

# Rearing of White Leg Shrimp *Litopenaeus vannamei* (Boone, 1931) in Biofloc and Substrate Systems: Microbial Community of Water, Growth and Immune Response of Shrimp

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## Abstract

The microbial composition of rearing water, growth and immune status of *Litopenaeus vannamei* juveniles in biofloc (T1), substrate-integrated biofloc (T2), substrate (T3) systems and a control with four replicates each were evaluated in a 49-day indoor trail. In each HDPE tank of 70 L capacity filled with 10 g L<sup>-1</sup> salinity water, ten shrimp (4.56±0.13 g) were stocked. The C: N ratio of 15:1 was maintained in T1 and T2 using wheat flour as carbon source for production of biofloc. The TAN, NO<sub>2</sub> and NO<sub>3</sub> were lower (P<0.05) in treatment tanks than that in control. It was also observed that the counts of *Bacillus*, *Lactobacillus*, *Vibrio* and zooplankton were high in T2 than T1, T3 and control. There was higher net weight gain (10.38±0.14 g) and lowest FCR (1.27±0.12) with T2 when compared to T1, T3 and control tanks. Moreover, the survival rate is significantly higher in treatments than control. Significant increase in THC (47.24±4.49 x 10<sup>6</sup> cells ml<sup>-1</sup>), serum protein (82.67±0.01 mg/ml), Phenoloxidase (0.73±0.03, OD 490 nm) and Lysozyme activity (56.32±0.03%) was observed in T2 than T1, T3 and control. The result shows that substrate-integrated biofloc system assures higher growth, survival and better immune response in *L. vannamei*.

## Introduction

Among the cultivable shrimps, the Pacific white shrimp *Litopenaeus vannamei* has been farmed intensively in many parts of the world (FAO, 2012). The intensive shrimp rearing systems are using large volumes of water for exchange and excessive use of high protein feeds has caused adverse environmental impacts (Martinez-Porchas et al., 2012). Hence, emphasis should be given to produce high quality shrimp in an eco-friendly manner at relatively lower production cost. Therefore, industry needs to develop an alternative technique to the existing intensive culture systems. Biofloc technology (BFT) has been developed as an intensive rearing system to make shrimp farming

more cost effective and eco-friendly by means of reduced water use and effluent discharges with improved biosecurity (Wasielesky et al., 2006; Khanjani and Sharifinia, 2020). Bioflocs are heterogeneous mixture of bacteria, filamentous microalgae, protozoans, rotifers, worms and nonliving components (Ju et al., 2008; Ballester et al., 2010; Galvez, 2015; Panigrahi et al., 2017; Manan et al., 2017). Bacterial populations such as *Bacillus* sp., *Lactobacillus* sp., and *Vibrio* sp., were reported in biofloc systems (Anand et al., 2014; Kumar et al., 2015), besides bioactive compounds like carotenoids (Xu & Pan 2013) and were known for probiotic (Crab et al., 2010) and immunostimulant (Anguilera-Rivera et al., 2014) properties. In shrimp farming, bioflocs could reduce

ammonia and nitrite in water (De Schryver et al., 2008; Ray et al., 2011; Xu et al., 2012a; Kamilya et al., 2017; Abbaszadeh et al., 2019; Khanjani et al., 2016, 2019); augment growth (Ballester et al., 2010; Emerenciano et al., 2012; Xu & Pan 2012; Xu et al., 2012; Abbaszadeh et al., 2019) and non-specific immunity, there by enhanced protection from pathogenic bacteria (Smith et al., 2003; Vazquez et al., 2009; Ekasari et al., 2014). The bioflocs have 'natural probiotic' effect and it could act both internally and/or externally against to the *Vibrio* sp. in shrimp (Panigrahi et al., 2014). The microorganisms in the biofloc and their cellular material or cell metabolites could act as immunostimulators that can improve the innate immune system of shrimp and provide protection against the bacterial pathogens (Smith et al., 2003; Vazquez et al., 2009).

The use of submerged artificial substrates in biofloc system has been projected as another way to enhance production of shrimp, as the submerged substrates supply sites for the production of autotrophic and heterotrophic bacterial community which maintain quality of water and serve as natural food for culturing animals (Langis et al., 1988; Ramesh et al., 1999; Thompson et al., 2002; Panigrahi et al., 2017). In the submerged substrate-based culture system/periphyton-based culture system, periphyton or biofilm on substrate usually consists of microscopic organisms like algae, bacteria, fungi, protozoa, zooplankton, other invertebrates and detritus (Azim et al., 2001; Azim & Wahab 2005; Anand et al., 2013). Periphyton developed on the submerged substrates can uptake nitrogenous compounds such as ammonia and nitrite, control the concentration of dissolved oxygen (DO) and maintain pH of the surrounding water (Azim et al., 2002; Dodds, 2003; Bender et al., 2004; Schweitzer et al., 2013; Anand et al., 2019; Shilta, et al., 2020).

Earlier studies on the effect of biofloc systems on water quality, growth, survival and production has been documented to some extent in tiger shrimp (Hargreaves 2013; Anand et al., 2014; Kumar et al., 2015), *L. vannamei* (Khanjani et al., 2016, 2019; Panigrahi et al., 2017, 2019; Abbaszadeh et al., 2019; Tong et al., 2020; Huang et al., 2021; Silva et al., 2021), *Fenneropenaeus indicus* (Effendy et al., 2016), *Farfantepenaeus paulensis* (Ballester et al., 2010) and *Marsupenaeus japonicus* (Zhao et al., 2012) and to a limited extent on physiological health status of *P. monodon* (Kumar et al., 2015) and *L. vannamei* (Xu & Pan 2013; Panigrahi et al., 2017). Furthermore, the use of artificial substrates in shrimp culture has been studied only for the growth performance and water quality (Audelo-Naranjio et al., 2010; Zhang, 2011; Zhang et al., 2014; Anand et al., 2019; Shilta et al., 2020). The effect of adding submerged substrates in biofloc systems on the growth of *L. vannamei* was studied by Olier et al. (2020). Sundaram et al. (2021) studied the effect of natural and artificial periphytic substrates in biofloc systems on both growth and immune response of *L. vannamei*. The present study aimed to understand the water quality

and its microbial composition, growth and immune response of *L. vannamei* juveniles reared in substrate-integrated bifloc system over only biofloc system, only substrate system and a control system without biofloc and substrate.

