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# Bio-augmentation of heterotrophic bacteria in biofloc system improves growth, survival, and immunity of Indian white shrimp *Penaeus indicus*

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ABSTRACT

Effect of bio-augmentation of *Bacillus spp* in biofloc on growth, survival and immunity in Indian white shrimp *Penaeus indicus* was evaluated. Nine *Bacillus* strains were isolated and screened individually as well as in the form of a consortia. To maintain a C:N ratio of 12:1 a blend of carbohydrate sources was used. Bio-augmentation with bacterial consortium and *Virgibacillus sp.* produced improved growth and immunity. Shrimp survival ranged from 80 to 95% among treatments. Production was higher (35%) in the biofloc tanks with an average body weight (ABW) of 10.89 ± 1.2 g. On evaluating the immune responses, it was found that trypsin significantly ($P < 0.05$) enhanced Prophenoloxidase (PO) activity in *Lysinibacillus*, *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus subtilis* bio-augmented groups. Laminarin induced PO activity was observed in groups supplemented with *Oceanobacillus sp.*, *Bacillus sp.*and *Bacillus megaterium*. The lysozyme (LZ) activity was significantly ($P < 0.05$) higher in *B. cereus* and Microbial Consortia (MC), while other treatments were less effective. Total hemocyte count (THC) significantly ($P < 0.05$) increased in all treatment groups compared to the control. Hyaline hemocyte (HH) count was significantly ($P < 0.05$) higher in the control group (14.43%). Semi granular hemocytes (SGH) was higher in groups treated with *Lysinibacillus, Bacillus sp., B. licheniformis* and *B. subtilis.* The granular hemocyte (GH) count was significantly ($P < 0.05$) higher in *Virgibacillus sp.*, *B. cereus*, *B.megaterium* and *Oceanobacillus sp.* The biofloc alone (BF), treated and augmented with *B. megaterium* significantly ($P < 0.05$) increased phagocytic activity. Highly significant phagocytic index (PI) was observed in bio-augmented groups, BF and MC. The relative expression levels of immune genes were found to be significantly up-regulated in shrimps grown in bio-augmented groups. Enhanced immunological parameters implies that bio-augmentation of biofloc with *Bacillus spp.* improved immunity in shrimps. Hence, bio-augmentation of probiotics in biofloc may be useful in improving culture conditions to produce *P. indicus.*

## 1. Introduction

In an aquatic environment, microbial community play an essential role in aquatic animal health, since microbes respond quickly to environmental changes. Probiotics are living microbial supplements that provides a healthy environment to the host by modifying the host-associated or ambient microbial community [1]. These changes can be subtle and manifested as activation or inactivation of specific metabolic pathways in bacterial community or change in their composition and functionality. The same changes happen in an aquaculture production system where diverse microorganisms can act positively in the transformation of harmful organic waste into beneficial products. Beneficial effects of probiotics are not only limited to environment manipulation but also provide additional health benefits such as improved immunity and enhanced nutrition through production of supplemental digestive enzymes, an eco-friendly alternative to combat disease [1–5]. Bio-augmented zero water exchange aquaculture systems are dominated by ammonia-oxidizing archaeal communities [6]. Hence it appears to have a beneficial effect on the reduction of ammonia in aquaculture systems.

The crustacean immune system is mostly dependent on nonspecific immunity for internal defense against parasites and pathogenic microbes as they do not possess specific immunity. Hence it is difficult to develop vaccines for crustacean diseases [7,8]. Therefore, as an alternative, probiotic bacteria could be utilised to control infectious diseases not only in shrimps but also in other farmed aquatic species too [1]. Previous reports suggest that dietary administration of *Psychrobacter sp.,* [9] *Lactobacillus plantarum* [10,11] and *Bacillus subtilis* [12–14] improved the feed utilization, enzyme activity, immune response and

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growth either in fishes or shellfishes.The ability of *Bacillus* species to produce an array of extracellular substances and antimicrobial peptides against aquatic microorganisms is well documented [15]. The *Bacillus spp.* increase survival, growth, and stimulate the digestive system in Indian white shrimp *Penaeus indicus* [16].

Biofloc based aquaculture is gaining importance recently due to several advantages. Being a zero or minimal water exchange system, Biofloc technology (BFT) provides biosecurity besides improved water quality through the self-generated bioremediation process and improved growth and immunity in shrimps through diverse heterotrophic bacterial community. In addition to this, biofloc also serves as a highly nutrient food source, rich in amino acids, proteins, fatty acids and lipids in the form of different microorganisms, substantially reducing external feed supply to make it more economical. The diverse microbial community of the flocs acts as consumers of dissolved oxygen, nutrient recyclers and enhance the secondary productivity [17–21]. The composition, structure, and stability of bioflocs are defined by the ratio of organic carbon sources (molasses, corn, wheat, glucose acetate, glycerol, and tapioca) used for generating the floc. The nutrient profile of biofloc depends mostly on the carbon-nitrogen ratio [21–24]. In addition, through the balanced addition of carbon, biofloc aids in the removal of nitrogen from the culture water [23]. However, the added value that bioflocs bring to the aquaculture systems requires detailed studies.

The objective of the present study was to investigate the effect of bio-augmentation of selected bacterial isolates in the biofloc system on growth, survival and immune parameters of *Penaeus indicus.* Accordingly, nine different probiotic strains separately as well as in a consortium along with manipulation of carbon source, was evaluated. Bacterial probiotics used in this study comprised of *Bacillus* group (*Virgibacillus sp*., *Oceanobacillus sp*., *Bacillus sp., Bacillus megaterium, Bacillus marisflavi, Lysinibacillus, Bacillus cereus, Bacillus licheniformis,* and *Bacilus subtilis* strains.

## 2. Materials and methods

### 2.1. Bacterial strains

Bacterial strains used in this study were isolated from experimental Biofloc shrimp ponds at the Muttukadu Experimental Stations of ICAR-Central Institute of Brackishwater Aquaculture, Chennai. Accordingly, nine bacterial species belonging to genus *Bacillus* were isolated in Zobell Marine Agar, characterized and stock cultures were stored at −80 °C till use. Mass culture of bacterial strains was carried out in 1 L flasks using Zobell marine broth and the cultures were incubated in a shaker incubator for 48 h at 28 °C. Subsequently the cultures were harvested by centrifuging at 5000 g for 10 min and resuspending in sterile sea water so as to obtain a cell density of $5.4 \times 10^9$ CFU/ml.

Bacterial cultures were identified through 16S rDNA sequencing. The DNA was extracted using water DNA isolation kit (HiMedia, India) following manufacturer's protocol. The PCR amplification was performed using Ampliqon-Taq DNA Polymerase Master Mix (Ampliqon, Denmark). Universal bacterial 16S rDNA primers 27F (5′-AGAGTTTG ATCMTGGCTCAG-3') and 1492R (5′-TACGGYTACCTTGTTACGA-CTT-3′) were used to amplify the DNA. The PCR amplification was performed in VeritiTM Thermal Cycler (Applied Biosystems, USA). The reaction conditions are as follows: an initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. The amplification products were separated by electrophoresis on a 1% agarose gel and purified using NucleoSpin® Gel and PCR Clean-Up (Takara, Japan) according to manufacturer’s protocol. The purified PCR products were sequenced and analyzed. The nucleotide sequences were compared with the NCBI data base and sequences were deposited in the NCBI.

