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In vivo efficiency of *Bacillus* sp. isolated from biofloc system on growth, haematological, immunological and antioxidant status of genetically improved farmed tilapia (GIFT)

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In aquaculture, Biofloc technology is emerging as one of the successful sustainable technologies to increase production. It is known to possess several immunostimulatory compounds exhibiting possible probiotic effect in culture. Here, we explored the *in vivo* efficiency of *Bacillus infantis* (T1), *B. subtilis* (T2), *Exiguobacterium profundum* (T3) and *B. megaterium* (T4) isolated from biofloc systems for improving the growth and immune performance of genetically improved farmed tilapia (GIFT). Animals (10±0.08 g) were stocked at a density of 100 per m^{-3} in 500 L FRP tanks for 42 days in triplicate. All the four probiotics (OD =1) were mixed with basal diet in treatments and feed without probiotic maintained as control. A significant difference ($P \leq 0.05$) in weight gain, specific growth rate and FCR were observed between treatments and control with 100% survival. Serum albumin, globulin, protein, total blood count, glucose, myeloperoxidase activity and SOD were significantly different ($P < 0.05$) between treatments and control. T4 and T2 showed better immunological and anti-oxidant ability when compared to other strains. Results from principal component analysis demonstrated that *B. megaterium* and *B. subtilis* can be the promising probiotic bacteria isolated from biofloc systems exhibiting multiple benefits with improved growth and health of the culture animals.

Keywords: Immune response, Nile tilapia, *Oreochromis niloticus*, PCA analysis, Probiotic

Tilapias [*Oreochromis niloticus* (L.) Fam. Cichlidae], the second most farmed freshwater fish, have intrinsic feature like fast growth rate, disease resistance ability, low trophic level feeding and good flesh quality. The total production of tilapia in was 6.8 million tonnes in 2018[1] and expected to produce 7.3 million tonnes by 2030[2]. The rapid expansion and intensification of aquaculture resulted in the outbreak of diseases leading to considerable economic losses and thereby hindering the sustainable development of the industry[3,4].

To prevent and treat diseases in aquatic animals, antibiotics have been used for improving aquaculture production. Indiscriminate–usage of antibiotics may result in the development of antibiotic resistant bacteria, antibiotic residues in the flesh and the microbial population destruction in the aquatic environment[5,6]. Hence, green alternatives viz. pre- and probiotics have come into use in place to development of antibiotics.

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Probiotics, live microbes when administered as a dietary supplement in *Oreochromis niloticus*, confer benefits to the host by improving the balance of the intestinal microbiota, exclusion of pathogens and growth performance. The commercially available probiotics used for tilapia culture were isolated from a wide range of sources such as tilapia culture water, sediment and its intestine[7-10]. *Bacillus* sp. is one of the most commonly used probiotics compared to other species due to its active role in enhancing immune mechanism of tilapia[11]. *Bacillus* sp. has the efficient enzymatic pathway in breaking the complex carbohydrates, proteins and lipids[12] and its spore-forming ability[13] adds an extra advantage of using it in the commercial aquaculture practices. In recent years, adoption of biofloc technology, a minimal water exchange technology promotes heterotrophic bacteria has replaced the commercial probiotics usage as it exerts the possible probiotic effect in various aquaculture animals[14]. However, reports on identification and isolation of *Bacillus* sp. from biofloc based tilapia culture are limited. This study

attempts to determine the efficiency of four *Bacillus* spp. namely, *Bacillus subtilis, B. megaterium, B. infantis* and *Exiguobacterium profundum* from biofloc systems of GIFT tilapia. This study also involves the strict process of selection of probiotics from biofloc systems using *in vivo* test associated with extensive evaluation in different aspects such as immunological, haematological parameters and antioxidant status of genetically improved farmed tilapia (GIFT).

Materials and Methods

Isolation of probiotic bacteria from biofloc culture water

The biofloc was developed and maintained in the 500 L FRP tank according to Taw[15]. Distillery spentwash as a carbon source was used to maintain C:N ratio at 10:1. The biofloc water sample was fortnightly screened for probiotic bacteria by spread plating on MRS agar. The morphologically different isolates were characterized according to Bergey's Manual of Determinative Bacteriology. DNA was isolated from the bacterial culture using phenol-chloroform method later amplified for its 16s rRNA region using Forward primer -5′AGAGTTTGATCC TGGCTCAG3′ and Reverse primer- 5′CGGTTA CCTTGTTACGACTT3′. The amplified DNA was sequenced using Sanger's method and obtained sequences were aligned and submitted in Genbank. The accession numbers for the *Bacillus* sp. were obtained as *Bacillus infantis*- MH424755 *Bacillus subtilis*- MH424900, *Exiguobacterium profundum*- MH424898 and *Bacillus megaterium*- MH424904.

