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Influence of differential protein levels of feed on production performance and immune response of pacific white leg shrimp in a biofloc–based system

A. Panigrahi[a,*], Sundaram M.[a], Saranya C.[a], Satish Kumar R.[b], Syama Dayal J.[a], Saraswathy R.[a], Otta S.K.[a], Shyne Anand P.S.[a], Nila Rekha P.[a], Gopal C.[a]

[a] Crustacean Culture Division, ICAR-Central institute of Brackishwater Aquaculture, #75, Santhome High Road, Raja Annamalai Puram, Chennai, Tamil Nadu 600028, India
[b] School of Chemical Engineering, Yeungnam University, Gyeongsan 38541, Republic of Korea

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ABSTRACT

A130-day culture experiment was performed to compare the effects of rearing *Litopenaeus vannamei* in a biofloc system and conventional autotrophic system, supplemented with diets having graded protein levels on growth performance, non-specific immune response and immunomodulatory activity. The experiment groups consisted of four protein-level treatments with four replicates each, and was performed in 16 experimental units (7.5-Tcapacity) stocked with juvenile L. *vannamei* (initial average weight 1.48 ± 0.4 g) at a density of 150 juvenile shrimps /m^3. Biofloc-based groups fed with supplementary feed containing varying levels of crude protein (CP) BFT40 (40% CP), BFT32 (32% CP) and BFT24 (24% CP), and a control group (CP 40%), were maintained in an autotrophic system. At the end of the experimental period, there was an improvement of 32.6–52.6% in terms of productivity, 22–27.6% average body weight; 8.7–19.6% survival rate and 10–31% feed conversion ratio (FCR) in shrimp maintained in biofloc systems as compared to control. Similarly, the protein efficiency ratio (PER) in biofloc treatment groups was32 to 83% higher than control. The shrimp also showed higher resistance to disease in the biofloc treatment when challenged with pathogenic *Vibrio parahaemolyticus,* as survival of juveniles was significantly higher ($p < 0.05$) in the biofloc treatments than in the control group. The total hemocyte count and proPhenoloxidase activity were significantly increased in the biofloc treatments when compared to control. The mRNA profiling of important immune genes in the shrimps showed higher levels of expression in the 32 and 40% CP feed treatments. Superoxide dismutase (SOD) mRNA transcript levels were highly up-regulated in the BFT40 treatment (7-fold), moderately upregulated in the BFT32 treatment (2-fold), and downregulated in the BFT24 treatment (0.9-fold). Transcription of antimicrobial peptides like crustin showed significant up-regulation in BFT40 (10.31-fold) followed by BFT32 (6.11-fold) and BFT24 (1.07-fold). Likewise, other immune genes, such as MnSOD, hemocyanin, proPhenoloxidase (proPO), peroxinectin (PX) and serine protease (SP) showed similar trends, indicating immunomodulation in the biofloc groups. We concluded that the biofloc system has the potential to improve growth performance, immune response and disease resistance in Pacific white shrimp in spite of the low-protein supplemented diet.

1. Introduction

Bioflocs are a consortium of particulate matter formed predominantly by a biota of aerobic and heterotrophic bacteria, protozoa, microalgae, metazoans, exoskeletons, faeces and remains of dead organisms. The biofloc principle combines the removal of nitrogenous metabolites from the water with the production of microbial biomass under strong aeration, which then can be used by the cultured species as an additional food source. Under an optimum carbon:nitrogen (C:N) ratio, inorganic nitrogen is immobilized into bacterial cells while organic substrates are metabolized. This technique was developed in Israel and subsequently has spread to many other countries due to its several advantages (Avnimelech, 1999). Biofloc technology (BFT) limits water exchange and water usage in aquaculture systems by maintaining adequate water quality within the system. Conversion of waste nitrogen metabolites intobiofloc provides a feed supplement for aquatic organisms (Avnimelech, 2007; Crab et al., 2007, 2009, 2010; Ferreira et al., 2015; Cardona et al., 2015; Sakkaravarthi, 2015; Ekasari et al., 2016). BFT is an innovative concept, which adapts the rearing of aquatic animals by manipulating the microbial population under controlled

* Corresponding author.
E-mail address: panigrahi@ciba.res.in (A. Panigrahi).

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conditions (Xu and Pan, 2012; Schveitzer et al., 2013; De Schryver et al., 2008; Crab et al., 2010). This practice assists the extensive production of marine animals at high stocking densities in a biosecure approach (McAbee et al., 2003; McNeil, 2000; Vinatea et al., 2009).