### 2.2. Experimental design

The experiments were conducted at Muttukadu Experimental Station of ICAR-CIBA for 90 days. Experimental tanks of 1-ton capacity Fiber Reinforced Plastic (FRP) were utilised. All the tanks were covered with protective sheets to prevent the escape of animals. Initially, the tanks were filled with sand filtered and pre-chlorinated seawater (30 ppt). During the course of the experiment, 30% of water was exchanged on a weekly basis.

### 2.3. Generation of biofloc

Prior to start of the experiment, biofloc conditions were generated with a C: N ratio of 12:1 in all the tanks. Initially, the tanks were treated with agricultural lime (CaCO3) at the rate of 20 ppm, inorganic fertilizers (urea: 20 ppm) and single superphosphate (15 ppm) to develop the system autotrophically. This was followed by addition of carbohydrate juice fermented with isolated bacterial probiotics for 24 h in a 10 L container. In this study, a blend of carbon sources (CHO) [24] comprising molasses, wheat flour, rice, corn, refined wheat, flour and flour of finger millet and gram were used as CHO.Two grams of each carbon source was mixed togather to make a slurry. The resultant slurray was soaked overnight with 50 ml of respective probiotics ($5.4 \times 10^9$ CFU/ml) prepared in 5 L of autoclaved seawater. Next day, the juice was filtered through a 100 μm mesh hand net and added to the respective experimental tank twice daily (10.00 a.m. and 3.00 p.m.) at 0.3 g/1 g feed. A control group was maintained in an autotrophic manner by developing the bloom using the aforementioned fertilizers. The biofloc control contained the carbon source without the addition of probiotics. To maintain 6–8 ppm oxygen level, continuous aeration was provided from a 5HP blower at 7.5 $m^3$ air/tank/minute. Once a fortnight, the sludge was removed and 30% water exchange was carried out in biofloc treatments, whereas in controls, 50% of water was exchanged once a week. A total of 12 treatments were maintained in triplicate. The treatments included: Control, BF: Biofloc stand alone (only carbon sources added), MC: BFT and a consortium of nine bacterial strains, 1: BFT and *Virgibacillus sp.*, 2: BFT and *Oceanobacillus sp.,* 3: BFT and *Bacillus sp.*, 4: BFT and *B. megaterium*, 5: BFT and *B. marisflavi*, 6: BFT and *Lysinibacillus,* 7: BFT and *B. cereus*, 8: BFT and *B. licheniformis* and 9: BFT and *B. subtilis* strain.

### 2.4. Animal stocking and feed management

Specific pathogen free (SPF) *Penaeus indicus* postlarvae (PL-15) were initially maintained in the hatchery for a fortnight after which they weighed on an average 0.15 g. Subsequently, experimental tanks (1000 L working volume) were stocked with *P.indicus* juveniles with a stocking density of 300 shrimps/tank. Shrimps were fed with formulated pellet feed containing 35% of crude protein (CP) at 10–12% of body weight/day, and gradually reduced to 3% towards the end of the experiment.

### 2.5. Analysis of water quality parameters

Water quality parameters viz. temperature, pH (using pH-Scan-Eutech instruments, Singapore), salinity (using hand refractometer), total ammonia nitrogen (TAN:Phenol hypochlorite method), $NO_2$–N, $NO_3$–N, phosphate- P ($PO_4$–P), total alkalinity, turbidity and dissolved oxygen were recorded on a weekly basis, following standard procedures. Total suspended solids was determined once a fortnight and biofloc volume was quantified on a daily basis using Imhoff cone.

### 2.6. Biometry and sampling

Every week, 50 shrimps from each treatment were sampled for recording growth. Accordingly, growth traits viz. length, weight gain (%),

feed efficiency ratio (FER), feed conversion ratio (FCR), and specific growth rate (%) (SGR) were computed as follows:

Weight gain (%) = ((FW-IW)/IW)*100

FCR = Feed given (DW)/bodyweight gain (WW),

FER = 1/FCR, SGR (%) = [ln (FW) − ln (IW)/N] × 100.

Where, FW = final weight, IW = initial weight, DW = dry weight, WW = wet weight, ln = natural log and N = days of culture.

### 2.7. Immunological parameters

Five shrimps from each tank were sampled and hemolymph was collected and used for assaying total protein, phenoloxidase activity, lysozyme activity, total hemocyte count, differential hemocyte counts and phagocytic index. Hemolymph (500 µl) was collected without anticoagulant to analyse total protein, phenoloxidase (PO) and lysozyme activity. The pooled hemolymph samples was allowed to clot at −80 °C for 6 h and centrifuged at 12,000 rpm for 30 min at 4 °C, and serum (supernatant) was collected and stored at −80 °C until assayed. The total serum protein concentration was determined following the method of [25] and using bovine serum albumin as a standard, the phenoloxidase activity was measured. Briefly, 25 µl of serum was preincubated with 25 µl of either trypsin (5 mg/ml) or laminarin (5 mg/ml), and 1 ml of 5 mM L-DOPA (Sigma, St. Louis, MO, USA) was added and incubated for 20 min at 25 °C. The optical density was measured spectrophotometrically at 490 nm, and the results were expressed in PO activity (Optical density) OD at 490 nm.

A turbidometric assay was performed to asses Lysozyme (LZ) activity. Briefly, a standard suspension of 0.2 mg.ml-1 *Micrococcus lysodeikticus* (Sigma Aldrich, St Louis, MO) cells (175 µ1) suspended in potassium phosphate buffer (50 mM; pH 6.5) was added to 25 µ1 of serum. The reaction was carried out at room temperature for 15 min, and the absorbance at 450 nm was recorded in a microplate reader (Biorad, 680) and the results expressed as LZ activity OD at 450 nm.

About 100 µl of hemolymph sample from each shrimp was rapidly withdrawn from the heart using a 2 ml sterile polypropylene syringe containing 1.9 ml of ice-cold cysteine anticoagulant buffer (10 mM HEPES, 10 mM KCl, 365 mM NaCl, 1 mM cysteine (free-base), pH 7.2, 780 mOsm) and used for total hemocyte count and differential hemocyte count (DHC) including hyaline hemocytes (HHC), semigranular hemocytes (SGHC) and granular hemocytes (GHC). The hemolymph and buffer were mixed by shaking the syringe gently. From this, a drop of hemolymph was placed on neubauer hemocytometer and the cells were counted on a phase-contrast microscope (Nikon ECLIPSE E200) at 20X magnification. Similarly, DHC, HHC, SGHC, and GHC were counted at 40X magnification, and the results are expressed as cells ml.1-1.

For the phagocytic assay, about 100 µl of hemolymph collected in 1.9 ml of ice-cold cysteine anticoagulant buffer was spread on an alcohol-washed, clean, dry glass slide over an area of 2 $cm^2$ and incubated in a moist chamber for 10 min at 23 °C to obtain hemocyte monolayer (50 µl). Human A blood group collected in Alsever's medium was fixed in glutaraldehyde. The phagocytosis assay was conducted through the phagocytosis of human A erythrocyte in three hemocyte monolayers/sample. The first and second pair of monolayers were overlaid with 50 µl human A erythrocyte (0.05%) and observed at 5 min interval for 1 h under a light microscope at 40X magnification. The mean of 5 readings was taken per sample, and the phagocytic activity was expressed as relative engulfed human A erythrocytes per hemocyte, according to Ref. [26] as follows:

Percentage phagocytosis = No. of phagocytic hemocytes/Total no. of hemocytes × 100

% PI (Phagocytic Index) = (No. of hemocytes ingesting Human A (red blood cells) RBCs/no. of hemocytes observed) × (no. of Human A RBCs ingested/no. of hemocytes observed) × 100