In vivo fish feeding trial

Experimental design

A commercial fish feed (30% protein, 4% fat, 4% fibre and 14% ash of Grobest Feed, India) was used as the basal diet. The experimental diet includes the basal diet supplemented with four bacterial cultures isolated from biofloc water which were grown in LB medium (37 °C for 16 h). The cell pellets were washed and resuspended in PBS to attain the OD value of 1.00. Later bacterial suspension was homogenized and sprayed on the experimental diets at a rate of 100ml bacterial suspension/kg of feed[16]. Without bacteria the same volume of PBS was added to the basal diet for control. The viability of bacteria in the experimental diets remains stable for seven days based on the confirmation with plating on the nutrient agar. Hence, these diets were prepared weekly once to warrant the bacterial performance.

GIFT tilapia juveniles (10±0.08 g) were stocked in fifteen FRP tanks of 500 litres capacity (50 animals per tank) filled with freshwater. The experiment was conducted for 42 days and treatments include: Control (basal diet without bacteria), T1 (basal diet supplemented with *Bacillus infantis*), T2 (basal diet supplemented with *Bacillus subtilis*), T3 (basal diet supplemented with *Exiguobacterium profundum*) and T4 (basal diet supplemented with *Bacillus megaterium*) in triplicates. The animals were fed with two rations at 3% of average body weight throughout the experimental trial. Regular siphoning of water from the experimental units was carried out to maintain the optimum water quality.

Growth performance

The growth performance and survival of juvenile fishes for all groups were calculated using the following equations:

Weight gain (WG in g) = $W_t - W_0$

Specific growth rate (SGR, % day^{-1}) = $[\ln W_t - \ln W_0]/t \times 100$

FCR = feed offered (dried wt.)/weight gain (wet wt.);

Survival rate (in %) = $(Nt \times 100)/N_0$

where W_0 and W_t are the initial weight (g) and final weight (g) of fish, N_0 and N_t are initial and final number of fish, and t represents culture duration in days.

Water quality parameters

Temperature (mercury thermometer) and pH (Labtronics pH meter) were measured on a daily basis. As per APHA[17], parameters like dissolved oxygen, free carbon dioxide, alkalinity, hardness, calcium and magnesium ion concentration were measured weekly. Nitrate-N (NO_3 -N) and nitrite nitrogen (NO_2 -N) were estimated using the filtered water samples and analysed using Resorcinol method. Ammonia was estimated by phenol hypochlorite method and orthophosphate according to APHA[17] every week.

Haematological, immunological and antioxidant indicators

Fishes were anesthetized using clove oil and the blood samples were drawn from the caudal vein. The blood was then transferred to EDTA (an anticoagulant) coated vials and for serum separation the blood sample was allowed to clot for 15 min, centrifuged and used for further analysis.

Total blood count, albumin and globulin content

The total blood count of the experimental animals was enumerated using Giemsa staining method. To the 10 µL of serum, 2.5 mL of reagent R containing

200 mM/L of succinate buffer, 0.4 mM/L of bromocresol green and 4 mM/L of sodium azide was added. 10 µL of albumin (4 g/dL) served as standard. The mixture was incubated for 5 min at 20-25°C. The absorbance was read at 623nm against blank[18].

Albumin concentration (g/dL) = ($A_{specimen}/A_{standard}$) × 4
Globulin= Total serum protein – albumin

Respiratory burst activity

Respiratory burst activity was performed following the modified method of Anderson & Siwiki[19]. 0.2% of nitroblue tetrazolium (NBT) solution was added to the 0.1 mL blood sample and incubated for 30 min at room temperature (30°C). about 1.0 mL N, N-dimethyl formamide (DMF) was added to the 0.05 mL of the NBT blood cell suspension and centrifuged for 5 min at 5000 rpm. The collected supernatant was read on a spectrophotometer at 540 nm.

Myeloperoxidase activity

Total MPO content present in serum was measured according to Quade and Roth[20] with slight modification. Ten µL of serum sample collected was diluted with 90 µL of Hank's balanced salt solution (HBSS). About 35 µL of 20 mM 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB) and five mM H_2O_2 (freshly prepared) were added to the serum. The colour change reaction was stopped after 2 min by adding 35 µL of 4 M sulphuric acid (H_2SO_4) and the OD was read at 450 nm.

