Received: 17 April 2019 | Revised: 6 June 2019 | Accepted: 20 June 2019

DOI: 10.1111/are.14256

ORIGINAL ARTICLE

Aquaculture Research WILEY

Integration of substrate in biofloc based system: Effects on growth performance, water quality and immune responses in black tiger shrimp, *Penaeus monodon* culture

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Funding information
Indian Council of Agricultural Research

Abstract

To evaluate effect of substrate integration in biofloc based system, a 52-day growth experiment was conducted using black tiger shrimp, *Penaeus monodon* juveniles (3.32 ± 0.07 g). The factorial design consisted of floc, F (with or without) as first factor and substrate (bamboo mat, B; nylon mesh, N; and without substrate) as second factor. This resulted six treatments; F + B, F + N, F, B, N and a control without biofloc and substrate. Shrimps were stocked at 110 nos. m^{-3} in Fibre Reinforced Plastic (FRP) tanks and, rice flour was used as carbon source in biofloc based treatments. Incorporation of nylon mesh and bamboo mat in biofloc system trapped 31.3%–38.6% and 8.5%–13.5% total suspended solids respectively and reduced bottom solid deposition. Among the substrate based groups, significantly better development of biofilm with higher microbial population noticed in F + B compared with nylon mesh. Similarly, significantly higher final growth ($p < 0.01$) was recorded in F + B system followed by F + N while no significant difference in body weight recorded among floc, F or substrate based groups (B, N). Biofloc and substrate integration (F + B and F + N) resulted significantly ($p < 0.01$) lower feed conversion ratio compared to control and floc. Incoporation of bamboo substrate in biofloc, (F + B) improved shrimp immune responses through higher hemocyte counts and prophenoloxidase activity compared to other treatments. The study revealed that integration of substrate in the biofloc system improved growth performance, FCR and immune parameters in shrimp by trapping the suspended biofloc particles, better water quality parameters, enhanced biofilm growth and provision of quality natural food.

KEYWORDS
biofloc, black tiger shrimp, growth performance, immune response, *Penaeus monodon*, substrate

1 | INTRODUCTION

Shrimp culture being the commercial face of aquaculture contributes to nutritional, livelihood security, and export earnings in developing countries. At present global shrimp industry is dominated by specific pathogen free pacific white shrimp, *Penaeus vannamei* which forms major share to total global shrimp production, 4.6 million metric ton (FAO, 2018) followed by black tiger shrimp, *P. monodon*. Economics of shrimp farming is largely dependent on the feed which constitutes 50%–60% of operational expenses (Tan et al., 2005). However,

shrimp retain only 20%–30% nutrients, and remaining 70%–80% is excreted and accumulated in the aquatic environment which many times leads to deterioration of soil and water quality (Crab, Defoirdt, Bossier, & Verstraete, 2012; Funge-Smith & Briggs, 1998). Intensification of shrimp culture is also related to physiological stress to the cultured animals and increased disease incidence of viral and bacterial origin (Kautsky, Ronnback, Tedengren, & Troell, 2000). These issues demand a paradigm shift in culture practices for sustainable shrimp culture.

Biofloc technology (BFT) is widely recognised as promising culture technique in aquaculture (Avnimelech, 2012; Azim & Little, 2008; Crab et al., 2012). It is based on the manipulation of C:N ratio by application of external carbohydrate which converts toxic ammonia nitrogen into microbial biofloc (Avnimelech, 1999; Hari, Kurup, Varghese, Schrama, & Verdegem, 2004; De Schryver, Crab, Defoirdt, Boon, & Verstraete, 2008). Biofloc consists of varieties of bacteria, microalgae, fungi, detritus and other suspended organisms (Anand et al., 2014; Azim & Little, 2008; Hargreaves, 2006). It improves water quality by removing toxic ammonia nitrogen and other excess nutrients (Anand et al., 2013; Crab et al., 2012) and serves as a good source of proteins, lipids, vitamins, and minerals to the cultured aquatic animals (Anand et al., 2014; Emerenciano, Ballester, Cavalli, & Wasielesky, 2011; Ju et al., 2008). It is reported that lipopolysaccharides (LPS), peptidoglycans and β-1,3-glucans present in bacterial or fungal cell wall activates the nonspecific innate immune system of crustaceans (Cerenius, Lee, & Söderhäll, 2008; Rengpipat, Rukpratanporn, Piyatiratitivorakul, & Menasaveta, 2000; Tassanakajon, Somboonwiwat, Supungul, & Tang, 2013). Several studies indicated that biofloc based culture system, being a diverse group of microbial community enhances shrimp immune system by improving hemocyte phagocytic activity, prophenoloxidase and superoxide dismutase activity (Ekasari et al., 2014; Kumar et al., 2017; Xu & Pan, 2013). Despite several reported benefits, the BFT finds technical hurdles at field level application (Crab et al., 2012). The biggest challenge lies in controlling the higher level of suspended particles and settlement of floc particles at the pond bottom. This leads to deterioration of water quality which negatively affects the health and growth of cultured shrimp (Gaona, Almeida, Viau, Poersch, & Wasielesky, 2017; Ray, Lewis, Browdy, & Leffler, 2010). To periodically remove excess unutilised floc particles various strategies such as central drainage system (Avnimelech, 2012) and settling tanks (Arantes, Schveitzer, Magnotti, Lapa, & Vinatea, 2017; Ray, Dillon, & Lotz, 2011) have been advised, these demands added facility and expenditure apart from wastage of microbial floc generated by additional carbohydrate application.

Application of submersed substrate in shrimp culture system helps to harness natural productivity and results in the formation of periphyton, a complex of microalgae and microorganism developed over submerged substrates (Anand et al., 2013; Audelo-Naranjo, Martínez-Córdova, Gómez-Jiménez, & Voltolina, 2017; Azim, Verdegem, Dam, & Beveridge, 2005; Keshavanath et al., 2001) or biofilms (Abreu et al., 2007; Khatoon, Yusoff, Banerjee, Shariff, & Mohamed, 2007; Sharma et al., 2010; Thompson, Abreu, & Wasielesky, 2002). These submerged substrates apart from being a constant source of quality natural food, improves nutrient recycling (Ballester, Wasielesky, Cavalli, & Abreu, 2007), and act as a refuge for shrimp during moulting and reduces the negative effect of high stocking densities (Arnold, Coman, Jackson, & Groves, 2009; Ballester et al., 2007). Similarly, better, growth performance (Anand et al., 2013) and immune response of shrimp are reported from substrate based shrimp culture (Anand et al., 2015; Kumar et al., 2015).

Integration of submerged substrates in biofloc system reported to have multiple advantages like better water quality and growth performance of shrimp (Anand et al., 2013; Ferreira, Lara, Wasielesky, & Abreu, 2016; Schveitzer et al., 2013). Though an array of reports on biofloc and substrate-based aquaculture documented, there is scarcity of data on the integration of different types of submerged substrates in biofloc system and its role in trapping flocculated particles and its microbial composition. Against this backdrop, the present study was taken to elucidate the integrated biofloc substrate system in growth performance, immune response, microbial and water quality dynamics in black tiger shrimp culture.

2 | MATERIALS AND METHODS

2.1 | Experimental design and set up

A 52-day on-station factorial experiment was conducted using black tiger shrimp juveniles in 18 fiberglass reinforced plastic (FRP) rectangular tanks (70 × 50 cm; 100 L) at Kakdwip Research Centre, ICAR-Central Institute of Brackishwater Aquaculture, Kakdwip (21°51′N; 88°11′E), West Bengal, India.

