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Enhanced growth and immuno-physiological response of Genetically Improved Farmed Tilapia in indoor biofloc units at different stocking densities

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Abstract

An experimental trial was conducted for 90 days to evaluate the growth performance, immunophysiological response of GIFT strain of Tilapia in biofloc-based rearing system and to assess the relative percentage survival in 3 days after challenging with the virulent strain of *Aeromonas hydrophila*. Fingerlings with an average body weight 0.98 ± 0.06 g were stocked in triplicate at different stocking densities of 200 (SD1), 250 (SD2), 300 (SD3) and 350 (SD4) m^{-3} in biofloc-based treatments and 150 (C) m^{-3} in control (clear water). Biofloc-based units (SD1 and SD2) obtained significantly better ($P < 0.05$) growth performances at the end of the experimental period. Mean body weight of fish in biofloc-based units showed a decreasing trend with increase in stocking density with 100% survival in all units including control. The stress parameters were significantly lower in biofloc-based rearing units especially in treatments SD1 and SD2 as compared to the control. The fish from the biofloc-based units (SD1 and SD2) possessed significantly ($P < 0.05$) higher immune status as compared to control and other biofloc treatments in terms of respiratory burst, serum lysozyme and myeloperoxidase activity. Relative survival percentages were significantly better in biofloc treatments with highest in SD1 and SD2 (83.33%) after challenge study. GIFT strain of Tilapia at higher stocking densities 200–250 nos m^{-3} can be taken as optimum stocking density whereas higher stocking densities up to 350 nos m^{-3} can be reared in the biofloc systems without compromising the growth and immunity.

Keywords: Genetically Improved Farmed Tilapia, Biofloc Technology, Immunity, *Aeromonas hydrophila*, Challenge study

Introduction

Stagnation of world capture fisheries is creating pressure on the aquaculture to adopt intensification, which in turn can result in water scarcity and pollution of the aquatic environment. There is urgent need of improvement of the present culture practices with minimal water exchange and biosecure systems that would limit the pollution as well as use of other resources in a sustainable way. Biofloc technology (BFT) is an eco-friendly and sustainable solution to adopt intensive aquacultural practices with minimal or zero-water exchange (Ekasari & Maryam 2012). Biofloc can be defined as protein-rich 'natural food' which is a macro-aggregate of organic material and microorganisms including diatoms, macro-algae, feed and faecal remnants, bacteria and invertebrates (Avnimelech 1999, 2006; Burford, Thompson, McIntosh, Bauman & Pearson 2003; Hargreaves 2006; Jatobá, da Silva, da Silva, do Nascimento Vieira, Mouriño, Seiffert & Toledo 2014).

Being a biosecure system, BFT ensures to avert the escape of cultured organism into the natural water body which could results in the genetic contamination of the wild population (Emerenciano, Gaxiola & Cuzon 2013). Uneaten feed and nutrient-rich excreta (major pollutants) can be used up by the heterotrophic bacteria in the presence of an external carbon source provided in the biofloc

system, and the microbial protein thus formed acts as a potential food and all time available protein source for the cultured organism (De Schryver, Crab, Defoirdt, Boon & Verstraete 2008).

Another major problem that hinders the intensification of tropical aquaculture is heavy mortality and severe economic losses due to diseases (Thangaviji, Michaelbabu, Anand, Gunasekaran & Citarasu 2012). Immunostimulants have proved to be better preventive measure to avoid disease outbreaks (Barros, Falcon, de Oliveira Orsi, Pezzato, Fernandes, Guimarães & Sartori 2014; Hassanin, Hakim & Badawi 2014). Bioflocs 'natural probiotic' effect due to the presence of immunostimulating and antistress factors helps to keep the fish healthy and resistant to diseases (Crab 2010; Crab, Defoirdt, Bossier & Verstraete 2012; Emerenciano *et al.* 2013; Pandey, Bharti & Kumar 2014).