### 2.8. Gene expression analysis by qRT-PCR

Transcript levels of differentially expressed immune genes in experimental animals were analyzed after Panigrahi et al., 2019. Once the experiment was complete, six shrimps were randomly sampled from each experimental tank, hepatopancreas tissue was dissected out, pooled and preserved in *RNAlater* and stored at $-20^{O}$C till further use. Total RNA was isolated using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma, USA) according to the manufacturer's protocol. The cDNA synthesis was performed using the Verso cDNA Synthesis Kit (Thermofisher, USA). Details regarding oligonucleotide primer sequences used for immune gene expression analysis are summarized in Table 3. Real-time amplification of immune genes, melt curve analysis and fold change analysis and calculation of gene expression was performed on a thermal cycler (Applied Biosystem's Real-Time PCR system Step One Plus®) employing SYBR Green chemistry. Briefly, the temperature cycling parameters included initial holding stage of 10 min at 95 °C, 45 cycles of denaturation at 95 °C for 00.15 s and isothermal annealing and extension at 60 °C for 1 min. At the end of each cycle, melt curve analysis (60–95 °C) was carried out to ensure specificity of the primer. A 20 µL PCR reaction was prepared with each tube containing 10 µL of 2X SYBR® Green qPCR master mix (Bio-Rad, USA), 1 µL of each forward and reverse primers (10 pmol), 1 µL of template DNA (30–60 ng) and 7 µL of PCR grade water. All the samples were analyzed in triplicate, and the relative expression was calculated by the comparative threshold value (CT) and (2−ΔΔCT method).

### 2.9. Statistical analysis

The descriptive statistics have been depicted in the form of means and their standard errors. The statistical analysis was carried out using the software SPSS 17.0. Analyses of variance was carried out on survival, length, body weight, water quality and immunomodulatory parameters and the level of significance was taken as $P < 0.05$.

## 3. Results

### 3.1. Molecular identification

Nine bacterial strains belonging to *Bacillus* genera isolated from Biofloc solid decanter were identified through 16S rDNA sequencing as *Virgibacillus sp.* MK966348, *Oceanobacillus sp*. MK966362, *Bacillus sp.* MK966345, *Bacillus megaterium* MK966368, *Bacillus marisflavi* MK966343, *Lysinibacillus* MK966358, *Bacillus cereus* MK966367, *Bacillus licheniformis* MK966350 and *Bacilus subtilis* MK966347strains.

### 3.2. Water quality parameters

There was no significant difference in salinity and temperature between treatments and control. The pH significantly decreased in biofloc treatments compared to control. Similarly, TDS and TSS values significantly increased in biofloc and biofloc supplemented with probiotics groups. However, TAN, $NO_2$–N and $NO_3$–N were significantly higher in the control group compared to those in the treatments (Table 1).

### 3.3. Floc volume

Floc volume was calculated once in 10 days. The floc volume never exceeded 30 mL/L as shown in the graph. Higher floc volume was observed in biofloc and biofloc supplemented with probiotic groups (Fig. 1).

**Table 1**
Water quality parameters expressed in means ± standard error.

| Water quality parameters | Treatments | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | BF | MC | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Salinity | 29.2 ± 1.4 | 29.2 ± 1.4 | 29.07 ± 1.3 | 29.01 ± 1.3 | 29.2 ± 1.3 | 29.3 ± 1.35 | 29.3 ± 1.34 | 29.38 ± 1.24 | 29.5 ± 1.2 | 29.3 ± 1.23 | 29.15 ± 1.4 | 29.1 ± 1.24 |
| Temperature | 29.23 | 30.2 ± 1.09 | 29.5 ± 1.34 | 30 ± 1.07 | 29.5 ± 1.2 | 29.2 ± 1.4 | 30.2 ± 1.21 | 29.5 ± 1.5 | 30.5 ± 1.4 | 30.2 ± 1.4 | 29.5 ± 1.21 | 29.5 ± 1.03 |
| pH | 8.16 ± 1.36 | 8.13 ± 0.3 | 8.11 ± 0.3 | 8.09 ± 0.39 | 8.13 ± 0.39 | 8.14 ± 0.39 | 8.12 ± 0.38 | 8.11 ± 0.37 | 8.11 ± 0.37 | 8.10 ± 0.38 | 8.13 ± 0.38 | 8.12 ± 0.38 |
| EC | 30.22 ± 3.56 | 40.00 ± 3.41 | 38.24 ± 5.7 | 43.66 ± 7.19 | 39.04 ± 6.19 | 42.61 ± 7.54 | 44.07 ± 5.06 | 39.04 ± 5.88 | 42.26 ± 1.48 | 36.55 ± 6.18 | 35.14 ± 6.31 | 37.26 ± 6.11 |
| TSS | 65.76 ± 6.55 | 154.89 ± 10.6 | 160.87 ± 11 | 163.155 ± 14 | 155.73 ± 9.43 | 160.34 ± 10.94 | 156.39 ± 11.82 | 162.19 ± 9.0 | 151.93 ± 5.12 | 159.91 ± 11 | 159.82 ± 10 | 160.66 ± 9.53 |
| TDS | 9.32 ± 1.55 | 13.78 ± 3.55 | 12.48 ± 2.3 | 12.94 ± 3.09 | 12.04 ± 2.97 | 12.15 ± 2.87 | 12.09 ± 3.01 | 11.87 ± 2.68 | 11.82 ± 2.89 | 11.90 ± 2.75 | 12.039 ± 2.6 | 12.18 ± 2.73 |
| TAN | 1.27 ± 668 | 0.239 ± 0.07 | 0.251 ± 0.1 | 0.275 ± 0.17 | 0.300 ± 0.14 | 0.283 ± 668 | 0.410 ± 0.19 | 0.368 ± 0.10 | 0.331 ± 0.14 | 0.393 ± 0.31 | 0.311 ± 0.23 | 0.38 ± 0.19 |
| $NO_2^-$ | 0.226 ± 0.09 | 0.132 ± 0.05 | 0.122 ± 0.02 | 0.114 ± 0.03 | 0.200 ± 0.010 | 0.146 ± 0.08 | 0.145 ± 0.08 | 0.155 ± 0.09 | 0.121 ± 0.04 | 0.139 ± 0.02 | 0.146 ± 0.09 | 0.135 ± 0.05 |
| $NO_3^-$ | 0.76 ± 0.89 | 0.86 ± 0.96 | 0.83 ± 0.89 | 0.81 ± 0.28 | 1.12 ± 0.29 | 0.955 ± 0.89 | 0.76 ± 0.09 | 0.85 ± 0.078 | 1.22 ± 0.89 | 0.898 ± 0.23 | 0.812 ± 0.34 | 0.981 ± 0.123 |
| PO4 | 0.39 ± 0.10 | 0.47 ± 0.16 | 0.50 ± 0.13 | 0.51 ± 0.16 | 0.49 ± 0.15 | 0.51 ± 0.12 | 0.45 ± 0.13 | 0.54 ± 0.13 | 0.55 ± 0.16 | 0.53 ± 0.12 | 0.55 ± 0.12 | 0.54 ± 0.10 |
| DO | 6.7 ± 0.5 | 5.8 ± 0.78 | 5.8 ± 0.5 | 5.7 ± 0.4 | 5.9 ± 0.75 | 5.76 ± 0.51 | 5.91 ± 0.57 | 5.43 ± 0.55 | 5.98 ± 0.57 | 5.6 ± 0.54 | 5.83 ± 0.62 | 5.56 ± 0.37 |
| COD | 33.39 ± 7.19 | 41.48 ± 7.70 | 49.11 ± 6.8 | 43.53 ± 9.26 | 46.72 ± 9.84 | 46.25 ± 10.11 | 42.84 ± 9.43 | 43.28 ± 10.8 | 40.78 ± 10.08 | 46.88 ± 7.77 | 44.75 ± | 47.66 ± 9.43 |
| Total Alkalinity | 153.12 ± 9.06 | 127.87 ± 8.45 | 131.25 ± 9 | 130.87 ± 7.7 | 128.75 ± 8.71 | 129.12 ± 8.70 | 133.75 ± 11.8 | 133.125 ± 14 | 129.12 ± 9.06 | 132.25 ± 9.1 | 128.25 ± 9.3 | 134.37 ± 12.5 |
| Biofloc volume | 4.99 ± 2.97 | 5.73 ± 3.44 | 4.73 ± 2.7 | 4.56 ± 3.02 | 4.78 ± 2.88 | 4.33 ± 2.84 | 4.37 ± 2.35 | 4.01 ± 2.86 | 4.67 ± 2.87 | 4.63 ± 3.01 | 4.597 ± 1.22 | 4.56 ± 2.33 |