The experimental design consisted of biofloc (with or without floc) as first factor and substrate (with or without) as second factor. This leads to six treatments comprising floc (F), floc with bamboo mat (F + B), floc with nylon mesh (F + N), bamboo mat (B), nylon mesh (N), and a group without floc and without substrate as control (C). All the groups received 40% crude protein diet while only floc groups (F, F + B, and F + N) received additional rice flour as a carbon source for biofloc production.

Healthy juvenile shrimp, *P. monodon* were obtained from a commercial shrimp farm (South 24 Parganas, West Bengal, India). Shrimp were acclimatized for 14 days and fed with control diet (40% crude protein) two times daily, before the start of the experiment. One hundred and ninety-eight *P. monodon* juveniles (3.32 ± 0.07 g) were randomly distributed into 18 FRP tanks (100 L) at 110 nos. m^{-3} following a completely randomized design.

Each FRP tank in the B and F + B treatment groups were provided with two bamboo mats, each having an area of 1,307.8 ± 10.3 cm^2. The bamboo mat was positioned at 30° slanting position in rectangular FRP tanks. Similarly, two nylon nets of 40 mesh, each having the area of 1,312.2 ± 21.5 cm^2 was placed in N and F + N treatment tanks. The nylon mesh was positioned 5 cm from the side and bottom of rectangular FRP tanks. Provison of submerged substrtates resulted in an additional surface area of 74.7%–75.0% of the tank surface area.

Shrimp in all the experimental groups were fed with the diet having 40% crude protein level. The feeding started with 7% of body weight and was reduced gradually to 5% of body weight at the end of the experiment. An equal amount of feed was fed to shrimp in all the experimental tanks, twice daily at 10:00 and 18.00 hr for 52 days. Rice flour (carbohydrate source) was added externally in biofloc treatment groups (F, F + B, and F + N) for converting total ammonia nitrogen into biofloc. To convert 1 g of total ammonia nitrogen (TAN) produced from excretion and uneaten feed, 10 g carbon or 20 g carbohydrate is required (Avnimelech, 1999). Rice flour (40% carbon) was added at the rate of 0.6 g for each 1.0 g of feed. The control group (C) received only feed and was neither provided with carbohydrate nor substrate. Continuous aeration and agitation were provided to each tank by two air stones, each diffusing 3.33 m^3 air $tank^{-1}$ min^{-1}. The loss of water due to evaporation and experimental analysis was replenished by addition of external water. Apart from this, no water exchange was done during the entire experimental period.

2.2 | Experimental diet

Experimental diet with 40% crude protein level was prepared. The composition of the experimental diet is presented in Table 1. Ingredients like wheat flour, fish meal, soybean meal, shrimp meal, guar gum, and lecithin were mixed with water to make the dough. The dough was steam cooked for 20 min in a pressure cooker at 10 psi. After cooling, additives like cholesterol, butylated hydroxytoluene (BHT), oil and the vitamin-mineral mixture was mixed with the test diet. The dough was pressed through a pelletizer with 2 mm die. The feed was dried at 60°C till the desired moisture level (<10%) reached.

TABLE 1 Composition of experimental diet on dry matter basis (g/kg)

Feed ingredients	Experimental diet
Fish meal	400
Shrimp meal	150
Soyabean meal	245.3
Wheat flour	115.2
Soya oil	30
Cod liver oil	10
Lecithin	10
Cholesterol	1
Vitamin and mineral mix[a]	16
Butylated hydroxytoluene	0.5
Guar gum	20
Vitamic C	1

[a]Composition of vitamin mineral mix (Supplevite-M) (quantity/kg): Vitamin A, 20,00,000 IU; Vitamin D3, 400,000 IU; Vitamin E, 300 unit; Vitamin B2, 0.8 g; Vitamin B6, 0.4 g; Vitamin K, 0.4 g; Vitamin B12, 2.4 mcg; Calcium Pantothenate, 1 g; Nicotinamide, 4 g; Choline Chloride, 60 g; Mn, 10.8 g; Iodine, 4 g; Fe, 3 g; Zn, 6 g; Cu, 0.8 g; Co, 0.18 g.

2.3 | Proximate composition of the experimental diet

Proximate composition of the experimental diets was determined following the standard method of AOAC (1995). Moisture content was estimated by oven drying at 105°C to a constant weight. Crude protein (N × 6.25) was estimated by the Kjeldahl method after acid digestion using an auto Kjeldahl system (Kelplus, DXVA, Pelican Equipments, India). Crude lipid was determined by ether extraction method using a Soxtec (Socs plus, SCS–6, Pelican Equipments). Ash content was estimated by incineration at 600°C for 6 hr in a muffle furnace. Crude fiber was estimated by sequential digestion with H_2SO_4 and NaOH using Fibertec (Foss Tecator 2022, Sweden). Nitrogen-free extract (NFE) and gross energy were determined by Jantrarotai, Sitasit, and Rajchapakdee (1994) and N.R.C. (1993) formulae. The carbon content in the feed and rice flour was determined by the formula of Hart, Lovis, Schulenberg, and Urquhart (2007).

$$\text{Nitrogen-free extract} = 100 - (\text{crude protein} + \text{crude lipid} + \text{ash} + \text{crude fibre} + \text{moisture})$$

$$\text{Gross energy (Kcal/100 g)} = (\text{Protein \%} \times 5.6) + (\text{Lipid \%} \times 9.44) + (\text{Crude fibre \%} \times 4.1) + (\text{NFE \%} \times 4.1)$$

$$\text{Carbon} = (0.80 \times \text{Lipid}) + (0.53 \times \text{Protein}) + (0.42 \times \text{Carbohydrate}) + (0.42 \times \text{Fibre})$$

2.4 | Determination of water quality parameters

The water quality parameters measured at regular intervals (0, 14, 24, 34 and 50 days) between 09:00 and 10:00 hr. Salinity, pH and dissolved oxygen (DO) measured with conductivity probe (CDC401), pH probe (PHC281) and luminescent dissolved oxygen (LDO) probe respectively, using Hach multi-parameter kit (HQ30D, Hach, USA). The total ammonia nitrogen (TAN) was measured by the phenol-hypochlorite method (Solorzano, 1969). The nitrite–N (NO_2–N), nitrate–N (NO_3–N), and phosphate–P were analyzed spectrophotometrically by colorimetry, hydrazine reduction, and ascorbic acid method respectively as per the protocols described in APHA (1998).

2.5 | Biofloc quantification

Biofloc was quantified at regular intervals (0, 14, 24, 34 and 50 days) by sampling 1,000 ml water in a series of Imhoff cone (Tarson, India). The floc accumulated at the bottom of the cone after 20 min was expressed as floc volume (ml/L; Avnimelech, 2012). On 28 and 50th day of the experiment, a detailed analysis of solid particles was carried out. The floc particles in suspension (total suspended solids), settled at the tank bottom (total bottom solid) and attached over bamboo mat or nylon mesh (total substrate solid) were analyzed. To estimate total suspended solids, water samples (125 ml) from the treatment tanks were collected without agitating the tank water and filtered under vacuum through pre-dried pre-weighed GF/C filter paper. The filter paper was

dried overnight at 105°C. The difference in weight was expressed as total suspended solids (mg/L). After carefully removing the substrate, water was thoroughly mixed to bring all the bottom settled particles in suspension. A 125 ml water sample was processed as described earlier. Fully agitated water provided the estimation for mixed solid (total suspended solids and total bottom solid). The deduction of total suspended solids from the mixed solid gave the estimation for total bottom solid. For estimating the total substrate solid, the substrate was carefully removed. The attached solid particles over substrate were scrapped using a sterile scalpel from 2.0 × 2.0 cm^2 area. The scrapped samples collected from 5 different locations/substrate were pooled together and dried overnight at 105°C in a pre-dried and pre-weighed container. The difference in final and initial weight was expressed as total substrate solid (mg/cm^2).

