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Stocking density affects immune and stress-related gene expression of Butter catfish (*Ompok bimaculatus*) fry in biofloc landscapes

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ABSTRACT

Scientific research into fish wellness is critical, and the concerns about crowding-related stress due to increased stocking density are inevitable. Taking this into consideration, the study defines the physiological signature of *Ompok bimaculatus* (Butter catfish) in a biofloc system when subjected to varying levels of stocking density. Fish (mean weight = 1.21 $g \pm 0.08$, $n = 600$) were randomly stocked in 40-L glass aquaria at stocking densities of 0.5 g/L (T1), 1 g/L (T2), 1.5 g/L (T3), and 2 g/L (T4) and fed a 35% protein diet. After the 90-day trial, the physio-biochemical, molecular, and tissue-level changes were assessed. An integrated biomarker response (IBR) analysis for the key stress indicators aided us in better understanding them. There was a significant difference in blood count between T1 and T4 (total erythrocyte count, hemoglobin, and packed cell volume). T1 had higher levels of globulin and total plasma protein, but T2 had higher levels of albumin. Only in T1 did the respiratory burst and lysozyme activity appear to be higher ($p < 0.05$). Increased stocking densities had a significant impact on the liver function enzymes, GOT and GPT ($p < 0.05$). In comparison to lower densities (T1 & T2), higher stocking density (T3 & T4) was found to raise glucose and cortisol levels ($p < 0.05$). Antioxidant enzymes such as catalase, glutathione-S-transferase, and malondialdehyde were found to be more pronounced in lower density tissues (T1). Furthermore, the IBR plots show that lower densities have better health than higher densities. At higher stocking densities, mRNA expression of *HSP70*, IL-1, and IL-20 increased ($p < 0.05$) in kidney and liver tissues. The Nrf-2 and Tlr-9 genes were also upregulated. Also, when stocking density was increased, tissue-level histo-architectural changes were more pronounced than when stocking density was kept low. The findings of this study show that the welfare of Butter catfish cultured at high density in biofloc systems suffers from severe stress, and therefore draw more attention to the development of a species-specific standard rearing methodology in the pursuit of a profitable aqua-farming enterprise.

1. Introduction

The expansion of aquaculture industry is hampered by resource constraints and related sustainability concerns. Despite this, the business is booming with technological assistance in order to feed the global galloping population, and so play a significant role in food production. Furthermore, the industry offers the opportunity to restructure one's livelihood through entrepreneurship. Despite the high harvest and economic gains reported across the areas, many social concerns exist, necessitating the implementation of strict solutions. Among them, fish well-being in intensive systems is frequently overlooked as the sector pursues profit-driven aquaculture. The public has been increasingly pressuring food producers and scientists to consider animal well-being in their research [1]. Fish in intensive culture systems are increasingly exposed to many stressors as technology develops [2]. Despite this, aquatic animals are seen as low in our moral circle from this perspective [3], and are frequently omitted from animal welfare regulations in many parts of the world [4].Given this, it is imperative to comprehend the level of stress experienced by aquatic animals in captivity in order to initiate an appropriate management methods and rearing protocols.

Biofloc technology (BFT) has evolved as a promising and sustainable resource utilization and management method over the last decade. The

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system mainly deals with heterotrophic bacterial growth, which results in nitrogen consumption and microbial protein production [5] by maintaining a carbon to nitrogen (C/N) ratio balance. Carbon can be added from outside sources or increased in the feed [6]. As a result, nitrogen intake is higher and ammonium levels are lower [7]. In an effort to standardize biofloc, a slew of studies has been undertaken to assess species, carbon sources, and system microbial flexibility.

In biofloc systems, the concomitant increase in stocking density of fishes is targeted to yield maximum crop per drop. Additionally, it is a key determinantof the overall productivity and cost-effectiveness in commercial level set-ups. Thus, the description of optimum density is indispensable to judge the economic sustainability of the aquaculture production system [8].The over estimation of production target of the fishes cultured in biofloc system as it is witnessed today, often opens the sluice of total failure of the system, besides compensating the fish well-being.

Physiological abnormalities have been reported in fish, as measured by integrated biomarker response [9]. In fish, overstocking causes chronic stress and suppresses both innate and adaptive immune responses via neuroendocrine pathways [10]. However, because a large number of useful microorganisms and their cell components aggregated in the bio-floc can act as probiotics, or immune-boosters to improve immune function, antioxidant status and disease resistance in aquatic organisms [11,12], the bio-floc system's increased density may be possible. As a result, a detailed assessment of the associated welfare status linked to the physiological changes when subjected to overcrowding conditions (as in a high stocking biofloc system) is required.

Many immunological and stress biomarkers (hematological, serological, molecular and histo-morphology) can provide an accurate picture of an individual's health status, reflecting their general well-being [13]. The consistent changes in these indicators as a result of stocking density may aid in understanding the mechanisms that lead to the undesirable outcome [13]. For example, fluctuations in the activity of important antioxidant enzymes are thought to be a reliable biomarker for toxic damage [14,15]. Furthermore, the tissue-level changes will be narrated by the cellular response to major immune and stress genes expressed in distinct animal tissues.

The primary concern therefore is to quantify the stress level *via* various gene regulations. *Hsp70* is one of the important proteins mostly activated upon stress induction and is well known for immunological and cyto-protective functions. Hence, recording the changes of this gene gave a clear insight of the response to increasing population density. Interleukins (ILs) are associated with immune response and the IL-20 is associated with tissue repair mechanisms. Studies show that it is strongly associated with intestinal inflammation in higher eukaryotes [16]. The present experiment also focused response of fish to biofloc, thereby intestinal immunity became one of the concerns. Hence, IL-20 was considered for this study. IL-1 is considered as a key regulator of stress response and adaptive immunity [17]. Similarly, *Tlr9* is associated with cytokine mediated immunity [18]. Biochemical alterations seen in the tissue, on the other hand, suggest tissue damage caused by enzyme activity suppression. Furthermore, despite its lack of specificity, histological analysis is a low-cost and useful tool for assessing ecological risk. By combining all these indicators, we will be able to better examine environmental and in-build risk assessments, allowing us to expand our knowledge base on fish well-being, particularly in high stocking biofloc situations.

Based on its prospects, research for the fish's use in biofloc has already begun. In our preliminary study, we improved fish compatibility in biofloc by adjusting the C/N ratio [9,19]. However, given the importance of the fish and the need to standardize the optimal amount of stocking of this fish in the BFT system, more research on numerous aspects of development and the physiological consequences of various stress factors such as stocking density is urgently needed. As a result, the relationship between the benefits of the technology and the appropriate stocking density should be investigated. In light of the foregoing, this study investigated the growth, survival, and changes in physiological functions (health status) of *O. bimaculatus* advanced fry at different stocking densities in a biofloc system.

2. Materials and methods

2.1. Experimental animals, facilities and design

Fish rearing protocol were done in accordance with the standard framework laid by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment & Forests (Animal Welfare Division) of the Government of India. The study was also approved by the Institutional Animal Ethics Committee (IAEC) of the College of Fisheries vide Letter No. CAU-CF/48/IAEC/2018/09a, dated 12/02/2021. The experimental trial was carried out for 90 days in glass aquaria housed in the indoor wet laboratory facility of the College of Fisheries, CAU, Lembucherra, Tripura, India.

Advanced fry of *O. bimaculatus* (mean weight = 0.1 $g \pm 0.001$) were transported live from a local hatchery in South Tripura, India, and further acclimatized. They are grown further for one week, until they reached an average size of 1.21 $g \pm 0.08$. Prior to introducing the fry to the acclimatization tanks, they were checked for size uniformity, health status, and given dip treatment in 5 ppm $KMnO_4$ solution to exclude any chances of disease incidence. Fishes were starved for 24 h after arrival. Six hundred uniform sized fish were selected from the stock and transferred to the individual experimental units. The experimental set-up follows a completely randomized design (CRD) using four treatment groups, performed in triplicates. Thus, a total of 12 aquarium tanks of 50 L volume (filled with 40 L water) (4 treatment × 3 replicates) were used in the experiment. Four different levels of stocking density were maintained to form the experimental groups as tabulated below (Table 1):

2.2. External carbon source and floc inoculation

A carbon to nitrogen (C/N) ratio of 20:1 was maintained throughout the study based on our previous work [9]. Carbon addition was based on the estimated N level of the feed and the C content of the exogenous carbon source supplied. Commercially available molasses, having a carbon content of 43% (wet weight basis), estimated using Walkley and Black's Rapid titration method [20] was used as the external carbon source. Floc generation was done in a mother tank (500 L) using the matured floc generated from our earlier trials as the inoculum [19]. Detailed procedure for floc preparation used in our laboratory was done as per [5] with suitable modifications. The mother floc was maintained with a continuous addition of 1.46 mL of molasses for every 1 g of feed supplied to the tanks. A start-up floc volume of 10 L each was removed from the mother tank, and added to the experimental tanks (previously filled up to 30 L). After one day of inoculum addition, *O. bimaculatus* fry were introduced in the tanks in the designated densities and fed on a commercial fish feed (35% protein, Charoen Pokphand Foods). Further maintenance of the desired C/N value of 20:1 throughout the experiment was done based on the total ammonia-nitrogen (TAN) levels in the tanks following methods of Abakari et al. [21] as detailed below (Table 2):

Table 1
Experimental groups and corresponding densities (biomass and number per unit volume).

