**Effect of *insitu* and *exsitu* biofloc on antioxidant, immunological, growth and gene expression responses of Genetically Improved Farm Tilapia (GIFT) and its resistance to *Aeromonas hydrophila***

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**Abstract**

The present study aimed to investigate the effect of biofloc intake of GIFT Tilapia, developed within the system and its supplementation in feed on water quality, growth performance, immunological parameters, antioxidant status, immune gene expression, and its resistance to *A. hydrophila* infection. GIFT Tilapia juveniles (5.1±0.05g) were stocked at a density of 15/m3 in 300 m2 lined ponds in triplicates for 180 days. The experimental groups consist 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. pH, BOD, free CO2, 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, RBT and Myeloperoxidase) and anti oxidant indicators (Glucose, 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 αin liver and intestine for both control and treatment. However the upregulated expression of targeted genes except tumour necrosis factor α were 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 the 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 *insitu* (T1)based biofloc method to obtain the better performance of GIFT Tilapia culture.

**Keywords**: Biofloc, *insitu*, *exsitu*, Immune response and Gene expression

**Introduction**

Tilapia- a hardy species is the second most widely cultured fish next to carps globally. In 2016, the total production of tilapia was roughly about 6.69 million tonnes [1] and has been expected to rise to 7.3 million tonnes by the end of 2030 [2]. The most distinct candidature traits of this species includes euryphagic feeding habit, captive breeding potential, tolerance to high stocking density and improved growth performance in various aquaculture systems. However, with the emerging disease problems and lack of fish seed availability posed the development of Genetically Improved Farm Tilapia (GIFT) strain using eight different species of Tilapia under selective breeding by World Fish Centre [3]. On the otherhand, growing global population increased the demand of aquaculture product including tilapia due to the decline of capture fisheries. The expansion of aquaculture limited to land and water utilization hinders the productivity of the aquaculture particularly in Tilapia farming [4-7]. To overcome these bottle necks, sustainable intensification by the adoption of advanced culture systems and technologies becomes inevitable to improve the production and productivity of the sector. One of such advanced technologies is biofloc technology, a minimal or zero water exchange technology, which allows the animals to stock at higher densities. Bioflocs are conglomerates of algae, bacteria, protozoans, fecal matter and uneaten feed which are held together in a loose matrix by the secretions of filamentous microorganisms or by electrostatic attraction [8]. This technology by maintaining the carbon and nitrogen in the culture water 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 microbial protein [10] faster than regular nitrification process [11].The nutrient profile of biofloc ranges from 25 to 50 percent of protein and 0.5 to 15 percent of fat on dry-weight basis. Bioflocs are also valuable source of limiting aminoacids such as methionine and lysine, vitamins (Vitamin C in range of 0 to 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.The discharge of nutrient rich culture water in the extensive and traditional systems can be reduced with no or little use of fishmeal by using algal supplement and other indigeneous organisms for the fish to feed[13]. The effluent waters from aquaculture systems was used for exsitu biofloc production in suspended growth bioreactors. The bioflocs produced can be dried and used as a feed ingredient for shrimp or fish [14].The use of biofloc as a feed has been studied in aquaculture and the uptake of biofloc as feed depends on the nature of species and its feeding ability, size of the animal and floc and the density of floc [15]. The findings from the previous study proved that freshwater prawn, shrimp and tilapia uptake the biofloc as the additional protein source indicating that it can be applicable 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 bioflocs also contain immunostimulatory compounds exhibiting possible probiotic effect [20]. However the relative efficiency of insitu and exsitu biofloc with respect to the immune gene expression of the animal has not been attempted so far particularly in GIFT Tilapia.Thus the objective of the study aimed to determine the intake of biofloc by GIFT Tilapia using different incorporation methods and its impact in animals immunological performance along with its gene expression.

**Materials and methods**

**Experimental Design**

A 180 days culture was carried out in Advanced Research Farm Facility, Madhavaram at Chennai (13.1478° N, 80.2310° E). The experimental group includes two treatments such as *insitu* biofloc developed within the culture systems as Treatment-1 (T1), biofloc incorporated fish feed developed by *exsitu* method as Treatment-2 (T2), and animals reared without biofloc as control (c) . Animals weighing (5.1 ±0.05g) were stocked at a density of 15/m3 in 300 m2 lined ponds in all the experimental groups in triplicates. The animals were fed with isoenergetic and isonitrogeneous diet as per the average body weight of the animals in all the treatments. The compositions of experimental diets are presented in Table 1.