**EC:** Electrical conductivity, **TSS:** Total suspended solids, **TDS:** Total dissolved solids, **TAN:** Total amoniacal nitrogen, **NO2⁻:** Nitrite, **NO3⁻:** Nitrate, **PO4:** Phosphate, **DO:** Dissolved oxygen, **COD:** Chemical oxygen demand, **BF:** Biofloc alone, **MC:** BFT supplemented with a consortia of 9 probiotic bacteria **and 1–9:** BFT supplemented with *Virgibacillus sp., Oceanobacillus sp., Bacillus sp., B. megaterium, B. marisflavi, Lysinibacillus, B. cereus, B. licheniformis,* and *B. subtilis.*

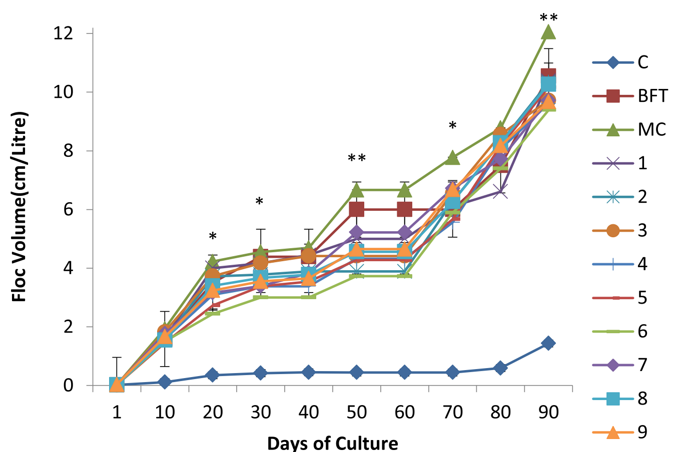


**Fig. 1. The floc volume calculated during the experiment:** C: Control, BF: biofloc, MC: BFT supplemented with consortia of 9 probiotic bacteria and 1–9: BFT supplemented with *Virgibacillus sp., Oceanobacillus sp., Bacillus sp., B. megaterium, B. marisflavi, Lysinibacillus, B. cereus, B. licheniformis,* and *B. subtilis* strain respectively. Data represents mean ± SE of 3 samples. Asterisk indicates statistical significance compared to control group (*p < 0.05).

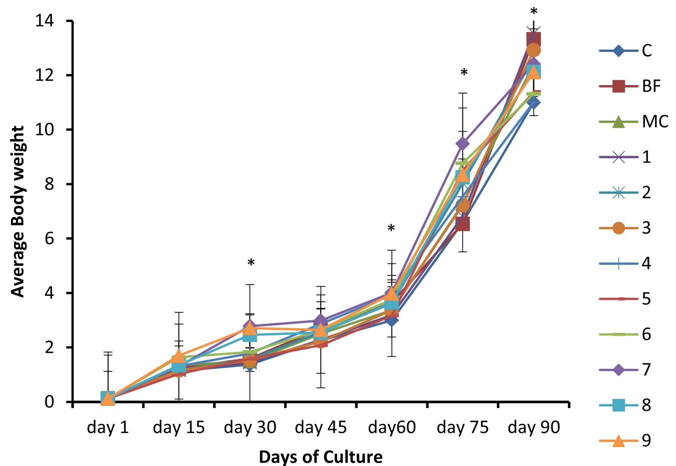


**Fig. 2. Growth *of Penaeus indicus* reared in different probiotics and Biofloc:** C: control, BF: biofloc, MC: BFT supplemented with Consortia of 9 probiotic bacteria and 1–9: BFT supplemented with *Virgibacillus sp., Oceanobacillus sp., Bacillus sp., B. megaterium, B. marisflavi, Lysinibacillus, B. cereus, B. licheniformis,* and *B. subtilis* strain respectively. Data represents mean ± SE of 3 samples. Asterisk indicates statistical significance compared to control group (*p < 0.05).

### *3.4. Growth and survival*

The average body weight (ABW) and average weight gain (Fig. 2 and Table 2) were found to be higher in BFT and BFT supplemented with the probiotic group. Survival was found to be higher in tanks treated with biofloc supplemented with customized probiotics and biofloc standalone tanks compared to that of control, The CHO supplementation resulted in significantly ($P < 0.05$) higher survival compared to the control. The survival computed at the end of the experiment ranged from 81 to 94% (Table 2). The biofloc supplemented with a consortium of nine probiotics exhibited the maximum survival of 94%. The groups with BFT, BFT supplemented with probiotics showed significantly ($P < 0.05$) higher survival compared to the control (Fig. 3).

### *3.5. Protein quantification*

The amount of total serum protein was estimated in BF, MC and BFT

**Table 3**
Oligonucleotide primers used for gene expression analysis.

| SI. No. | Target | Sequence | Product size |
|---|---|---|---|
| 1 | Transglutaminase | 5′- TTCACAAGCCTGACATCACC-3′<br>5′-GCAGCAGTGGGATAGGGTTA-3′ | 99 BP |
| 2 | Prophenoloxidase | 5′-TTCCAGCTCTTCTTCATGCT-3′<br>5′-TCGGGGTACTTGGCGTCCTG-3′ | 116 BP<br>243 BP |
| 3 | β-actin | 5′-CAACCGCGAGAAGATGACAC-3′<br>5′-TCGGTCAGGATCTTCATCAGG -3′ | |

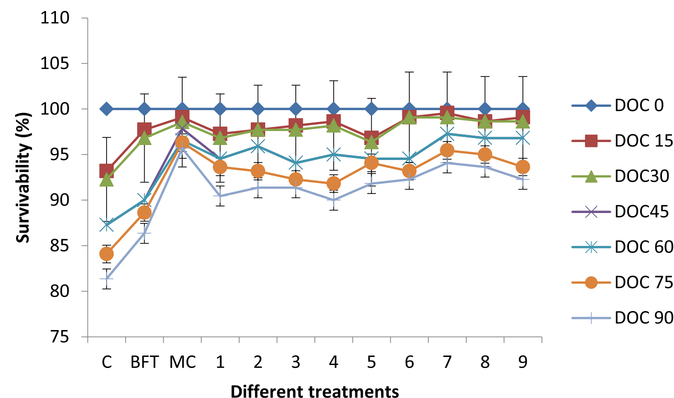


**Fig. 3. Percentage survival during the culture period:** C: control, BF: biofloc, MC: BFT supplemented with Consortia of 9 probiotic bacteria and 1–9: BFT supplemented with *Virgibacillus sp., Oceanobacillus sp., Bacillus sp., B. megaterium, B. marisflavi, Lysinibacillus, B. cereus, B. licheniformis,* and *B. subtilis* strain respectively. DOC indicate day of culture. Data represents mean ± SE of 3 samples. Asterisk indicates statistical significance compared to control group (*p < 0.05).

supplemented with nine different probiotic bacteria. Among them, BFT supplemented with *B. licheniformis* (287.21 mg) showed significant (P < 0.05) increase in the protein level compared to the control (275.29 mg). This was followed by BFT supplemented with *B. subtilis* (284.09 mg), *Oceanobacillus sp.* (281.99 mg), BF (281.65 mg) and *Virgibacillus sp.* (281.30 mg). On comparison with BF *B. licheniformis* and *B. subtilis* produced significantly higher serum protein level (Fig. 4A).