Tilapia being a hardy fish can adapt well to the crowded conditions and higher density with higher growth rate (FAO, 2001, 2012) and can offer a higher net production in biofloc system as it can assimilate biofloc as a food source (Avnimelech 2007; Azim & Little 2008). However, early maturity, differential growth and prolific breeding behaviour are the main drawbacks limiting its potential as a culture species. Improved strain of tilapia named as Genetically Improved Farmed Tilapia (GIFT) produced by world fish GIFT project (1988) with selective breeding is proved to give better growth and production compared to normal strains. Considering the above-mentioned factors, the aim of this study was to evaluate the growth parameters and the effect of biofloc on immune response of GIFT strain of Tilapia in biofloc rearing system at different stocking densities.

Material and methods

Experimental design and set-up

GIFT strain of Tilapia (n = 2000) for the experiment was obtained from Rajiv Gandhi Centre for Aquaculture, Andhra Pradesh, India, and the same were acclimatized for 25 days in 1000-L FRP (fibreglass reinforced plastic) tanks fed with a commercial diet having 35% crude protein. The experiment followed completely randomized design (CRD), with four biofloc treatments and one control (clear water), in triplicates. Experiment was conducted in 110-L capacity tanks. The carbon nitrogen ratio (C/N) in BFT were maintained at 15:1 (Hargreaves 2013). The carbon source used was commercial wheat flour with 50% C g^{-1} (Azim & Little 2008). The calculation of C/N ratio and preparation of biofloc were made following (Avnimelech 1999). Biofloc treatment tanks were stocked with fish weighing 0.98 ± 0.06 g, at the rate of 200 numbers (nos) m^{-3} (SD1), 250 nos m^{-3} (SD2), 300 nos m^{-3}(SD3) and 350 nos m^{-3}(SD4), and control tanks were stocked with 150 nos m^{-3}(C). The experiment was conducted in two phase, culture phase of 90 days and 3 days challenge study with pathogenic *A. hydrophila.*

Physico-chemical parameters of water

Water quality parameters like pH and temperature were monitored daily, while dissolved oxygen was monitored once in a week during the experimental period as per the standard procedures (APHA, 1998). Total ammonia-N, nitrite-N and nitrate-N were measured using test kits of HI83203 aquaculture photometer (Hanna Instruments, USA). Turbidity was measured by Nephelo turbidity meter (turbidimeter AQ4500). Floc volume was measured by allowing the floc to settle down in the Imhoff cone for 30 min without disturbance.

Growth analysis

Growth was measured at an interval of 15 days by collecting 20% of fish from each treatment randomly. Feeding was performed at 1–2% of biomass and was adjusted accordingly. Growth parameters such as percentage weight gain, specific growth rate (SGR), daily increment (DI), feed efficiency ratio (FER), protein efficiency ratio (PER), biomass and survival were measured using the following formulas.

$$\text{Percentage weight gain (\%)} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} \times 100$$

$$\text{SGR (\% day}^{-1}\text{)} = \frac{\text{Log}_e \text{Final weight} - \text{Log}_e \text{Initial weight}}{\text{Number of days}} \times 100$$

$$\text{DI (g day}^{-1}\text{)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Experimental period in days}}$$

$$\text{FER} = \frac{\text{Body weight gain (wet weight)}}{\text{Feed given (dry weight)}}$$

$$\text{PER} = \frac{\text{Body weight gain (wet weight)}}{\text{Crude Protein fed}}$$

$$\text{Survival (\%)} = \frac{\text{Total number of animal harvested}}{\text{Total number stocked animal}} \times 100$$

Immune parameters

Respiratory burst activity

The respiratory burst activity of the phagocytes was carried out by nitrobluetetrazolium (NBT) assay following the modified method (Anderson & Siwicki 1995). The sample absorbance was read on spectrophotometer at 540 nm.

Myeloperoxidase content

The total myeloperoxidase activity of serum was determined as described by Quade and Roth (1997) and partially modified by Sahoo, Kumari and Mishra (2005). The OD value was read at 450 nm in a microplate reader.