**Production of biofloc**

In T1, development and maintenance of biofloc in the freshwater culture ponds were adopted as suggested by Taw [21] at C: N ratio of 10:1.The addition of carbon source to maintain the C:N ratio was followed by adopting Avnimelech [15] for transition of the heterotrophic system from autotrophic system. For T2, biofloc production was carried out in two indoor raceway tanks (50tonnes;15m x 3m x 1m) in six batches at 10-days interval during January to March, 2018.Raceway tanks were filled with used culture water taken from the fish ponds and 100L biofloc inoculum developed in a separate tank was added to each raceways to improve the floc production. Spentwash obtained from M/s. Rajshree Biosolutions Private Ltd were used as a carbon source.The C:N ratio was maintained at 10:1 for the development of biofloc in the raceway tanks.Urea and spentwash was added as the nitrogen and carbon source for the conversion of inorganic nitrogen to microbial protein.On the 7thday, biofloc was collected using harvest pit by discharging the tank water through nylon filter bag of 10 μm pore size. The collected floc was centrifuged at 2000 rpm and washed twice with filtered freshwater to get rid of the traces of ammonia nitrogen if any in the biofloc. The flocs were shade dried to remove excess moisture content and followed by drying in hot air oven at 45 °C. Later the flocs were grounded to fine powder (<200 μm) and stored at 4°C for future use.

**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 replacing soyabean, corn meal and fish meal levels. The dough was prepared using all the ingredients other than biofloc powder, aminoacids, butylated hydroxyl toluene (BHT) and vitamin-mineral mixture. Later it was steam cooked using pressure cooker for 20 min at 15 psi. The dough were allowed for cooling followed by the addition of bioflocs and other additives. Then the mixed dough was used for preparing pellet feed using pelletizer with 2 mm die and dried at 60˚C.The prepared pelleted feed were stored in air tight containers in refrigerator.

**Table 1 Formulation and proximate composition of the diets used in the experiments (% dry matter)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Ingredients (%)** | **Control (C)** | **Insitu (T1)** | **Exsitu (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 |
| Rice bran | 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 |

**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 [22]. Nitrate-N (NO3–N), Nitrite-N (NO2–N) and Ammonia were estimated using the filtered water samples [22] on weekly basis.

**Immunological parameters and Antioxidant indicators**

Fishes were anesthetized to collect the blood samples from the caudal vein. The blood was collected in EDTA coated vials 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 [23]. Myeloperoxidase activity in serum was performed according to Quade and Roth [24] with slight modification. The serum sample was analysed for glucose level using Beacon diagnostics pvt.Ltd., kit. The protein estimation of fish serum was carried out by Lowry’s method [25]. Catalase stress enzyme assay and Super oxide Dismutase (SOD) Assay were performed by following the method of Takahara et al. [26] and Misra and Fridovich [27]. All these analyses were performed at the end of the experiment.

**Growth Parameters**

The growth parameters of GIFT Tilapia were monitored on 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

**Gene expression studies**

The Immune related gene expression were studied in Head kidney, liver, hepatopancreas and intestine of the experimental animals in all 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 the table 2. The cDNA obtained through reverse transcriptase PCR was serially diluted and used for amplification, melt curve analysis and relative quantification of the target genes were 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 sec, annealing and extension at 60 °C for 1min for 45 cycles. The PCR was performed with 20 µL total reaction volume containing 10µL of 2X SYBR®Green qPCR 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 2DDCT (Delta-Delta CT method) using b-actin as internal control [28].

