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Effect of *in-situ* and *ex-situ* biofloc on immune response of Genetically Improved Farmed Tilapia

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ABSTRACT

The present study is aimed to investigate the effect of biofloc intake on Genetically Improved Farmed Tilapia (GIFT), developed within the system and its influence as feed supplementation on water quality, growth performance, immunological parameters, antioxidant status, immune gene expression, and its resistance to *Aeromonas hydrophila* infection. GIFT Tilapia juveniles of 5.1 g (± 0.05) were stocked at a density of 15/m^3 in lined ponds of 300 m^2 in triplicates for 180 days. The experimental groups consisted of T1-biofloc developed within the culture systems (*insitu*), T2-biofloc supplementation in fish feed (*exsitu*) and C- Control without biofloc. Distillery Spent wash was used as a carbon source to maintain the C/N ratio of 10:1 for floc development in T1. Free CO_2, pH, BOD, dissolved oxygen, alkalinity, Calcium and Magnesium ions, Nitrate, Nitrite and ammonia were found to be significantly different between the treatments and control throughout the experiment. The immunological (Serum protein, Respiratory burst test (RBT) and Myeloperoxidase) and antioxidant indicators (Glucose, Superoxide dismutase (SOD) and catalase) were found to be significantly higher in T1 at the end of the trial. Increased weight gain, specific growth rate, survival and decreased feed conversion ratio was found in T1 when compared with the other experimental groups. Real time quantitative PCR analysis revealed that there was no folded expression of the immunological genes such as Metallothionein gene, cathepsin L, Toll like receptor 7, Interleukin 1 β and Tumour necrosis factor α in liver and intestine for both control and treatment. However, the upregulated expression of targeted genes except tumour necrosis factor α was found in head kidney of T1. At the end of the study, GIFT Tilapia when infected with *Aeromonas hydrophila* showed an improved immune response in T1 and T2 with lesser signs of infection than Control. The findings of the present study affirmed the importance of biofloc technology in triggering the immunomodulatory response of GIFT Tilapia with its upregulated immune gene expression and its role as an antimicrobial agent against *Aeromonas hydrophila*. This study suggests the adoption of *in-situ* (T1) based biofloc method to obtain better performance of GIFT Tilapia culture.

1. Introduction

Tilapia, considered to be a hardy species, is the second most cultured freshwater fish globally. In 2016, the total production of tilapia was roughly about 6.69 million tonnes [1] and is expected to rise to 7.3 million tonnes by the end of 2030 [2]. The most distinct characteristic traits of this species include its euryphagic feeding habit, captive breeding potential, tolerance to high stocking density and improved growth performance in various aquaculture systems. The Genetically Improved Farm Tilapia (GIFT) strain has been developed using eight different species of Tilapia under selective breeding by World Fish Centre [3] as a consequence to the emergence of new diseases and lack of fish seed availability. The rapidly rising global population and decline in capture fisheries has accorded greater significance to aquaculture than ever. However, the expansion of aquaculture is limited to land and water utilization which hinders the productivity of aquaculture activities, particularly in Tilapia farming [4–7]. To overcome these bottlenecks, sustainable intensification by the adoption of advanced culture systems and technologies becomes inevitable to improve the production and productivity of the sector.

One of the best bets is on the biofloc technology which requires a minimal or zero water exchange and allows stocking of animals at higher densities. Biofloc are conglomerates of algae, bacteria, protozoans, fecal matter and uneaten feed which are held together in a loose

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matrix by the secretions of filamentous microorganisms or by electrostatic attraction [8]. This technology maintains the carbon and nitrogen content in the culture water and uses the dense microbial biomass to strip the ammonia and serves as a nutritional supplement [9]. The external addition of carbon sources to the culture water stimulates the growth of heterotrophic bacteria and its uptake of nitrogen by the production of the microbial protein [10] faster than regular nitrification process [11]. The nutrient profile of biofloc ranges from 25 to 50% of protein and 0.5 to 15% of fat on a dry-weight basis. Bioflocs are also a valuable source of limiting amino acids such as methionine and lysine, vitamins (Vitamin C in the range of 0–54 μg/g dry matter) and limiting mineral such as phosphorus [12]. In aquafeeds, dried biofloc can be used possibly to replace fishmeal or soybean meal as cheaper sources of protein. Extensive and traditional systems with no or little use of fishmeal supplies nutrient-rich materials to the culture water enhancing the growth of algae and other indigenous organisms on which the fish can feed [13]. The effluent waters from aquaculture systems are used for *ex-situ* biofloc production in suspended growth bioreactors. The biofloc produced can be dried and used as a feed supplement for shrimp or fish [14]. But the uptake of biofloc as feed depends on the nature of species, its feeding ability, size of the animal, and size and density of the floc [15].

According to the findings from the previous study, the uptake of biofloc as an additional protein source by freshwater prawn, shrimp, and tilapia indicates that the technology can be applied to both freshwater and seawater culture [12,16,17]. Biofloc helps in the potential feed gain with decreased production cost [18] which can be estimated to be in the order of 10–20% [19]. As biofloc technology deals with bacteria and bacterial products, it is likely to come across immunostimulatory compounds exhibiting possible probiotic effects. However, the relative efficiency of *in-situ* and *ex-situ* biofloc with respect to the immune gene expression of the animal has not been attempted so far particularly in GIFT Tilapia. The objective of this study is thus aimed to determine the intake of biofloc by GIFT Tilapia using different incorporation methods and its impact on animal immunological performance along with its gene expression.