Treatments	Stocking biomass (g/L)	Stocking number (nos./L)
Group 1 (T1)	0.5 g/L	20
Group 2 (T2)	1.0 g/L	40
Group 3 (T3)	1.5 g/L	60
Group 4 (T4)	2.0 g/L	80

Table 2
TAN values (in mg/L) in the experimental groups observed during the sampling periods.

Treatment/ period	0th day	15th day	30th day	45th day	60th day	75th day	90th day
T1	0.01	0.03	0.2	0.25	0.44	0.54	0.77
T2	0.02	0.13	0.19	0.5	0.5	0.67	0.83
T3	0.02	0.26	0.3	1.00	1.00	1.17	1.2
T4	0.02	0.14	0.18	0.83	0.83	0.83	0.83
Calculated amount of carbon (molasses) (g) to be added in each experimental group							
T1	0.015	0.05	0.33	0.4	0.71	0.84	1.16
T2	0.03	0.19	0.28	0.8	0.8	1.02	1.27
T3	0.03	0.42	0.47	1.6	1.6	1.79	1.86
T4	0.03	0.2	0.29	1.27	1.27	1.27	1.27

2.3. Feeding of animals and tank maintenance

After introduction of fishes, feeding was done using commercial sinking feed with 35% crude protein. Initially, feeding was done at 5% body weight thrice dailyfor the first 15 days, and at 3% for the next 15 days. After 30 days, rate was reduced to 2% of the total biomass for next 15 days, and finally fed at 1% of the body weight for the rest 45 days. The daily feeding ration for each treatment was calculated and adjusted by estimating the 15 days sampled mean biomass. Pre-weighted external carbon source (molasses) was added as per calculation depicted in Table 2 by mixing with water and aerating in a beaker for 24 h and sprinkling directly into the water column in the evening to maintain the required C/N ratio. No water exchange was performed, except for maintaining the desired water level due to evaporation losses. Excess sludge was removed from the bottom of the cultured tank by siphoning.

2.4. Blood and tissue sampling

From each tank, six fishes ($n = 6$) were selected randomly, and anaesthetized with clove oil @ 50 μg/L. One ml hypodermal medical syringe (24-gage needle) previously rinsed in a 2.7% EDTA solution was used to draw blood from the caudal peduncle region of the fish. Blood samples were pooled andtransferredto the EDTA coated centrifuge tubes, which were then gently shaken to avoid haemolysis. Forplasma, blood was drawn intocentrifuge tubes (without EDTA) which was immediately centrifuged at 3000 rpm for 10–15 min. The supernatant plasma was then pooled, and stored inthe deep freezer at − 20 °C, until use. For enzymatic analysis, tissues (gut, liver and muscle) were collected aseptically by dissecting over a cold plate. Using 0.25 M chilled sucrose solution, 5% tissue homogenate was made, and centrifuged at 5000 rpm (15 min) and then stored at −20 °C.

2.5. Hematological indices

Total red blood cell (RBC) and white blood cell (WBC) count were estimated using a haemocytometer observed under a microscope. Blood was diluted in Hayem's fluid (Qualigens, India) and Turk's fluid for RBC and WBC, respectively. Sahli' shaemometer was used for the estimation of Hemoglobin (g%). Haematocrit (Ht) or packed cell volume (PCV) was estimated as described by Schaperclaus et al. [22]. For this, whole blood was centrifuged using heparinized micro-hematocrit tubes at 3000 rpm for 3 min.

2.6. Immunological response biomarker

A diagnostic kit (Medsource ozone biomedicals, India) based on Biuret method was used to determine total plasma protein. Albumin diagnostic kit (Medsource ozone biomedicals, India) was used for albumin estimation which was based on bromocresol green method [23]. Globulin was determined by subtracting albumin from total plasma protein whereas A/G ratio was estimated by dividing albumin with globulin [15]. Respiratory burst activity was estimated with the help of nitro blue tetrazolium (NBT) assay as per Secombes [24] with some modifications. Lysozyme activity of blood plasma was determined following the method of Anderson et al. [25].

2.7. Plasma glucose and cortisol

A diagnostic kit (Coral Clinical Systems, India) based on the GOD/ POD method was used to determine plasma glucose level [26]. The assay is based on the formation of red color edquinoneimine dye complex which is measured at 505 nm using an UV-spectro-photometer (UV-1800, Shimadzu). Plasma cortisol was quantified using a commercial cortisol ELISA kit (Calbiotech Cortisol ELISA CO368S).

2.8. Hepatic function test

A diagnostic kit (Diatek Healthcare Pvt. Ltd., Kolkata, India) based on UV- Kinetic method was used for the determination of both serum Glutamate-oxaloacetate transaminase (sGOT) and Glutamate pyruvate transaminase (sGPT) activity as per [27].

2.9. Assay of oxidative stress biomarker

Key oxidative stress biomarker like catalase (CAT), superoxide dismutase (SOD), Glutathione-S-transferase (GST) and Malondialdehyde (MDA) were examined in liver, gut and muscle tissues. CAT activity was assayed based on the start of the reactionwith the addition of 1 mL H_2O_2 solution as per [28]. SOD was assayed according to [29]. The reaction was initiated with the addition of 0.4 mL of epinephrine and measured the change in optical density per min at 480 nm for 2 min in a UV-spectrophotometer. One unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine auto oxidation. Glutathione-S-transferase (GST) was determined as per [30]. Malondialdehyde (MDA) activity was assayed through a commercially available kit following the manufacturer's protocol. This was based on the fact that MDA produced colored thiobarbituric-acid-reacting substances (TBARS) and was measured at 532 nm.

2.10. Integrated biomarker response (IBR)

The Integrated biomarker responses (IBRs) index was calculated for specified biomarkers such as plasma glucose, serum cortisol, oxidative stress enzymes (CAT, SOD, MDA and GPT) in liver, muscle and gut tissues. Evaluations were performed based on the methods of [31], and further modified by [32]. Star plots for each biomarker were portrayed in different tissues to elucidate the multi-biomarker response in the fish. The IBR index for each treatment for different tissue (liver, muscle and gut) and serum was then standardized to calculate the mean value of each biomarker. Online software designed by Universitede Lorraine was used for the calculations.

2.11. Expression of key immune and stress genes

Total RNA sample was collected from the liver and kidney of the fish with the help of TRIzol reagent (Invitrogen, USA) following manufacturer's guidelines. The isolated RNA samples were treated with DNase (MBlFermentas, USA) to avoid any sort of genomic DNA contamination. Quantification of the isolated RNA was measured using Biospectrometer (Eppendorf, USA). The OD260/ OD280 absorption ratio provided the estimate of the purity of the isolated RNA. The integrity of the isolated RNA was checked by gel electrophoresis (prepared from 2% agarose gel). The samples with intense bands of 18 s and 28 s were used for further analysis. The mRNA was transcribed to its complementary DNA using the First Strand cDNA Synthesis kit (Fermentas, USA) following the manufacturer's guidelines. For the real-time PCR of Nrf2, *HSP70*, IL-1b, Tlr9 and IL20 gene in *O. bimaculatus*, primers were designed from the

NCBI data base. The primer sequences of each gene are listed in Table 3. Rt-PCR based on SYBR green chemistry was done on a Step OneReal-Time PCR system (Applied Biosystems, USA) to study the expression analysis of the genes. Housekeeping gene (β-actin) was utilized for expression level normalization. The relative expression level of the target gene was obtained using $2^{-\Delta\Delta CT}$ method [33]. For the comparison of the expression levels of the target genes in the treatment groups, a control tank with optimal stocking density and rearing conditions was used as reference.

2.12. Histo-architectural changes

Histopathological examination of gills and livers tissues of *O. bimaculatus* in different groups were carried out following standard methods described in [34]. Gill and liver tissues were collected aseptically and fixed in the Bouin's fixative (5% glacial acetic acid, 9% formaldehyde and 0.9% picric acid) for 24 h. After this, the fixed tissues were processed in an Automatic Tissue Processor (Thermo-Scientific, Shandon Citadel 2000), followed by paraffin block preparation in Histocenter (Thermo-Scientific, Shandon Histocentre 3). The tissue embedded in paraffin blocks were sectioned with the help of Rotary Microtome (Leica, RM2245) in sections of 5–8 μm thickness, followed by staining with hematoxylin & eosin (Hi-Media) and, further mounted with dibutylpthalate polystyrene xylene (DPX). The mounted slides were observed under a light microscope (Lieca DM750) at 40 × magnification for assessment of the tissues.

2.13. Statistical data analysis

Observed data were subjected to Shapiro-Wilk, Levene's test for checking normality and homogeneity of variances. All the data were analyzed using statistical software "Statistical Package for the Social Sciences (SPSS), Version 25 (SPSS, Chicago, IL). One-way analysis of variance (one-way ANOVA) was performed to describe the statistical significance and a value of $p < 0.05$ was considered statistically significant. It was followed by Duncan New Multiple Range Test, which was done to compare mean values betweenexperimental groups. The observed immune-biochemical parameters were subjected to factor and principle component analysis (PCA) to look for the variables which may likely be responsible for the co-variation among the data obtained. Here, higher factor loading designates the supremacy of the variable. A loading assigned (+) shows direct relationship, while, opposites are indicated as (-). All the data were expressed as mean ± standard error (S. E.).