## Material and Methods

### Experimental Design

Sixteen rectangular flat bottom HDPE tanks of 0.6m x 0.4m x 0.3m (L x W x H) size, filled with 50 L of 10 g L<sup>-1</sup> salinity water were used for the experiment conducted at the wet lab of College of Fishery Science, Muthukur, SPSR Nellore district, Andhra Pradesh, India. Sea water collected from the Krishnapatnam beach (14.2546° N and 80.1094° E) of Bay of Bengal was disinfected with sodium hypochlorite and diluted with freshwater to get salinity of 10 g L<sup>-1</sup>. Healthy *L. vannamei* juveniles (3.10±0.2 g) were procured from a private shrimp farm located at Kogili village, SPSR Nellore district, Andhra Pradesh. The juveniles were acclimatized in rectangular flat bottom fiber glass reinforced plastic (FRP) reservoir tank of one-ton capacity for a period of three weeks prior to stocking in experimental tanks. The experiment consisted of four treatments including control (clear water; C), T1 (only biofloc), T2 (substrate-integrated biofloc) and T3 (only substrate) with zero water exchange. Four replicates were maintained for each treatment. All the tanks were well aerated using 3 aeration pipes with air stones and regulator in each tank through a centralized 0.5 Hp capacity roots air blower. Bamboo mat substrate of 40×28 cm size was designed as submerged substrates for T2 and T3. Each replicate tank of the treatments T2 and T3 were placed vertically with a bamboo mat substrate. Wheat flour was used as carbon source for the development of biofloc in treatments T1 and T2 in view of its efficacy in production of good quality floc (Azim and little, 2008; Ballester et al., 2010; Anand et al., 2014; Raj Kumar et al., 2015; Kim et al., 2021). The biofloc inoculum was prepared by adding 10 mg of ammonium sulphate and 400 mg of wheat flour as carbohydrate source in glass beakers having one-liter water of 10 g L<sup>-1</sup> salinity with aeration. After 48 h, the biofloc inoculum from one glass beaker was transferred to each replicate tank of the treatments T1 and T2. Immediately after transfer of biofloc inoculum, each of the T1 and T2 treatment tanks were added with 10 ppm ammonium sulphate and 30 ppm of wheat flour for maintaining the biofloc. The acclimatized shrimp (4.56±0.13 g) were then stocked in both treatment and control tanks at 10 juveniles per tank. A commercial pellet feed (Charoen Pokphand (CP) India Pvt. Ltd, Chennai, India) with 35.3 % crude protein was fed to shrimp daily at 08:00 Am and 08:00 Pm @ 3.0% body weight. The details of biochemical composition of the pellet feed and wheat flour (carbon source) are given in Table 1.

**Table 1.** Proximate composition of experimental feed and wheat flour (carbon source)

Proximate Composition	Experimental Feed	Wheat Flour
Protein (%)	35.30	12.80
Fat (%)	6.10	1.80
Ash (%)	5.05	13.30
Fiber (%)	4.12	1.20
Moisture	8.72	6.72

### Maintenance of C: N Ratio

The C/N ratio was manipulated by considering total ammonia nitrogen (TAN) level in treatment tanks by adopting Avinimelech (1999) method. Wheat flour as carbon source was added to the treatment tanks T1 and T2 for maintaining the C/N ratio of 15:1 at every three-day interval, after checking the total ammonia nitrogen (TAN) levels. In general, it is assumed that the carbohydrate source contains minimum 50% carbon, thus taking the microbial growth requirement of C/N ratio 15, about 30 ppm wheat flour was added to reduce TAN concentration by 1 ppm N. The feed used in this study had a calculated C/N ratio of 9:1, assuming the feed had 50% carbon and 5.65% of nitrogen. The control tanks (C:N-9) were maintained with no supplementation of carbon source. Freshwater was added in all the experimental tanks once a week to make up water loss due to evaporation.

### Physico-chemical Water Parameters

During the study period, water pH (universal pH indicator), dissolved oxygen (Aqua check DO test kit, HiMedia Laboratories Pvt. Ltd., Mumbai, India) and temperature (Mercury bulb thermometer) were measured daily at 10:00 Am. Total ammonia nitrogen (TAN) was measured once in every three days at 640 nm; nitrite and- nitrate were measured at weekly intervals at 543 nm by Spectrophotometric (LMSP-UV 1200, LABMAN Scientific Instruments) method (APHA, 2012). Salinity was measured with portable refractometer (ERMA, RHS-28) once in a week while adding freshwater to compensate evaporation loss.

### Qualitative Analysis of Planktonic Organisms in Biofloc and Periphyton

The biofloc in the water and periphyton developed on a submerged bamboo substrate in treatment tanks were collected for the analysis of planktonic organisms like zooplankton. The samples were suspended in distilled water and fixed in 5 % buffered formalin for few minutes followed by spinning for 3 min to separate the complex materials in biofloc and periphyton. Then microscopic fauna was observed under binocular microscope (Olympus India Pvt. Ltd., CX21iLEDFS1) and organisms were identified using the taxonomic keys (Brusca and Brusca 2003; Ruppert et al. 2005) and illustrations (APHA, 2012).

### Microbial Composition of Biofloc

At the end of the experiment, total plate count (heterotrophic bacteria), *Bacillus*, *Lactobacillus* and *Vibrio* species were isolated from experimental tanks as per the method described by Kumar et al. (2015) with minor modifications. Microbial populations were collected from each treatment tank by collecting 100 ml of water sample and subjected to centrifugation at 10,000 rpm for 40 sec. The pellet formed was then subjected to serial dilutions for fifteen fold using physiological saline. 0.1 ml of dilutions was spread plated on Tryptone soya agar (1.0 % w/v NaCl) for total heterotrophic bacteria (THB) counts. Further, *Vibrio*, *Bacillus* and *Lactobacillus* were isolated on TCBS agar, *Bacillus cereus* agar and *Lactobacillus* MRS agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) respectively. All the inoculated plates were incubated aerobically at 28°C for 24-48 h. However, *Lactobacillus* MRS agar plates were incubated in microaerophilic condition. The incubated plates having 30 to 300 colonies were counted as colony forming units (cfu) per ml.