Units/mL enzyme = (A470 nm Test at 1.0 min; A470 nm Blank at 1.0 min) (df)/(1.0) (0.035)
df = Dilution factor; 1.0 = The increase in A470 nm/min per unit of enzyme; and 0.035 = Volume of enzyme used (mL)

Glucose and Protein estimation

The serum sample was analysed for glucose level using Beacon Diagnostics Pvt. Ltd., kit. The protein estimation in blood as well in serum was done by Lowry's method[21].

Catalase stress enzyme assay

The blood sample (10-50 µL) was added to 2.5 mL of phosphate buffer (50 mM/pH7). A volume of 1.0 mL of 0.3% H_2O_2 (freshly prepared) was added to the above suspension. The decrease in the absorbance was read at 240 nm for 3 min at 30 s interval[22].

CAT (units/mg protein) = [OD/min (3) × total volume]/ [34 × sample volume × protein] × 1000

Super oxide dismutase (SOD) assay

The blood sample (10-50 µL) was added to 1.5 mL of carbonate buffer (0.1 M/ pH10.2). About 0.5 mL of epinephrine (freshly prepared) was added to the above suspension. The increase in the absorbance was read at 480 nm for 3 min at 30 s interval[23]. Inhibition % = OD blank-change in OD/min/OD blank × 100; SoD unit/mg of protein = inhibition %/50 × sample vol × 18 × mg of protein

Statistical analysis

One-way ANOVA was performed to find the significant difference between the treatments and control using SPSS version 20.0 for the immunological, haematological, growth and water quality parameters. Statistical difference was found at P <0.05. The Principal component analysis was performed to find the overall immune performance of probiotic isolates.

Results

Growth performance

The weight gain, specific growth rate and feed conversion ratio of GIFT tilapia fed with different experimental diets along with the statistical analysis were shown in the Table 1. All the growth parameters showed significant difference between control and treatments (P <0.05). *Bacillus megaterium* and *Bacillus subtilis* fed animals possessed higher weight gain with improved FCR.

Water quality parameters

The various water quality parameters along with the statistical analysis were shown in the Table 2. There was no significant difference between control and treatments for all the parameters analysed.

Haematological, immunological and antioxidant indicators

The immunological and haematological parameters were analysed and the graphs along with the standard

Table 1 — Growth Performance of GIFT Tilapia fed with different experimental diets at the end of the culture trial

Parameters	Control	*B. infantis*	*B. subtilis*	*E. profundum*	*B. megaterium*
Initial Weight (g)	10±0.05[a]	10±0.08[a]	10± 0.06[a]	10±0.02[a]	10±0.04[a]
Final Weight (g)	20.6±1.13[a]	26.3±1.64[b]	27.2±0.81[b]	25.9± 0.32[b]	27.96±1.00[b]
Weight gain (g)	10.6±1.13[a]	16.3±1.64[b]	17.2±0.81[b]	15.9± 0.32[b]	17.96±1.00[b]
Specific growth rate	1.73±0.16[a]	2.28±0.33[b]	2.41±0.15[b]	2.29±0.11[b]	2.45±0.16[b]
Feed conversion ratio	2.55± 0.15[a]	2.12± 0.24[b]	2.1± 0.2[b]	2.21± 0.18[b]	2.09± 0.24[b]

[Data assigned with different superscripts denote significant difference in a row (P <0.05)]

Table 2 — Water quality parameters of experimental groups in the 42 days culture trial of GIFT Tilapia