3. Results

3.1. Effect on hematological parameters

The assessment of blood health was done on the basis of the results obtained from the analysed parameters, i.e., TEC, TLC, Hb and PCV. Groups with lowest stocking density (SD) had a significantly higher ($p < 0.05$) hemoglobin (3.77 ± 0.29) and PCV (78.78 ± 6.1) levels as shown in Fig. 1, whereas, the treatment with highest SD had a lower value. In case of TEC, there was no significant difference ($p > 0.05$) among T1, T2 and T3, however, T4 was significantly lower (3.29 ± 0.45) compared to the other groups. The highest TLC count was found in T4 (45.45 ± 3.8), whereas, in T2 the value was found to be significantly low ($p < 0.05$).

3.2. Lysozyme and respiratory burst activity

The lowest SD group(T1) had the highest ($p < 0.05$) lysozyme level (636.00 ± 60.4), whereas, T4 had the lowest (74.00 ± 10.4) (Fig. 2). Similarly, in case of respiratory burst activity, T2 and T3 did not show any significant difference ($p > 0.05$), whereas T1 and T4 representedhighest (0.467 ± 0.04) and lowest value (0.21 ± 0.04), respectively.

3.3. Activities of enzymes related to hepatic function

The changes in the key enzymes relating to hepatic function is presented in Fig. 3. The highest sGPT level was found in T4 (25.32 ± 0.9), whereas the lowest level was found in T1 (8.67 ± 1.6)($p < 0.05$). Similarly, in case of sGOT, T4 reported highervalue (61.11 ± 1.2), whereas, T1 reported significantly lower value (16.41 ± 3.8)($p < 0.05$).

3.4. Plasma biochemistry

The observed results of plasma biochemistry is presented in Fig. 4. Total plasma protein was observed highest in T1 (3.89 ± 0.32). Among groups, T3 hadthe lowest value (2.8 ± 0.48). The albumin content of T1 was found ($p < 0.05$) higher (1.5 ± 0.05), as compared to T2 and T4, wherein, T4 recorded the lowest (0.8 ± 0.05). In case of globulin, T3 had the lowest (1.5 ± 0.09) value ($p < 0.05$) compared to others. No significant difference ($p > 0.05$) among T1, T2 and T4 was observed. The A/G ratio was significantly higher in T3 (0.87 ± 0.03), whereas, T4 showed significantly lower value (0.36 ± 0.02).

3.5. Glucose and cortisol levels

The plasma glucose and cortisol levels followed a similar trend among the treatments as depicted in Fig. 5. T4 had the highest ($p < 0.05$) plasma glucose level (217.36 ± 19.3) among the treatments, whereas, T1 had the lowest level (94.39 ± 14.25). Similarly, the highest cortisol level was observed in T4 (22.75 ± 0.02), whereas significantly lower ($p < 0.05$) value was found in T1 (9.13 ± 0.01).

3.6. Oxidative biomarkers

The activities of key oxidative enzymes(CAT, SOD, GSTand MDA) in gut, liver and muscle tissues of *O. bimaculatus* reared in different SDs is presented Fig. 6 (A-D).Activities of the enzymes was observed higher ($p < 0.05$) in the liver, compared to gut and muscle. In liver, the CAT activity was found higher ($p < 0.05$) in the T1 (17.09 ± 0.5) while in gut no significance was noted. In the muscle, higher values in T1 and T2 was observed.As regards the SOD activity, in liver it was observed to be significantlyhigh ($p < 0.05$) in T1 (18.6 ± 0.2), whereas low in T4 (13.84 ± 0.6). SOD activity in gut and muscle was observed to be significantly lower ($p < 0.05$) in T4 than that of the others. GST activity in liver was observedhighest ($p < 0.05$) in T1 (5.9 ± 0.5) and lowest in T4 (4.86 ± 0.8). But the activity in gut and muscle was reported to be lowest in T4. Similarly, MDA activity in liver was observed to be high ($p < 0.05$) in T1

Table 3
Primers used for the immune and stress gene expression of *O. bimaculatus* reared under different stocking density in biofloc system.

Targets genes	Forward primer 5′→3′	Reverse primer→
Nrf2	GGAGGAGAAGGATCGTTTGATG	TGAAGGGAGTAGTCGTTAGGG
HSP70	CAAACGCAACACCACTATTCC	GGGATGCCTGTCAGATCAAA
IL-1β	GTGACCAGGAGCTCTTCAATATC	AAGCGAGCAGAAGAGGAAAC
Tlr9	CCTGAATCACAGACAGGAGTAAA	ACAGGTCCCAGCCATATAGA
IL20	GGGAGAAGAAGGAGAAGATGAAATA	CATCCCTTCCCTTGTTCCATTA

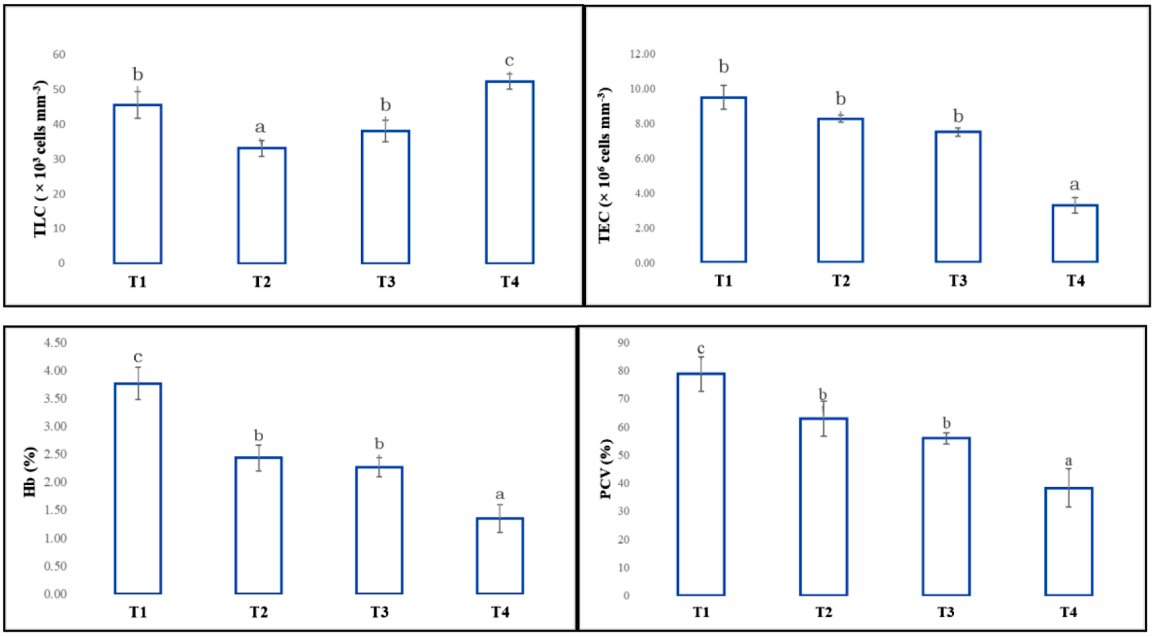

Fig. 1. Hematological indices (TEC, TLC, Hb and PCV) of *O. bimaculatus* reared in biofloc systems at different stocking density for 90 days period. Data are represented as mean ± S.E ($n = 6$). Different superscripts indicate significant difference ($p < 0.05$) among the experimental groups.

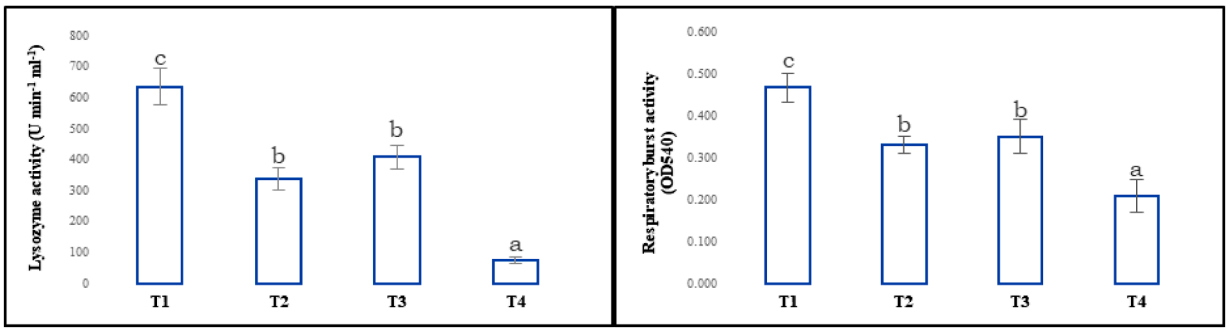

Fig. 2. Lysozyme and Respiratory burst activity of *O. bimaculatus* reared in biofloc systems at different stocking density for 90 days period. Data- are represented as mean ± S.E ($n = 6$). Different superscripts indicate significant difference ($p < 0.05$) among the experimental groups.

