionally, **Haghparast *et al.* (2020)** reported the highest RBCs, hematocrit, and hemoglobin in biofloc group than control. In contrast to these results, no statistical variation in the RBCs, hemoglobin, hematocrit and WBCs was recorded in fish between BFTs and CWs group **(Kim *et al.*, 2018; Hoang *et al.*, 2020)**. Stress conditions has wide eefects on the health of raised fish **(Jeyachandran *et al.*, 2023)**. Serum cortisol concentrations of raised fish positively correlate with the degree of stress on fish **(El-Khaldi, 2010)**. Plasma transaminases levels are the major indicators of stress and the condition of fish health **(Suárez-Causado *et al.*, 2015)**. Consisted with reduced oxidative

stress illustrated by higher antioxidants activity in BFTs group in our study, the results of this study revealed a statistically reduced stress status in terms of lower cortisol, alanine aminotransferase in addition to aspartate aminotransferase levels in BFTs group than CWs group. In agreement with these results, lower cortisol and glucose concentrations were reported in fish raised in biofloc conditions than clear water conditions **(Menaga *et al.*, 2019)**. In addition, a notable reduction (P<0.05) in the plasma levels of alanine aminotransferase and aspartate aminotransferase have been reported in BFTs based treatments than control **(Hoang *et al.*, 2020)**. Several serological parameters have major function in fish non-specific immunity like, serum proteins including total proteins, albumin, globulin, immunoglobulin and lysozymes **(Rao *et al.*, 2006)**. Regarding serum proteins, the results revealed the highest total protein and globulin concentrations in BFTs system than CWs. Comparable outcomes were noted in the studies of **Haghparast *et al.* (2020)** and **Haridas *et al.* (2021)**. Significantly higher lysozymes levels were detected in BFTs group compared to CWs group. Close results were recorded by **Yu *et al.* (2020)** and **Adeih *et al.* (2022)**. Enhanced respiratory burst activity represented by higher levels of nitro blue tetrazolium was found in BFTs group which is consistent with the finding of **Verma *et al.* (2016)**. BFTs treatment exhibited higher phagocytic activity and phagocytic index which concurs with the outcome of **Serradell *et al.* (2020)**. An increased immunoglobulin concentration has been noticed in BFTs fish than CW group which was also documented by **Verma *et al.* (2016)** and **Hoang *et al.* (2020)**. An enhancement in hematological, serological and antioxidants parameters in fish raised in BFTs may be addressed to the abundance of beneficial microorganisms, bioactive and prebiotic factors available in biofloc **(Liu *et al.*, 2018)**.

Intestinal histomorphometric results showed significantly higher *villi* length and width, crypt depth and higher count of IELs and goblet cells in the intestines of the keeled mullet reared in BFTs compared to CWs. Increasing *villi* length and width give a plenty surface in the intestine for nutrient absorption that may lead to enhanced feed utilization and growth **(Pirarat *et al.*, 2011)** and also can enhance osmoregulation and the immunity of fish **(Klahan *et al.*, 2023)**. Goblet cells secret mucus rich in antimicrobial molecules **(Alesci *et al.*, 2022)**. Intestinal epithelial cells (IELs) stimulate the gut mucosa's immunological response **(Stosik *et al.*, 2023)**. The enhancement of intestinal histomorphmetric observations in BFTs supports the conclusions of **Haraz *et al.* (2023)**, who noted increased villus height and width, goblet cell numbers, and crypt depth in fish intestine in BFTs compared to control group. Furthermore, **Gomes *et al.* (2023)** observed that biofloc treatment resulted in increasing the *vilii* length and width. This improvement may be because of the proliferation of different beneficial microorganisms with many bioactive and immunostimulatory compounds **(Suloma *et al.*, 2021)**.

The findings revealed significantly increased total bacterial enumeration and total Bacillus count BFTs compared to CWs. Close to our findings, **Yuvarajan (2021)** found higher total bacterial number and *Bacillus* counts in the rearing water and intestine of fish

kept under biofloc system than control. The increased bacterial number in fish intestine reared in BFTs possibly cause the buildup of organic matter in the culture water of biofloc treatment that encourages the heterotrophic bacterial growth **(Emerenciano *et al.*, 2017)**. The higher number of *Bacillus* count in BFTs compared to control may have resulted in higher growth performance and immunological response enhancement reported in this study. The beneficial effect of *Bacillus* spp. has been reported by **Said *et al.* (2022)** as they found that the probiotic (*Bacillus* spp.) added to tilapia diets enhanced growth, survival and prevented the growth of pathogenic bacteria.

Regarding gene expression results, the highest mRNA transcript level for all studied immune genes in fish liver was elevated in BFTs than CWs which represents how effective BFTs are in improving the immunity of farmed fish. *IL-1β* and *IL-8* are vital to stimulate immune response by inducing lymphocytes activity, bactericidal actions, phagocytosis, and promoting different cytokines production **(Wang *et al.*, 2019)**. Another pro-inflammatory cytokine is *TNF-α*, engaged an inflammatory process to the defense against microbial invasion **(Wang & Secombes, 2013)**. Consistent with our findings, **Kheti *et al.* (2017)** revealed that *TNF-α*, *IL-10* and *IL-1β* genes were up-regulated in fish liver when treated by biofloc meal rather than traditional change water units. In the same context, **Elayaraja *et al.* (2020)** observed superior expressions of *IL-1β* and *TNFs* in fish liver stocked under biofloc system. Increased expression of genes related to growth and immunity in BFTs treatment may be attributed to the presence of probiotic organisms such as (*Bacillus, Lactobacillus, Clostridium butyricum*, etc.) bacteria which improve intestinal health, strengthen the immunity and induce the anti-inflammatory response **(Balzaretti *et al.*, 2017)**. Higher expressions of growth gene (*GH* and *IGF-1*) were reported in fish reared under biofloc system compared to control. *GH* and *IGF-1* genes are from the major genes controlling growth and metabolism **(Norbeck *et al.*, 2007)**. Consistent with the results of our experiment, **El-Hawarry *et al.* (2021)** assured that applying starch to biofloc system significantly increased the levels of *GH*, and *IGF-1* genes.

CONCLUSION

It can be concluded that BFTs is a sustainable system which is suitable for the cultivation of the keeled mullet with multiple benefits. It serves as an additional food source that contains macro and micronutrient necessary for the cultured fish, improved growth, the expression of growth related genes, survival, body chemical analysis and digestive enzymes activity in fish. BFTs can induce the immune system by enhancing antioxidants activity, hematology, serum immunological parameters, the expression of immune genes and decrease stress enzymes of the cultured keeled mullet. BFTs, moreover, resulted in better gut health in the cultured fish by increasing total bacterial and *Bacillus* count in the gut compared to control, as well as showing a significantly

better gut histomorphmetric analysis for the cultured fish compared to the control. Further investigation is recommended for the culture of the keeled mullet species in biofloc systems for longer periods with different water salinities and carbon sources.

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