Parameters	Control	*B. infantis*	*B. subtilis*	*E. profundum*	*B. megaterium*
pH	7.24±0.1[a] (7.12-7.36)	7.22±0.15[a] (7.14-7.3)	7.37±0.17[a] (7.33-7.79)	7.33±0.21[a] (7.16-7.5)	7.42±0.22[a] (7.25-7.59)
Temperature (°C)	27.45±0.19[a] (27-30)	27.26±0.12[a] (27-30)	27.45±0.1[a] (27– 30)	27.26±0.12[a] (27-30)	27.56±0.17[a] (27-30)
DO (mg/L)	5.45±0.04[a] (4.1-5.7)	5.7±0.04[a] (4.2-5.9)	5.55±0.04[a] (4.3 -5.7)	5.72±0.04[a] (4.5 - 5.9)	5.65±0.04[a] (4.4 - 5.8)
Free CO_2 (mg/L)	2.4± 0.15[a] (1.9-3.2)	2.33±0.1[a] (1.7 - 3.3)	2.65±0.15[a] (1.7 - 3.4)	2.77±0.15[a] (1.9-3.5)	2.55±0.12[a] (1.8- 3.3)
Alkalinity (mg/L)	55±2.5[a] (45-65)	57.89±2.9[a] (38-79)	56.8±2.45[a] (36-65)	57.25±2.6[a] (39-78)	56.89±2.75[a] (37-75)
Hardness (mg/L)	250±3.6[a] (236-292)	249±5.6[a] (219-279)	252±3.9[a] (225-277)	248±5.1[a] (210-269)	251±5.15[a] (241-267)
Calcium ions (mg/L)	55.59±4.75[a] (44-75)	55±3.77[a] (34-65)	53.59±4.54[a] (46-69)	53.1±4.98[a] (34-68)	54.5±3.98[a] (38-70)
Magnesium ions (mg/L)	79±2.7[a] (45-85)	78±2.7[a] (42-88)	79.55±2.74[a] (42-80)	77.5±2.55[a] (41– 85)	78.12±2.9[a] (42-88)
Nitrate (mg/L)	0.007±0.0[a] (0.004-0.012)	0.006±0.0[a] (0.004-0.015)	0.007±0.0[a] (0.005-0.008)	0.006±0.0[a] (0.003-0.01)	0.008±0.0[a] (0.004-0.015)
Nitrite (mg/L)	0.014±0.0[a] (0.007-0.019)	0.011±0.0[a] (0.006-0.015)	0.013±0.0[a] (0.008-0.019)	0.012±0.0[a] (0.005-0.015)	0.011±0.0[a] (0.004-0.015)
Ammonia (mg/L)	0.07±0.02[a] (0.05-0.19)	0.05±0.025[a] (0.04– 0.26)	0.04±0.03[a] (0.04-0.19)	0.06±0.032[a] (0.04– 0.29)	0.05±0.024[a] (0.05– 0.25)
Phosphate (mg/L)	0.08±0.02[a] (0.05-0.15)	0.085±0.01[a] (0.04-0.17)	0.082± 0.02[a] (0.033-0.12)	0.09±0.015[a] (0.044-0.16)	0.083±0.018[a] (0.04-0.19)

[Data assigned with different superscripts denote significant difference in a row (P <0.05)]

deviation were constructed which were represented in the Fig. 1.

From the One way ANOVA performed, the results confer that there is a significant difference between treatments (T1, T2, T3 and T4) and control (C) for serum albumin, globulin, protein, total blood count, glucose, myeloperoxidase and SOD. No significant difference was found in the blood protein levels of animals fed with *Bacillus infantis* and *Bacillus subtilis* diets. Respiratory burst activity of the experimental animals fed with *Bacillus subtilis* and *Exiguobacterium profundum* showed no significant differences.

The first four principal components (PCs) accounted for 92.45% of the total variance which was listed in the table 3. The distribution plot variables of PC 1 and PC 2 on the plane, which are mainly related to serum protein and myeloperoxidase are presented in the figure.2. These two selected components made up to 50.79 and 17.40% of the variance in the model, suggesting that they were key factors in distinguishing the different strains used. It could be inferred that strains presented in quadrant IV were significant as they showed high correlation with respect to variables. *Bacillus megaterium* and *B. subtilis* had the highest contribution to PC 1 and may be preferentially used as potential probiotics among all the strains.

Table 3 — Correlation of variables obtained from total variance with the factors of the PCA analysis

	PC 1	PC 2	PC 3	PC 4
Albumin	0.408	0.037	0.201	0.129
Globulin	0.406	0.014	0.195	0.133
Serum protein	0.417	0.030	0.200	0.114
Blood Protein	0.379	-0.152	0.016	-0.373
Total blood count (*103 cells/µL)	0.321	0.402	0.193	-0.312
Glucose	-0.283	0.558	0.071	-0.098
Myeloperoxidase	0.060	0.575	-0.476	0.069
RBT	0.321	0.281	-0.275	0.105
Catalase	0.211	-0.206	-0.541	0.498
SOD	-0.125	0.221	0.495	0.665
% variance	50.79	17.40	13.22	11.04

Discussion

Growth performance of GIFT tilapia fed with probiotics showed positive results and ascertained with the fact of early findings[24-28]. Higher growth rate was found in *B. megaterium* fed animals and lower growth rate was seen in control. All the experimental groups showed 100% survival which could be due to