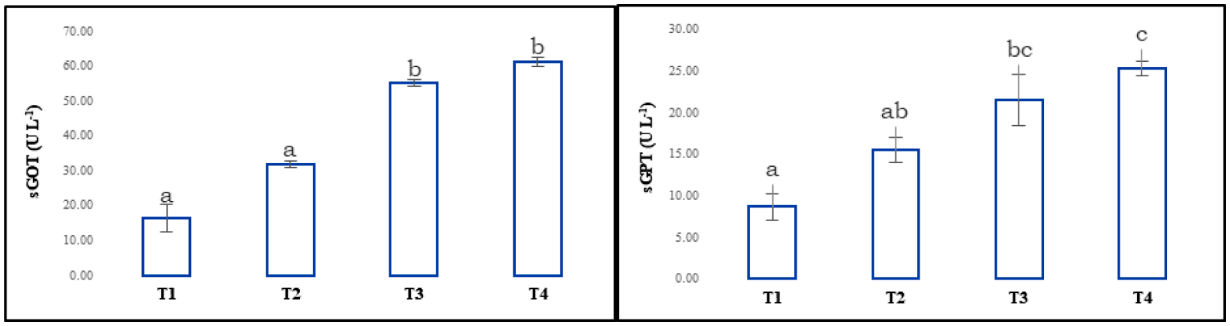

Fig. 3. Hepatic function (sGOT and sGPT) of *O. bimaculatus* reared in biofloc systems at different stocking density for 90days period. Data are represented as mean ± S.E ($n = 6$). Different superscripts indicate significant difference ($p < 0.05$) among the experimental groups.

(14.84 ± 1.29). Activity in muscle was found to be high in T2 (10.54 ± 1.01), whereas in gut it was found to be high in both T1 (5.67 ± 0.4) and T2 (5.6 ± 0.5).

3.7. Principal component analysis (PCA)

The factor analysis and PCA biplot of the examined immune-biochemical parameters showed three major factors accounting to 70.78% of the total variation (Table 4; Fig. 7). Factor 1 explained 48.10% of the variance which are positively influenced by parameters viz., TP, NBT, mCAT and negatively influenced by COR, GLU,sGPT and ALB. Factor 2 contributes to 14.48% of the total and mostly explained by A/G, mGST, and gSOD (+ve), while, GLB, LYZ and mCAT contributed negatively.

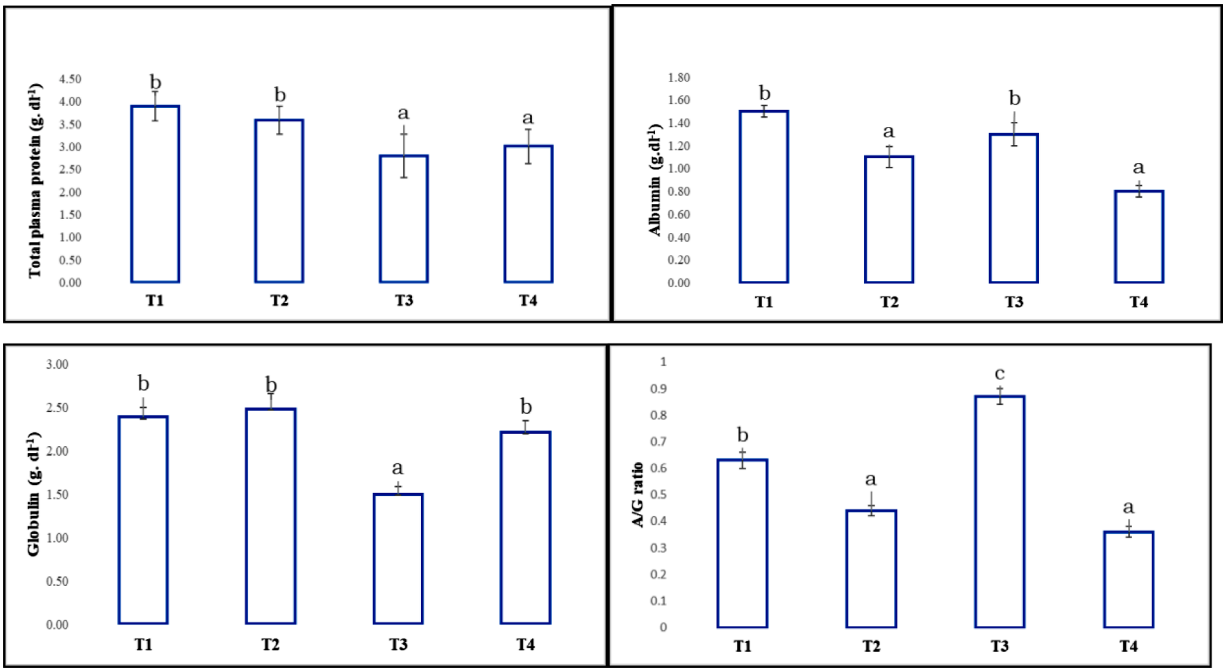

Fig. 4. Plasma biochemical indices (Total plasma protein, albumin, globulin and albumin/globulin ratio) of *O. bimaculatus* reared in biofloc systems at different stocking density for 90 days period. Data are represented as mean ± S.E ($n = 6$). Different superscripts indicate significant difference ($p < 0.05$) among the experimental groups.

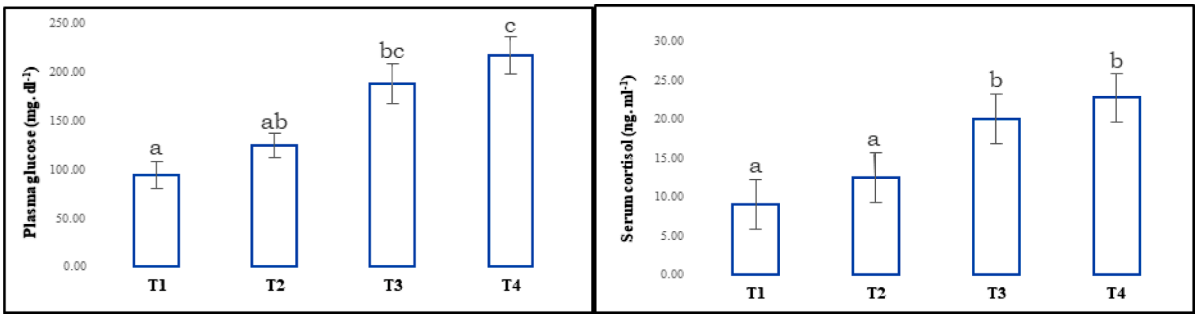

Fig. 5. Glucose and cortisol levels of *O. bimaculatus* reared in biofloc systems for 90 days period. Data are represented as mean ± S.E ($n = 6$). Different superscripts indicate significant difference ($p < 0.05$) among the experimental groups.

3.8. Expression of key stressand immune genes

The expression pattern (fold changes against the control) of the investigated genes are depicted below in Figs. 8–12.

3.8.1. HSP70 gene

The *HSP70* gene was differentially expressed in kidney and liver in different treatmentscompared to the control.Transcript level of the gene was found lowest in fishes under normal condition (with a mean fold of control ≈0.05) and highest (with a mean fold of control ≈1) inT4.

3.8.2. IL-1β gene

Higher expression ($p < 0.05$) level of IL-1β was observed in kidney tissue of T2, T3 and T4 groups, and T4 showed highest expression among the treatments. In liver tissue, its expression was lower in control, T1 and T3 groups, whereas, T4 showed significantly highest level ($p < 0.05$).

3.8.3. IL-20 gene

The expression of *IL-20* gene was determined in kidney and liver. This gene was differentially expressed in kidney of fishes under different treatments and also in the normal fish. The expression was highest in both T2 and T4 (with mean fold of control ≈1) fishes and lowest in T1 (with mean fold of control ≈0.55). In case of liver tissue, *IL-20* expression was lowest in T1 and highest in T2.

3.8.4. Nrf-2 gene

In the kidney, the expression of *Nrf-2* gene was found to be lowestof T1 group ($p < 0.05$). Among the groups, T2 showed the highest expression level ($p < 0.05$). Similarly, the expression in liver tissue was observed to be highest in T4, while T1 showed a significantly lowest value ($p < 0.05$).

3.8.5. Tlr-9 gene

In kidney, the expression was highest in the T4 ($p < 0.05$).The expression in the control group of fishes was observed to be significantly higher in comparison to the T1 group of fishes. In case of liver, the expression was found to be significantly lowest ($p < 0.05$) in T1 whereas, T4 showed significantly highest value.

3.9. Integrated biomarker response

The observed IBR means in different tissues is presented in Table 5.