The different types of solids were estimated by the following formulae.

$$\text{Total suspended solid}\ (\text{mg/L}) = 1000 \times \frac{\text{Wt of dried filter paper with filtrtae containg suspended particles in water coulmn} - \text{wt of dried empty filter paper}}{\text{volume of sample}}$$

$$\text{Total mixed solid}\ (\text{mg/L}) = 1000 \times \frac{\text{wt of dried filter paper with filtrate containg both suspended and bottom settled floc particles} - \text{wt of dried empty filter paper}}{\text{volume of sample}}$$

$$\text{Total bottom solid}\ (\text{mg/L}) = \text{Total mixed solid} - \text{Total suspended solid}$$

$$\text{Total substrate solid}\ \left(\text{mg/cm}^2\right) = \frac{\text{Dried wt of scrapped sample}}{\text{Total scrapped area}}$$

$$\text{Total solid}\ (\text{mg/L}) = \text{Total suspended solid} + \text{Total bottom solid} + \left(\text{Total substrate solid}\ \left(\text{mg/cm}^2\right) * \text{surface area of substrate}\right) / \text{volume of water}$$

2.6 | Growth performance and survival

At the end of the trial, weekly growth performance parameters and survival were estimated using the following formulae.

Feed conversion ratio (FCR) = Feed applied/live weight gain
Protein efficiency ratio (PER) = Gain in body mass/protein applied
Specific growth rate (SGR) = (ln final weight − ln initial weight) × 100/days of experiment
Survival % = (Total number of shrimp survived/Total number of shrimp stocked) × 100
Weight gain/day = (Final weight − Initial weight)/days of experiment

2.7 | Estimation of biofloc microbial community

Water and substrate scrapped samples were processed for microbial analysis as per earlier described methods (Anand et al., 2013; Kumar et al., 2017). In brief, 200 ml of a water sample from treatment tanks was homogenized for 30 s in a blender for dissociating the bacteria from the flocculated material. Similarly, from substrates, the scrapped substrate sample (2 g) after mixing with 200 ml of normal saline solution (NSS) was homogenized for 1 min. The homogenized samples were inoculated on tryptone soya agar (1.0% w/v NaCl) for total heterotrophic bacterial count and thiosulfate citrate bile salt sucrose (TCBS) agar for *Vibrio spp*. *Bacillus*, *Lactobacillus* and yeast counts were carried out using *Bacillus cereus* agar, *Lactobacillus* MRS agar and Sabouraud dextrose agar (HiMedia, Mumbai, India), respectively. Mat samples were analyzed for microbial parameters on 28th and 50th days of the experiment. The water samples were analyzed at 14 days interval. Microbial plates were incubated in aerobic condition at 28°C except for *Lactobacillus* MRS agar plates which were incubated in microaerophilic condition. Colony in the range of 30 to 300 counted and expressed as colony forming unit (CFU/mL).

2.8 | Hemolymph collection and hemocyte lysate supernatant preparation

After completion of the feeding experiment, 9 inter-molt shrimps from each treatment group (3 from each replicate) were anesthetized with clove oil (50 µl/L). Hemolymph was collected from the ventral sinus of each shrimp using 26 Gauge, 1 ml syringe and mixed with cooled anticoagulant (30 mM trisodium citrate, 388 mM sodium chloride, 0.12 M glucose, 10 mM EDTA, and pH 7.55). About 300 µl hemolymph was collected from each shrimp.

Hemocyte lysate supernatant (HLS) was prepared using methods described by Smith and Söderhäll (1991). In brief, hemolymph was centrifuged at 300 *g* for 10 min at 4°C and the pellet was washed with 1 ml cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M NaCl, 0.1 M trisodium citrate; pH 7.0). After centrifugation the pellet was re-suspended in chilled 1:10 cacodylate (CAC) buffer (0.01 M sodium cacodylate, 0.45 M NaCl, 10 mM CaCl2, 26 mM MgCl2; pH 7.0) and homogenized with a probe sonicator (PCI Analytics, India) at 20 KHz amplitude. The lysate was centrifuged at 15,000 *g* for 15 min at 4°C and the collected supernatant were stored at 4°C till further use.

2.9 | Hemocyte count and immunological parameters

Hemolymph mixed with cooled anticoagulant solution was counted in improved Neubauer bright-line chamber under 400 × magnifications by phase contrast microscope (Carl Zeiss). The cells were differentiated into granulocyte and hyaline cells based upon the granular content and size of the cells (Sritunyalucksana, Gangnonngiw, Archakunakorn, Fegan, & Flegel, 2005). Cells were expressed as total hemocyte count/mL, total granulocyte count/mL and total hyaline cells count/mL. The prophenoloxidase and superoxide dismutase activity were determined as per our earlier described methods (Kumar et al., 2017).

2.10 | Statistical analysis

Growth performance, water quality, microbial analysis, immune response parameters were analyzed by two-way ANOVA using biofloc as first factor and different substrates as second factor. To see the difference among the treatments, growth performance and immune response parameters were further analyzed by one-way ANOVA. The level of significance was made at 95% and 99% level. Before all analysis, data was checked for normality by probability plots and homogeneity of variances by Levene's test. All analysis was performed using the statistical software package SAS v.9.2 program (SAS Institute, Cary, NC, USA).

3 | RESULTS

3.1 | Nutrient composition of diet and carbon supplements

The protein content of the experimental diet was 40.45 ± 0.28 (Table 2) and lipid content was 5.96 ± 0.45. Rice flour used as carbon source for biofloc formation had 8.1% protein and 81% nitrogen-free extract.

3.2 | Water quality parameters

Average water quality parameters (mean ± *SE*) of biofloc and substrate added groups during experimental period are presented in Table 3. Though carbohydrate addition does not significantly reduced inorganic nitrogenous compounds such as TAN, NO_2-N, NO_3-N, comparatively lower level was recorded among floc based groups. However, substrate addition has resulted significant reduction ($p < 0.05$) in TAN among the treatment. The level of total ammonia nitrogen (TAN) over the time period indicated reduction in TAN level on 14th and 24th day of sampling in carbohydrate supplemented floc groups (F, F + B, F + N) compared to control (Figure 1a).

TABLE 2 Proximate composition of experimental diet and carbon supplement (mean ± *SD*)

Nutrients	Feed	Rice flour
Crude protein	40.45 ± 0.28	8.14 ± 0.00
Crude lipid	5.96 ± 0.45	0.80 ± 0.03
Crude fibre	12.35 ± 0.10	1.54 ± 0.11
Total ash	15.21 ± 0.13	0.99 ± 0.00
Acid insoluble ash	3.05 ± 0.04	0.27 ± 0.02
Moisture	6.45 ± 0.14	7.46 ± 0.05
Nitrogen free extract[a]	19.58 ± 1.10	81.07 ± 0.18
Gross energy (KCal/100 g)[b]	413.70 ± 1.74	391.81 ± 0.07

[a]Nitrogen free extract = 100 − (Crude protein + Crude fat + Crude fibre + Ash +Moisture).
[b]Gross energy = (Crude protein × 5.6) + (Crude fat × 9.44) + (Crude fibre × 4.1) + (NFE × 4.1) Kcal/100 g (NRC, 1993).