### 3.6. Phenoloxidase (PO) activity

The serum PO activity in shrimps showed significant variation between the treatments and control. The serum PO activity on trypsin in tests and control was highly significant (P < 0.05), whereas, it was in the following order in *B. subtilis* (0.914), *Lysinibacillus* (0.896), *B. cereus* (0.888) and *B. licheniformis* (0.816) when compared to control (0.650). All other treatments showed reduced PO activity. Similarly, the laminarin significantly (P < 0.05) enhanced the PO activity in *B. megaterium*, (0.705) followed by *Bacillus sp.* (0.699), *Oceanobacillus sp.* (0.681) and *B. subtilis* strains (0.635) compared to control (0.555). The other probiotic bacterial treatments too enhanced PO activity but not significantly. Compared to Biofloc alone (BF), *Lysinibacillus, B. cereus, B. licheniformis,* and *B. subtilis* produced significant increase in trypsin PO activity. However, laminarin induced PO activity was not significant (Fig. 4B).

### 3.7. Lysozyme activity

The results indicated high levels of lysozyme activity in shrimps supplemented with *B. cereus* (37.92%) followed by *B. licheniformis* (30.82%) *B. subtilis* strain (27.24) and *Lysinibacillus* (26.02%) compared to the control. On the other hand, a low activity level (12.69–22.91%) was observed in other treatments. *B. megaterium, Lysinibacillus, B.*

**Table 2**
Growth parameters observed during the culture period (a total of 300 shrimp stocked in all treatments including control). The results are represented as mean ± standard error.

| Growth parameters | Treatments | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | BF | MC | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| survival (%) | 81 ± 2.55 | 92 ± 3.0 | 94 ± 1.5 | 86 ± 2.5 | 90 ± 1.6 | 92 ± 2.6 | 91 ± 1.6 | 89 ± 3.5 | 93 ± 2.6 | 90 ± 1.5 | 86 ± 2.5 | 89 ± 2.5 |
| Initial weight(g) | 0.13 ± 0.02 | 0.12 ± 0.014 | 0.12 ± 0.014 | 0.12 ± 0.01 | 0.12 ± 0.01 | 0.12 ± 0.014 | 0.12 ± 0.01 | 0.12 ± 0.014 | 0.12 ± 0.014 | 0.12 ± 0.01 | 0.12 ± 0.014 | 0.12 ± 0.014 |
| Final weight(g) | 10.92 ± 1.3 | 13.31 ± 2.65 | 14.56 ± 2.88 | 13.53 ± 1.21 | 12.64 ± 1.35 | 12.92 ± 1.5 | 11.0 ± 1.2 | 11.37 ± 1.97 | 11.31 ± 1.35 | 12.41 ± 1.8 | 12.12 ± 1.35 | 12.10 ± 1.86 |
| Weight gain(g) | 10.77 ± 0.8 | 13.16 ± 1.2 | 14.35 ± 0.98 | 13.38 ± 0.85 | 12.49 ± 1.23 | 12.77 ± 0.75 | 10.85 ± 1.25 | 11.21 ± 0.86 | 11.16 ± 0.25 | 12.26 ± 1.2 | 11.97 ± 1.21 | 11.95 ± 1.22 |
| Total biomass produced(kg) | 2.6 | 3.6 | 4.1 | 3.48 | 3.41 | 3.56 | 3.0 | 3.03 | 3.15 | 3.35 | 3.12 | 3.23 |
| Total feed given (kg) | 5.5 | 5 | 4.8 | 5 | 5.2 | 5 | 4.5 | 4.8 | 5 | 4.8 | 4.5 | 4.8 |
| FCR | 1.6 | 1.38 | 1.37 | 1.43 | 1.52 | 1.4 | 1.5 | 1.58 | 1.5 | 1.45 | 1.45 | 1.5 |
| ADG (g) | 0.119 | 0.146 | 0.15 | 0.148 | 0.138 | 0.141 | 0.12 | 0.124 | 0.123 | 0.136 | 0.133 | 0.132 |

**FCR:** Feed conversion ratio, **ADG:** Average daily growth, **BF:** Biofloc alone, **MC:** BFT supplemented with a consortia of 9 probiotic bacteria and **1–9:** BFT supplemented with *Virgibacillus sp., Oceanobacillus sp., Bacillus sp., Bacillus megaterium, B. marisflavi, Lysinibacillus, B. cereus, B. licheniformis,* and *B. subtilis.*

 