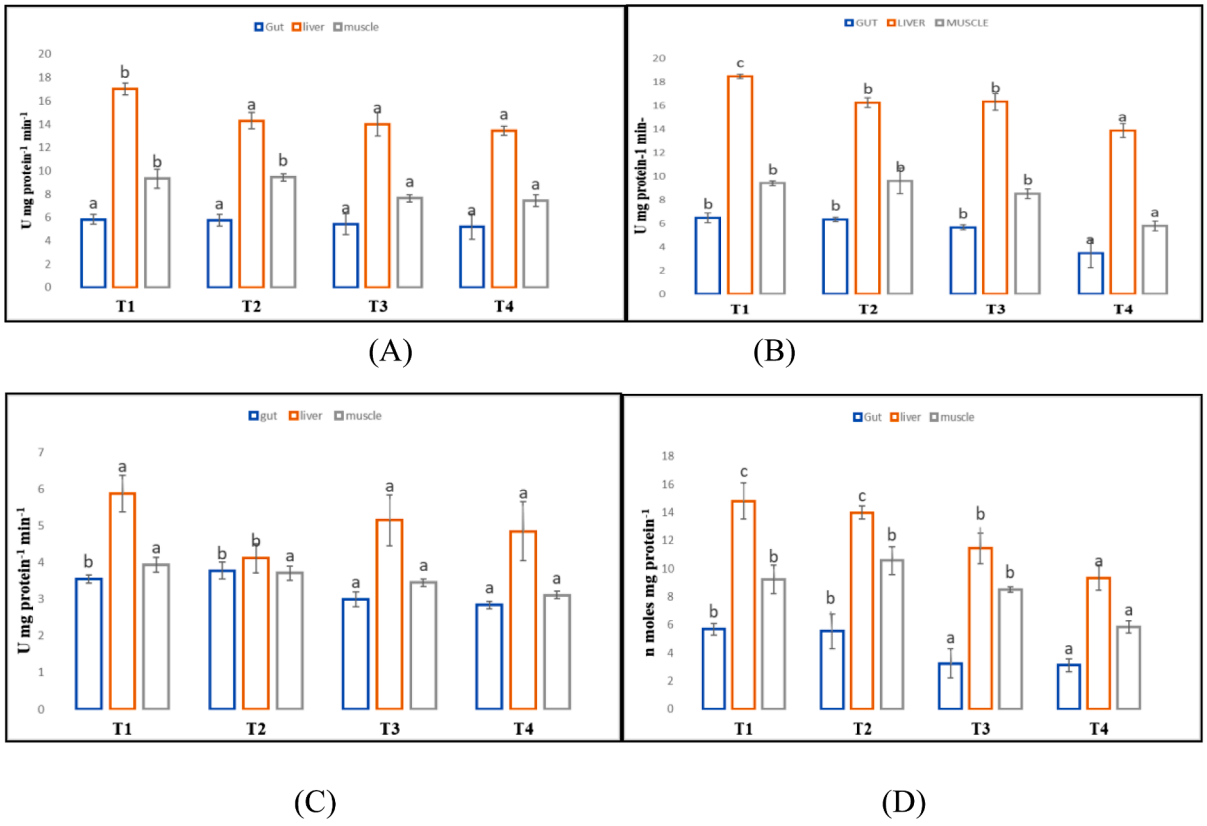

Fig. 6. Antioxidant enzymes (A. Catalase: CAT; B. Superoxide dismutase: SOD; C. Glutathione-S-transferase: GST &D. Malondialdehyde: MDA) activity of *O. bimaculatus* reared in BFT systems at different stocking density for 90 days. Data are represented as mean ± S.E. Different superscripts indicate significant difference ($p < 0.05$) among the experimental groups.

Table 4
Factor analysis of key immune-biochemical parameters recorded in *O. bimaculatus* stocked at varied densities in a biofloc system for 90-days.

Immune-biochemicalparameters	Factor1	Factor2	Factor3
TP	**0.27972**	−0.0425	−0.1618
ALB	**−0.24938**	−0.0905	0.20218
GLB	0.08797	**−0.48433**	0.02469
A/G	0.04618	**0.49778**	0.01996
GLU	**−0.27099**	−0.05109	0.02224
sGOT	−0.15729	0.23012	**−0.37905**
sGPT	**−0.27468**	0.00905	0.03191
LZY	0.18384	**−0.22176**	−0.03138
COR	**−0.29913**	0.03525	0.06161
NBT	**0.27304**	−0.07819	0.28173
gMDA	0.2479	−0.09855	0.13924
lMDA	0.25232	−0.05654	−0.00198
mMDA	0.17451	0.01053	0.07423
gSOD	0.22447	**0.27445**	−0.07149
lSOD	0.20701	0.15687	**0.2991**
mSOD	0.21609	0.05458	−0.16752
gCAT	0.0957	−0.03452	**−0.40152**
lCAT	0.23356	−0.09018	0.07528
mCAT	**0.2415**	**−0.19528**	−0.18992
gGST	0.19023	0.24132	−0.32264
lGST	0.08736	0.13483	**0.45286**
mGST	0.12993	**0.39454**	0.20717
Eigenvalue	10.57215	3.18704	1.81362
%ofvariance	48.05523	14.48655	8.24373
%Cumulative	48.05523	62.54178	70.78552

Notations: TP: Total protein; ALB: Albumin; GLB: globulin; A/G: Albumin globulin ratio; GLU: glucose; GOT: Glutamate-oxaloacetate transaminase; GPT: Glutamate pyruvate transaminase; s: serum; LZY: lysozyme; COR: cortisol; NBT: nitro blue tetrazolium; MDA: Malondialdehyde; SOD: Superoxide dismutase; CAT: Catalase; GST: Glutathione-S-transferase; m,g,l: muscle, gut and liver.

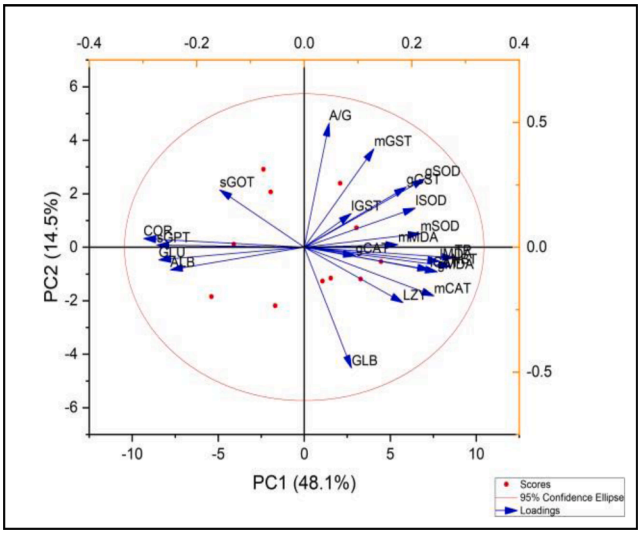

Fig. 7. Principal Component Analysis (PCA) biplot of key immune-biochemical parameters recorded in *O. bimaculatus* stocked at varied densities in a biofloc system. Blue arrows specify the increasing values for each variable.

In all tissues, means were higher in lowest SD (T1) as compared to the corresponding higher SD groups. Again, among the tissues, liver had a higher mean (10.82 ± 0.01) compared to gut (7.81 ± 0.08) and muscle (7.91 ± 0.08) tissue. Lowest mean value in all tissues was noticed in T4 (0.00 ± 0.00). Further, the star plots (Fig. 13) were observed to be high in T1 and T2 groups for all the three examined tissues, compared to the other two groups. Among all the groups, T1 had the best plot.

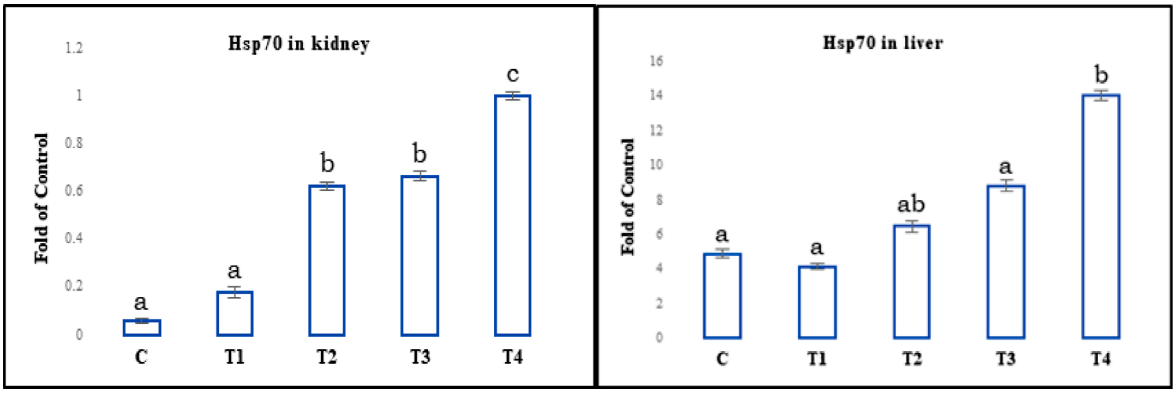

Fig. 8. Relative expression levels of *HSP70* mRNA in the kidney (A) and liver (B) of *O. bimaculatus* subjected to different stocking densities in biofloc system. Data are expressed as the mean ± S.E. ($n = 6$). Different letters indicate significant differencesbetween groups ($p < 0.05$).

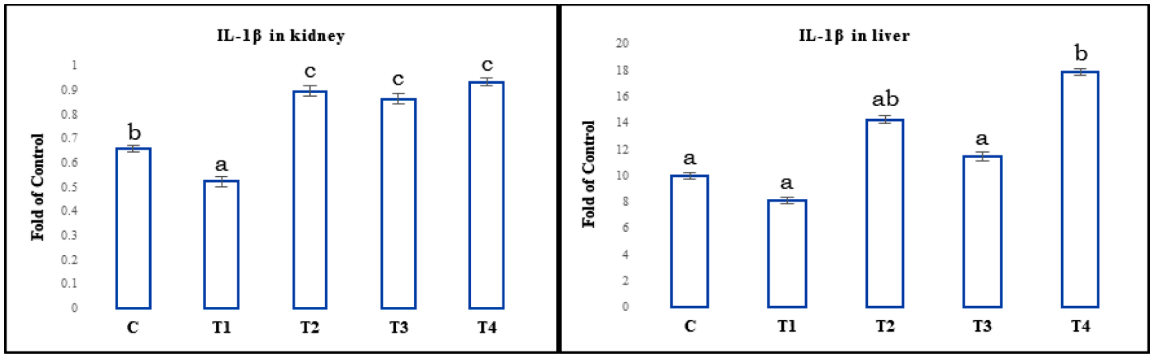

Fig. 9. Relative expression levels of *IL-1β* mRNA in the kidney (A) and liver (B) of *O. bimaculatus* subjected to different stocking densities in biofloc system. Data are expressed as the mean ± S.E. ($n = 6$). Different letters indicate significant differencesbetween groups ($p < 0.05$).