2. Materials and methods

2.1. Experimental design

A 180-day culture was carried out in the Advanced Research Farm Facility, Madhavaram in Chennai (13.1478° N, 80.2310° E). The experimental group included *in-situ* biofloc developed within the culture systems - Treatment-1 (T1), biofloc incorporated fish feed developed by *ex-situ* method as Treatment-2 (T2), and animals reared without biofloc as control (C). Animals weighing 5.1 g (± 0.05) were stocked at a density of 15/m^3 in lined ponds of 300 m^2, in all the experimental groups in triplicates. The animals were fed with isoenergetic and isonitrogeneous diet as per their average body weight in all the treatments. The proximate composition of the biofloc and the experimental diets are presented in Tables 1 and 2.

2.2. Production of biofloc

In T1, development and maintenance of biofloc in the freshwater culture ponds was adopted as suggested by Taw [20] at C:N ratio of 10:1. The addition of carbon source to maintain the C:N ratio was followed using the method of Avnimelech [15] for the transition of the heterotrophic system. For T2, biofloc production was carried out in two indoor raceway tanks (50 tonnes; 15 m × 3 m x 1 m) in six batches at 10-days interval during January to March 2018. Tanks were filled with used culture water taken from the fish ponds and 100 L biofloc inoculum with bacterial floc developed in a separate tank was added to each raceway. Spentwash obtained from M/s. Rajshree Biosolutions Private Ltd was used as a carbon source. The addition of carbon source promotes the heterotrophic bacteria to reduce the organic matter and assimilate the nitrogen waste into microbial protein. The C:N ratio was maintained at 10:1 for the development of biofloc and addition of urea for nitrogen source. Spentwash as carbon source was added for the maximum utilization of leftover inorganic nitrogen and to reduce the chance of occurrence of inorganic nitrogen in the form of total ammonia nitrogen in the collected biofloc. On the 7thday, biofloc was collected using harvest pit by closing the aeration and subsequently harvested by passing water in a nylon filter bag with 10 μm pore size. The collected floc was centrifuged at 2000 rpm and the supernatant water was discarded. To remove the traces of ammonia nitrogen level, bioflocs were washed twice with filtered freshwater. Flocs were dried in a hot air oven at 45 °C. The dried flocs were ground into fine powder (less than 200 μm), packed in airtight containers and kept in refrigerator until experimental diets were made.

Table 1
Proximate composition of biofloc.

Nutritional Parameters	Composition (%)
Crude protein	29.82 ± 0.60
Crude lipid	4.45 ± 0.5
Crude Fibre	3.51 ± 0.32
Ash	33.2 ± 0.7
Acid insoluble ash	11.25 ± 0.5
Moisture	8.34 ± 0.64
Organic matter	66.8 ± 0.32
Total NFE	26.35 ± 0.58
Gross Energy Kcal/100 g	331.42 ± 5.5

Organic matter (OM) = 100- Ash.
Nitrogen free extract (NFE) = 100- (CP + CL + CF + Ash + Moisture).
Gross energy (GE) = (CP X 5.6) + (CL X 9.44) + (CF X 4.1) + (NFE X 4.1) K Cal/100 g

Table 2
Formulation and proximate composition of the diets used in the experiments (% dry matter).

Ingredients (%)	Control (C)	*In-situ* (T1)	*Ex-situ* (T2)
Soybean meal	43.55	43.55	33.55
Corn	15.99	15.99	09.10
Fish meal	10.00	10.00	0.00
Biofloc meal	0.00	0.00	26.89
Ricebran	10.00	10.00	10.00
Bentonite	8.54	8.54	8.54
Limestone	4.57	4.57	4.57
Dicalcium phosphate	4.65	4.65	4.65
Cellulose	0.40	0.40	0.40
Sodium chloride	0.50	0.50	0.50
Vitamin & mineral supplemental mix	0.40	0.40	0.40
L-Lysine	0.95	0.95	0.95
DL-Methionine	0.35	0.35	0.35
Vitamin –C	0.07	0.07	0.07
BHT (Butylated Hydroxy toluene)	0.02	0.02	0.02
Dry matter	92.34	92.34	92.79
Digestible dry matter (%)	56.45	56.45	55.13
Crude protein (%)	30.15	30.15	30.10
Digestible protein (%)	27.65	27.65	27.11
Gross energy (KJ/g)	14.36	14.36	14.53
Digestible energy (KJ/g)	11.49	11.49	11.78
Ether extract (%)	2.01	2.01	2.04
Ash (%)	19.74	19.74	19.96

2.3. Experimental diets used in the trial

A diet without biofloc used in C and T1 was compared against the biofloc incorporated diet in T2 by manipulating soyabean meal, cornmeal and fish meal levels. All the ingredients except biofloc powder, amino acids, butylated hydroxyl toluene (BHT) and vitamin-mineral

mixture were mixed with water to make dough. The dough was steam cooked using a pressure cooker for 20 min at 15 psi. Bioflocs and other additives were mixed after cooling and the dough was pressed through a pelletizer with 2 mm die and then dried at 60 °C till the desired moisture level was reached. The feed was then stored at 4 °C until use.