Table 2 Primers used for five immune related genes in qRT-PCR

|  |  |  |  |
| --- | --- | --- | --- |
| S.No | Gene name | Primers | Base pair |
| 1 | Metallothionein gene  XM\_003447045.5 | GCCACTCCTACACCGTCATTC (FP) | 63 |
| CTGGCGTTGCTCTTGTCTCTT (RP) |
| 2 | Cathepsin L  XM\_003444107.5 | TGTCTTGCTCGTGGGCTATG (FP) | 63 |
| CAGCTATTTTTCACCAGCCAGTAG (RP) |
| 3 | Toll like receptor 7  XM\_019352834.2 | CCTATTTTGGCAACTGGCATCT (FP) | 78 |
| CACTTCACTCCCATTGTTGATCTC (RP) |
| 4 | Interleukin 1 β  KF747686.1 | TGTCGCTCTGGGCATCAA (FP) | 63 |
| GGCTTGTCGTCATCCTTGTGA (RP) |
| 5 | Tumour necrosis factor α  XM\_003438427.5 | GCTACGACTCCCAGCACTTTG (FP) | 72 |
| GCGGTACTGCTCGGATCTCT (RP) |

**Histopathology studies**

At the end of the 180 days 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 grown in tryptic soy broth (TSB Hi Media, India) for 24 h (30-31 oC) was centrifuged at 10,000 rpm for 10 min followed by pellet resuspension in phosphate buffered saline (PBS, pH 7.2). The suspension in sterile PBS was injected intramuscularly (0.1ml) in healthy tilapia [29] from all the treatments delivering 107 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 hrs.The fixed tissues were washed in a series of alcohol concentration (70%, 80%, 90%, and 100%, respectively), and embedded in paraffin wax.The tissues were sectioned at 5 mm, later stained with hematoxylin-eosin (H&E) [30].The histopathological analysis was performed in Department of Pathology, Madras Veterinary College, Chennai.

**Statistical analysis**

Water quality,growth, survival, immunological parameters, antioxidant status and gene expression of the culture animals were analysed using one way 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 were performed using SPSS software version 20.0.The significant differences were calculated at 5% level.

**Results**

**Water quality parameters**

The various water quality parameters along with the statistical analysis were shown in the table.3.

Table 3–Water quality parameters of experimental groups in the 180 days culture trial of GIFT Tilapia

|  |  |  |  |
| --- | --- | --- | --- |
| **Parameters** | **C** | **T1** | **T2** |
| pH | 7.51± 0.01a  (7.22-7.4) | 7.31± 0.02b  (7.36-7.66) | 7.47± 0.01c  (7.37-7.75) |
| Temperature (°C) | 30.36± 0.29a  (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±0.0004c  (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 denotes the significant difference (P<0.05) between groups for each parameter.

Temperature found to have no significant difference between the treatments and control. pH, BOD, Free CO2, 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 control and T2.The floc volume in T1 was maintained at 15ml/L for the first 60 days of the culture and it was increased upto 45ml/L at the end of the experiment.

**Immunological and Antioxidant indicators**

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

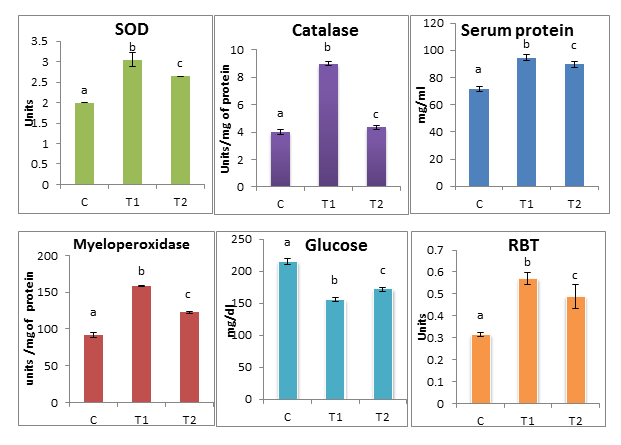


Figure 1 Immunological and antioxidant indicators of GIFT tilapia in various treatments

Different superscripts denotes the significant difference (P<0.05) between groups for each parameters.

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.

**Growth performance**

The weight gain, specific growth rate, feed conversion ratio and survival rate of GIFT Tilapia along with the statistical analysis were shown in the table.4.

Table.4 - 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 denotes 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.

**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 experimental groups. The gene expression in head kidney was found to be significantly different between the treatments and control with the higher level of expression in T1.In head kidney, relative mRNA expression of target genes was upregulated except tumor necrosis factor alpha gene. Mellathionein is expressed 3 fold in T2 whereas in T1 higher expression of 7 fold of this gene was observed. Cathesipin L is expressed 4 fold in T2 and 6 fold expression in T1 respectively. Toll like receptor expression levels were up-regulated in both T1 and T2. Interleukin 1 beta gene expressions levels were 1-3 fold higher in T1 and T2 compared to C. Tumor 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 was shown in the figure 2.