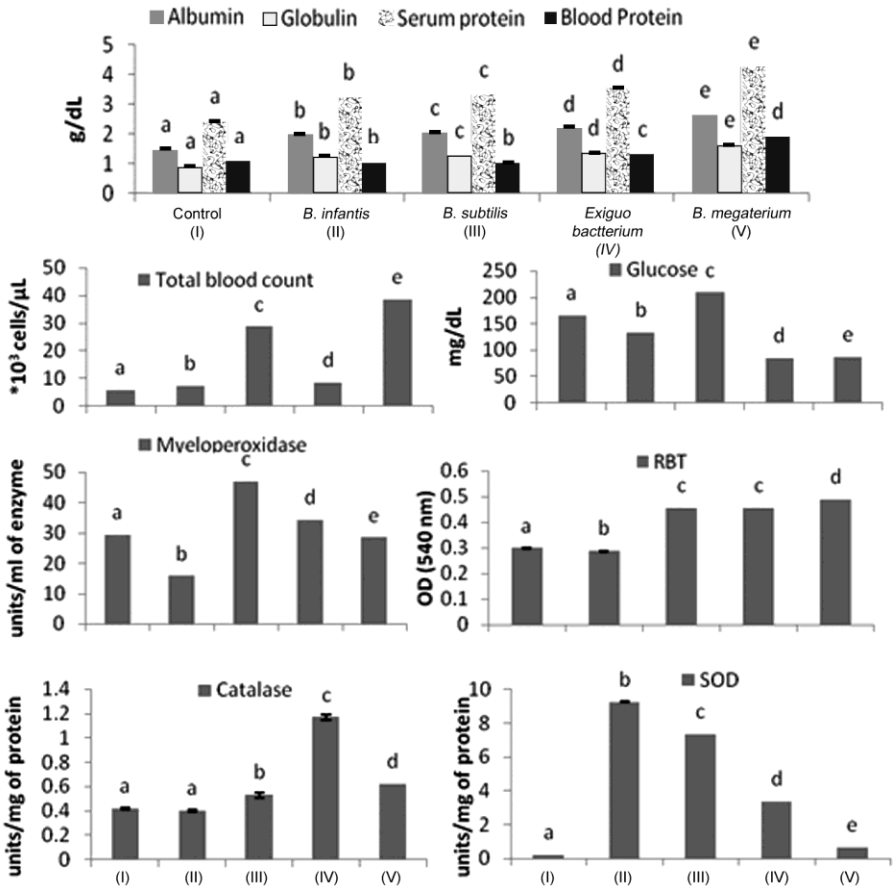

Fig. 1 — Haematological, immunological and anti oxidant indicators of GIFT Tilapia in the *in vivo* feeding trial of 42 days.

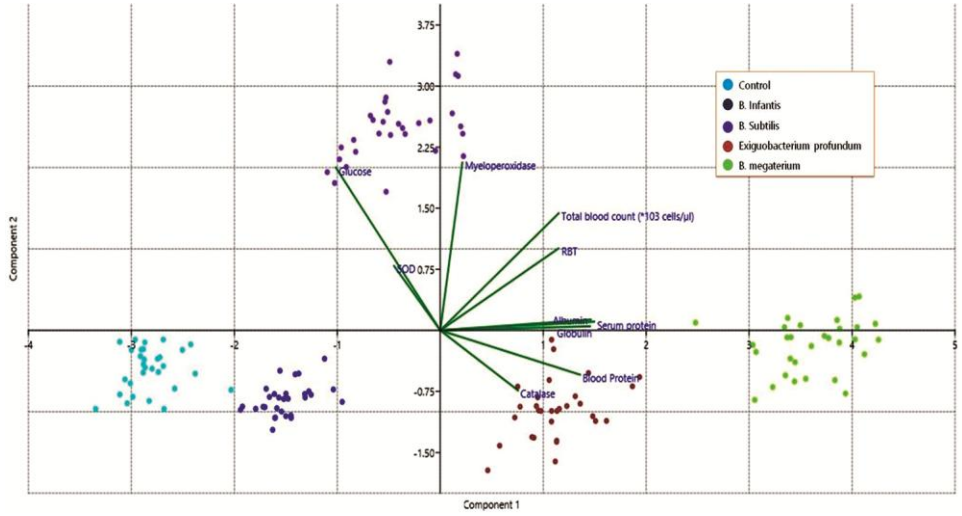

Fig. 2 — Principal component analysis based on four probiotic strains and tested parameters

the regular water exchange favouring good environmental condition for the animals. Water quality parameters showed no significant differences with the supplementation of probiotics along with the feed.