### Growth Performance and Survival Rate

The experimental shrimp were sampled at weekly intervals and weighed using an electronic balance (A & D, GR 200) accurate to 0.01 g for assessment of growth. The shrimps were starved for 24 h on 49<sup>th</sup> day, weighed for growth assessment and counted for survival rate. The final growth and survival rate was calculated by using the following formulae (Panigrahi et al., 2017):

$$\text{Net weight gain (NWG, g)} = \text{Final live body weight (g)} - \text{Initial live body weight (g)}$$

$$\text{Specific growth rate (SGR)} = 100 \times [\text{Ln (final body weight)} - \text{Ln (initial body weight)}] / \text{No. of days of experiment}$$

$$\text{Feed conversion ratio (FCR)} = \text{Total feed consumed (dry weight)} / \text{Net wet weight of shrimp}$$

$$\text{Survival Rate (\%)} = (\text{Total number of shrimps survived} / \text{Total number of shrimps stocked}) \times 100$$

### Collection of Haemolymph and Serum Samples

For all the immunological assays, haemolymph were collected from *L. vannamei* at the end of the

experiment. A total of twelve inter-moult shrimps per treatment i.e., 3 shrimp from each replicate tank collected were anaesthetized using clove oil (50  $\mu\text{L L}^{-1}$ ). Around 50  $\mu\text{L}$  haemolymph was collected from the ventral sinus of each shrimp using anticoagulant (30 mM tris-sodium citrate, 388 mM sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55) coated 1 ml syringe fitted with 26-gauge needle. The collected haemolymph was transferred to 1.5 ml centrifuge tube containing 450  $\mu\text{L}$  of pre-cooled (4°C) anticoagulant. The anticoagulant mixed haemolymph samples of each treatment were then pooled into a sterile centrifuge tube and kept on ice for further analysis. Further, haemolymph drawn from the 3 shrimps per replicate of each treatment for serum collection was transferred to anticoagulant free centrifuge tubes and kept undisturbed at 4°C. After 6 hr, the clotted haemolymph tubes were centrifuged at 10,000 rpm for 30 min followed by collection of supernatant as serum and stored at -80°C until further use.

### Estimation of Immune Parameters

Haemolymph (150  $\mu\text{L}$ ) collected from 3 shrimps per replicate of each treatment mixed with 1350  $\mu\text{L}$  pre-cooled anticoagulant was transferred to a Neubauer haemocytometer and kept under Olympus light microscope (CX21i, LED) at 400x magnification for observing total and differential haemocyte counts. Total haemocyte count (THC) was expressed as total haemocyte cells  $\text{ml}^{-1}$  haemolymph. Further, haemocytes were differentiated into granulocytes and agranulocytes (hyaline cells) based on the granular content of the cell (Le Moullac, Klein, Sellos and Van Wormhoudt 1997) were termed as total granulocyte cells  $\text{ml}^{-1}$  (TGC  $\text{ml}^{-1}$ ) and total agranulocyte cells count (TAC  $\text{ml}^{-1}$ ). The total serum protein content was assessed by Bradford (1976) method. Bradford reagent added to 1  $\mu\text{L}$  serum sample in each tube was well mixed and incubated at room temperature for 20 min followed by measurement of the absorbance at 595 nm using spectrophotometer (T60, LABINDA). The prophenol oxidase (PO) activity was measured by Cheng and Chen (2000) method, where 25  $\mu\text{L}$  of serum in 150  $\mu\text{L}$  of substrate (5 mM L-3,4, dihydroxy-phenylalanine) made up to 2 ml using tris buffer (50 mM Tris-HCl; pH 7.5) was incubated for 15 min at room temperature. The colour developed in the reaction wells was measured in ELISA plate reader (POWERWAVE XS, BIOTEK, USA) at 490 nm against substrate solution as reagent blank and the OD at 490 nm was expressed as PO activity.

The serum lysozyme activity was analysed by Ellis (1990) turbidimetric method. The standard solution at various concentrations was prepared using chicken egg white lysozyme (Sigma-Aldrich, St. Louis, MO, USA) diluted with 0.04 M phosphate buffer. The *Micrococcus lysodeikticus* (Sigma-Aldrich, St. Louis, MO, USA) is used as a substrate (0.2 mg  $\text{ml}^{-1}$  suspension of *M. lysodeikticus* in 200  $\mu\text{L}$  of 0.1M PBS, pH 6.5). 10  $\mu\text{L}$  of

serum with 190  $\mu\text{L}$  *M. lysodeikticus* was added to each well of a 96-well microtiter plate and incubated for 10 min at 37°C. The absorbance at 450 nm was read at 5 min and then at 15 min intervals. The lysozyme activity in the sample was calculated using the following formula of the standard curve.

$$\text{Lysozyme activity (\% inhibition)} = (\text{OD of sample at 450 nm} / \text{OD of positive control at 450 nm}) \times 100$$

### Statistical Analysis

The experimental results were statistically analyzed using statistical software SPSS version 20 (IBM, 2012). The data on water quality, growth, microbial load and immune parameters were analyzed by one-way analysis of variance (ANOVA) to test difference among the treatments. Statistical significance level was set at  $P < 0.05$  and the difference between the treatments means were tested by Duncan's multiple range test.

## Results

### Physico-chemical Water Parameters

The physico-chemical parameters of water were found to be within optimal range for *L. vannamei* in all the experimental tanks and are presented in Table 2 and Figure 1. Water temperature was not significantly different ( $P > 0.05$ ) among the experimental tanks. However, DO, pH, TAN, Nitrate-N and Nitrite-N showed significant difference ( $P < 0.05$ ), where the experimental tank T2 had significantly lower DO (5.50  $\text{mg L}^{-1}$ ) and pH (7.53) than that of other treatments and control. The TAN, Nitrate-N and Nitrite-N concentrations are significantly higher in control ( $P < 0.05$ ) than treatment tanks except Nitrite-N in T3 tank. Among the treatments significantly higher TAN (0.44  $\text{mg L}^{-1}$ ), Nitrate-N (1.92  $\text{mg L}^{-1}$ ) and Nitrite-N (0.13  $\text{mg L}^{-1}$ ) concentrations were recorded in substrate system (T3).

### Qualitative Analysis of Planktonic Organisms

Zooplankton communities were mainly identified in biofloc and periphyton. The bioflocs mainly contained rotifers, copepods, cladocerans, nematodes and ciliated protozoans. The periphyton was composed of rotifers, copepods, ciliates and nematods. Ciliates, rotifers and copepods were dominant groups of microscopic organisms in the all the treatment tanks.