Figure 2 Gene expression levels in head kidney of GIFT Tilapia in experimental groups

Different superscripts denotes the significant difference (P<0.05) between groups for each parameters.

**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 the figures 3, 4 and 5.

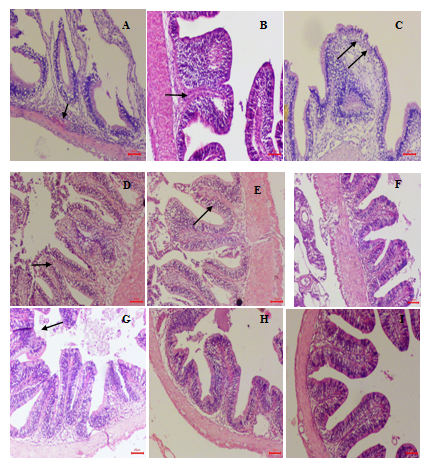
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Figure 3: **A**: Intestine Control **-** Congestion and mild degeneration of villi; **B**: Intestine control- Fusion of villi and the separation of lamella propria from the epithelium; **C**: Intestine control- Mild degenerative necrosis of mucosoepithelial 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 cellsC:\Users\home\Desktop\PhD\Data\Histology Slides\Insitu\Intestine\mil inflam infil cells.jpg and **I**: T2 Intestine- NAD

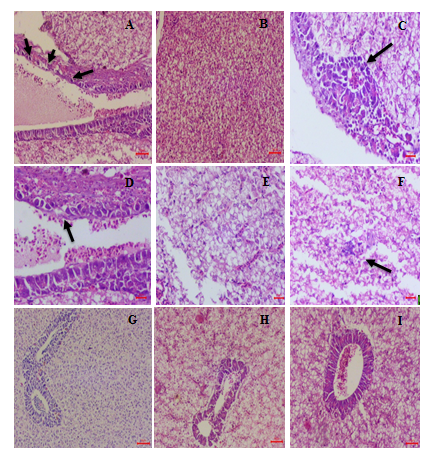


Figure 4: **A**: Liver Control **-** Degenerative Necrosis & Congestion of haemorrhages; **B**: Liver control- Fatty Degeneration of hepatocytes; **C**: Liver control- Mild haemocytic infiltration & degenerative haemorrhages; **D**: Hepatopancreas Control - Degenerative Pancreatic Cell Haemorrhages; **E**: Hepatopancreas Control - Degenerative Sinusoidal Congestion C:\Users\home\Desktop\PhD\Data\Histology Slides\Insitu\Intestine\mil inflam infil cells.jpg; **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.

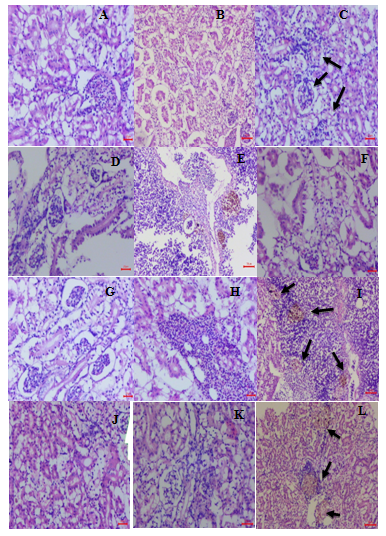


Figure 5: **A**: Kidney Control **-** Congestion and vacuolar degeneration of nephritic tubules; **B**: Kidney Control - Degenerative necrosis tubular epithelial cells; **C**: Kidney Control - Hyperimia of glomeruli; **D**: Kidney Control - Mild dilatation of bowman’s capsule; **E**: Kidney Control -Melanomacrophage 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