2.4. Water quality parameters

Temperature (mercury thermometer) and pH (Labtronics) were monitored daily. Dissolved oxygen, BOD, Free Carbon dioxide, Alkalinity, Calcium and Magnesium ion concentration were measured on weekly basis [21]. Nitrate-N (NO_3–N), Nitrite-N (NO_2–N) and Ammonia were estimated using the filtered water samples [21] on a weekly basis.

2.5. Immunological parameters and antioxidant indicators

Fish were anesthetized to collect blood samples from the caudal vein. EDTA coated vials were used to collect the blood and to separate the serum, the blood was allowed to clot and centrifuged. Respiratory burst activity was analysed using the modified method of Anderson and Siwiki [22]. Myeloperoxidase activity in serum was performed according to Quade and Roth [23] with slight modifications. The serum sample was analysed for glucose level using a kit from Beacon diagnostics Pvt. Ltd. The protein estimation of fish serum was carried out by Lowry's method [24]. Catalase stress enzyme assay and Superoxide Dismutase (SOD) assay were performed by following the method of Takahara et al. [25] and Misra and Fridovich [26]. All these analyses were performed at the end of the experiment.

2.6. Growth parameters

The growth parameters of GIFT Tilapia were monitored on a weekly basis and various growth indices were calculated:

Weight gain (WG in g) = Final weight- Initial weight

Feed conversion ratio = Feed given /Body weight gain

Specific growth rate (%) = Ln (Final weight) –Ln (Initial weight) x 100 /Number of days

Survival rate (%) = Total number of Fish harvested/Total number of Fish stocked x 100

2.7. Gene expression studies

The Immune-related gene expression was studied in Head kidney, liver and intestine of the experimental animals in all the treatments. The tissue sample was homogenized in TRI Reagent for RNA isolation and the isolated RNA was stored in −20 °C for further use. The RNA isolated was converted to cDNA for Metallothionein gene, Cathepsin L, Toll like receptor 7, Interleukin 1 β and Tumour necrosis factor α using the primers listed in Table 3. The cDNA obtained through reverse transcriptase PCR was serially diluted and used for amplification, melt curve analysis and relative quantification of the target genes was carried out using the Real-Time PCR (Applied Biosystem's Real-Time PCR system StepOnePlus®). The temperature cycling parameters for the two-step PCR reaction were as follows: Initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1min for 45 cycles. The PCR was performed with 20 μL total reaction volume containing 10 μL of 2X SYBR®Greenq PCR master mix (Bio-Rad, USA), 1 μL each of forward and reverse primers (10 pmol), 1 μL of template DNA (30–60 ng) and 7 μL of Nuclease free water. The samples were analysed in triplicates and the relative expression was determined by the comparative threshold cycle method 2^{DDCT} (Delta-Delta CT method) using b-actin as internal control [27].

2.8. Histopathology studies

The animals were stocked at 1.25 kg/m^3 in the 2000 L FRP tanks in triplicates from all the experimental groups for the challenge study. Before the challenge study, the Lethal dose (LD_{50}) has been derived based on the experiments carried out with four different dosages delivering the bacteria (10^4, 10^5, 10^6 and 10^7). Low relative percent survival was found in tilapias when they were infected with bacteria of 10^7concentrations. The pathogenic dose has been arrived at, based on these results. At the end of the 180-day culture, the experimental animals were challenged with *Aeromonas hydrophila* pathogen obtained from State Referral Laboratory under Tamil Nadu Dr J. Jayalalithaa Fisheries University. The isolate was grown in tryptic soy broth (TSB Hi- Media, India) for 24 h (30–31 °C) and was harvested by centrifugation at 10,000 rpm for 10 min. This was followed by re-suspending the pellet in phosphate buffered saline (PBS, pH 7.2). The suspension in sterile PBS was injected intramuscularly (0.1 ml) in healthy tilapia [28] from all the treatments delivering 10^7 CFU/fish. The infected moribund fish with typical haemorrhagic wounds at the site of injection were sacrificed for the histopathological study after 4 dpi. Kidney, liver, hepatopancreas and intestine were dissected, rinsed in normal saline and fixed in 10% formalin buffer for 24 h. After fixation, the tissues were dehydrated in a series of alcohol concentration (70%, 80%, 90%, and 100% respectively), embedded in paraffin, sectioned at 5 mm and later stained with hematoxylin-eosin (H& E) [29]. The histopathological analysis was performed in the Department of Pathology, Madras Veterinary College, Chennai.

2.9. Statistical analysis

Water quality, growth, survival, immunological parameters, antioxidant status and gene expression of the culture animals were analysed using ANOVA to find out any significant difference between the treatments and control and post hoc analysis using Duncan Multiple range test for the significant values. Statistical analysis was performed using SPSS software version 20.0. The significant differences were calculated at 5% level.

Table 3
Primers used for five immune-related genes in qRT-PCR.