To promote the biofloc system, a high C/N ratio of 10:1 to 20:1 is suggested, as the growth of heterotrophic bacteria considered an intrinsic element this system is elevated if the system is provided with substrate having a C:N ratio greater than or equal to 10:1 (Avnimelech, 1999; Hargreaves, 2006; Asaduzzaman et al., 2008). Earlier studies showed that the C:N ratio could be augmented by either decreasing the crude protein level of the feed or by increasing the carbohydrate level (Avnimelech, 1999; Hari et al., 2004; Anand et al., 2014). Protein is considered the most expensive component of shrimp feed, and several recent studies have been undertaken to decrease the cost of feed by decreasing the level of protein. Additionally, studies have revealed that with higher C/N ratio, the biofloc community can be increased without negatively affecting the nutritional quality of the biofloc (Azim and Little, 2008; Asaduzzaman et al., 2010). Thus, the biofloc system allows the use of diets with lower crude protein (CP) content, which helps minimize production costs and reduce environmental impact due to the reduction of nitrogen input and consumption of fishmeal in the diet (Crab et al., 2007; Crab et al., 2010; Xu et al., 2013).

Previous studies showed that 29% of food consumed by the shrimp L. *vannamei* could be derived from the biofloc present in the heterotrophic medium (Burford et al., 2004). Further, microbial interventions are considered as one of the preeminent strategies for managing aquatic diseases (Panigrahi et al., 2009). The biofloc has intrinsic antagonistic affects against viral and bacterial pathogens and can induce immunity, disease resistance and growth performance in the cultured animals (Crab et al., 2012; Ekasari et al., 2014). BFT systems induce the activation of the host immune system when the disease-causing pathogen is in close proximity. The biofloc provides cultured shrimp with pattern recognition and induces several other pathways that ultimately lead to stimulation of the non-specific immune system by downstream activation of a cascade of signals, which results in melanization via elevated production of proPhenoloxidase. The process is a rapid defence mechanism initiated by the shrimps against bacterial and fungal pathogens (Charoensapsri, 2014; Kim et al., 2014). Biofloc could enhance the cellular immune response and antioxidant response of the cultured shrimp, probably because it is rich in natural microorganisms and bioactive compounds (Cardona et al., 2015; Ju et al., 2008; Xu et al., 2013; Panigrahi et al., 2018). Similarly, several other biochemical pathways are triggered when shrimps are reared in a BFT system. Understanding of the molecular expression patterns of immunological genes involved in the process is still incomplete. There has been little study to establish the immunomodulatory effect *vis-à-vis* performance of shrimp with respect to different protein levels in feed. The objectives of this study were to evaluate the performances, immunomodulation and protective response of L. *vannamei* juveniles reared in a BFT system at high stocking density fed at three different protein levels.

2. Materials and methods

2.1. Experimental design

A 130-day outdoor experimental trial was conducted with nursery-grown juveniles of *Litopenaeus vannamei* (1.48 ± 0.4 g) with stocking density of 150 juvenile shrimp /m^3 in quadruplicated cement tanks (7.5 m^{-3}), i.e., Control (autotrophic condition +40% CP), BFT24 (biofloc +24% CP), BFT32 (biofloc +32% CP), BFT40 (biofloc +40% CP).All the tanks were filled with disinfected seawater (chlorinated at 30 ppm), and provided with a 5-HP blower to provide sufficient oxygen to keep the biofloc in suspension. In order to maintain the carbon:nitrogen ratio of 15:1, molasses was used as a exogenous carbon source and added according to the recommendation of Avnimelech (1999). Each biofloc treatment was inoculated with *Bacillus subtilis* (MTCC 2756) and *Saccharomyces cerevisiae* (IAM 14383T) cells at the rate of 1×10^6 cfu/ml at the beginning of the experiment for initial generation of biofloc.

Table 1
Ingredients and proximate composition (% as feed basis) of test diets.

	BFT24	BFT32	BFT40
Composition			
Protein base[a]	30	50	70
Carbohydrate base[b]	63	43.5	24
Fish oil	3	2.5	2
Lecithin	1	1	1
Vitamin and mineral			
Mix[c]	2	2	2
Binder[d]	1	1	1
Proximate composition			
Moisture	9.38	9.42	9.49
Crude protein	24.87	32.18	39.56
Ether extract	6.42	6.56	6.72
Crude fibre	4.21	3.83	3.52
Total ash	7.62	9.14	12.42
NFE[e]	47.50	38.87	28.29