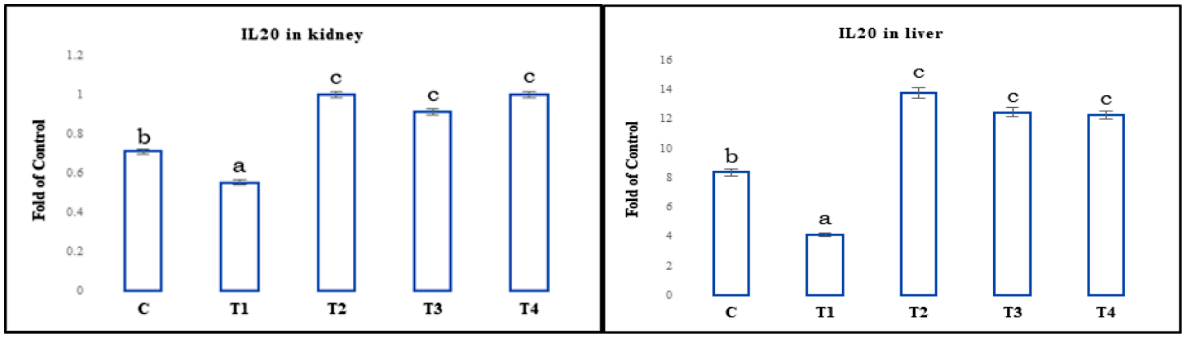

Fig. 10. Relative expression levels of *IL-20* mRNA in the kidney (A) and liver (B) of *O. bimaculatus* subjected to different stocking densities in biofloc system. Data are expressed as the mean ± S.E. ($n = 6$). Different letters indicate significant differencesbetween groups ($p < 0.05$).

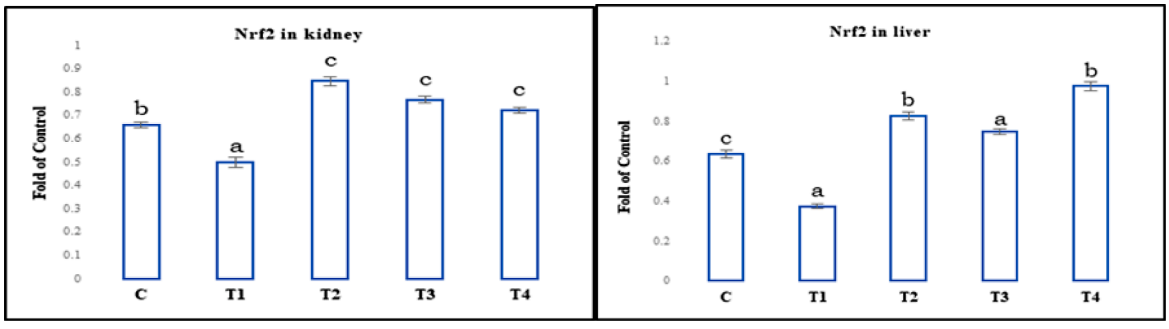

Fig. 11. Relative expression levels of *Nrf-2* mRNA in the kidney (A) and liver (B) of *O. bimaculatus* subjected to different stocking densities in biofloc system. Data are expressed as the mean ± S.E. ($n = 6$). Different letters indicate significant differences between groups ($p < 0.05$).

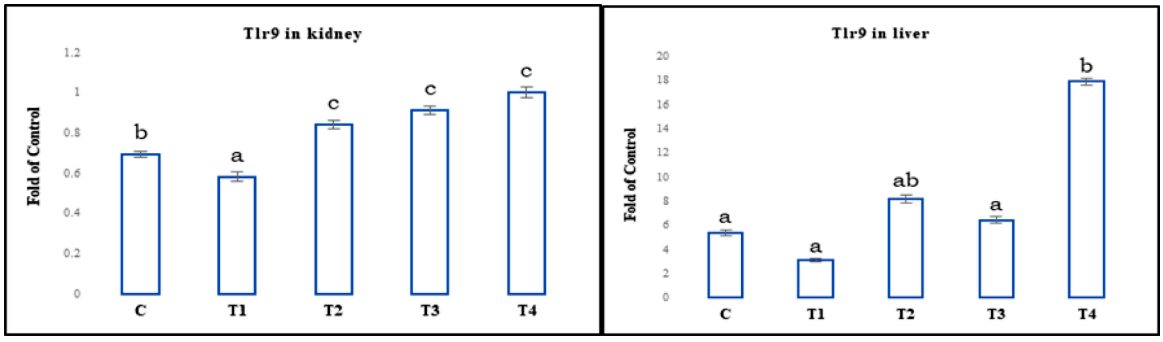

Fig. 12. Relative expression levels of *Tlr-9* mRNA in the kidney (A) and liver (B) of *O. bimaculatus* subjected to different stocking densities in biofloc system. Data are expressed as the mean ± S.E. ($n = 6$). Different letters indicate significant differencesbetween groups ($p < 0.05$).

Table 5
Summary of IBR Mean ± S.D. of different oxidative biomarkers in differenttissues of *O. bimaculatus* stocked in varied densities.

Tissues	Treatments			
	T1	T2	T3	T4
Liver	10.82 ± 0.01	1.33 ± 0.46	1.77 ± 0.12	0.00 ± 0.00
Gut	7.81 ± 0.08	6.84 ± 0.03	0.82 ± 0.46	0.00 ± 0.00
Muscle	7.91 ± 0.08	7.72 ± 0.06	1.86 ± 0.18	0.00 ± 0.00

3.10. Histopathology of gills and livers

Histopathological assessment of gill and liver tissues of the fish stocked in different stocking density (SD) were carried out using H&E stain and the observed histological alterations are displayed in Figs. 14 and 15. Alterations in gills of the fish stocked in different SD,with an increased alteration with increasing SD. Least alteration wasnoticed in T1 (lowest SD). Gill tissues in T1 showed epithelial lifting (Fig. 14A). Gill tissues in T2 showed telangiectasis, shortening of secondary lamellae and chloride cell hyperplasia (Fig. 14B). Gill tissues in T3 showed epithelial lifting, epithelial cell hyperplasia, chloride cell hyperplasia and cellular necrosis (Fig. 14C) while the gill tissues in T4 showed curling of secondary lamellae, lamellar swelling, desquamation, necrosis and degeneration (Fig. 14D).Similarly, liver tissues of the fish stocked in increasing stocking densities showed several cellular alterations which were found to be increased with increase in density. T1 displayed normal liver architecture (Fig. 15A). T2 displayed nuclear hypertrophy and congestion of sinusoid (Fig. 15B). T3 displayed nuclear pyknosis, cellular necrosis and cloudy swelling (Fig. 15C) while T4 displayed hepatocytes hypertrophy, cellular degeneration, hydropic degenerations, blood congestion and vacuolar degeneration (Fig. 15D).

4. Discussion

Over-stocking can cause stress and decrease the host immunological capability in intensive systems, making aquatic animal health and well-being critical [35,36]. Modern aquaculture systems, such as biofloc, have been shown to boost the immune systems of the host animals. It is necessary to understand how much stocking biofloc can handle without jeopardizing fish welfare. In fish physiological studies, a wide range of biological levels, including biochemical, molecular, and histo-pathological alterations, are used to explain the organism's response to various stresses [37]. The ability of floc-proteins to boost the immune system should be obvious as a result of overcrowding. Biological responses should be studied simultaneously and at various levels of biological organization [18]. Our research used a variety of well-being indicators to pinpoint a specific biological reaction in *O. bimaculatus*, which helped to ground this hypothetical situation.

4.1. Host immunity

The blood profile of farmed fish can be used as an accurate indicator of the fish's health [38]. Increased stocking density decreased TEC, Hb, and PCV while increasing TLC, which is related to innate immune function [39]. Lower SD (0.5 g/L) indicated improved O. bimaculatus health, with higher levels of the parameters detected correlating with that improvement. Heterotrophic bacterial aggregation in flocs, which act as probiotics and are well known for their immune-stimulating properties [40]. The amount of immunological improvement does not provide 100% protection for fish at high stocking densities (1.0, 1.5, and 2 g/L). Lower blood parameters may be due to higher density cage culture, which has previously been linked to increased erythrocyte hypertrophy [41,42]. Bioactive substances found in bio-flocs that improve fish immune function include chlorophylls, carotenoids, bromophenols,

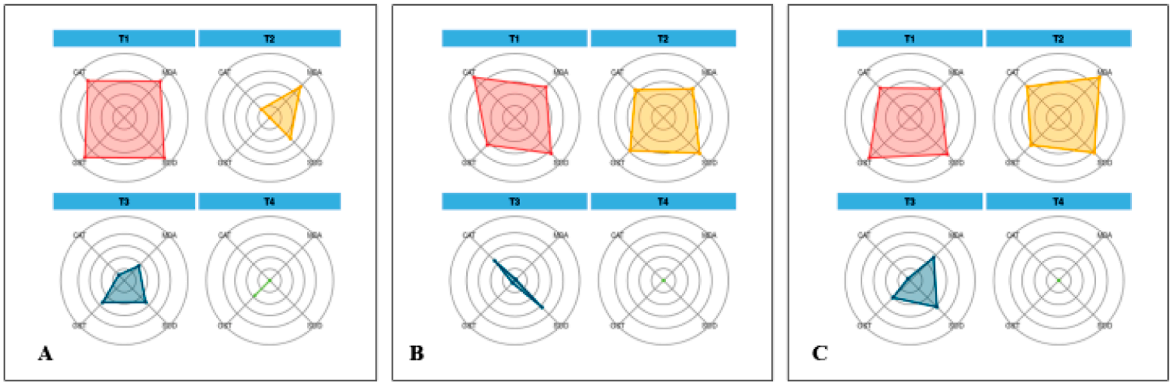

Fig. 13. Integrated bio-marker response (IBR) star plots of the oxidative enzymes in different tissues (A. Liver, B. Gut and C. Muscle) of *O. bimaculatus* stocked at different densities in a bio-floc system.