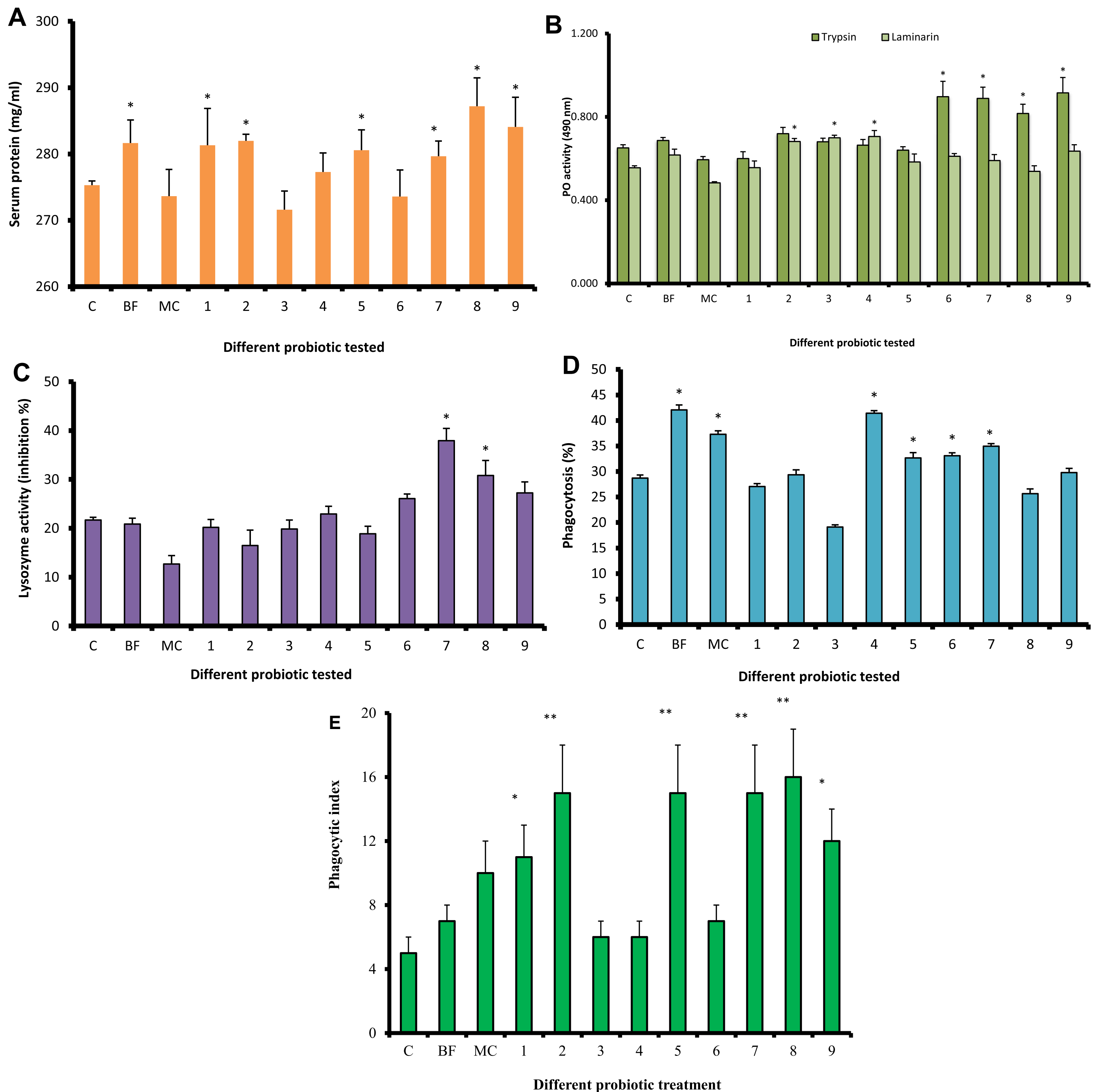


**Fig. 4. A) Serum protein levels B) ProPO activity of serum C) Lysozyme activity D) Phagocytosis % E) Phagocytic index of hemolymph collected from different treatments.** C: control, BF: biofloc, MC: BFT supplemented with a consortia of 9 probiotic bacteria and 1–9: BFT supplemented with *Virgibacillus sp., Oceanobacillus sp., Bacillus sp., B. megaterium, B. marisflavi, Lysinibacillus, B. cereus, B. licheniformis,* and *B. subtilis* strain respectively. Data represents mean ± SD of 3 samples. Asterisk indicates statistical significance compared to control group (*p < 0.05).

*cereus, B. licheniformis,* and *B. subtilis* produced enhanced lysozyme activity than that of BF (Fig. 4C).

### 3.8. In vitro phagocytosis and phagocytic index

The highest percentage of phagocytic effect was evident in BF (42.07%) and *B. megaterium* treatment (41.43%) followed by MC (37.29%) and *B. cereus* (34.95%) compared to control (32.02%). The other probiotic supplemented groups when compared to control did not show any increase in phagocytic activity. When assessing phagocytic index, it was found that *B. licheniformis*, *Oceanobacillus sp.*, *B. marisflavi* and *B. cereus* treatment groups engulfed the maximum number of RBCs (15–16) whereas all other treatments showed lower RBC engulfing property. On comparison with BF, phagocytic index was higher in MC, *Virgibacillus sp., Oceanobacillus sp., B. marisflavi, B. cereus, B. licheniformis,* and *B. subtilis* (Fig. 4D and E).

### 3.9. Total and differential hemocyte count

At the end of the experiment, both in probiotic supplemented BFT and non-supplemented BF groups; there was a significant ($P < 0.05$) increase in total hemocyte count (THC) compared to control. The

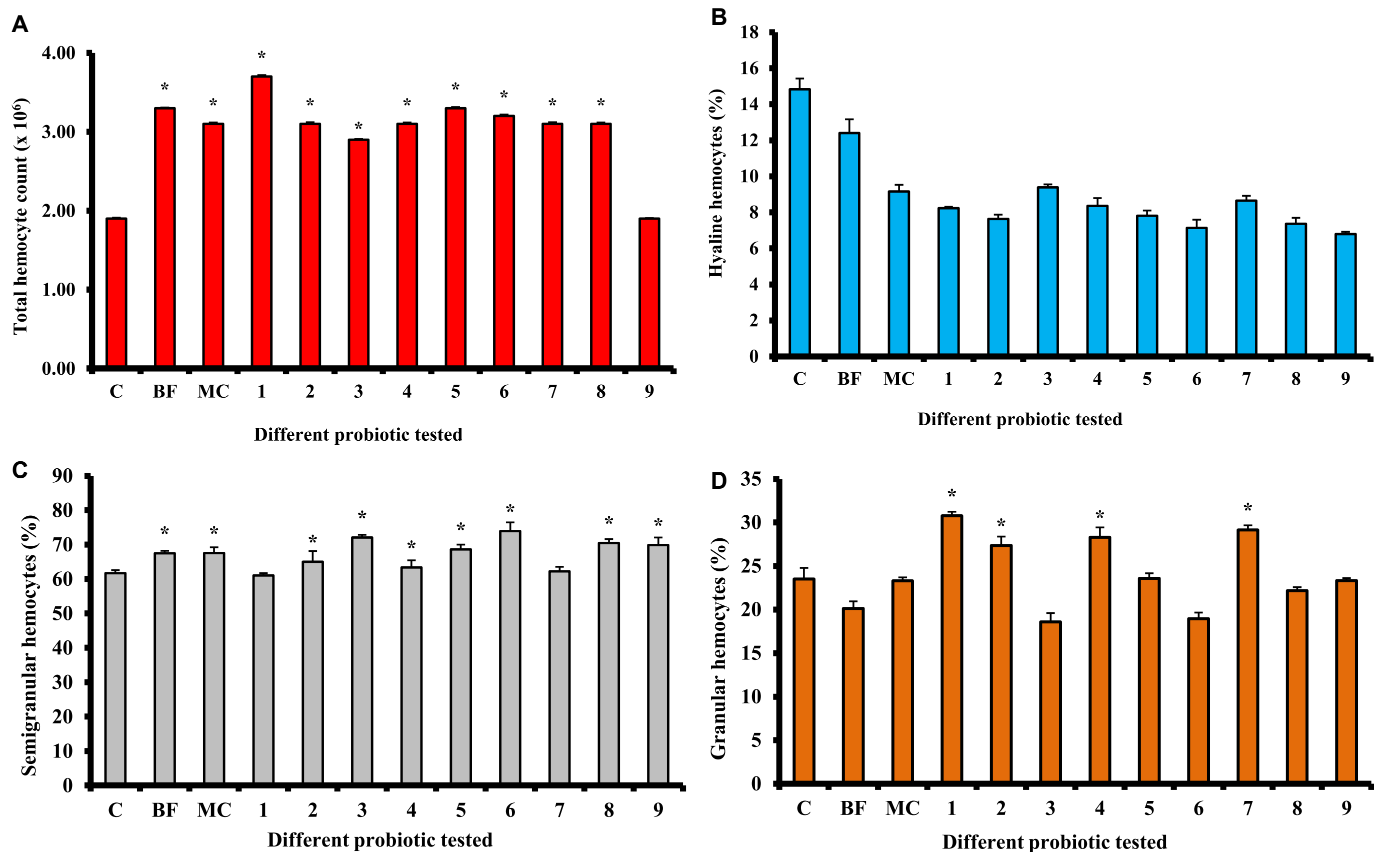


**Fig. 5. A) Total hemocytes count B) Differential hemocyte count of hyaline hemocytes C) Differential hemocyte count of semi granular hemocytes (SGH) D) Differential hemocyte count of granular hemocytes in shrimp hemolymph collected from different treatments.** C: control, BF: biofloc, MC: BFT supplemented with a consortia of 9 probiotic bacteria and 1–9: BFT supplemented with *Virgibacillus sp., Oceanobacillus sp., Bacillus sp., B. megaterium, B. marisflavi, Lysinibacillus, B. cereus, B. licheniformis,* and *B. subtilis* strain respectively. Data represents mean ± SD of 3 samples. Asterisk indicates statistical significance compared to control group (*p < 0.05).