Though comparatively lower, substrate based groups such as bamboo mat (B) and nylon mesh (N) recorded lower TAN level during initial samplings. The nitrite-N and nitrate-N level started increasing from the 14th day with steepest rise observed on 24th-day sampling in all the treatment groups. The rise in nitrite-N and nitrate-N levels was concurrently associated with a reduction in TAN level on the 24th day.

The dissolved oxygen level in all the treatments were in the safe limit, 6.1 to 7.2 ppm, and was influenced by carbohydrate addition as treatments with floc (F, F + B and F + N) recorded lower DO levels throughout the experiment compared to control (C; without floc and substrate; Figure 1d).

3.3 | Floc quantification

The level of floc volume (FV) was higher throughout the experimental periods in floc based groups (F, F + B, and F + N) compared to without carbohydrate supplemented groups (C, B, and N; Figure 1e). Among floc based treatments, the highest level of FV was observed on the 50th day, in treatment F (8.7 ml/L), followed by bamboo integrated floc system, F + B (8.2 ml/L) and nylon integrated floc system, F + N (6.8 ml/L). Among the groups without carbohydrate supplements (C, B, and N), the FV level ranged between 0 and 3.5 ml/L, with the lowest level recorded in nylon mesh (N) group.

The generated flocculated particles in experimental tanks were categorized as total suspended solid (TSS), total bottom solid (TBS) and total substrate solid (TSbS; Table 4). The addition of substrate had significantly reduced ($p < 0.01$) the TSS and TBS. Similar to floc volume, the level of total suspended solid was significantly higher in floc based groups, F and F + B compared to without carbon added groups (C, B, and N). Incorporation of submerged substrates in biofloc system (F + B and F + N) resulted significantly lower level ($p < 0.05$) of TBS, and TSS compared to only floc based treatment (F). Overall, the integration of substrate in biofloc system, F + B and F + N groups reduced 49.43% and 37.79% deposition of bottom solid, respectively. Similarly, there was 30.43% and 34.32% reduction in TSS level due to incorporation of submerged substrates; bamboo mat (F + B) and nylon mesh (F + N) compared with floc based treatment, F. Among the substrate, biofilm development was faster in bamboo mat compared with nylon mesh.

Taking the entire solid into consideration, incorporation of nylon mesh in biofloc, F + N retained approximately one third of solid over surface (31.3%–38.6%), 9.4%–35.7% in suspension and 33.1%–52.0% at bottom while the floc group (F) recorded 21.5%–30.4% in suspension and rest at the tank bottom (Figure 2). Compared to F + N, the bamboo integrated biofloc system (F + B) retained comparatively less over bamboo mat (8.5%–13.5%).

3.4 | Microbial count

The microbial count developed in water coulmn and over the substrate is presented in Tables 5 and 6, respectively. Among the measured water microbial population, the *Bacillus* count was influenced

TABLE 3 Effects of substrate and biofloc on water quality parameters (mean ± *SE*) in *Penaeus monodon* experiment based on two-way ANOVA

	Without Floc			Floc			Interaction effects		
Parameters	**C**	**B**	**N**	**F**	**F + B**	**F + N**	**F**	**S**	**F × S**
Temperature (°C)	30.83 ± 0.04	30.85 ± 0.07	30.81 ± 0.07	30.94 ± 0.07	30.93 ± 0.07	30.96 ± 0.10	NS	*	NS
Salinity (ppt)	13.76 ± 0.22	13.77 ± 0.21	13.77 ± 0.21	13.69 ± 0.20	13.76 ± 0.19	13.73 ± 0.20	NS	NS	NS
pH	7.57 ± 0.13	7.68 ± 0.09	7.78 ± 0.04	7.68 ± 0.09	7.82 ± 0.04	7.77 ± 0.03	NS	NS	NS
Dissolved oxygen (ppm)	6.91 ± 0.05	6.72 ± 0.08	6.69 ± 0.08	6.68 ± 0.06	6.62 ± 0.07	6.64 ± 0.07	NS	*	NS
Alkalinity (mg $CaCo_3$)	103.56 ± 4.96	112.89 ± 1.60	105.33 ± 3.53	96.89 ± 6.07	101.78 ± 2.99	106.22 ± 2.91	NS	NS	NS
Total Ammonia Nitrogen(TAN) (mg/L)	0.69 ± 0.15	0.60 ± 0.11	0.61 ± 0.11	0.40 ± 0.06	0.52 ± 0.08	0.39 ± 0.05	NS	*	NS
Nitrite-N (mg/L)	0.64 ± 0.07	0.71 ± 0.06	0.69 ± 0.04	0.63 ± 0.09	0.69 ± 0.07	0.69 ± 0.07	NS	NS	NS
Nitrate-N (mg/L)	2.88 ± 0.42	3.06 ± 0.42	2.82 ± 0.36	2.92 ± 0.55	3.06 ± 0.50	2.88 ± 0.44	NS	NS	NS
Phosphate-P (mg/L)	0.57 ± 0.10	0.63 ± 0.12	0.63 ± 0.13	0.59 ± 0.11	0.60 ± 0.11	0.68 ± 0.13	NS	NS	NS

Note: The level of significance is indicated by *<0.05; NS, not significant. The treatment groups are control (C), floc (F), bamboo mat (B), nylon mesh (N), floc with bamboo mat (F + B) and floc with nylon mesh (F + N).
Abbreviations: F: Floc; F × S = Floc × Substrate; S: Substrate.

($p < 0.05$) by carbohydrate supplementation with higher level recorded in biofloc based treatments. In comparison, yeast count showed significant difference ($p < 0.05$) among the treatments with higher level recorded in bamboo mat (B) and floc integrated bamboo mat (F + B).

Microbial counts over substrate revealed that *Bacillus* ($p < 0.05$), *Lactobacillus* ($p < 0.01$) and yeast ($p < 0.01$) populations showed significant difference among the treatments with the highest level recorded in F + B group. Similarly, floc integrated with nylon (F + N) recorded a comparatively higher level of total bacterial count, *Bacillus* and *Lactobacillus* counts compared to without biofloc substrate treatments (B and N). Among different microbes, the most predominant bacterial species over mat surface was *Bacillus* (10.3%–13.6%).

3.5 | Growth performance

Growth performance parameters of *P. monodon* are presented in Table 7. The results indicated that provision of substrate in biofloc positively influenced ($p < 0.01$) the final body weight and weight gain/day. The highest final body weight, 7.99 ± 0.30 g was noticed in floc integrated bamboo mat system (F + B) followed by floc integrated nylon (F + N; 7.51 ± 0.12 g) which was significantly higher ($p < 0.01$) compared with control, C (5.67 ± 0.24 g). Similarly, significantly higher weight gain was observed in bamboo mat (B; 6.92 ± 0.26 g) and floc, F (7.03 ± 0.34 g) compared with control. The body weight gain day^{-1} was almost 100% higher in F + B (89.88 mg/day) compared with control, C (45.16 mg/day).