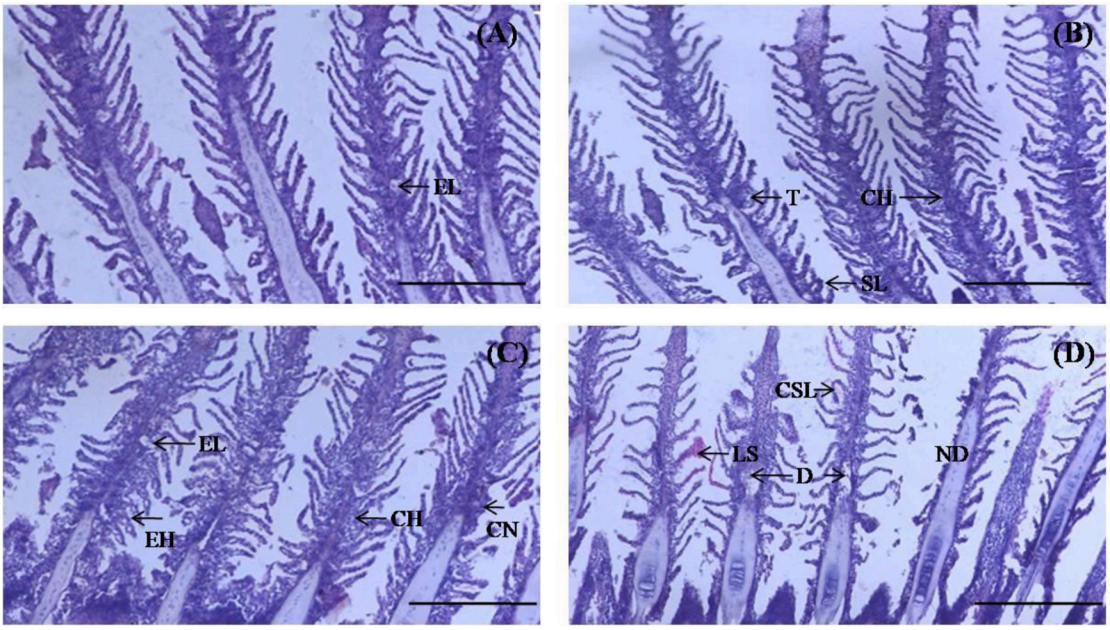

Fig. 14. Photomicrographs of H & E stained gills of fish *O. bimaculatus*. (A) epithelial lifting (EL); (B) telangiectasis (T), shortening of secondary lamellae (SL), chloride cell hyperplasia(CH); (C) epithelial lifting (EL), epithelial cell hyperplasia (EH), chloride cell hyperplasia (CH), cellular necrosis (CN); (D) curling of secondary lamellae (CSL), lamellar swelling (LS), desquamation (D), necrosis and degeneration (ND). Bar scale: 50 μm, 40X.

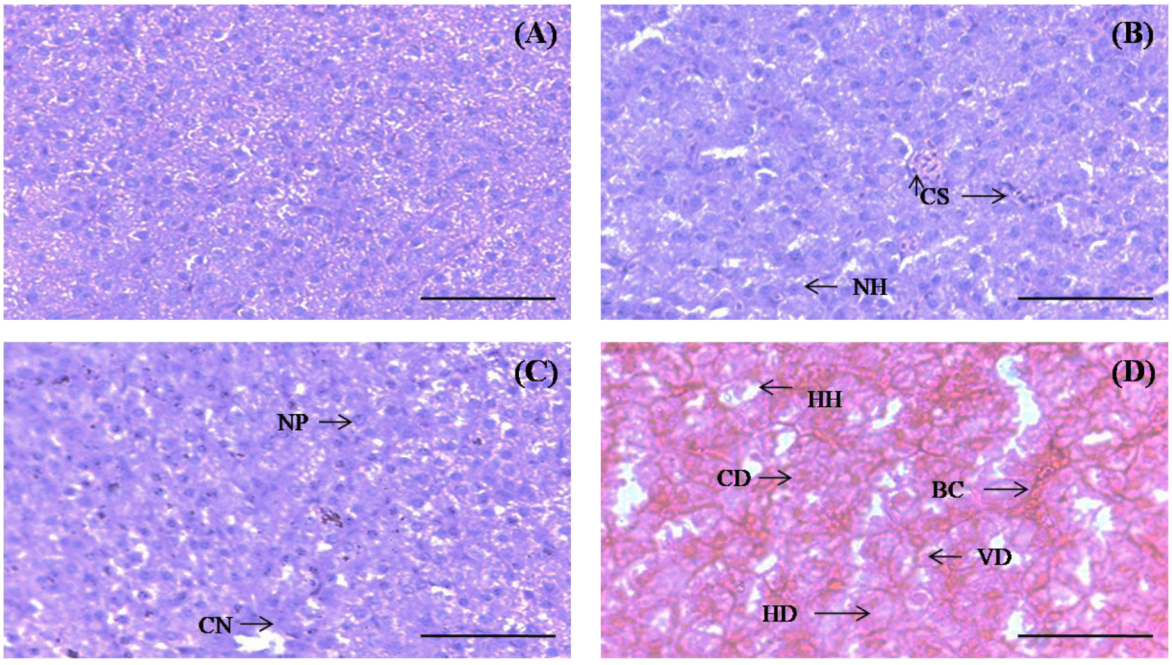

Fig. 15. Photomicrographs of H & E stained livers of fish *O. bimaculatus*. (A) normal liver architect; (B) nuclear hypertrophy (NH) and congestion of sinusoid (CS); (C) nuclear pyknosis (NP), cellular necrosis and cloudy swelling (CN); (D) hepatocytes hypertrophy (HH), cellular degeneration (CD), hydropic degenerations (HD), blood congestion, and vacuolar degeneration(VD). Bar scale: 50 μm, 40 X.

phytosterols, amino sugars, and anti-bacterial chemicals [43]. Previous research has found that growing O. bimaculatus seeds in the bio-floc method results in better immunological status and blood chemistry [9]. According to [44], when there are more common carps (*C. carpio*) around, their immune system improves.

If fish populations are over-crowded and hazardous nitrogenous substances accumulate together, the normal physiological stability of fishes is put at risk. The fish's plasma biochemistry changes as a result of this, which is an excellent measure of their well-being [45]. Fish health and immunity are closely linked to the amount of plasma protein in their blood [46]. A high quantity of albumin and globulin in the blood is associated with a powerful innate immune system [47]. The lowest density (0.5 g/L) had higher levels of total plasma protein, albumin, globulin and the A/G ratio, all of which indicate a healthier state of affairs. Adineh et al. [44] found that in a bio-floc system, serum total protein and albumin levels were considerably higher in low SD. For more information, the mitochondrion enzymes sGOT and sGPT are universal amino-transferases that can be employed as accurate stress

indicators for the hepato-pancreas system [48]. We found that stocking density more than 1 g/L resulted in an increase in levels of sGOT and sGPT, whereas stocking density below that level resulted in a decrease. This suggests that hepatic damage and poor liver function are connected to overstocking stress. Both jade perch, *Scortum barcoo* [49] and *Puntius sarana* [42] had lower immunological levels when cultivated under high SD conditions. Because of this, it appears the utilization of the flocs offered may not alleviate the additional stress produced by crowding, as noted here.

An increase in the levels of blood-lysozyme enzyme, which lyses bacteria, is considered a natural defense mechanism in fishes [15]. There was a significant difference between the lowest and highest SD groups in this study. Immunological suppression occurs even in a bio-floc system, as evidenced by the fact that the degree of stocking may affect the non-specific immune characteristics generated by the flocs available. In bio-floc, this is conceivable, as demonstrated by the steady decline in serum lysozyme levels in Nile Tilapia, *O. niloticus*, in a high stocking bio-floc system [50,51]. In the NBT experiment, phagocytic cells to create oxygen radicals because of their capacity to induce respiratory burst activity [24]. There have been previous reports of the ability of the bio-floc system for immunological regulation in *O. bimaculatus* fry under normal SD, as demonstrated by Debbarma et al. [9]. With increasing SD, we noted that burst activity decreased significantly at greater densities. This was in accordance with our findings. Another study by Kheti et al. [52], found that supplementing rohu, L. *rohita,* with bio-floc meal gave strong immunity and elevated NBT activity. Because the fish's activity decreased as SD rose, we believe that its immune system had become compromised to the point where it could no longer manage it.