**Discussion**

Temperature and DO (> 3mg/L) were maintained at the optimum levels in the experimental groups which is ideal for GIFT Tilapia growth [31]. Lower levels of alkalinity was found in T1 due to the presence of dominant heterotrophic bacterial groups which are responsible for nitrogen uptake due to carbon supplementation which was in agreement with the studies of Ebeling et al. [32]. As alkalinity concentration tends to change the buffering capacity of the water it was found that in T1 the effect of low alkalinity leads to lower pH levels. The higher concentration of free CO2 and BOD with lower levels of dissolved oxygen in T1was also found. This may be due to the respiration by the fishes as well as microbes present in the biofloc however lower levels of CO2 and BOD were found in control due to its photosynthetic oxygen production. The improved levels of Calcium and Magnesium was found 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 (< 0.02mg/L) throughout the culture trial. The increased levels of Nitrate-N and Nitrite-N in control and T2 indicates 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 [33]. 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, not only enhances the nutrition of the animal but also stimulates the fish cellular defence mechanism in the mode of respiratory burst and phagocytosis [34, 35]. The myeloperoxidase (MPO), an antimicrobial enzyme produce hypochlorous acid by utilizing one of the oxidative radicals. The increased MPO activity was seen in T1 than the other experimental groups which were concurrent with the findings of Long et al. [36] who reported the increased MPO activity in GIFT when grown in biofloc system for a period of 8 weeks. Increased glycogenolysis and the glucose synthesis from extra hepatic tissue proteins and amino acids increases the glucose content in blood as an indicator of stress in animals [37]. In the present study, T1 was found to have lesser glucose level when compared with other treatments which inturn indicates the reduced stress level in animals. Biofloc reduced the physiological stress in GIFT which agrees with the studies of Verma et al. [38] who reported the reduced levels of Cortisol and glucose in *Labeo rohita* when reared in biofloc systems. The results from the present study revealed that in T1 the increased SOD and catalase level was found followed by T2 and C. The increased levels of SOD and catalase improves the antioxidant status of the animal by preventing the lipid peroxidation by converting the superoxide anion to water and oxygen [39].Similar studies were done by Yilmaz [40] 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 condition are involved in the antioxidant defence system by removing and detoxifying oxygen radicals generated within the cells under normal or stressful conditions [41]. Lower levels of SOD and catalase indicates the cell damage due to the high-level free radical accumulation in cells affecting the quality and palatability of fishes which impacts on human consumption. Thus GIFT Tilapia in T1 & T2 reared under biofloc technology showed improved antioxidant status with increased SOD and catalase levels for its easy consumer acceptance.

T1 was 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 are produced as cellular protein by the assimilation of waste nitrogen by the culture animal. The enhanced floc production by the heterotrophic bacterial population in T1 paved the way for increased intake of animal in the culture ponds [42]. 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 animals response in T1 was not high and this may be due to the existence of biofloc in the culture system continuously throughout the experiment. The total feed applied in T2 and control disappeared in short span of time, whereas increased feed retention was observed in T1. This paved the way for 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 which was also proven from the studies of Kheti et al. [43] who reported when 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. [44]. IL-1β activates the lymphocytes and stimulates the release of other cytokines during the microbial invasion or when there is a tissue injury [45]. TNFs plays a role in inflammatory response, proliferation and differentiation of cells, and stimulation of the immune system [46, 47]. The pattern of these cytokine gene expression predicts the changes in immune response.The upregulated expression of these immune genes in T1 enhance the immune cells secretions such as proinflammatory cytokines like TNF-α and IL-1β to modulate the innate immune response of the culture animals. However, there were no much 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 [48]. Degenerative necrosis of tubular epithelial cells along with the melanomacrophage centre aggregation were the major histopathological observations 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 production of toxins and extracellular products such as hemolysin, protease, and elastase by *A.hydrophila* causing severe necrosis in liver and other tissues [49].

The infection in T1 fishes were found to be lesser due to the production of immunostimulatory compounds [50,51] 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, 52, 53].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 higher immune potential towards the infection followed by T2 and control. Similar study was performed by Kheti et al. [43] 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 revealed the improved performance of insitu based biofloc compared to exsitu feeding as it exhibits an 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 the pathogenic infection.

**Conclusion**

Biofloc technology is one of the advanced culture technologies adopted for tilapia farming due to its innumerable benefits as it serves as feed for the culture animals in improving the biosecurity of the farm with minimal or zero water exchange. The present findings of the study becomes the first study to know the effect of biofloc intake relating to the immunological performance of GIFT Tilapia with gene expression. This gives the 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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