S.No	Gene name	Accession Number	Primers	Base pair
1	Metallothionein gene	XM_003447045.5	GCCACTCCTACACCGTCATTC (FP) CTGGCGTTGCTCTTGTCTCTT (RP)	63
2	Cathepsin L	XM_003444107.5	TGTCTTGCTCGTGGGCTATG (FP) CAGCTATTTTTCACCAGCCAGTAG (RP)	63
3	Toll like receptor 7	XM_019352834.2	CCTATTTTGGCAACTGGCATCT (FP) CACTTCACTCCCATTGTTGATCTC (RP)	78
4	Interleukin 1 β	KF747686.1	TGTCGCTCTGGGCATCAA (FP) GGCTTGTCGTCATCCTTGTGA (RP)	63
5	Tumour necrosis factor α	XM_003438427.5	GCTACGACTCCCAGCACTTTG (FP) GCGGTACTGCTCGGATCTCT (RP)	72

Table 4
Water quality parameters of experimental groups in the 180 days culture trial of GIFT Tilapia.

Parameters	C	T1	T2
pH	7.51 ± 0.01[a] (7.22–7.4)	7.31 ± 0.02[b] (7.36–7.66)	7.47 ± 0.01[c] (7.37–7.75)
Temperature (°C)	30.36 ± 0.29[a] (28.02–31.4)	30.52 ± 0.32[a] (28.0–31.3)	30.62 ± 0.30[a] (28.03–31.3)
DO (mg/l)	6.12 ± 0.05[a] (4.12–6.34)	5.36 ± 0.04[b] (3.29–5.78)	5.87 ± 0.04[c] (3.17–5.92)
Free carbon di oxide (mg/l)	5.82 ± 0.58[a] (4.06–8.4)	6.65 ± 0.82[b] (4.15–8.73)	6.04 ± 0.78[c] (5.06–8.53)
Alkalinity (mg/l)	70.58 ± 0.61[a] (45.13–81.6)	65.08 ± 0.60[b] (45.86–84.3)	67.71 ± 0.75[c] (54.03–86.45)
Calcium ions (mg/l)	54.48 ± 0.57[a] (50.53–63.41)	57.70 ± 0.58[b] (49.6–65.73)	55.14 ± 0.66[a] (47.5–60.24)
Magnesium ions (mg/l)	46.01 ± 0.61[a] (30.63–62.83)	49 ± 0.61[b] (31.7–67.1)	45.23 ± 0.62[a] (32.6–64.2)
Nitrate (mg/l)	0.163 ± 0.0004[a] (0.001–0.17)	0.124 ± 0.0004[b] (0.002–0.18)	0.174 ± 0.0004[c] (0.001–0.18)
Nitrite (mg/l)	0.017 ± 0.001[a] (0.002–0.02)	0.004 ± 0.0004[b] (0.002–0.01)	0.007 ± 0.002[c] (0.002–0.01)
Ammonia (mg/l)	0.154 ± 0.0002[a] (0.001–0.16)	0.073 ± 0.0003[b] (0.001–0.08)	0.120 ± 0.0004[c] (0.001–0.21)
BOD (mg/l)	6.30 ± 0.39[a] (3.53–8.73)	6.85 ± 0.76[b] (4.05–8.03)	6.57 ± 0.65[c] (5.4–7.46)

Different superscripts denote the significant difference ($P < 0.05$) between groups for each parameter.
Temperature found to have no significant difference between the treatments and control. Free CO_2, pH, BOD, Dissolved oxygen, alkalinity, Nitrate-N, Nitrite-N and Ammonia-N were found to be significantly different between the treatments and control. Calcium and Magnesium ion concentrations were found to be significantly higher in T1 than in control and T2. The floc volume in T1 was maintained at 15 ml/L for the first 60 days of the culture and it was increased to 45 ml/L at the end of the experiment.

3. Results

3.1. Water quality parameters

The various water quality parameters along with statistical analysis are shown in Table 4.

3.2. Immunological and antioxidant indicators

The immunological and antioxidant indicators were analysed and the graphs along with the standard deviation were constructed which are represented in Fig. 1.

3.3. Growth performance

The weight gain, specific growth rate, feed conversion ratio and survival rate of GIFT Tilapia along with the statistical analysis are shown in Table 5.

Table 5
Growth Performance of GIFT Tilapia at the end of the culture trial.

Parameters	C	T1	T2
Initial Weight (gm)	5.12 ± 0.04[a]	5.23 ± 0.05[a]	5.18 ± 0.04[a]
Final Weight (gm)	253.33 ± 4.4[a]	323 ± 4.16[b]	282.33 ± 4.33[c]
Weight gain (gm)	248.21 ± 4.39[a]	317.77 ± 4.12[b]	277.15 ± 4.3[c]
Specific growth rate	2.16 ± 0.37[a]	2.29 ± 0.01[b]	2.22 ± 0.03[c]
Feed conversion ratio	1.42 ± 0.01[a]	1.27 ± 0.01[b]	1.31 ± 0.005[c]
Survival rate	83 ± 1.85[a]	91 ± 1.52[b]	89 ± 1.15[c]

Different superscripts denote the significant difference ($P < 0.05$) between groups for each parameter.
Weight gain, specific growth rate, feed conversion ratio and survival rate were found to be significantly different between control and treatments. The results of the study showed improved performance of GIFT Tilapia in T1 compared to T2.