The haematological parameters such as albumin, globulin and total blood count were found to be more pronounced in probiotics fed tilapia than control. Albumin and globulins are the two major groups of serum proteins and taken as total protein. Blood

globulin contents are inevitable for the healthy culture of animals with improved immune functions. However, for sustaining the osmotic pressure, albumin is the vital need for proper distribution of body fluids as it acts as a plasma carrier[29]. High concentrations of total protein in fish serum can be correlated with enhancement of the non-specific immune response. The results indicated higher (P <0.05) albumin and globulin concentrations of tilapia blood serum were obtained in probiotic fed fishes compared with that of the control in the present study. Higher levels of albumin and globulin were found in *Bacillus megaterium* and this may be due to the presence of carotenoids and its antioxidant potential[30]. The increase in blood cell count in tilapia due to probiotic feed indicates an immunostimulant effect. The possible explanation for the variation of blood cells could be attributed to the various probiotic feed.

Immunological parameters, such as respiratory burst and myeloperoxidase activity was significantly higher in the probiotic mixed diets than control. Immune systems can be activated in several ways like enhancing the number of phagocytes, activating phagocytes or increasing the synthesis of the involved molecules. Increased bacterial pathogen killing ability of phagocytes can be inferred from increased respiratory burst activity which is a most important bactericidal mechanism in fishes[31]. It had been demonstrated that the RBT of tilapia when administered with bacterial probiotics isolated from biofloc culture systems showed an improved performance than control. Similar results were observed in the administration of *Lactobacillus rhamnosus* (strain ATCC 53103) in rainbow trout (*Oncorhynchus mykiss*)[32]. The myeloperoxidase (MPO) utilizes one of the oxidative radicals to produce hypochlorous acid thereby acting as an antimicrobial enzyme. The MPO was mostly released by the azurophilic granules of neutrophils during oxidative respiratory burst. The improved MPO activity was seen in treatment with addition of *B. subtilis* as a probiotic to tilapia. This was in concurrent with the findings of Wang *et al*.[33] on the addition of *E. faecium* ZJ4 to the aquaria.

Antioxidant status such as glucose, SOD and catalase activity was significantly different in the treatment than control. Intensive glycogenolysis and the synthesis of glucose from extra hepatic tissue proteins and amino acids increases the glucose content in blood as an indicator of stress in animals[34]. Nakno and Tomlinson *et al*.[35] observed that all types of stress elevated the secretion of catecholamine which stimulates the breakdown of glycogen thereby increasing blood glucose level. In the present study, *B. megaterium* fed tilapia has less glucose level when compared with other treatments indicating the lesser stress levels in animals.

SOD and catalase are two important enzymes in the cellular antioxidant defence system, means of dealing with oxidative stress. SOD and catalase remove oxygen radicals produced within the cells[36] and are expected to increase under hypoxia to detoxify ROS. Lower levels of SOD and catalase are indication for cell damage due to the accumulation of the high-level free radical. The experimental animals fed with probiotic supplemented diets possessed increased SOD and catalase levels compared to control. However, within the treatments, *Exiguobacterium profundum* and *Bacillus infantis* supplemented diets enhanced the SOD and catalase levels of the animals. The overall performance of the *Bacillus* sp. examined using Principal component analysis (PCA) of *in vivo* characteristics particularly immunological, haematological and anti-oxidant status revealed *Bacillus megaterium* and *Bacillus subtilis* as a superior strain with desirable qualities compared to the other strains for the GIFT tilapia culture. The results from the present study also confirmed the probiotic effect of biofloc as the strains were isolated from the biofloc systems probing the ecological health of culture animals.

Conclusion

The four bacterial species isolated from the biofloc water sample were tested for *in vivo* probiotic efficiency in GIFT tilapia. Improved performance of the culture animals was found in probiotics fed animal and among the four bacillus strains, *Bacillus megaterium* and *B. subtilis* can be used as potential probiotics as they exhibited better performance in terms of immunological, haematological and growth parameters throughout the experimental trial. These two strains can further be commercialised and used as effective probiotics in aquaculture. The different physical forms of the tested probiotics and its combined performance would further help to determine its efficiency for the mass scale production in aquaculture.

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Ethical statement

Control and supervision of experiments on animals were carried as per the clause of 15 of prevention of cruelty to Animals act 1960. SOP procedures were adopted as per the Institutional Animals Ethics Committee.

Conflict of Interest

The authors declare no conflict of interests.

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