Factorial ANOVA indicated a significant interaction between substrate and floc on feed conversion ratio (FCR). Substrate integrated biofloc systems (F + B and F + N) had significantly lower FCR, and higher protein efficiency ratio (PER) compared to other treatments. However, no significant difference in FCR and PER were recorded among F or B or N treatments, and were significantly better ($p < 0.05$) than control. Provision of substrate and floc significantly influenced ($p < 0.01$) the survival of shrimp juveniles. At the end of the experiment, substrate integrated biofloc groups (F + B and F + N), and bamboo alone (B) recorded the highest level of survival (93.9%) compared to nylon alone (81.8), Floc (78.8) and control (63.6%).

3.6 | Immunological parameters

Integration of substrates in floc system resulted better cellular immune responses through significantly higher level of total hemocyte ($p < 0.01$), granulocyte ($p < 0.01$) and hyaline cell ($p < 0.01$) counts compared to those without substrate based treatments (Figure 3a–c). Among the treatments, floc with the bamboo substrate (F + B) recorded the highest total hemocyte count (63.2 ± 1.3 × 10^6 cells/ml) which differed significantly from floc, F (35.0 ± 3.1 × 10^6 cells/ml) and control (20.8 ± 2.2 × 10^6 cells/ml). Among the substrate based treatments, bamboo group (B) recorded higher hemocyte ($p < 0.05$), granulocyte ($p > 0.05$) and hyaline cell ($p < 0.05$) counts compared with control. Though insignificant ($p > 0.05$), the level of hemocyte, granulocyte and hyaline cell counts in bamboo substrate group (B) were higher compared to floc (F) and floc integrated nylon (F + N) groups.

Immunological parameters such as prophenoloxidase (proPO) activity and anti-oxidative enzyme superoxide dismutase (SOD) activity was measured in hemocyte lysate supernatant (HLS) and is presented in Figure 3d,e. Substrate provision significantly increased the proPO activity ($p < 0.01$) and SOD ($p < 0.05$) compared

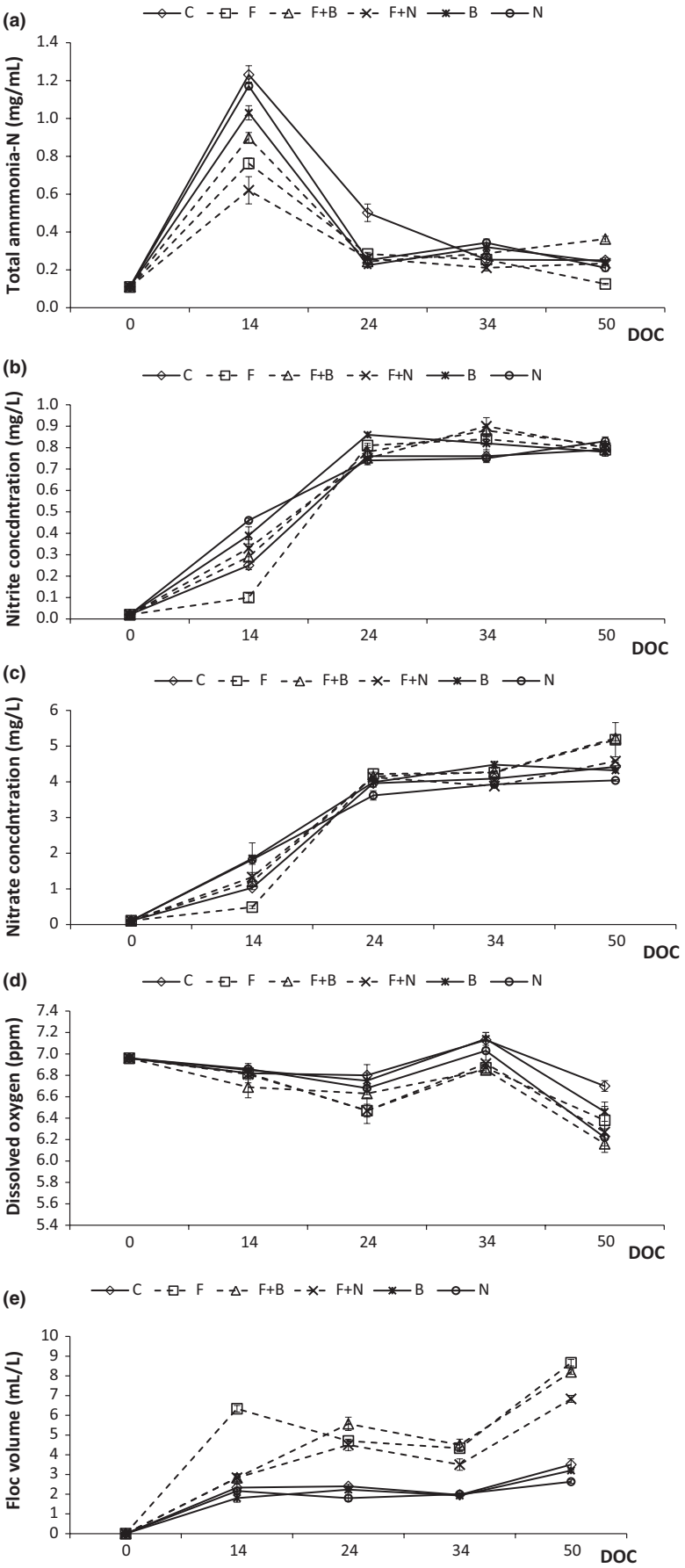

FIGURE 1 Water quality parameters (a) Total ammonia-N (b) Nitrite-N (c) Nitrate-N (d) Dissolved oxygen (e) Floc volume measured over the time period (mean ± *SE*) in experimental groups with biofloc and substrate combination, floc (F), bamboo mat (B), nylon mesh (N), floc with bamboo mat (F + B) and floc with nylon mesh (F + N) and control (C)

TABLE 4 Effects of substrate and biofloc on floc volume and solid particles parameters (mean ± *SE*) in *Penaeus monodon* experiment based on two-way ANOVA

	Without Floc			Floc			Interaction effects		
Parameters	C	B	N	F	F + B	F + N	F	S	F × S
Floc volume (ml/L)	2.6 ± 0.2	2.3 ± 0.2	2.2 ± 0.1	6.0 ± 0.5	5.3 ± 0.6	4.4 ± 0.5	*	**	NS
Total suspended solid (mg/L)	244.6 ± 13.1	245.3 ± 13.5	246.6 ± 24.7	603.7 ± 23.1	420.4 ± 43.6	396.5 ± 39.3	NS	**	NS
Total bottom solid (mg/L)	162.7 ± 22.4	163.8 ± 4.6	178.2 ± 25.3	446.7 ± 23.8	225.9 ± 78.3	277.9 ± 58.6	*	**	NS
Total substrate solid (mg/cm^2)	–	0.9 ± 0.1	3.9 ± 0.2	–	1.9 ± 0.1	8.4 ± 1.4	**	**	NS
Total solid (mg/L)	244.6 ± 13.1	269.3 ± 11.1	347.8 ± 23.4	603.7 ± 23.1	470.2 ± 44.0	618.1 ± 74.2	*	**	NS

Note: The level of significance is indicated by *<0.05; **<0.01; NS, not significant. The treatment groups are control (C), floc (F), bamboo mat (B), nylon mesh (N), floc with bamboo mat (F + B) and floc with nylon mesh (F + N).
Abbreviatons: F: Floc; F × S = Floc × Substrate; S: Substrate.