3.4. Gene expression studies

The results of the gene expression showed upregulated immune gene expression in head kidney compared to liver and intestine in all the

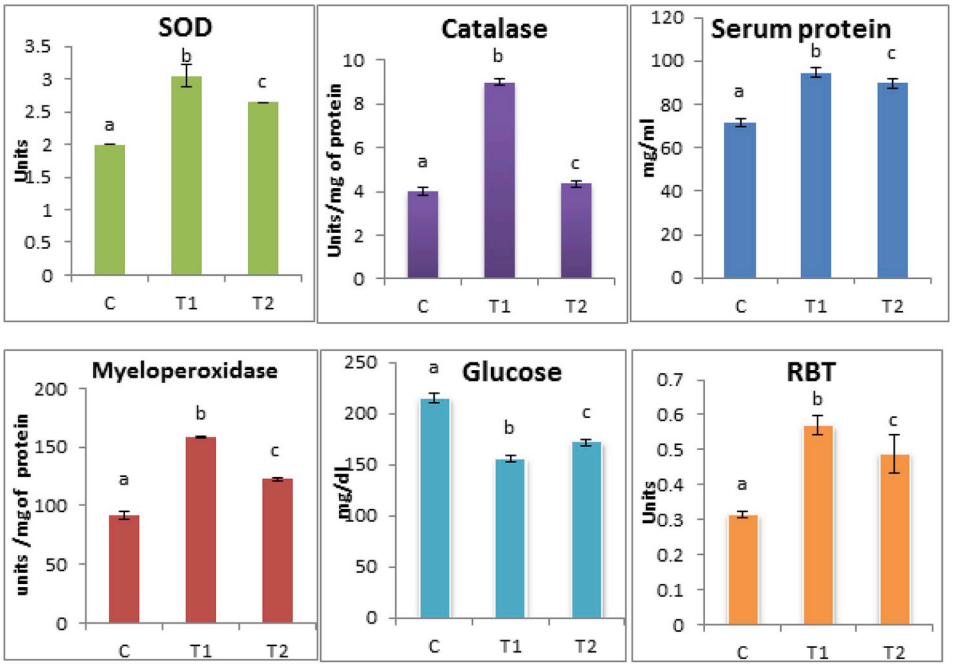

Fig. 1. Immunological and antioxidant indicators of GIFT tilapia in various treatments.
Different superscripts denote the significant difference ($P < 0.05$) between groups for each parameter.
At the end of the study, serum protein, RBT, glucose levels, catalase, SOD and Myeloperoxidase were found to be significantly different between control and treatments.

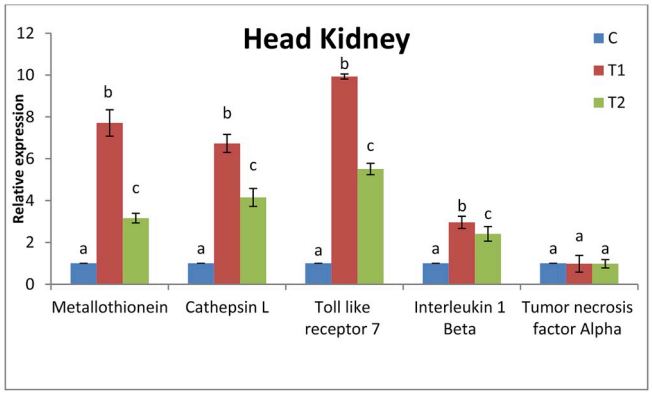

Fig. 2. Gene expression levels in the head kidney of GIFT Tilapia in experimental groups.
Different superscripts denote the significant difference ($P < 0.05$) between groups for each parameter.

experimental groups. The gene expression in the head kidney was found to be significantly different between the treatments and control with a higher level of expression in T1. In head kidney, relative mRNA expression of target genes was upregulated except tumour necrosis factor alpha gene. Metallothionein is expressed threefold in T2 whereas in T1, a sevenfold higher expression of this gene was observed. Cathepsin L is expressed fourfold in T2 and sixfold in T1 respectively. Toll like receptor expression levels was up-regulated in both T1 and T2. Interleukin 1 beta gene expression levels were one to threefold higher in T1 and T2 compared to C. Tumour necrosis factor Alpha gene showed no marked level of expression in all the experimental groups. In liver and intestine there was no folded expression of targeted genes in both control and treatment. The gene expression levels in head kidney are shown in Fig. 2.

3.5. Histopathology studies

No mortality was observed when the cultured animals were challenged with *Aeromonas hydrophila* at the end of the trial. The results from histopathology showed the presence of lower degree levels of infection in T1 followed by T2 and C. The histopathological analysis of intestine, liver, hepatopancreas and kidney were shown in Figs. 3–5.