Serum lysozyme activity

A turbidimetric assay utilizing lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, Mumbai, India) was carried out to determine the lysozyme activity in serum as described by (Studnicka, Siwicki & Ryka 1986). Lysozyme activity was expressed as units mL^{-1} where one unit is defined as the decrease in absorbance of 0.001 min^{-1}.

Stress parameters

Serum cortisol

Serum cortisol levels were estimated using the Cortisol EIA Kit (Cayman chemicals, Mumbai, India), and the values were expressed in ng mL^{-1}.

Serum glucose

Serum glucose level was analysed using the glucose kit (Transasia Bio-Medicals, Mumbai, India), and the result was expressed in mg dL^{-1} of sample.

Antioxidant enzymes

Superoxide dismutase assay

The superoxide dismutase (SOD) activity in the liver tissues was estimated by the method of (Misra & Fridovich 1972). The change in OD at 480 nm for 2 min was measured in UV spectrophotometer.

Catalase assay

Catalase activity (CAT) in the liver tissues was estimated according to the method of Takahara, Hamilton, Neel, Kobara, Ogura and Nishimura (1960). The decrease in absorbance was measured at 240 nm at 30-s intervals for 2 min. The CAT activity was expressed as nmoles of H_2O_2 decomposed min^{-1} $protein^{-1}$.

Protein metabolic enzymes

Alanine transaminase activity (ALT)

ALT in liver tissues was estimated using the ALT assay kit (Cayman Chemicals, Ann Arbor, MI, USA). ALT in sample was measured by monitoring the rate NADH oxidation in a coupled reaction with lactate dehydrogenase (LDH). The oxidation of NADH to NAD+ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease is directly proportional to the ALT activity in the sample.

Aspartate aminotransferase activity (AST)

AST assay kit (Sigma-Aldrich) was used to estimate AST in liver samples. The conversion of amino group aspartate into α-keto-glutarate produces a coloured compound of glutamate. The colour intensity was measured at 450 nm of microplate reader, which is proportional to AST activity of the tissue. One unit of AST is the amount of enzyme that will generate 1.0 μmole of glutamate per minute at pH 8.0 at 37°C.

Challenge study

After the growth study of 90 days, 24 fish from each treatment groups and control were challenged with virulent *A. hydrophila* (ATCC 7966) obtained from Aquatic Environment and Health management Division of Central Institute of Fisheries Education, Mumbai. The final bacterial concentration was adjusted to 1×10^6 cfu mL^{-1} by serial dilution. About 100 μL of bacterial suspension was injected intra peritoneally to eight fish per replicate in each treatment as well as control using 1-mL tuberculin syringe. All challenged fish were released back to their respective tank and mortalities were observed for 3 days. Survival was measured in each day, and the cumulative number of dead fish was recorded each day. The relative percentage survival (RPS %) was calculated using the formula (Amend 1981).

$$\text{Relative percentage survival (RPS)} = \frac{\text{Number of surviving fish after challenge}}{\text{Number of fish injected with bacteria}} \times 100$$

Statistical analysis

Statistical analysis of growth and immune parameters was performed using one-way ANOVA, and significant differences among treatments were measured by Duncan multiple range test ($P < 0.05$). All statistical analyses were performed using software SPSS (Release16 SPSS, Chicago, IL, USA).

Results

Water quality parameters

The range of variation observed in pH was less in the case of control (7–7.5), whereas the biofloc treatments show wide fluctuation in pH values (5–8). Dissolved oxygen levels showed a significant difference between control and biofloc treatment groups with higher average values of DO (7.31 ± 0.20 mg L^{-1}) in control, whereas in biofloc treatment groups, it was 5.6 mg L^{-1}. Lower values of ammonia were found in control (0.43 ± 0.16 mg L^{-1}) as well as SD1 (0.38 ± 0.18 mg L^{-1}) compared to other treatments where it was in the range of 0.56–0.93 mg L^{-1}. Floc volume in biofloc tanks ranged from 8 to 100 mL L^{-1} during the culture period. Highest floc volume was observed in SD4 which was having higher stocking density. Turbidity was in the range of 1–5 NTU in control, whereas it was in the range of 130–330 NTU in biofloc tanks (Table 1).