highest THC values were observed in *Virgibacillus sp.* supplemented groups (3.7 × 10$^6$) compared to control and BF (1.9 × 10$^6$). However, the THC value of *B. subtilis* strain did not differ from that in the control. The percentage of SGH and GH in the control groups were 61.67 and 23.50% respectively. Similarly, among treatments, *B. cereus* (73.92%) followed by *Bacillus sp.* (72.02%), *B. licheniformis* (70.45%) and *B. subtilis* strains (69.88%) showed significantly ($P < 0.05$) higher percentage of SGH compared to control. Percentage of Hyaline hemocte count (HC) was lowest in all treated groups except control and BF. The lowest percentage of SGH was found in *B. cereus* supplemented group. However, the rest of the probiotic treatments showed a slight level of increase in the SGH percentage. On the other hand, the GH count in test groups was significantly ($P < 0.05$) different when compared to the control. The highest percentage of GH count was in *Virgibacillus sp.* supplemented (30.76%) followed by *B. cereus* (29.15%), *B. megaterium* (28.31%) and *Oceanobacillus sp.* (27.36%). In all other probiotic bacterial treatments, GH did not increase significantly compared to control In BF treated groups, GH percentage was lower than control, MC, *Virgibacillus sp., Oceanobacillus sp., B. megaterium, B. marisflavi, B. cereus, B. licheniformis,* and *B. subtilis* (Fig. 5A, B, C, D).

### 3.10. Immune gene expression

Immune-related gene expression analysis through real-time PCR, revealed the upregulation in mRNA expression of transglutaminase genes and PPAE (ProPhenoloxidase activating enzyme) genes. There was a five-fold increase in transcript levels in *Virgibacillus sp.* supplemented treatment and six-fold increase in *B. megaterium* treatment, 5.6 fold increase in *B. cereus* treatment, all of which were significantly higher than the values in the control and BF. Both in control and BF groups, transglutaminase gene transcripts were lower than the other groups whereas MC and *Bacillus sp.* showed higher levels of PPAE gene transcripts than BF. The enhanced immune regulation in biofloc with probiotics was higher compared to that in the control (Fig. 6).

## 4. Discussion

The Indian white shrimp *P. indicus* (formerly *Fenneropenaeus indicus),* is one of the dominant commercial shrimp species exported worldwide. Biofloc technology is a modern aquaculture farming system practiced in many parts of the world to benefit the environment, drastically reducing effluent discharges, increasing biosecurity, optimizing feed management, intensifying production and ultimately the economy of shrimp farmers. Biofloc conditions provide a nutrient-rich food source to the cultured animals, thereby reducing the feed input and ultimately the cost of production. Biofloc is rich in amino acids, native proteins, fatty acids and lipids in the form of different microorganisms. The diverse microbial community of Biofloc not only provides supplemental nutrition, but also acts as nutrient recyclers and as a food source for the large spectrum of microbial community. The supplemented organic carbon sources such as molasses, corn, wheat, glucose acetate, glycerol and tapioca in optimum ratios determine the composition, structure, nutrient composition (carbohydrate, protein, lipid, and fatty acid), microbial community structure and stability of bioflocs [17,21,22,27–29].

Probiotics are extensively used in aquaculture [30] to enhance the growth and survival of shrimp [31]. Introduction of diets supplemented with *B. subtilis* and *Lactobacillus rhamnosus* improve PO, phagocytosis,

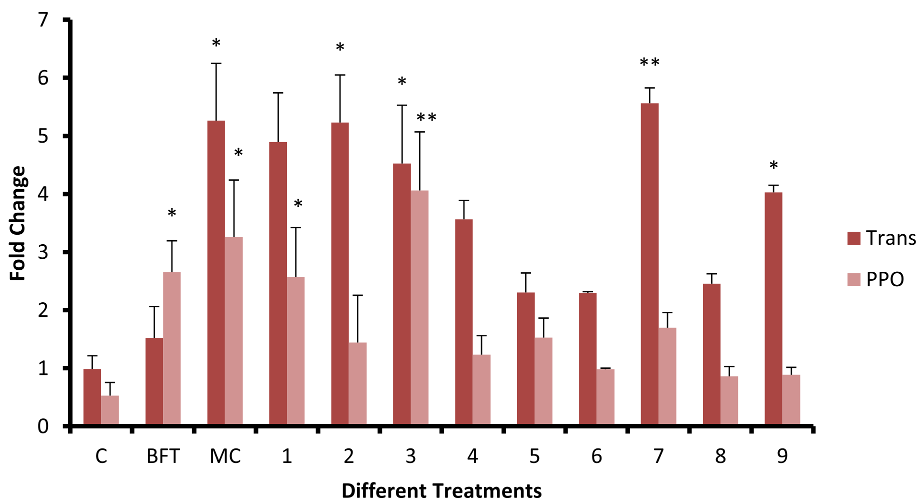


**Fig. 6. Comparative mRNA expression levels (fold change) of Transglutaminase genes and Prophenoloxidase activating enzyme (PPAE) gene in *P. indicus* under experimental conditions** C: control, BF: biofloc, MC: BFT supplemented with a consortia of 9 probiotic bacteria and 1–9: BFT supplemented with *Virgibacillus sp., Oceanobacillus sp., Bacillus sp., B. megaterium, B. marisflavi, Lysinibacillus, B. cereus, B. licheniformis* and *B. subtilis* strain respectively. Five individual shrimps were analyzed from the control and biofloc probiotic groups. Data represented are mean ± SD of gene expression in the different treatments. Asterisk indicates statistical significance compared to control group (*$p < 0.05$).

phagocytic index (PI) and inhibit the lysozyme activity in cultivable penaeid shrimps like *P. monodon* and *P. indicus* [32]. Bio-augmentation is a bioremediation strategy wherein beneficial microorganisms and/or metabolites are introduced to accelerate the removal of contaminants [33,34]. The present study was undertaken to ascertain the bio-augmentation potential of selected probiotic bacteria (*Virgibacillus sp., Oceanobacillus sp., Bacillus sp., B. megaterium, B. marisflavi, Lysinibacillus, B. cereus, B. licheniformis and B. subtilis* strains) under Biofloc rearing conditions on the growth, survival and immunity of *P.indicus*. Simultaneously, an assessment of the influence of bio-augmentation on key water quality parameters was also carried out.

It was observed that there was a reduction in pH, TAN, $NO_2$–N, $NO_3$–N, and increased TDS and TSS values in biofloc and biofloc supplemented with probiotics groups when compared to the control group, indicating an improvement in water quality. Adoption of biofloc technology could help in maintaining a pH of 7.5–8.4 and ammonia concentration under 1.1 mg $ml^{-1}$ [35]. Suita et al., 2015 [36] reported that floc generated with dextrose has a better effect on growth and culture water quality in *Litopenaeus vannamei*.