4. Discussion

Temperature and DO (> 3 mg/L) in the experimental groups were maintained at levels ideal for the growth of GIFT tilapia [30]. Lower levels of alkalinity were found in T1 due to the presence of dominant heterotrophic bacterial groups which are responsible for nitrogen uptake due to carbon supplementation. This was in agreement with the studies of Ebeling et al. [31]. As alkalinity concentration alters the buffering capacity of the water it was found that in T1 the effect of low alkalinity leads to lower pH levels. A higher concentration of free CO_2 and BOD with lower levels of dissolved oxygen in T1 was also found. This may be due to respiration by the fish as well as microbes present in the biofloc. However lower levels of CO_2 and BOD were found in control due to its photosynthetic oxygen production. The levels of Calcium and Magnesium were found to be improved in T1 as this ionic concentration influences the floc formation [19] and adhesion by neutralizing the negative charges of the particles. Ammonia-N in T1 remained stable (< 0.02 mg/L) throughout the culture trial. The increased levels of Nitrate-N and Nitrite-N in control and T2 indicate the existence of autotrophic nitrification.

The higher level of serum protein in T1 helps to reduce the dietary protein levels of the pelleted feed with the enhancement of the non-specific immune response [32]. In this study, the RBT of tilapia showed an improved performance in T1 than C and T2. This may be related to the intake of biofloc by the culture animals in T1, which not only boosts the nutrition of the animal but also stimulates the fish cellular defence mechanism in the mode of respiratory burst and phagocytosis [33,34]. The myeloperoxidase (MPO), an antimicrobial enzyme acts by utilizing one of the oxidative radicals to produce hypochlorous acid. The increased MPO activity was seen more in T1 than the other experimental groups. This was concurrent with the findings of Long et al. [35] who reported increased MPO activity in GIFT when grown in biofloc system for a period of 8 weeks. Increased glycogenolysis and the glucose synthesis from extrahepatic tissue proteins and amino acids aggravates the glucose content in blood as an indicator of stress in animals [36]. In the present study, T1 was found to have lesser glucose level when compared with other treatments which in turn indicates the reduced stress level in animals. Biofloc reduced the physiological stress in GIFT which agrees with the studies of Verma et al. [37] who reported the reduced levels of Cortisol and glucose in *Labeo rohita* when reared in biofloc systems.

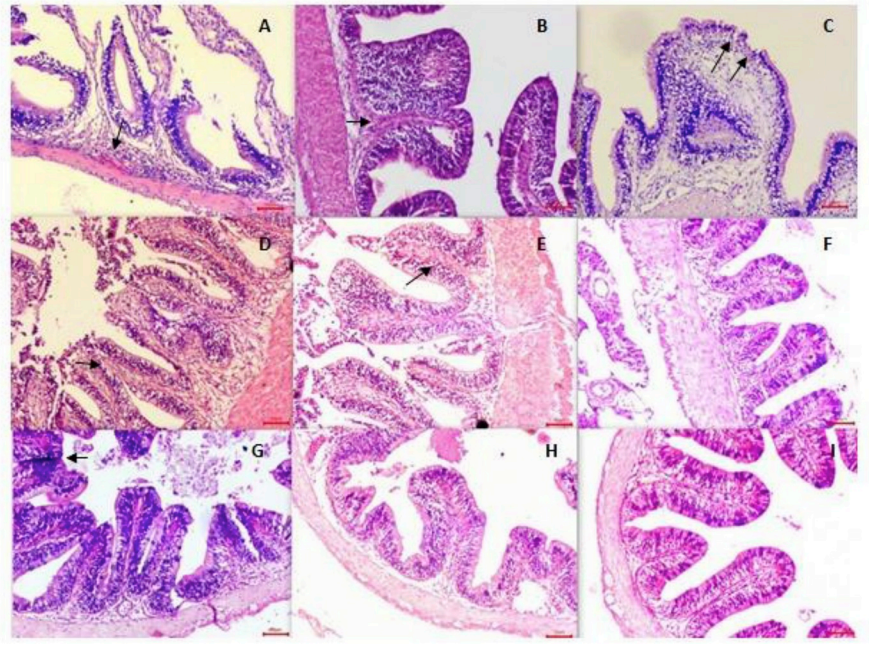

Fig. 3. A: Intestine Control - Congestion and mild degeneration of villi; **B**: Intestine control- Fusion of villi and the separation of lamella propria from the epithelium; **C**: Intestine control- Mild degenerative necrosis of mucosoepithelial cells; **D**: T1 Intestine- Mild Inflammation of infiltration cells; **E**: T1-Intestine- Mild Infiltration of Inflammatory Cells; **F**: T1 Intestine- NAD; **G**: T2 Intestine- Fusion of villi and mild degeneration of mucosal epithelium; **H**:T2 Intestine- Mild inflammation of infiltration cells; and **I**: T2 Intestine- NAD.