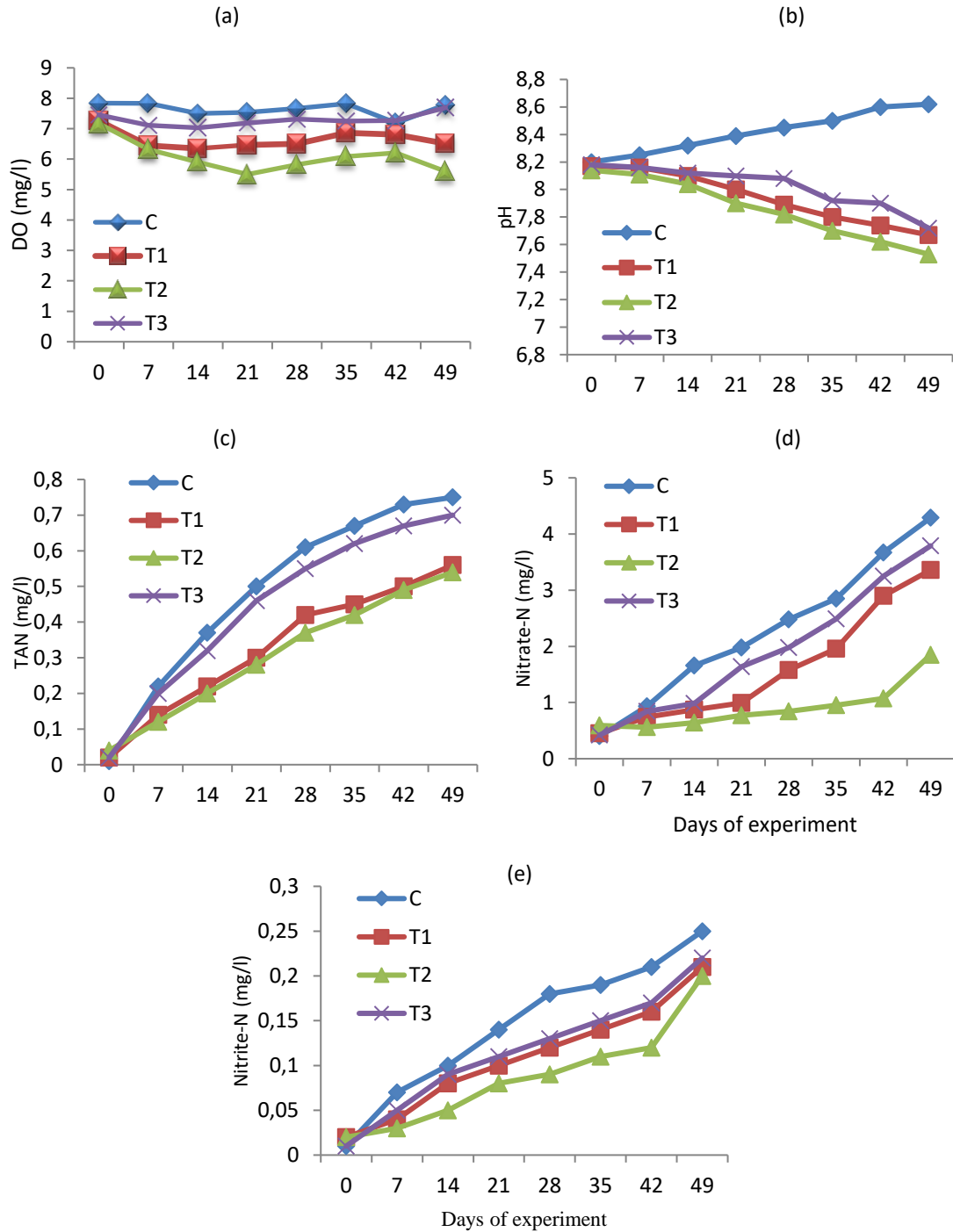
### Microbial Load

The total heterotrophic bacteria, *Bacillus sp.*, *Lactobacillus sp.* and *Vibrio sp.*, counts among the treatment and control groups are given in Table 3 and Figure 2. Significantly higher ( $P < 0.05$ ) bacterial loads were recorded in all treatment tanks than the control group. Among the treatments, T2 had higher ( $P < 0.05$ )

**Table 2.** Water quality parameters (mean±SD) of different experimental groups

Parameters	Control	Treatments		
		T1	T2	T3
Temperature(°C)	28.79±0.32 <sup>a</sup>	28.80±0.50 <sup>a</sup>	28.79±0.46 <sup>a</sup>	28.80±0.59 <sup>a</sup>
DO(mg L <sup>-1</sup> )	7.64±0.26 <sup>d</sup>	6.65±0.22 <sup>b</sup>	6.05±0.14 <sup>a</sup>	7.28±0.14 <sup>c</sup>
pH	8.41±0.13 <sup>d</sup>	7.93±0.07 <sup>b</sup>	7.85±0.16 <sup>a</sup>	8.02±0.09 <sup>c</sup>
Total ammonia nitrogen(mg L <sup>-1</sup> )	0.49±0.08 <sup>c</sup>	0.31±0.02 <sup>a</sup>	0.30±0.04 <sup>a</sup>	0.44±0.08 <sup>b</sup>
Nitrate-N(mg L <sup>-1</sup> )	2.28±0.11 <sup>d</sup>	1.60±0.09 <sup>b</sup>	0.90±0.40 <sup>a</sup>	1.92±0.20 <sup>c</sup>
Nitrite-N(mg L <sup>-1</sup> )	0.14±0.06 <sup>b</sup>	0.10±0.02 <sup>a</sup>	0.09±0.03 <sup>a</sup>	0.13±0.04 <sup>b</sup>

T1, only biofloc; T2, substrate-integrated biofloc; T3, only substrate.  
 Mean values in the same row with different superscript differ significantly (P<0.05).