The results suggest that supplementation of probiotic bacteria in biofloc improved the survival of *P. indicus*. Similarly, average body weight and average weight gain were also found to be higher in BFT and BFT supplemented with the probiotic groups. Of the treatments, supplementation of BFT with consortia of 9 bacterial strains showed better survival and growth [37]. reported that the addition of *Bacillus sp., Lactobacillus,* and *Rhodobacter sp*. in biofloc could improve the growth of *Fennerpenaeus chinensis.* However in contrast [38], reported that addition of commercial probiotics in biofloc system was efficient in controlling vibriosis in *Litopenaeus vannamei*, but not in increasing growth. The findngs in our study are in agreement with those reported by Ref. [39] thereby confirming that significant improvement in growth and health of shrimp could be achieved through biofloc technology [40]. observed a 30% increase in growth and survival in *Litopenaeus vannamei* reared in biofloc [41]. reported higher growth in shrimps at a low culture density under biofloc culture condition through the addition of dextrose and molasses. Substitution of fish meal with biofloc flour could increase survival in *Litopenaeus vannamei* [42] thereby indicating the suitability of biofloc in improving the growth of shrimp and ultimately production.

Nonspecific Immune responses, such as phenoloxidase activity, lysozyme activity, total hemocyte count, differential hemocyte counts, and phagocytic index were analyzed in response to biofloc and biofloc supplemented with nine *Bacillus* strains. In this study, it was revealed that bio-augmentation of selected *Bacillus spp.* under Biofloc conditions aids in enhancing the shrimp innate immune responses such as PO enzyme, total and differential hemocyte count, phagocytosis and lysozyme activity. Several studies have shown that *Bacillus spp.* could be used as probiotic bacteria to enhance immunity and resistance to pathogens in aquatic animals [43,44] [20,32,45,10,14,46].

Analyses of hematological parameters revealed that biofloc and the addition of probiotic bacteria in a biofloc system could significantly increase the total hemocyte counts (THC) in *Penaeus indicus*. These findings are in agreement with those reported by Ref. [47] Li et al., 2007 in *L. vannamei* wherein varying concentrations of *B. licheniformis* were tried. The role of hemocytes in immunity and protection against microbial intruders is well known [48]. Increase in THC in crustaceans is an indication of improved immunity [49], as hemocytes are responsible for various nonspecific immune cell reactions such as phagocytosis, encapsulation, and storage and release of the prophenoloxidase system [50]. Prolonged application of probiotic bacteria results in colonization of beneficial bacteria in the digestive tract or the test water. This ultimately leads to the induction of more hemocytes to attach to the epidermis to offer better protection, as demonstrated by Ref. [10].

Bio-augmentation of selected *Bacillus spp.* in biofloc results in enhanced levels of PO enzyme in *P.indicus*. In the present study, there was a significant alteration in the serum PO activity of trypsin when bio-augmented with bacterial probiotics, *B. cereus, B. licheniformis* and *B. subtilis* compared to the control group. In this study we have used different bacterial strains belonging to *Bacillus sp., Virgibacillus sp., Oceanobacillus sp.,* and *Lysinibacillus* etc. as probiotic. The major cell wall component of these bacteria are peptidoglycan type. However composition of cell wall components such glucose (Glc), galactose (Gal), N-acetyl mannosamine (ManNAc), and N-acetylglucosamine (GlcNAc) may vary in each bacteria as reported by Ref. [51]. This might be one of the reasons besides growth and proliferation in the system for different activites by them. Laminarin, with cell wall component the β-1,3 glucan polymer was found to activate the serum PO activity in *Oceanobacillus sp.*, *Bacillussp.* and *B. megaterium* bioaugmented groups. Phenol oxidase is a crucial enzyme in the melanization cascade, which plays a key role in microbial defense of invertebrates. Prophenoloxidase (proPO) is an inactive proenzyme that is converted to PO and exists in hemolymph as a result of endogenous or exogenous activators [52,53]. Bacterial probiotics are able to alter PO enzyme activity and hence improve immunity against pathogens in *Litopenaeus vannamei* and *Penaeus monodon* [14,45,54–56]. The enhanced PO activity may partly be attributed to BFT and bio-augmentation of *Bacillus spp*. resulting in the consumption of the microbial floc in the system [23,57]. This has resulted in the enhancement of THC and PO activity, leading to increased shrimp immunity. Bioaugmentation in BF also resulted in the increased phagocytic activity. The effect of C:N ratio manipulation on Biofloc driven immunostimulation in *Litopenaeus vannamei* was studied and

revealed that C:N ratio manipulation results in elevated phagocytosis percentage in shrimps [23].

Our findings revealed that the lysozyme activity of serum in experimental shrimps was significantly elevated through supplementation of *B. cereus, B. licheniformis*, *B. subtilis* strain and *Lysinibacillus*. Lysozyme is a nonspecific innate immune molecule that hydrolyzes the bacterial cell and inhibits pathogens [1]. Similar findings were also reported [58] in *P. monodon* wherein the addition of *B. subtilis* strains elevated lysozyme activity protecting shrimps from *Vibrio harveyi* infection. The mRNA transcripts of Transglutaminase and Prophenol oxidase genes were found to be highly up-regulated in *Bacillus* bio augmented groups and BF groups compared to the control [37], who reported similar findings related to Prophenol oxidase gene in *Litopenaeus vannamei* postlarvae.

Overall, immunology results suggest that different probiotics have different effects on nonspecific immunity. This may be due to the fact that different probiotic bacteria under biofloc condition act differently. The biofloc is a complex of micro and macroorganisms interacting each other [17–21]. In present study same source of carbohydrate and methodology was adopted for all the treatments to generate the Biofloc. However the different probiotic strains used for different treatments had different effect on the nonspecific immunity. Besides the cell wall components, these bacterial strains differ in their enzyme production capability and metabolic pathways too. Our earlier findings proved that colonization of the micro flora and production of amylase, protease and cellulase aids to the digestion of external carbon sources and feed supplied to the culture shrimp in the Biofloc system [59]. These enzyme producing bacterial flora are often reported in shrimp and fish intestine and benefits the host [60–63]. Enzymatic probiotic strains have important role in BFT system. Probiotics strains in bioflc utilize the input carbohydrate and in turn give better performance and immunity to the reared animals [64]. In general, our findings revealed that Bioaugmentation of Biofloc with *Bacillus* species could improve water quality, survival, growth and yield, reduction in supplemented feed and improved immunity in *Penaeus indicus*. However, the mechanism of action of Biofloc in improving shrimp health and productivity is unknown. It was reported that heterotrophic growth of protozoa, rotifers, cyanobacteria, and diatoms in biofloc system may contribute to improved growth, survival, and commercial food reduction [65]. Use of *Bacillus* species as a probiotic in aquaculture to improve survival, growth, activate digestive enzymes, enhance the immune responses to inhibit the pathogen, and physical stress [1,13,66–69] is already documented. Further, *Bacillus* is known for its antagonistic activity against aquaculture pathogens [70,71]. It was reported that the surface antigens of *Bacillus* and their metabolites could serve as immunogens and the cell wall peptidoglycan may trigger immune functions in shrimp [45,72]. Hence, bioaugmentation of Biofloc system with *Bacillus* can improve shrimp production and enhance immunity under Biofloc culture conditions. Addition of commercial probiotics in the biofloc system could reduce *Vibrio parahaemolyticus* infection in *Litopenaeus vannamei* [38]. Miaou et al. [73] used *Bacillus subtilis* and *Lactobacillus* in *Macrobrachium rosenbergii* along with biofloc condition to improve humoral immune response. Supplementation of *Bacillus sp., Lactobacillus sp.* and *Rhodobacter sp.* in biofloc, improved growth, immune response and reduced oxidative stress [74] in *Fennerpenaeus chinensis*.

The findings in our study reveal that bioaugmentation of biofloc with beneficial bacteria could improve shrimp production that can be attributed to its multiple benefits such as enhanced immunity and survival, improved water quality, reduced feed supplementation and better growth and the most important factor of all, profitability to the farmer. However, the exact mode of action of biofloc is complex and warrants more studies to unravel the intricacies.

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