4.2. Levels of glucose and cortisol

Chronic stresses like over-crowding impact the fish's immune system and tolerance capacity through a variety of physiological pathways. Bifurcated findings have been noted in papers about the effects of the bio-floc system on fish's stress tolerance. In addition to the immunostimulatory effect, the level of cortisol and glucose in fish in the bio-floc system may explain well the likelihood of stress. To manufacture cortisol, the anterior pituitary gland releases adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which stimulates the inter-renal tissue to make cortisol [53]. Cortisol mobilizes and increase glucose synthesis either through glycogenesis or glycogenolysis in response to catecholamine hormoneslike adrenaline and nor-adrenaline generatedby chromaffin tissue under stressful conditions [54]. There was a decrease in glucose and cortisol levels as the SD increased in the current investigation. This suggests that higher levels of density interfere with the body's ability to operate optimally, and the stored energy is used to maintain the body's homeostasis via the production of glucose. In L. *rohita* under stress, Ahmad et al. [55] found that the values of these parameters rose significantly. Also, reports by Adineh et al. [44] in *C. carpio* reared in high SD supported the existing findings.

4.3. Oxidative stress response

In the biofloc system, oxidative stress and ammonia toxicity are linked. A high concentration of ammonia nitrogen in the aqueous environment inhibits ammonia elimination by fish, resulting in the build-up of ammonia in fish, which results in reactive oxygen species generation (ROS). When rearing in high stockings, ROS level rise, generating oxidative stress, which has detrimental effects on cell proteins, lipids and DNA [56]. With the aid of enzyme scavengers, an antioxidant defense mechanism counteracts the build-up of ROS. Enzymes such as SOD, CAT, MDA and GST serve as the first line of defense against free radicals [57]. SOD is the first line of defense against oxidative stress, since it is primarily responsible for converting superoxide into peroxide [58]. According to our findings, increased stocking densities resulted in lower levels of the four enzymes tested. Even though bio-floc created *in-situ* exhibited an "anti-stress" impact, further intensification in density might reduce the antioxidant response. In a bio-floc system, Liu et al. [51] found that stocking density had a significant impact on the levels of these oxidative enzymes in *O. niloticus*. Similarly, Bauelos-Vargas et al. [59] found that high densities lowered Red Tilapia's anti-oxidant defences, resulting in lower CAT and GPX activities. But Wang et al. [41] observed that an increase in density, there was no evidence of activity modification in GIFT tilapia, deviating other studies. Comparing our findings to previous research demonstrates that fish species and stocking amounts have a significant impact on the fish's ability to cope with stress. This, however, requires more research.

4.4. Integrated biomarker response

As a follow-up to this study's focus on individual biomarkers, we studied the viability of an integrated strategy to define the stress of high stocking density on *O. bimaculatus*. That's because it's difficult to comprehend the results of biomarker-based monitoring since it lacks an integrated statistical analysis [60]. As a result, we used the integrated biomarker response (IBR) score to get a better idea of how much stress the animal experience from high-density. All three tissues (liver, gut and muscle) showed elevated levels of biomarkers at 0.5 g/L when examined. However, as compared to 1.0 g/L, the change in the gut and muscle was small. Stocking density of 1.0 g/L results in impaired hepatic function, which is why a lower stocking density is suggested for the sake of the fish's well-being and productivity. As previously reported by Debbarma et al. [9], this finding confirms the use of IBR indices as a new confirmatory analysis for fish welfare sustainability in bio-floc systems.

4.5. Principal component analysis

The PCA bi-plots and factor analysis results show a clear grouping of the parameters, which very well justifies the variable degree of stress response caused by gradual elevation in stocking density in this study. The combined contribution of individual parameters from biochemical and immunological data provides a precise understanding of the welfare status of Butter catfish cultured at various SD in a bio-floc system. Previously, Islam et al. [61] used PCA bi-plots to reveal the metabolic, osmotic, and molecular stress responses in European seabass, *Dicentrarchus labrax*, under low salinity.

4.6. Expression of key stressandimmune genes

HSP70, a member of the HSP family, aids in the folding of polypeptide chains, the repair or destruction of damaged proteins, and cellular processes [62]. In this study, the way the *HSP70* gene is regulated provides information on productivity and stress, two topics that are frequently linked. This gene has been identified as an important participant in the stress response. Stress response mechanisms in the fish in this study may have been activated by environmental variables such as stocking density, as evidenced by higher levels of *HSP70* mRNA compared to the control group. Higher SD can boost productivity, but only if it does not harm the animal's health. As a result, SD increased in groups T2, T3, and T4, leading to an increase in *HSP70* expression. In contrast to our findings, [63] discovered that high SD increased gene expression in the *Scophthalmus maximus*. Increasing stress levels are linked to higher levels of the *HSP70* stress response gene, a biomarker. Furthermore, by inducing cytokine factors, the *HSP70* gene activates the fish's immune system in response to stress. In stressful situations, immune-related cytokines regulate fish immunity and anti-inflammatory responses [64]. We concentrated on the cytokine factors IL1, IL20, and Tlr9 for this study. Esam et al. [65] discovered an increase in the levels of IL-8, IL-1, and Tnf in the liver tissues of Nile tilapia exposed to ammonia and high temperatures, which could be attributed to liver damage caused by oxidative stress [66]. TLRs, a

family of trans-membrane proteins, play an important role in the initiation of adaptive immune responses in response to stimuli [67]. In response to an increase in *HSP70*, the expression of these genes increases over time. A high number of these genes indicates a strong immune response. Despite this, it is impossible to deny that the fish are under a great deal of stress, which is harming them. The expression patterns of these genes are strikingly similar to those of *HSP70*. When cytokine activity is high, as it was in the T1 group, immunity improves. The increased immunological response at higher SD, on the other hand, could be a sign of illness or stress. The Nrf-2 gene, which is important in dealing with oxidative stress, was also considered. The Nrf-2-keap1 signaling pathway regulates antioxidant responses, and Nrf-2 acts as a transcription factor for cellular Nrf-2 [68]. It is also important to note that Nrf-2 deficiency increases liver lipid peroxidation, which is harmful to an individual's health [68,69]. Oxidative stress activated the genes for antioxidant enzymes, and Nrf-2 was transported to the nucleus, where it uncoupled from Keap1 and increased antioxidant enzyme activity to combat ROS-induced oxidative damage. Previously, Liu et al. [70] discovered that ammonia treatment of golden pompano decreased Nrf-2 and increased Keap1. However, the current study found that oxidative stress increased with increasing stocking density, which was unexpected. The activity of the Nrf-2 gene increases in response to oxidative stress, indicating an increased response and resistance development in the treated fish.

4.7. Histological changes

High SD in biofloc aquaculture systems, as seen by histological changes in gill and liver samples, may lead to stress-induced changes in the cells of important organs like the gills and liver. As the first organ to respond to environmental stress, fish's gills are well-known as potential targets for parasite attack [71]. The gills of different fish species change in comparable ways when exposed to environmental stress, according to a number of studies. In [72], secondary lamellae were shown to have epithelial hyperplasia and curling, swelling at the terminals of multiple secondary lamellae, and clump-shaped secondary lamellae. When exposed to cyphenothrin, the gills of *Lebistes reticulates* developed clup-shaped lamellae, a necrosis and degeneration of secondary lamellae and a shortening of secondary lamellae, according to [73]. The telangiectasis necrosis, and lifting of the epithelium seen in this study has been previously documented in other species [74]. Due to the stressors in this investigation, we noticed necrosis and desquamation of the gill epithelium. Toxicants have to travel a longer distance to reach the blood stream when the epithelium is lifted; gill hyperplasia reduces respiratory surface area and thus increases the toxicant-blood diffusion distance [75]. Cellular collapse and loss of integrity of the vascular system may have contributed to an increased incidence of telangiectasis in lamellae [76]. The lamellar epithelium would have been pushed outward by the massive blood flow that would have resulted from this. Toxic material excretion, xenobiotic detoxification and metabolic processes are all carried out primarily by the liver [77]. We found that the pathological abnormalities detected in the present study were comparable to those previously described by other researchers. These include nuclear congestion and pyknosis as well as vacuolar degeneration and hypertrophy of hepatocytes. According to Velmurugan et al. [78,79], these liver alterations were similar. The liver of the *Ctenopharyngodon idellus* was studied by Tilak et al. [80], who found that fen-valerate caused hepatocyte degeneration, necrosis and the removal of the hepatocyte wall. Velisek et al. [81] also reported the degeneration of hepatocytes. The results of this study explains that *O. bimaculatus* shows extreme sensitiveness to a rise in SD in the biofloc aquaculture system.

5. Conclusion

Biofloc production could be the wave of the future for many types of commercial fish. To better understand how to standardize a stress-free, reliable culture method, we investigate the health status of butter catfish in high-intensive practices like biofloc. This study reveals a broad spectrum of responses in Butter catfish when grown at different densities. The stocking density have an effect on fish health and fitness. Stress in fish is revealed in its entirety when a temporal connection is established between immunological capability, stress biomarkers, tissue-level modifications, and molecular biomarker genes. In this case, the integrated approach based on IBR analysis could be used to assess overall physiology at an advanced level. Even though most research suggests that fish health can be improved by keeping the water clean through the simultaneous and faster assimilation of nitrogenous waste using green technology like biofloc, this study shows that there must be a maximum density limit to protect fish health. Fishes stocked at 0.5 g/L were better than those stocked at higher concentrations across a range of molecular, histological, and physio-biochemical indices. These findings have the potential to influence our understanding of the significance of internal changes in fish well-being, which is particularly important in the context of modern fish farming, where welfare interventions are often necessary.

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Declaration of Competing Interest

No potential conflicts of interest were reported by the authors.

Data availability

Data will be made available on request.

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