Growth performance

Growth parameters such as average body weight (ABW) (Figure 1), specific growth rate (SGR), percentage weight gain, daily increment (DI), feed efficiency ratio (FER), protein efficiency ratio (PER) and survival of tilapia cultured in different biofloc treatment and control were measured (Table 2). After 90 days of trial, ABW, SGR, PWG and DI were found to be significantly higher in all the bio-

Table 1 Water quality parameters of the experimental units observed during the culture period of 90 days

Parameter	Control	T1	T2	T3	T4
Temperature (^{0}C)	24.51 ± 0.18^a	24.62 ± 0.18^a	24.66 ± 0.18^a	24.77 ± 0.19^a	24.89 ± 0.19^a
pH	7.10 ± 0.07^a	7.14 ± 0.26^a	7.12 ± 0.28^a	7.06 ± 0.25^a	6.93 ± 0.31^a
Dissolved oxygen (mg L^{-1})	7.31 ± 0.20^b	5.60 ± 0.09^a	5.82 ± 0.17^a	5.67 ± 0.16^a	5.42 ± 0.19^a
Ammonia (mg L^{-1})	0.43 ± 0.16^{ab}	0.38 ± 0.18^a	0.56 ± 0.27^b	0.56 ± 0.27^b	0.93 ± 0.30^c
Nitrite – N (mg L^{-1})	0.07 ± 0.004^a	0.36 ± 0.035^b	0.39 ± 0.036^b	0.41 ± 0.038^b	0.46 ± 0.040^b
Nitrate – N (mg L^{-1})	1.19 ± 0.47^a	6.76 ± 3.42^b	6.74 ± 3.36^b	7.29 ± 3.67^b	8.09 ± 3.57^b
Floc volume (mL L^{-1})	0.00^a	26.00 ± 3.04^c	24.63 ± 3.27^{bc}	18.58 ± 1.67^b	21.89 ± 2.37^{bc}
Turbidity (NTU)	2.78 ± 0.50^a	213.17 ± 13.02^{bc}	183.67 ± 13.56^b	234.17 ± 24.92^d	205.67 ± 13.08^{bc}

Values in the same row with different superscripts differ significantly ($P < 0.05$) for each parameter. One-way ANOVA was used following Duncan multiple range test in SPSS-16.0.

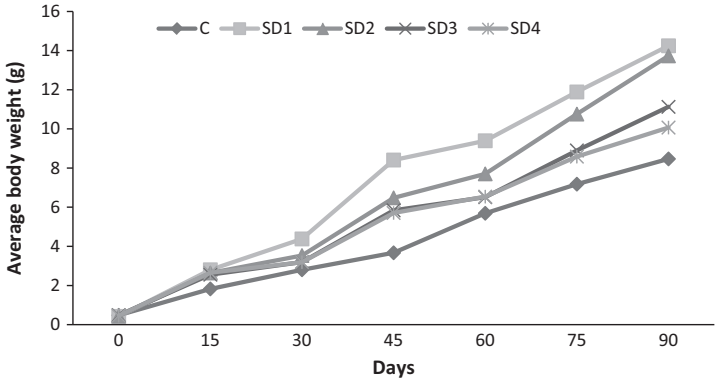

Figure 1 Average body weight (g) of GIFT strain of Tilapia during the culture period of 90 days.

Table 2 Growth, feed utilization and survival of GIFT strain of tilapia in different treatment groups at the end of 90 days experiment