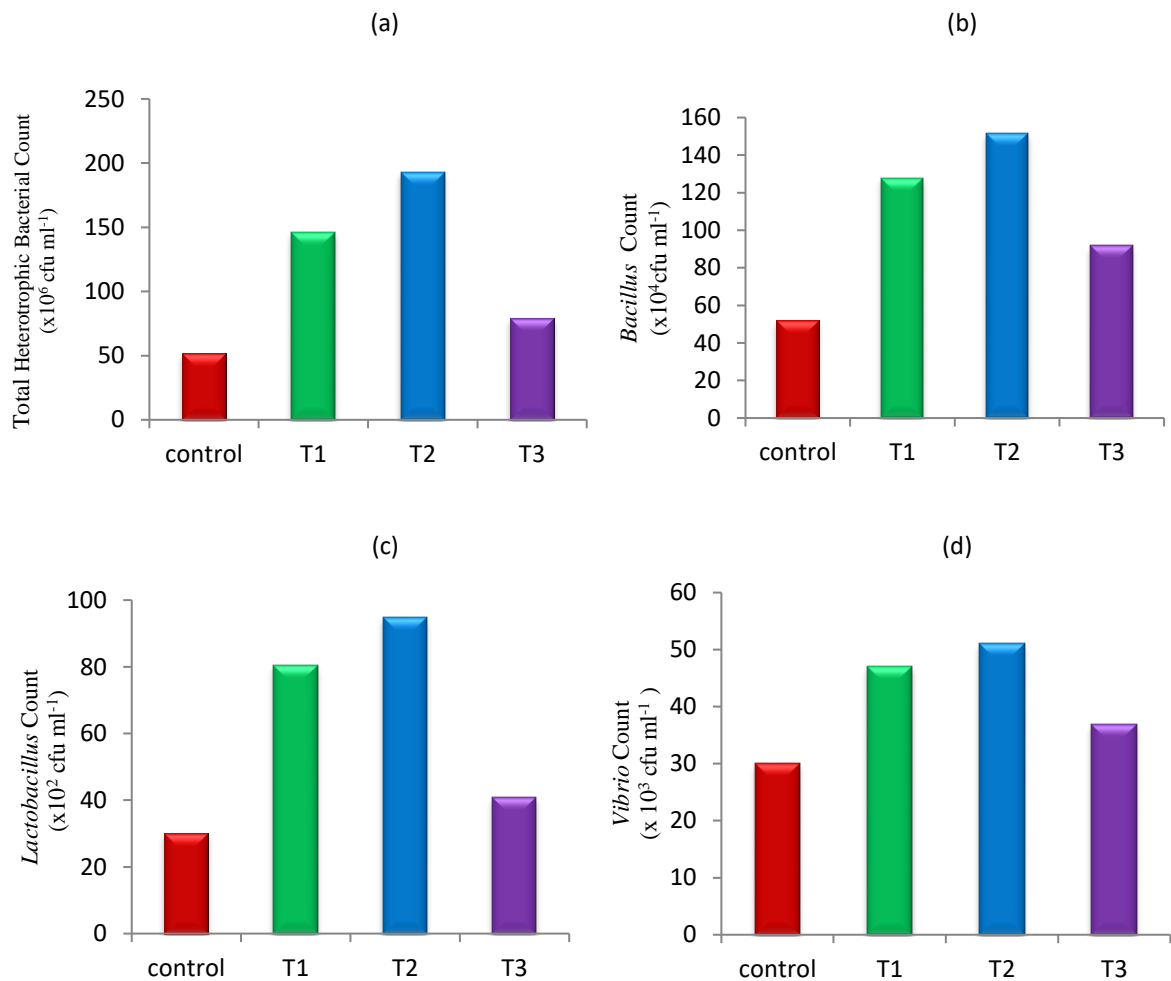
**Figure 1.** Dissolved oxygen (DO) (a), pH (b), Total ammonia nitrogen (TAN) (c), Nitrate-N (d) and Nitrite-N (e) in different experimental groups. Data represents mean±SD of four replicates.

**Table 3.** Microbial count (mean±SD) in the experimental tanks

Parameters	Control	Treatments		
		T1	T2	T3
Total Heterotrophic Bacterial Count (x10 <sup>6</sup> cfu ml <sup>-1</sup> )	51.50±3.20 <sup>a</sup>	146.30±23.50 <sup>c</sup>	192.70±34.50 <sup>d</sup>	79.60±13.10 <sup>b</sup>
<i>Bacillus</i> Count (x 10 <sup>4</sup> cfu ml <sup>-1</sup> )	51.79±5.10 <sup>a</sup>	127.76±32.30 <sup>c</sup>	151.65±40.40 <sup>d</sup>	91.67±18.60 <sup>b</sup>
<i>Lactobacillus</i> Count (x 10 <sup>2</sup> cfu ml <sup>-1</sup> )	30.21±11.30 <sup>a</sup>	80.50±35.65 <sup>c</sup>	94.80±47.10 <sup>d</sup>	40.85±22.20 <sup>b</sup>
<i>Vibrio</i> Count (x 10 <sup>3</sup> cfu ml <sup>-1</sup> )	30.16±2.70 <sup>a</sup>	47.01±4.90 <sup>c</sup>	51.02±6.50 <sup>d</sup>	36.83±3.60 <sup>b</sup>

T1, only biofloc; T2, substrate-integrated biofloc; T3, only substrate.

Mean values in the same row with different superscript differ significantly (P<0.05). Values expressed in cfu ml<sup>-1</sup>.



**Figure 2.** Bacterial count in the experimental groups. (a) Total heterotrophic bacterial count (THB) (x10<sup>6</sup> cfu ml<sup>-1</sup>), (b) *Bacillus* count (x10<sup>4</sup> cfu ml<sup>-1</sup>), (c) *Lactobacillus* count (x10<sup>2</sup> cfu ml<sup>-1</sup>) and (d) *Vibrio* count (x10<sup>3</sup> cfu ml<sup>-1</sup>).

loads of THB (192.70 ± 34.50 x10<sup>6</sup> cfu ml<sup>-1</sup>), *Bacillus* (151.65 ± 40.40 x10<sup>4</sup> cfu ml<sup>-1</sup>), *Lactobacillus* (94.80 ± 47.10 x10<sup>2</sup> cfu ml<sup>-1</sup>) and *Vibrio* (51.02 ± 6.50 x10<sup>3</sup> cfu ml<sup>-1</sup>) when compared to other two treatments. Among the isolated bacterial communities, *Bacillus* sp. was the dominant group.

**Growth Parameters**

The growth parameters like NWG, SGR, FCR and survival rate was significantly lower (P<0.05) in control group than the treatments. Among the treatments, T2 has significantly better (P<0.05) NWG, SGR and FCR in

comparison with the other two treatments. However, survival rate was not significantly different (P>0.05) among all the treatment groups. Growth parameters of *L. vannamei* recorded in all the experimental groups are shown in Table 4.

**Immune Parameters**

In *L. vannamei*, the total haemocyte count, total hyaline cells and total granular cells, serum protein, phenoloxidase (PO) activity, serum lysozyme activity was significantly higher (P<0.05) in treatment groups than the control (Table 5 and Figure 3). Among the

treatment groups, T2 had significantly higher ( $P<0.05$ ) THC ( $47.24\pm 4.49 \times 10^6$  cells/ml), hyaline cell count ( $18.21\pm 2.22 \times 10^6$  cells/ml) and granulocytes count ( $29.04\pm 2.89 \times 10^6$  cells/ml) than T1 and T3. Similarly, highest serum protein level, phenoloxidase (PO) activity, serum lysozyme activity of shrimp was observed in substrate-integrated biofloc (T2) group than only biofloc (T1) and only substrate (T3) groups.