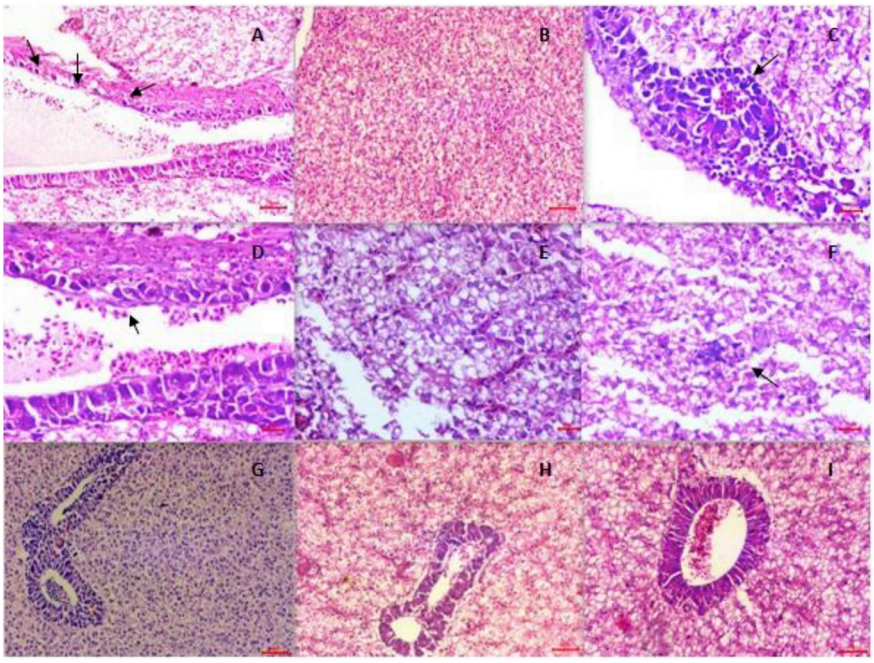

Fig. 4. A: Liver Control - Degenerative Necrosis & Congestion of haemorrhages; **B**: Liver control- Fatty Degeneration of hepatocytes; **C**: Liver control- Mild haemocytic infiltration & degenerative haemorrhages; **D**: Hepatopancreas Control - Degenerative Pancreatic Cell Haemorrhages; **E**: Hepatopancreas Control - Degenerative Sinusoidal Congestion; **F**: Hepatopancreas Control -Mild haemocytic infiltration; **G**: T2 Liver- Mild degenerative changes of hepatocytes; **H**: T2 Liver- Sinusoidal congestion & mild fatty degeneration and **I**: T2 Liver - Very mild degeneration of pancreatic cells.

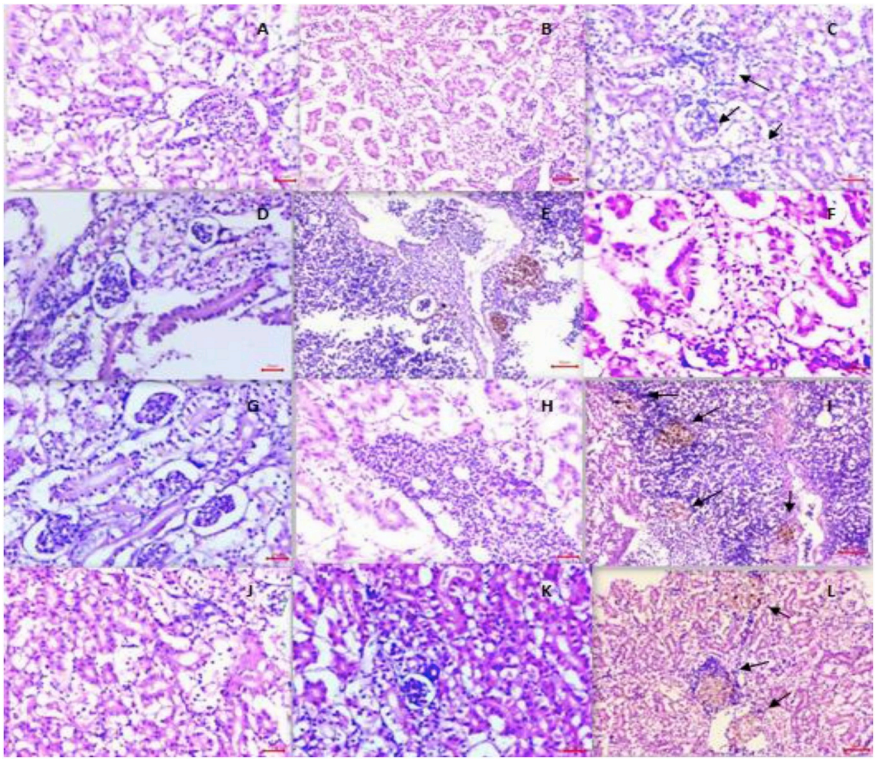

Fig. 5. A: Kidney Control - Congestion and vacuolar degeneration of nephritic tubules; **B**: Kidney Control - Degenerative necrosis tubular epithelial cells; **C**: Kidney Control - Hyperemia of glomeruli; **D**: Kidney Control - Mild dilatation of bowman's capsule; **E**: Kidney Control -Melanomacrophage aggregation and infiltration; **F**: Kidney Control -Necrosis of tubular epithelial cells along with pyknotic nuclei; **G**: Kidney Control - Partial loss of glomeruli tuft; **H**: Kidney Control - Haemorrhages; **I**: Kidney Control - Melanomacrophage centres and congestion; **J**: T1 Kidney - Mild tubular degeneration of epithelial cells; **K**: T2 Kidney- Mild Degenerative tubular epithelial cells; **L**: T2 Kidney- Few Melanomacrophage centre aggregation.