Parameter	Control	SD1	SD2	SD3	SD4
SGR (% day^{-1})	2.41 ± 0.06^{a}	2.99 ± 0.01^{c}	2.93 ± 0.01^{c}	2.68 ± 0.03^{b}	2.60 ± 0.04^{b}
PWG (%)	778.86 ± 47.96^{a}	1371.40 ± 6.05^{c}	1300.60 ± 10.30^{c}	1015.50 ± 33.17^{b}	936.40 ± 38.94^{b}
DI (g day^{-1})	0.08 ± 0.005^{a}	0.15 ± 0.001^{c}	0.14 ± 0.001^{c}	0.11 ± 0.003^{b}	0.10 ± 0.004^{b}
FER	0.20 ± 0.0132^{a}	0.85 ± 0.0058^{b}	0.93 ± 0.007^{c}	0.85 ± 0.033^{b}	0.81 ± 0.033^{b}
PER	0.58 ± 0.038^{a}	2.43 ± 0.017^{b}	2.65 ± 0.020^{c}	2.44 ± 0.096^{b}	2.32 ± 0.095^{b}
Biomass	126.84	285.46	343.13	333.5	355.72
Survival (%)	100	100	100	100	100

SGR, Specific growth rate; PWG, percentage weight gain; DI, daily increment; FER, feed efficiency ratio; PER, protein efficiency ratio. Values in the same row with different superscripts differ significantly ($P < 0.05$) for each parameter. Values are presented as mean ± standard error.

floc treatment groups than control ($P < 0.05$). Among the biofloc treatment groups, highest ABW, SGR, % weight gain and DI were observed in SD1. The highest FER was noted in SD2 (0.93 ± 0.01), and the lowest (0.20 ± 0.013) was registered in control group. The mean PER value was significantly different ($P < 0.05$) among the different treatment groups, the highest being in SD2 (250 nos m^{-3}) and lowest in control group. All the experimental groups recorded 100% survival at the end of the experiment.

Immune parameters

Respiratory burst activity (OD at 540 nm), myeloperoxidase activity (OD at 450 nm) and serum lysozyme activity (unit mL^{-1}) were significantly higher ($P < 0.05$) in biofloc treatment groups compared to control (Table 3). Fish reared in SD1 group exhibited significantly higher immunity compared to the other biofloc groups.

Stress parameters

Significantly lower level of serum cortisol was observed in SD1 (30.52 ± 1.47 ng mL^{-1}) and SD2 (31.96 ± 0.37 ng mL^{-1}) than the other biofloc groups but the highest was in control (56.09 ± 6.59 ng mL^{-1}). The serum glucose level varied significantly among the treatment groups with the highest being in control (90.50 ± 2.52 mg dL^{-1}) and the lowest in SD1 (50.24 ± 2.79 mg dL^{-1}) group (Table 4).

The SOD and catalase activities were significantly different ($P < 0.05$) among the treatment groups with the highest in control (117.43 ± 7.22 units mg protein^{-1},1.77 ± 0.47 units mg protein^{-1}, respectively) and lowest value was

Table 3 Respiratory burst activity, myeloperoxidase activity and serum lysozyme activity in different biofloc-based treatments

Parameter	Control	SD1	SD2	SD3	SD4
NBT (OD at 540 nm)	0.17 ± .009^{a}	0.31 ± .012^{c}	0.29 ± .008^{bc}	0.26 ± 021^{b}	0.25 ± .021^{b}
MPO (OD at 450 nm)	0.24 ± .030^{a}	0.70 ± .047^{c}	0.65 ± 093^{c}	0.53 ± .025^{bc}	0.39 ± 060^{ab}
Lysozyme (Units mL^{-1})	77.67 ± 2.85^{a}	155.33 ± 13.28^{c}	143.33 ± 4.81^{bc}	121.00 ± 8.50^{b}	117.67 ± 8.25^{b}

NBT, Respiratory burst activity; MPO, myeloperoxidase activity.
Values in the same row with different superscripts differ significantly ($P < 0.05$) for each parameter. Values are presented as mean ± standard error.

Table 4 Serum cortisol (ng mL^{-1}) and glucose level (mg dL^{-1}) of Tilapia at the end of culture period of 90 days

Parameter	Control	SD1	SD2	SD3	SD4
Cortisol (ng mL^{-1})	56.09 ± 6.59^{b}	30.52 ± 1.47^{a}	31.96 ± 0.37^{a}	37.92 ± 4.11^{a}	39.17 ± 2.09^{a}
Glucose(mg dL^{-1})	90.50 ± 2.52^{b}	50.24 ± 2.79^{a}	58.94 ± 5.02^{a}	61.12 ± 1.82^{a}	63.12 ± 7.57^{a}

Values in the same row with different superscripts differ significantly ($P < 0.05$) for each parameter. Values are presented as mean ± standard error.