## Discussion

### Water Quality Parameters

The observed physico-chemical water parameters in all the experimental groups were in suitable ranges for *L. vannamei* culture (Van et al., 1999; Lin & Chen, 2003). According to Chen (1985), optimum DO level for the weight increment and survival of shrimp was  $5 \text{ mg L}^{-1}$ . The lowest level of DO recorded in this study in substrate-integrated biofloc treatment (T2) was sufficient (6.05 ppm) for healthy growth of *L. vannamei*. Immobilization of inorganic nitrogen by heterotrophic bacteria in biofloc, substrate and substrate integrated-biofloc treatments of the present study demanded more DO than the conventional clear water control system as stated by Hargreaves (2006). In the treatment tanks, pH was lower than that of control tanks. The lowest pH was recorded in substrate-integrated biofloc treatment (T2). This might be due to the high nitrification and respiration rates by microorganisms resulted in increased carbon dioxide ( $\text{CO}_2$ ) levels in substrate-integrated biofloc (T2) followed by only biofloc and only substrate (T3) treatments (Wasielesky et al., 2006; Zhang et al., 2014; Khanjani et al., 2016, 2019). According to Hargreaves (2013) and Tong et al. (2020) increasing inputs of C:N ratio between 12 and 15 helps the heterotrophic bacteria for more ammonia ( $\text{NH}_3$ ) control in biofloc systems. Similarly, in substrate based ponds, periphyton develops on substrate act as habitat for nitrifying bacteria which enhance nitrification keeping ammonia at low level (Anand et al., 2013; Zhang et al., 2014). Earlier studies on water quality in biofloc and substrate systems avoiding water exchange reported lower concentrations of ammonia ( $\text{NH}_3$ ), nitrite and nitrate (Raj Kumar et al., 2015; Brito et al., 2016; Khanjani et al., 2016; Bossier and Ekasari et al., 2017; Panigrahi et al., 2017 & 2019; Anand et al., 2019; Khanjani et al., 2019; Khoa et al., 2020; Shilta et al.,

2020; Huang et al., 2021). The levels of TAN and nitrite in substrate-integrated biofloc (T2) and biofloc systems were significantly lower than that of only substrate (T3) and control. This study is in consonance with the observations of Ray et al. (2011), Rajkumar et al. (2015), Luis-Villaseñor et al. (2015) and Olier et al., (2020).

### Qualitative Analysis of Plankton in Biofloc and Periphyton

In the present study, rotifers, copepods, cladocerans, nematodes and ciliated protozoans observed in biofloc are in agreement with the findings of Hargeaves (2013), Panigrahi et al. (2014) and Raj Kumar et al. (2015). Ju et al. (2008) reported that bioflocs consist of 98% flagellates, 1.5% rotifer and 0.5% amoeba. Galvez (2015) recorded 13 genera of zooplankton of rotifera, copepoda, protozoa and cladocera groups in biofloc system in which rotifers remain the most abundant group. Manan et al. (2017) recorded nematodes, gastrotrichs, euplotes, protozoans, rotifers and copepods in the biofloc systems of *P. vannamei*. Khoa et al. (2020) also recorded diatoms, chlorophytes, protozoa, cyanobacteria, brachionidae, and copepoda in biofloc systems of *L. vannamei* rearing in outdoor culture systems. The periphyton community of the present study composed of rotifers, copepods, ciliates and nematods. Azim et al. (2001) studied periphyton community on bamboo substrates and reported about five genera of zooplankton which includes Rotifera, Copepoda and Cladocera. Anand et al. (2013) also reported that rotifer group dominated among the periphytic communities in the bamboo substrate system followed by nematods and crustaceans. Olier et al. (2020) recorded higher concentrations of diatoms followed by rotifers, nematodes, cladocerans, and ciliated protozoans in the biofloc with Geotextile stripe used as a substrate. In our study, ciliates, rotifers and copepods remain a dominant group in all the treatment tanks. Further, phytoplankton communities were absent in all the treatments since this study was conducted in indoor wet laboratory.

### Microbial Community

In biofloc system, microbial community develops and reaches to a density of  $10^7 \text{ cfu ml}^{-1}$  (Buford et al., 2003). Kumar et al. (2015) observed highest mean total

**Table 4.** Growth parameters of *L. vannamei* (mean $\pm$ SD) in the experimental groups

Parameters	Treatments			
	Control	T1	T2	T3
NWG (g)	5.88 $\pm$ 0.34 <sup>a</sup>	9.47 $\pm$ 0.12 <sup>c</sup>	10.38 $\pm$ 0.14 <sup>d</sup>	7.55 $\pm$ 0.36 <sup>b</sup>
SGR	1.81 $\pm$ 0.06 <sup>a</sup>	2.32 $\pm$ 0.01 <sup>c</sup>	2.52 $\pm$ 0.02 <sup>d</sup>	1.95 $\pm$ 0.02 <sup>b</sup>
FCR	2.05 $\pm$ 0.07 <sup>d</sup>	1.40 $\pm$ 0.08 <sup>b</sup>	1.27 $\pm$ 0.12 <sup>a</sup>	1.51 $\pm$ 0.02 <sup>c</sup>
Survival (%)	62.50 $\pm$ 0.81 <sup>a</sup>	75.10 $\pm$ 0.57 <sup>b</sup>	77.50 $\pm$ 0.95 <sup>b</sup>	74.60 $\pm$ 0.81 <sup>b</sup>