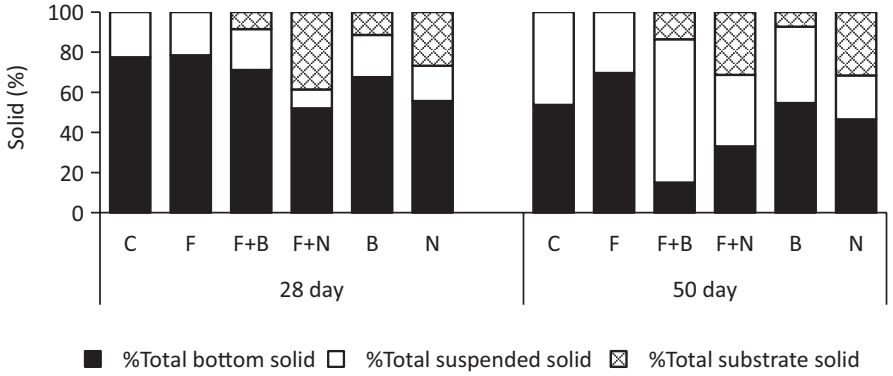

FIGURE 2 Percentage contribution by different types of solid (total suspended solid, total bottom solid and total substrate solid) recorded during 28 and 50th day of experiment with biofloc and substrate combination. The treatments were control (C), floc (F), bamboo mat (B), nylon mesh (N), floc with bamboo mat (F + B) and floc with nylon mesh (F + N).

TABLE 5 Water microbial load (mean ± *SE*) in treatments with biofloc and substrates combination in *Penaeus monodon* experiment based on two-way ANOVA

	Without Floc			Floc			Interaction effects		
Parameters	C	B	N	F	F + B	F + N	F	S	F × S
Total bacteria (×10^5 CFU/mL)	6.0 ± 1.9	12.8 ± 4.2	7.0 ± 1.9	13.3 ± 4.6	14.2 ± 2.0	9.7 ± 2.4	NS	NS	NS
Total *Vibrio* (×10^5 CFU/mL)	0.7 ± 0.4	1.7 ± 1.0	1.0 ± 0.6	0.7 ± 0.3	1.0 ± 0.4	1.8 ± 0.9	NS	NS	NS
Bacillus (×10^5 CFU/mL)	3.8 ± 0.8	2.9 ± 0.3	4.6 ± 1.1	5.8 ± 1.7	6.8 ± 1.5	4.3 ± 0.7	*	NS	NS
Lactobacillus (×10^2 CFU/mL)	37.8 ± 15.2	35.8 ± 9.5	18.3 ± 3.8	44.2 ± 10.2	52.5 ± 14.0	72.5 ± 30.1	NS	NS	NS
Yeast (×10^3 CFU/mL)	7.5 ± 2.5	50.0 ± 15.0	62.5 ± 12.5	30.0 ± 5.0	77.5 ± 12.5	60.0 ± 15.0	NS	*	NS

Note: The level of significance is indicated by *<0.05; NS, not significant. The treatment groups are control (C), floc (F), bamboo mat (B), nylon mesh (N), floc with bamboo mat (F + B) and floc with nylon mesh (F + N).
Abbreviations: F: Floc; F × S = Floc × Substrate; S: Substrate.

to without substrate based treatments. The higher proPO activity was observed in bamboo mat based treatments (F + B and B) which differed significantly ($p < 0.01$) from control (C) and floc (F) based treatment groups. Similarly, the highest level ($p < 0.05$) of anti-oxidative enzyme SOD was observed in F + B group. Nylon based substrate groups (N and F + N) showed comparatively higher level of

TABLE 6 Microbial counts (mean ± *SE*) developed over substrate in treatments with biofloc and substrates combination in *Penaeus monodon* experiment based on two-way ANOVA

	Without floc		Floc		Interaction effects		
Parameters	**B**	**N**	**F + B**	**F + N**	**F**	**S**	**F × S**
Total bacteria (×10^7 CFU/mL)	11.3 ± 4.3	13.8 ± 3.5	23.8 ± 3.8	15.7 ± 5.4	NS	NS	NS
Total *Vibrio* (×10^6 CFU/mL)	2.8 ± 0.8	4.8 ± 0.7	4.7 ± 1.5	4.2 ± 0.8	NS	NS	NS
Bacillus (×10^7 CFU/mL)	0.9 ± 0.1	1.7 ± 0.3	2.6 ± 0.5	2.4 ± 0.6	**	NS	NS
Lactobacillus (×10^4 CFU/mL)	1.2 ± 0.2	0.8 ± 0.2	3.2 ± 0.4	2.5 ± 0.6	**	NS	NS
Yeast (×10^5 CFU/mL)	3.2 ± 0.2	1.6 ± 0.1	6.5 ± 1.0	2.0 ± 0.3	**	**	*

Note: The level of significance is indicated by *<0.05; **<0.01; NS, not significant. The treatment groups are control (C), floc (F), bamboo mat (B), nylon mesh (N), floc with bamboo mat (F + B) and floc with nylon mesh (F + N).
Abbreviations: F: Floc; F × S = Floc × Substrate; S:Substrate.

TABLE 7 Growth performance (mean ± *SE*) of *Penaeus monodon* with biofloc and different substrates combination based on two-way ANOVA

	Without Floc			Floc			Interaction effects		
Parameters	**C**	**B**	**N**	**F**	**F + B**	**F + N**	**S**	**F**	**S × F**
Final Wt	5.67 ± 0.24^{c}	6.92 ± 0.26^{ab}	6.80 ± 0.27^{bc}	7.03 ± 0.34^{ab}	7.99 ± 0.30^{a}	7.51 ± 0.12^{ab}	**	**	NS
FCR	4.26 ± 0.19^{a}	2.36 ± 0.08^{bcd}	2.78 ± 0.17^{bc}	2.78 ± 0.14^{b}	2.04 ± 0.03^{d}	2.18 ± 0.09^{cd}	**	**	**
PER	0.59 ± 0.02^{c}	1.06 ± 0.03^{ab}	0.91 ± 0.06^{b}	0.90 ± 0.05^{b}	1.22 ± 0.02^{a}	1.15 ± 0.05^{a}	**	**	NS
SGR	0.95 ± 0.08^{b}	1.31 ± 0.04^{a}	1.28 ± 0.07^{a}	1.34 ± 0.09^{a}	1.57 ± 0.06^{a}	1.46 ± 0.03^{a}	**	**	NS
Wt gain/day (mg)	45.16 ± 4.56^{c}	69.29 ± 3.14^{ab}	66.95 ± 5.14^{bc}	71.34 ± 6.46^{ab}	89.88 ± 5.33^{a}	80.64 ± 2.27^{ab}	**	**	NS
Survival %	63.64 ± 0.00^{b}	93.94 ± 3.03^{a}	81.82 ± 5.25^{a}	78.79 ± 3.03^{ab}	93.94 ± 3.03^{a}	93.94 ± 3.03^{a}	**	**	NS

Note: The treatment groups are control (C), floc (F), bamboo mat (B), nylon mesh (N), floc with bamboo mat (F + B) and floc with nylon mesh (F + N). F: Floc; F × S = Floc × Substrate; S: Substrate.
Means in the same row having different superscript differ significantly. The level of significance is indicated by **<0.01; NS, not significant.