Table 5 Antioxidant and protein metabolic enzyme activity in the liver of GIFT strain of Tilapia during the culture period

Parameter	Control	SD1	SD2	SD3	SD4
SOD (U mg protein^{-1})	117.43 ± 7.22^{c}	43.72 ± 1.73^{a}	45.92 ± 1.72^{a}	51.92 ± 2.35^{a}	85.63 ± 11.08^{b}
Catalase (U mg protein^{-1})	8.45 ± 1.75^{b}	1.77 ± 0.47^{a}	2.88 ± 0.66^{a}	2.94 ± 1.34^{a}	4.43 ± 0.86^{a}
AST (U mL^{-1})	0.62 ± 0.11^{b}	0.14 ± 0.03^{a}	0.29 ± 0.07^{a}	0.54 ± 0.22^{a}	0.30 ± 0.06^{a}
ALT (U mL^{-1})	0.85 ± 0.09^{b}	0.52 ± 0.07^{a}	0.57 ± 0.05^{a}	0.58 ± 0.05^{a}	0.59 ± 0.03^{a}

SOD, superoxide dismutase; AST, Aspartate aminotransferase; ALT, alanine aminotransferase.
Values in the same row with different superscripts differ significantly ($P < 0.05$) for each parameter. Values are presented as mean ± standard error.

observed in SD1 (43.72 ± 1.73 units mg protein^{-1},1.77 ± 0.47 units mg protein^{-1}, respectively). The ALT and AST activity in the liver of GIFT tilapia showed highest values in control (0.85 ± 0.09 units mL^{-1}, 0.62 ± 0.11 units mL^{-1}) and lowest in SD1 (0.52 ± 0.07 units mL^{-1}, 0.14 ± 0.03 units mL^{-1}) with lowest stocking density in case of biofloc group, and the values showed an increasing trend with increasing stocking density (Table 5).

Challenge study

Fingerlings were challenged with virulent strain of *A. hydrophila,* and the highest RPS were observed in treatment groups SD1 and SD2 (83.33%) followed by SD3 (75%) SD4 (62.5%), whereas the lowest RPS was recorded in control group (37.50%) (Figure 2).

Discussion

Physiological adaptations allow shrimp and tilapia to consume biofloc and digest microbial protein (Azim & Little 2008; Hargreaves 2013). In the present study, the growth parameters were found to be better in biofloc units than the control. Azim and Little (2008) also reported that the tilapia reared in biofloc tank had 45% higher net fish production compared to clear water counterpart. The increased growth performance in the biofloc treatment groups with lowest stocking density may be due to maintenance of optimum water quality parameters and constant availability of nutritious floc for the animals. Better growth was observed in SD1 and SD2 indicating the optimal stocking density.

FER was found to be low for the control group, while the biofloc treatment group has shown higher values of FER, indicating the better utilization of the microbial protein by the fish in addition to the artificial feed. Similarly, Azim and Little (2008) reported low FER of around 0.2 in clear water control compared to the biofloc counterpart. Ekasari and Maryam (2012) found that the total feed used in BFT was lower suggesting that biofloc could be continuously harvested by the tilapia as other source of food. Hence, higher FER and PER seen in the biofloc treatments indicate utilization of bioflocs by the GIFT strain of Tilapia as an alternative food source. Higher growth of tilapia in