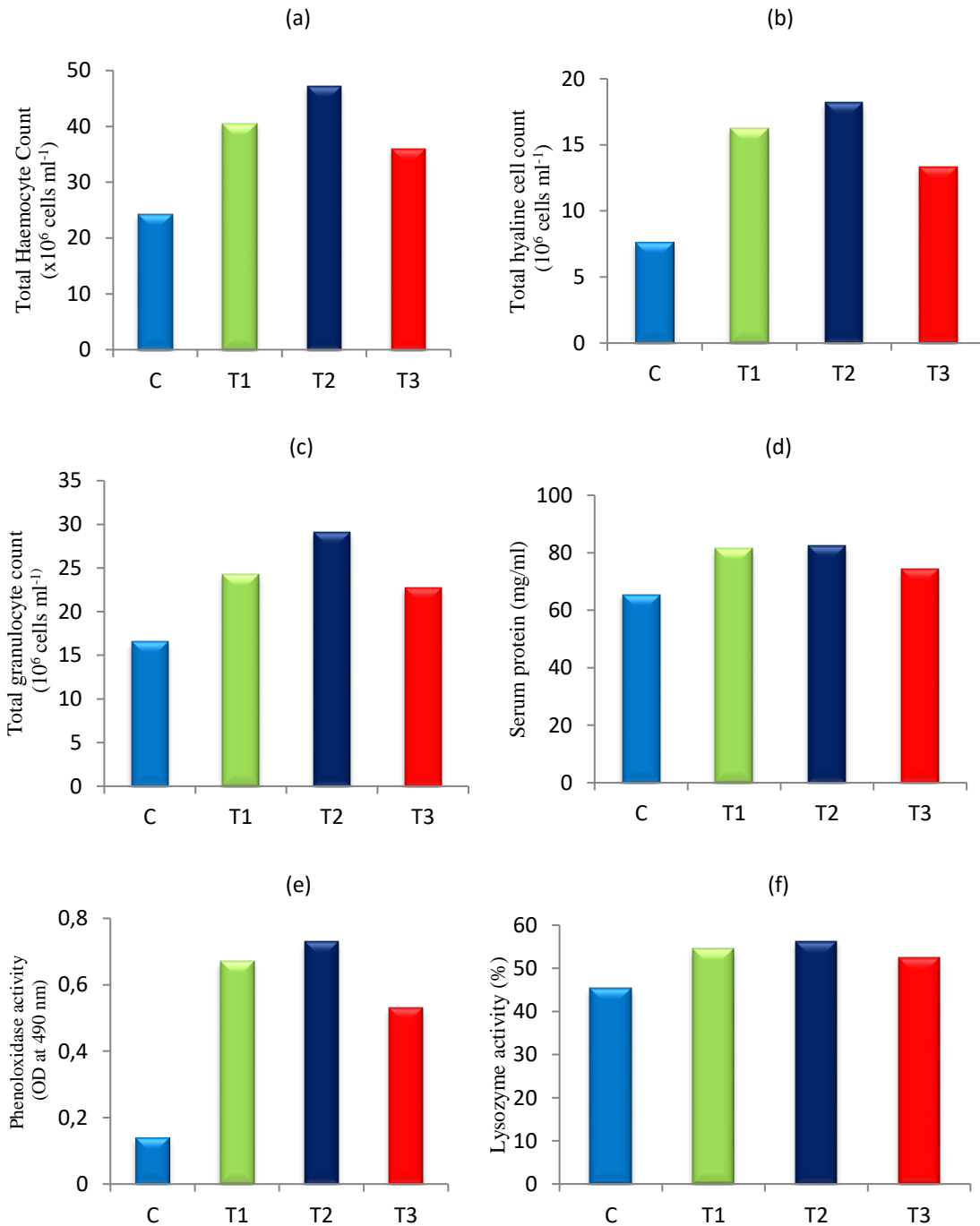
T1, only biofloc; T2, substrate-integrated biofloc; T3, only substrate.

Mean values in the same row with different superscript differ significantly ( $P<0.05$ ).

**Table 5.** Immune parameters (mean±SD) of *L. vannamei* in the experimental groups

Immune parameters	Treatments			
	Control	T1	T2	T3
Total Haemocyte count (x10 <sup>6</sup> )	24.17±1.78 <sup>a</sup>	40.53±3.41 <sup>c</sup>	47.24±4.49 <sup>d</sup>	36.01±2.30 <sup>b</sup>
Total Hyaline cell count (x10 <sup>6</sup> )	7.67±0.56 <sup>a</sup>	16.25±1.73 <sup>c</sup>	18.21±2.22 <sup>d</sup>	13.32±1.31 <sup>b</sup>
Total Granulocyte count (x10 <sup>6</sup> )	16.53±1.68 <sup>a</sup>	24.28±2.60 <sup>c</sup>	29.04±2.89 <sup>d</sup>	22.70±2.43 <sup>b</sup>
Serum protein (mg/ml)	65.50±0.03 <sup>a</sup>	81.60±0.01 <sup>c</sup>	82.67±0.01 <sup>d</sup>	74.35±0.04 <sup>b</sup>
Phenoloxidase activity (OD at 490 nm)	0.14±0.01 <sup>a</sup>	0.67±0.02 <sup>c</sup>	0.73±0.03 <sup>d</sup>	0.53±0.01 <sup>b</sup>
Lysozyme activity (%)	45.38±0.01 <sup>a</sup>	54.54±0.02 <sup>c</sup>	56.32±0.03 <sup>d</sup>	52.53±0.01 <sup>b</sup>

T1, only biofloc; T2, substrate integrated biofloc; T3, only substrate.  
 Mean values in the same row with different superscript differ significantly (P<0.05).



**Figure 3.** Total haemocyte count (x10<sup>6</sup> cells ml<sup>-1</sup>) (a), Total hyaline cell count (x10<sup>6</sup> cells ml<sup>-1</sup>) (b), total granulocyte count (x10<sup>6</sup> cells ml<sup>-1</sup>) (c), serum protein (mg/ml) (d), phenoloxidase activity (OD at 490 nm) (e) and lysozyme activity (%) (f) in *P. vannamei* juveniles reared in only biofloc, only substrate, substrate-integrated biofloc and clear water control (C) systems.



bacterial count in bioflocs generated from molasses with *Bacillus* being the most dominant bacterial group followed by *Vibrio* and *Lactobacillus*. Similarly, Panigrahi et al. (2019) also reported that carbohydrate supplementation systems significantly increasing the THB count ( $82.9 \pm 4.4\%$ ) in biofloc system when compared with control group. Recently, Khoa et al. (2020) found significantly high THB and *Vibrio* loads in biofloc treatment tanks when compared with control. Furthermore, Sundaram et al. (2020) recorded significantly high THB loads in substrate integrated biofloc systems than clear water control system. In our study, all the treatment tanks were observed with significantly higher counts of THB, *Bacillus*, *Lactobacillus* and *Vibrio* loads than that of control tanks. Substrate-integrated biofloc treatment (T2) has recorded with significantly higher THB, *Bacillus*, *Lactobacillus* and *Vibrio* counts than the only biofloc and only substrate treatments which might be due to the development of heterotrophic bacteria in higher abundance, both in the form of flocs and biofilm in T2.