SOD activity compared to both control and floc groups though the effect was not significant.

4 | DISCUSSION

The control of excess suspended solids is a major challenge in biofloc system (Gaona et al., 2017; Ray et al., 2010). The biofloc particles vary in size from few microns to more than 1 mm with larger particles tend to settle at the bottom even with heavy aeration (De Schryver et al., 2008). The overload of suspended biofloc particles can cause several adverse effects such as deterioration of water quality, growth retardation and clogging of shrimp respiratory systems (Gaona et al., 2017; Ray et al., 2010). The basic aim of the present experiment was to control the excess suspended particles generated in the biofloc system by using various substrate. Integration of submerged substrates like nylon mesh and bamboo mat trapped 31.3%–38.6% and 8.5%–13.5% biofloc particles, respectively. A higher level of suspended biofloc deposition in nylon can be attributed to the fine meshes in nylon which provided a better trapping material compared to the bamboo mat. This was further evident as it recorded a lower level of floc particles deposition at tank bottom (33.1%–52.0%) compared to biofloc without substrate (69.6%–78.5%). Several authors have reported that managing the total suspended solids is crucial for shrimp growth and system stability (Ebeling, Timmons, & Bisogni, 2006; Gaona et al., 2017; Ray et al., 2010; De Schryver et al., 2008). Though, removal of excess suspended solids in biofloc system is primarily achieved through central drainage system (Avnimelech, 2012), it demands modification of pond structure, energy for pumping out excess solid, and loss of underutilized nutrient rich biofloc coming by periodic addition of carbohydrate. Our earlier studies revealed that the nature of carbohydrate influences the quantum of biofloc production (Kumar et al., 2017). A carbon source with a complex polysaccharide such as rice flour produces a slow generation of biofloc compared to molasses having sucrose, a disaccharide. This indicate an appropriate carbon source and substrate integrated biofloc system could control suspended floc particles and its deposition at the pond bottom.

At high C:N ratio, heterotrophic bacteria utilize ammonium ion for production of microbial protein. This helps to reduce toxic total ammonia nitrogen (TAN) level in biofloc system (Avnimelech, 1999).

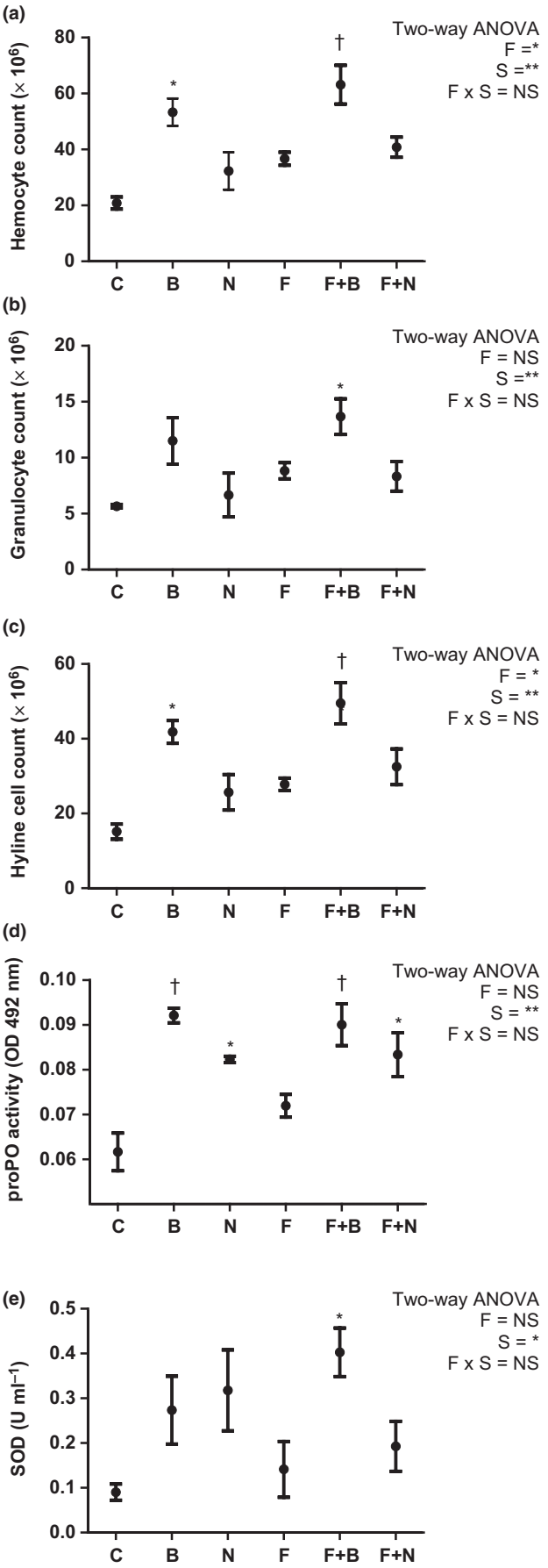

FIGURE 3 Immunological parameters (mean ± standard mean) measured at the end of experiment with biofloc and substrate combination. The effect of substrate (S) floc (F) and their interaction (S × F) have been presented in the form of significance. * = $p < 0.05$, ** = $<p < 0.01$. * indicate that the treatment is significantly different from control (C) while † indicate significant difference from both control and floc (F) group. The treatment groups are control (C), floc (F), bamboo mat (B), nylon mesh (N), floc with bamboo mat (F + B) and floc with nylon mesh (F + N).

In the present study, a lower level of TAN was recorded in floc based groups (F, F + B, and F + N) compared to control, C (without biofloc and substrate) upto initial 24th day of experiment which is in conformity with the earlier reports (Hari et al., 2004; Kumar et al., 2014). However, during the later period, the TAN level did not differ significantly between control and floc group. There are three principal pathways to remove toxic nitrogen metabolites in aquaculture (a) photoautotrophic removal by algae (b) Immobilization by heterotrophic bacteria into microbial protein (c) chemoautotrophic oxidation to nitrate by nitrifying bacteria (Ebeling et al., 2006). In the present experiment, removal of nitrogen metabolites by heterotrophic microbes through biofloc and autotrophic algal community via submerged substrate was encouraged. However, increasing nitrite-N and nitrate-N level from 14th day onwards with a peak on the 24th day, and concurrent reduction in the TAN level in all the treatment groups might be due to the dominance of nitrifying bacterial population. Earlier, Burford, Thompson, McIntosh, Bauman, and Pearson (2004) reported that during the later culture period nitrifying bacteria sets-in and take upper hand in the control of TAN level. Recently, Deng et al. (2018) reported a higher level of ammonia-oxidizing bacteria (AOB) in biofloc groups using a metagenomics approach with specifically designed primers for AOB. Hence, to have a better understanding of nitrogen removal in biofloc, periphyton, and integrated system, a holistic approach to delineate the role of photoautotrophs, chemoautotrophs and heterotrophs is worth for investigation.