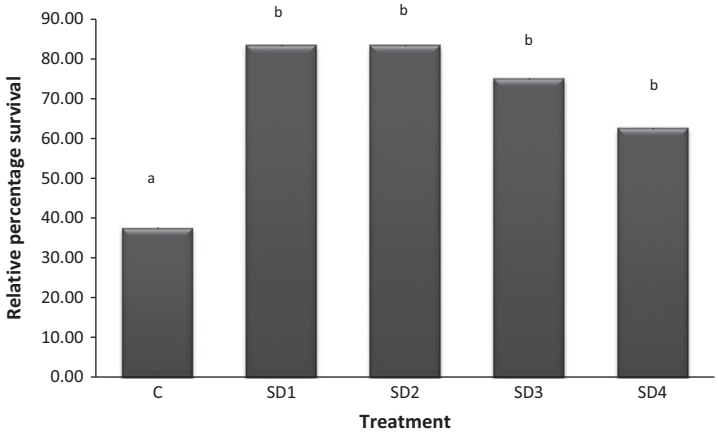

Figure 2 Relative percentage of survival (%) after challenge with *Aeromonas hydrophila* in GIFT strain of tilapia.

biofloc-based systems was also obtained in the earlier studies (Avnimelech 2007; Azim & Little 2008; Rakocy, Danaher, Bailey & Shultz 2008; Avnimelech & Kochba 2009; Crab, Kochva, Verstraete & Avnimelech 2009). Biofloc is reported to be the richest source of many essential fatty acids (Ekasari, Crab & Verstraete 2010; Toledo, Silva, Vieira, Mouriño & Seiffert 2016) which may result in the better growth of cultured organisms. Ju, Forster, Conquest and Dominy (2008) evaluated many bioactive compounds in biofloc such as carotenoids, chlorophylls and phytosteroids, which are expected to contribute better growth of cultured organisms in biofloc system. Likewise, Avnimelech (2007) reported that biofloc contribute to 50% of the feed protein requirement of tilapia. Avnimelech and Kochba (2009) evaluated nitrogen uptake and excretion by tilapia in biofloc tanks using ^{15}N tracing. They concluded that net uptake of microbial protein from a biofloc suspension contributes about 25% of the normal protein ration. Hence, higher growth observed in biofloc treatment compared to the control groups during the rearing period of 90 days indicated that the biofloc was utilized efficiently and effectively converted into body mass.

In the biofloc groups, 100% survival was observed which indicate that GIFT strain of tilapia can tolerate zero-water exchange system and a higher stocking density. Ekasari and Maryam (2012) also reported higher survival of red tilapia in biofloc compared to control. Moreover, the zero-water exchange-based culture units will ensure biosecurity, and hence, the threat to biodiversity through culturing invasive species like tilapia can be nullified.

Disease occurrence and treatment in aquaculture systems have effect on its production as well as profitability. Enhancing non-specific immunity of fish is a disease prophylaxis measure which can be opted safely to avoid the cause. Immunostimulants are the substance used to improve immunity in fish (Sharma, Deo, Riteshkumar, Chanu & Das 2010; Brogden, von Köckritz-Blickwede, Adamek, Reuner, Jung-Schroers, Naim & Steinhagen 2012; Acosta, Carpio, Valdés, Velázquez, Zamora, Morales & Estrada 2014). Crab (2010) suggested that bacteria, bacterial products, carbohydrates and nutritional factors play a key role in immunostimulatory action in normal cases; the presence of same in BFT may have contributed to enhanced immunity of the culture organisms. Emerenciano *et al.* (2013) suggested that the phenomenon of 'natural probiotic' effect is found in BFT, which could act internally and/or externally against pathogenic *Vibrio* sp. and ecto-parasites in BFT respectively. Immunity is enhanced by stimulating or activating immune systems in several ways: enhancing the number of phagocytes, activating phagocytes or increasing the synthesis of the involved molecules (Gültepe, Bilen, Yılmaz, Güroy & Aydın 2014). Phagocytosis is the second-line defence mechanism in fish which utilizes respiratory burst activity, myeloperoxidase activity, and lysozyme activity (Fischer, Utke, Somamoto, Köllner, Ototake & Nakanishi 2006). All the non-specific immune parameters measured in the present study showed significant difference among the experimental groups and lower values of immune parameters were observed in control group. Higher NBT, MPO and lysozyme values in biofloc support the hypothesis of natural probiotic effect, whereas the decrease in values with increasing stocking density may be due to the crowding stress.