The results from the present study revealed increased SOD and catalase level in T1, followed by T2 and C. A spurt in the levels of SOD and catalase improves the antioxidant status of the animal by preventing lipid peroxidation through conversion of superoxide anion to water and oxygen [38]. Similar studies were done by Yilmaz [39] where Nile tilapia when fed with 5 g/kg of caffeic acid as a dietary supplement for 60 days improved the fish immune parameters, antioxidant status, as well as survival rate against *A. veronii*. SOD and catalase under hypoxia conditions are involved in the antioxidant defence system by removing and detoxifying oxygen radicals generated within the cells under normal or stressful conditions [40]. Lower levels of SOD and catalase indicate cell damage due to the accumulation of high-level free radicals in cells affecting the quality and palatability of fish which impacts human consumption. GIFT Tilapia in T1& T2 reared under biofloc technology showed improved antioxidant status with increased SOD and catalase levels thus paving the way for easy consumer acceptance. Animals in T1 were found to have increased weight gain, specific growth rate, survival and decreased feed conversion ratio. This may be due to the consumption of microbial floc which is produced as cellular protein by the assimilation of waste nitrogen in the culture animal. The increased intake of the animal in the culture ponds is attributed to the enhanced floc production by the heterotrophic bacterial

population in T1 [41]. The feed response of biofloc incorporated diet in T2 and control was similar as animals tend to jump to fetch feed at the time of application. The animal's response in T1 was not high and this may be due to the existence of biofloc in the culture system consistently throughout the experiment. The total feed applied in T2 and control disappeared in a short span of time, whereas increased feed retention was observed in T1. This led to decreased pellet feeding to the animals in T1. These observations are similar to the findings of Avnimelech [11] as tilapia has the ability to harvest the flocs continuously for feeding in the culture ponds with decreased pellet feeding.

The up-regulation of IL- 1β was observed in head kidney, which indicates its influence in stimulation of immune response. This was also proven from the studies of Kheti et al. [42] who reported that microbial floc supplemented in the diet of rohu potentiates the expression of IL-1β and TNF-α in head kidney and liver. Similar kind of upregulated expression of IL-1β and TNF-α in intestinal tissue was found when *Echinacea purpurea* extract and/or vitamin C in combination or individually supplemented along with the basal diet by Rahman et al. [43]. IL-1β activates the lymphocytes and stimulates the release of other cytokines during the microbial invasion or when there is a tissue injury [44]. TNFs play a role in inflammatory response, proliferation and differentiation of cells, and stimulation of the immune system [45,46]. The pattern of this cytokine gene expression predicts the changes in immune response. The upregulated expression of these immune genes in T1 enhances the immune cell secretions such as proinflammatory cytokines like TNF-α and IL-1β to modulate the innate immune response of the culture animals. However, there are not too many previous studies reporting the immune gene expression in Tilapia either by rearing in biofloc based culture system or feeding with biofloc meal.

Histopathological manifestations in kidney, liver, pancreas and intestine of GIFT Tilapia against its challenge with *Aeromonas hydrophila* were similar to the observations of Roberts [47]. Degenerative necrosis of tubular epithelial cells along with the melanomacrophage centre aggregation was the major histopathological observation in the kidney. Fatty degeneration of hepatocytes with sinusoidal congestion was found in the liver and pancreas. Fusion of villi with inflammation of infiltration cells and infiltration of inflammatory cells were commonly seen in intestine. These major manifestations were observed with the higher degree of infection in control followed by T2 and T1. This may be due to the toxins and extracellular products produced by *A. hydrophila* such as hemolysin, protease, and elastase causing severe necrosis in the liver and other tissues [48]. The infection in T1 fish was found to be lesser due to the production of immunostimulatory compounds [49,50] by the heterotrophic bacteria in the biofloc produced within the culture ponds. Microbial floc has also been reported for the presence of bioactive compounds such as carotenoids, polysaccharides, phytosterols, taurine and poly-β-hydroxybutyrate (PHB) [19,51,52]. The results of the present study can be related to the antioxidant status of the animal and it is found that animals in T1 had a higher immune potential towards the infection followed by T2 and Control. Similar study was performed by Kheti et al. [42] who administered the microbial floc in the diets of Rohu and showed the increased survival rate when infected with *Edwardsiella tarda*.

Thus, from the above research findings, the present study reveals the improved performance of *in-situ* based biofloc compared to *ex-situ* feeding as it exhibits ideal water quality parameters, improved growth performance, modulatory immune response as well as the upregulated expression of genes responsible for immune system and the resistance towards pathogenic infection.

5. Conclusion

Biofloc technology is one of the advanced culture technologies adopted for tilapia farming due to its innumerable benefits. It serves as feed for the culture animals, improves the biosecurity of the farm with minimal or zero water exchange. This study and its findings are the first to know the effect of biofloc intake relating to the immunological performance of GIFT Tilapia with gene expression. This gives strong insights on the dietary supplementation of biofloc in feed and its development within the culture ponds for the maintenance of the optimum water quality parameters, growth performance and immune gene regulations in the grow out culture systems of GIFT Tilapia.

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