In comparison to control, the biofloc group (F) recorded better growth performance parameters in terms of body weight, FCR, PER and weight gain/day. In biofloc system, apart from commercial feed, growth is contributed by endogenously produced microbial biofloc (Anand et al., 2014; Azim & Little, 2008; Kumar et al., 2017). On an exclusive biofloc diet, Avnimelech and Kochba (2009) reported that tilapia consumed 25% of daily protein uptake from biofloc. The similar work conducted in shrimp reported 18%–29% of daily nitrogen retention was contributed by consumption of biofloc (Burford et al., 2004). There are several reports suggesting the positive influence of substrate-based system on growth and survival of shrimp (Anand et al., 2013; Kumar et al., 2015; Schveitzer et al., 2013). In the present study, bamboo (B) and nylon (N) substrate based treatments recorded better weight gain, survival and reduced FCR compared with control. It can be attributed to the fact that periphyton, a complex of microbial community, develops over the submerged substrate forms a quality natural food for the cultured shrimp (Anand et al., 2013;

Arnold et al., 2009; Audelo-Naranjo et al., 2017). Further, the growth performance was better in bamboo mat (B) compared to nylon mesh (N). It seems that bamboo mat primarily served as a substrate for growth of microbes in the form of a biofilm, whereas nylon mesh trapped suspended particles (Khatoon et al., 2007). It has been reported that nutrient leaching occurs when a biodegradable natural substrate such as bamboo are submerged in the water column which promotes faster biofilm formation compared to artificial substrate nylon (Azim et al., 2005). This probably allowed higher grazing tendency for shrimp towards bamboo mat biofilm, and resulted in decreased total substrate solid (biofilm dry matter) as culture duration increases.

Among the treatment, integration of substrate in biofloc system (F + B and F + N) recorded better growth performance compared to other treatments. Provision of substrate helped the attachment of biofloc particles (Simon, Grossart, Schweitzer, & Ploug, 2002) which results in better grazing at two-dimensional solid supports compared to suspended particles in three-dimensional water columns (Anand et al., 2013). It has been reported that maintenance of optimum level of total suspended solids is needed for better stability of the culture system (Ebeling et al., 2006; Ray et al., 2010; Schryver et al., 2008). Ray et al. (2010) controlled the level of suspended solids particles using external settling tank and reported 41% improved growth performance in *P. vannamei*. Hence, a marked reduction in total suspended solids and bottom solids and rise in substrate solid in substrate integrated biofloc groups (F + B and F + N) could have helped in improving the growth and survival of shrimps. Interestingly, the growth performance, in the present experiment, was better in the bamboo-based group (F + B) compared to nylon (F + N). Nature of substrate affects the trophic chain of the system. It seems, bamboo provide a better surface structure for attachment of periphytic species or may leach nutrients beneficial for the periphyton growth (Keshavanath et al., 2001). Further, incorporating rice flour as carbohydrate source increased the protein content in the biofloc based system. This, in turn, promoted the growth of bacteria and yeasts over the natural bamboo substrate resulting better growth and survival of shrimp in F + B group. Nevertheless quantitatively the total substrate solid or biomass developed in the form of periphyton did not differ significantly between bamboo substrate integrated biofloc (F + B) and Bamboo mat (B) group; the carbohydrate supplementation improved the quantity and diversity of the microbial community like total microbial count, *Bacillus* and *Lactobacillus* and yeast counts over the bamboo mat in F + B group. Higher secretion of digestive enzymes by many of the probiotic bacteria and yeast might have attributed to the growth performance, survival, and immune indicators in these groups (De, Ananda, Ghoshal, Mukherjee, & Vijayan, 2018; Farzanfar, 2006; Rengpipat et al., 2000; Zheng & Wang, 2017; Ziaei-Nejad et al., 2006). Further, the provision of the substrate increased the surface area of the tank by 75.0% which in turn effectively reduced the relative stocking density which appears to reduce the stress level in shrimp (Schveitzer et al., 2013) and improved shrimp survival rate. The provision of substrates in culture systems act as refuge and protect shrimp during molting, and from cannibalism leads to better survival (Anand et al., 2013; Audelo-Naranjo et al., 2017; Bratvold & Browdy, 2001).

There are several reports in recent years suggesting that bioflocs are able to induce immune response in shrimp (Anand et al. 2017; Crab et al., 2012; Ekasari et al., 2014; Kim et al., 2014; Kumar et al., 2017; Xu & Pan, 2013). In the present study, provision of substrate improved the shrimp immune response with the highest response observed in substrate integrated biofloc system, F + B due to the synergistic influence of substrate and biofloc compared to other treatments. The circulating hemocytes in shrimp perform several defense related functions such as phagocytosis, encapsulation, and storage and release of prophenoloxidase system (Bachère, 2000; Rodrıguez & Le Moullac, 2000). The phenol oxidase enzyme causes inactivation of foreign cells through melanization and prevent their spread throughout the body (Amparyup, Charoensapsri, & Tassanakajon, 2013). It has been reported that bacterial cell wall components such as lipopolysaccharide (LPS), β-1, 3 glucans and microalgal products enhance total hemocyte count and proPO activity in shrimp (Campa-Córdova, Hernández-Saavedra, De Philippis, & Ascencio, 2002; De et al., 2018; Ringo, Jose, Vecino, Wadsworth, & Song, 2012). As both periphyton and biofloc are primarily a microbial consortium developed in the form of biofilm over the bamboo mat, the best immune response in bamboo mat integrated biofloc (F + B) system is evident. This is supported by the highest level of microbial community recorded in F + B group over bamboo substrate. Further, biofloc is also known to contain many bioactive compounds such as carotenoids, chlorophylls, phytosterols, bromophenols, amino sugars which could exert an immuno-stimulatory effect on shrimp (Ju et al., 2008). Being a halophilic organism, *Vibrio* is the major microbial constituent in the brackishwater system. Higher microbial load noticed over submerged substrates indicates biofilm forming microbes which secrete extracellular polymeric substances (Abdallah, Chaieb, Zmantar, Kallel, & Bakhrouf, 2009; Sharma et al., 2010) which in turn results in strong immune enhancing ability in shrimp *P. monodon (*Sharma et al., 2010). Therefore, it could be concluded that a higher level of beneficial microbes over the bamboo substratum enhanced the immune system of shrimps. Interestingly, in the present study, though floc group (F) recorded improved immune response, the influence was lesser compared to bamboo mat (B). It suggests that solid three-dimensional supports helped the grazing of shrimp over quality biofilm, helping bamboo mat group (B) to outperform biofloc group (F).

5 | CONCLUSION

The present study reveals that integration of biofloc with the substrate is promising and a sustainable form of shrimp aquaculture. Nylon mesh trapped a substantial amount of floc particles and reduced floc deposition at the bottom while formation of biofilm development was better in bamboo mat. Provision of the submerged substrate in biofloc system trap the floc particles and allow to attach over two dimensional solid surfaces which can easily be consumed

by the shrimp and control higher level of suspended particle in floc system. This was reflected by improved growth performance and immune parameters.

ACKNOWLEDGEMENT

The authors are grateful to the Director, Central Institute of Brackishwater Aquaculture, Chennai for providing the required facilities to conduct this study. Support and help received from the laboratory staffs from Kakdwip Research Centre of CIBA, is duly acknowledged.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA ACCESSIBILITY

The primary data will be made available as and when required by contacting the corresponding author.

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How to cite this article: Kumar S, Shyne Anand PS, De D, Ghoshal TK, Alavandi SV, Vijayan KK. Integration of substrate in biofloc based system: Effects on growth performance, water quality and immune responses in black tiger shrimp, *Penaeus monodon* culture. *Aquac Res*. 2019;00:1–14. https://doi.org/10.1111/are.14256