Increased levels of serum cortisol and serum glucose are reported in Nile tilapia when exposed to different kinds of stress (Barreto & Volpato 2006; EL-Khaldi 2010). Significantly lower level of serum cortisol and glucose was observed in biofloc units (SD1 and SD2) compared to control. This indicates that the biofloc unit under zero-water exchange does not cause any stress-related problems to the fish even at stocking densities up to (250 nos m^{-3}). The results of the present study are comparable with earlier findings in Nile tilapia (Azim & Little 2008; Ahmed & Sadek 2014).

Antioxidant enzymes are key factors in animal defence system against oxidative stress, and its value tends to increase with stress (Zahran & Risha 2014). The antioxidant enzyme system including superoxide dismutase (SOD) and catalase (CAT) plays important roles in higher animals in sustaining the homoeostasis of the cell (Aruoma 1998). The SOD and catalase activity were significantly different among the treatment groups with the highest value noticed in control and lowest in SD1. Significantly lower activity of antioxidant enzymes in the fish reared in biofloc systems indicates the presence of antioxidative components in bioflocs, which may help to reduce the oxidative stress, and thus lower levels of antioxidant enzymes in the fish. Increase in value of antioxidant enzymes was obtained with increase in stocking density. However, antioxidant enzyme activity

at higher stocking density was lower as compared to the control. Ju *et al.* (2008) analysed the components in biofloc and mentioned the presence of antioxidant substance such as carotenoids which can be helpful to reduce the oxidative stress in biofloc reared animals. The results of the present study are in agreement with the assumption that there are antioxidative substances (bioactive compounds) in the biofloc which reduces the oxidative stress and thus leads to the lower production of antioxidant enzymes such as SOD and CAT.

Transaminases are enzymes that redistribute amino nitrogen among amino acids forming new amino acids (Mishra, Mishra, Lee & Tucker 2013). Transaminase enzymes such as AST and ALT in liver and serum can be used as an indicator of stress in animals. In the present study, AST and ALT activity was significantly different among the treatment groups and the biofloc groups, that is SD1 showed lower values compared to other biofloc treatment groups. Within the biofloc treatment groups, there was an increasing trend in the values of AST and ALT with increasing stocking density. Kpundeh, Xu, Yang, Qiang and He (2013) also reported the increased levels of AST and ALT in the serum of GIFT juveniles with increasing stocking density.

The enhanced immune status of biofloc reared fish was further confirmed by a challenge test using *A. hydrophila*. There were earlier reports of enhanced survival of Red tilapia against *A. hydrophila* supplemented with immunostimulants (Barros *et al.* 2014; Hassanin *et al.* 2014). Increased RPS (%) in biofloc groups in the present study compared to the control indicates the effect of biofloc in disease resistance as supported by (Irshad, Verma, Rani, Rathore, Saharan & Gora 2016). The increase in disease resistance in biofloc reared fish may be due to the enhanced immunity acquired with the constant exposure to microbial populations and its by-products in biofloc (Crab, Lambert, Defoirdt, Bossier & Verstraete 2010). Michaud, Blancheton, Bruni and Piedrahita (2006) also reported that heterotrophic microbial biomass has a controlling effect on pathogenic bacteria *A. hydrophila.* Further, the groups with higher stocking density recorded significantly lower RPS (%) and higher cumulative mortality percentage among the biofloc treatment group which can be attributed to the possible stress level which hampered the immune status and hence the disease resistance.

Conclusion

The result of the present study suggests that the optimum stocking density of GIFT tilapia in biofloc-based system should be 200–250 nos m^{-3} for optimum growth and improved immune status whereas the intensification up to 350 nos m^{-3} can be performed without affecting the growth, survival and immunity. This study led to the possibility of utilization of biofloc-based zero-water exchange system to ensure biosecurity and environmental sustainability in farming. Further research should focus on the biofloc composition, microbial characterization and ways to enhance the growth of beneficial microbes, species diversification as well as co culture of different species to utilize the maximum resources with minimum stress to the environment.

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