### Growth Parameters

Enhanced growth performance, survival and better FCR was observed in *L. vannamei* (Wasielesky et al., 2006; Becerra-Dorame et al., 2012; Xu & Pan, 2012; Emerenciano et al., 2013; Krummenauer et al., 2014; Rajkumar et al., 2015; Luis-villaseñor et al., 2015; Panigrahi et al., 2017 & 2019; Abbaszadeh et al., 2019; Khanjani et al., 2016 & 2019; Khoa et al., 2020; Tong et al., 2020; Huang et al., 2021; Silva et al., 2021), *P. monodon* (Kumar et al., 2015), *Fenneropenaeus indicus* (Effendy et al., 2016) reared in the biofloc systems than that of conventional systems. Similarly, the rearing of *P. monodon* (Arnold et al., 2006; Anand et al., 2013; Anand et al., 2019), *Farfantepenaeus paulensis* (Ballester et al., 2007), *Macrobrachium rosenbergii* (Uddin, 2007; Shilta et al., 2020) and *L. vannamei* (Zhang et al., 2014) in artificial submerged substrate systems has shown increased production and survivability. Sundaram et al. (2021) recorded highest weight gain of *L. vannamei* reared in submerged substrate-integrated biofloc systems. In the present study also, the growth performance and survival rate of *L. vannamei* was significantly higher in treatment groups than that of control. On the other hand, substrate-integrated biofloc system performed better than systems with the only biofloc and only substrate. Integration of substrates in biofloc system has recorded better growth performance and survivability in *M. rosenbergii* (Assaduzzaman et al., 2010), *L. vannamei* (Becerra-Dórame et al., 2012; Schweitzer et al., 2013; Ferreira et al., 2015; Olier et al., 2020) than that of biofloc systems alone. The present study results corroborate with the findings of above studies that shows more positive effect of submerged substrates when integrated in biofloc system on production of *L. vannamei* juvenile than the systems in which these techniques are used independently.

### Immunological Parameters

Haemolymph parameters are mostly used to monitor the physiological condition, nutritional quality and status of immune systems in crustaceans exposed to various stressors (Matozzo et al., 2011; Porchas-Cornejo et al., 2011). The circulating haemocyte count of crustaceans in terms of both increase in quantity and quality and proPO activity indicated enhanced immune status in crustaceans and hence disease resistance (Rodríguez & Le Moullac, 2000; Chiu et al., 2007). The higher serum protein levels were observed in *P. monodon* juveniles fed on biofloc incorporated diets (Anand, 2012) and as well reared in biofloc systems (Kumar et al., 2015). Similarly, higher serum protein levels in both haemolymph and muscle of *L. vannamei* were observed in submerged substrate based culture systems (Audilo-Naranjo et al., 2012). Sundaram et al. (2021) also reported significantly high serum protein levels of *L. vannamei* reared in substrate integrated biofloc systems than that of clear water control system. On the other hand, lysozymes are initial defenders against bacteria and remain as hall mark of non-specific immunity in crustaceans (Vazquez et al., 2009). In our study, substrate-integrated biofloc, only biofloc and only substrate treatments recorded with better immune response in *L. vannamei* in terms of total haemocytes, hyaline cells, granulocyte count, PO activity, serum protein and serum lysozyme activity when compared to control shrimp. An elevated level of THC has been reported after feeding shrimp with dietary supplements like probiotics (Rengpipat et al., 2000; Li et al., 2009; Abdollahi-Arpanahi et al., 2018),  $\beta$ -glucan (Lopez et al., 2003; Neto & Nunes, 2015), macroalgae and  $\beta$ -carotene supplemented diets (Supamattaya et al., 2005; Niu et al., 2014). Similarly, increased haemocyte count was reported in *L. vannamei* (Xu & Pan, 2013; Panigrahi et al., 2017, 2018 & 2019; Abbaszadeh et al., 2019; Tong et al., 2020;), *P. monodon* (Kumar et al., 2015) and *P. indicus* (Panigrahi et al., 2020) in biofloc based systems. Further, Zhang et al. (2010) reported that, microbial biofilms in periphyton, developed over submerged substrates, enhanced the non-specific immunity factors in penaeid shrimp. Shrimps evidently consume microbial floc *in situ* in biofloc systems (Crab et al., 2012), that increases THC and PO activity in vannamei biofloc systems (Kim et al., 2014), indicating higher expression levels of proPO1, proPO2 and PPAE1 genes. Similarly, higher PO activity was observed in *L. vannamei* (Ekasari et al., 2014; Panigrahi et al., 2017, 2018 & 2019), *P. monodon* (Kumar et al., 2015) and *P. indicus* (Panigrahi et al., 2020) when grown in biofloc systems than in clear water system. Sundaram et al. (2021) also reported high PO activity, superoxide dismutase assay of *L. vannamei*, reared in substrate-integrated biofloc systems than that of clear water system. Significantly higher lysozyme activity was observed in GIFT strain of *Oreochromis niloticus* (Long et al., 2015) and *Labeo rohita* (Ahmad et al., 2016) reared in biofloc culture systems. Vega et al.

(2006) reported active antibacterial activity of *L. vannamei* recombinant lysozyme (*c-lyz*) against *Vibrio alginolyticus*, *V. parahaemolyticus* and *V. cholera*. In the present study, among the treatment groups shrimp grown in substrate-integrated biofloc system elicited significantly better THC, hyaline cell count, granulocyte, PO activity, serum protein and lysozyme activity over only biofloc or only substrate systems. This improvement might be attributed to the consumption of large quantity of bacteria (*Bacillus* and *Lactobacillus*) associated with bioflocs and substrates by the shrimp that has probably released immune-stimulatory substances in the intestinal tract and could significantly enhanced the immune status in *L. vannamei* juveniles in substrate-integrated biofloc system.

## Conclusion

This study reveals that the substrate-integrated biofloc based system is more suitable for vannamei farming than the systems with only biofloc or only substrate for better production and immune status of shrimp with enhanced water quality. Hence, initiatives are to be taken to develop several nutritional procedures like biofloc and periphyton based systems which can maximize the contribution of higher quality *in situ* food in the same culture system besides maintaining water quality and enhanced immune status in *L. vannamei*. This would help in expanding zero-water exchange biosecured culture systems for vannamei shrimp to achieve maximum production. These are the results of a small scale indoor laboratory experiment. So, further studies are required to understand the suitability and economic viability of biofloc system, substrate system and substrate-integrated biofloc system at shrimp farm level on commercial scale.

## Ethical Statement

All applicable national guidelines for the care and use of animals were followed by the authors.

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## Author Contribution

FirstAuthor: Conceptualization, Writing -review and editing, Data Curation, Formal Analysis, Investigation, Methodology, Visualization and Writing -original draft.

Corresponding Author: Funding Acquisition, Project Administration, Resources, Supervision, Writing -review and editing.

All authors have made substantial contributions to each step of experimental study. The idea for this study was given by major guide Dr. Tambireddy Neeraja to Chethurajupalli Lavanya. The experiment was designed and performed under the guidance of T. Neeraja for carrying out the Master of Fisheries Science (MFSc) research work. This article was prepared by the first author and edited by second author.

## Conflict of Interest

The authors declare no conflict of interest.

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