[a] Protein base: Fish meal: *Acetes* sp.: soya cake: gingelly oil cake in the ratio of 4:2:3:1.
[b] Carbohydrate base: Wheat: broken rice: maida in the ratio of 4:2:3.
[c] Vitamins (mg kg-1): Vitamin A 20.0, Vitamin D 4.0, Vitamin E 120.0, Vitamin K 60.0, Choline chloride 6000.0, Thiamine 180.0, Riboflavin 240.0, Pyridoxine 180.0, Niacin 1080.0, Pantothenic acid 720.0, Biotin 2.0, Folic acid 30.0, Vitamin B12 0.150 Inositol 1500.0, Vitamin C 9000.0. Minerals (g kg-1): $CaCO_3$ 28.0, K_2SO_4 10.0, $MgSO_4$ 12.5, $CuSO_4$ 0.2, $FeCl_3$ 0.5, $MnSO_4$ 0.5, KI 0.01; ZnSO4 1.0, CoSO4 0.01, Cr_2SO_4 0.05, Bread flour 7.14.
[d] Poly Methylol Carbamide.
[e] Nitrogen-free extract calculated by difference = 100-(moisture% + Crude protein% + Crude fibre% + Ether extract% + Total ash%).

2.2. Feed formulation and management

Three practical experimental diets were formulated to contain crude protein levels of 24%, 32% or 40% as per the experimental design. The list of ingredients and feed proximate composition are shown in Table 1. The protein base of the diet contained a mixture of dried fishmeal, acetes, soya cake, and gingerly oil cake in the ratio of 4:2:3:1. A mixture of wheat, broken rice and maida in the ratio of 4:2:3 were used as the carbohydrate source for the feed preparation. For lipid source, both fish oil (cod liver oil) and soy lecithin were mixed. The formulated diet was fortified with minerals and vitamins as per the suggestions of Hu et al. (2008) and Xu and Pan (2014), respectively. Sinking pellets were produced in the ICAR-Central Institute of Brackishwater Aquaculture pilot-scale feed mill, Muttukadu, Chennai. All the coarse ingredients of the formula were powdered by two stages of grinding in a hammer mill and micro pulveriser and passed through a 0.5-mm screen. The powder along with the liquid ingredients and the binder were mixed into a horizontal ribbon mixture and thoroughly homogenized after adding 3 L of water per 100 kg material. The mash was pelletized in a ring-die pellet mill at 15–16% moisture and at 90 °C under steam conditioning. The pellets were produced using 1.8 and 2.0 mm dies and were dried in a conditioning chamber by using a dry heat exchanger, and then stored in a plastic bag (Dayal et al., 2017). The proximate compositions of the diets were estimated following recommendations of AOAC (1995). All the experimental animals were fed thrice daily, at 06.00, 11.00 and 18.00 h, initially for 2 months followed by one additional feeding ration at 22.00 h till the end of the experiment. The shrimps were fed at the rate of 8% of shrimp body weight, which was gradually reduced to 2.5% of total biomass towards the end of experiment as the shrimp grew.

2.3. Assessment of water quality parameters

Water quality parameters, i.e., temperature (mercury thermometer), pH (pH-Scan, Eutech Instruments, Singapore), salinity (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 at 09:00 h following the methods described in APHA et al. (1998). Total suspended solids was determined every fortnight (APHA, et al., 1998). Biofloc volume was quantified using an Imhoff cone on a daily basis as per Avnimelech and Kochba (2009).

2.4. Assessment of growth parameters

Shrimp ($n = 50$) from each treatment group were weighed for determining growth performance, quantified as weight gain (AWG) (g week − 1), final biomass (g m − 3), survival (%), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) following Panigrahi et al. (2017).

2.5. Bacterial quantification

Total heterotrophic bacteria and *Vibrio* abundance in the experimental unit were estimated every 10 days using the spread plate technique on Zobell marine agar (ZMA) and thiosulfate citrate bile salts sucrose agar (TCBS agar) (HIMEDIA®, Mumbai, India), respectively. Plates were incubated at room temperature for 48 h, and colonies were counted and expressed as number of bacterial colony-forming units (CFU) (Panigrahi et al., 2017). The ratio of *Vibrio*/ total bacterial count (V/T ratio) was determined by following formula of Ramaiah and Chandramohan (1993):

V/T ratio = 100*(total *Vibrio* count/total heterotrophic bacterial count)

2.6. Hemolymph collection and determination of immune parameters

Hemolymph samples were collected from the ventral sinus using a 21-G needle attached to a 2-ml sterile polypropylene syringe containing 1 ml of ice-cold cysteine anticoagulant saline in all treatments. Anticoagulant was prepared by adding 3 mg cysteine to 5 ml of physiological saline [NaCl (340 mM), KCl (13 mM), $MgSO_4$ (11 mM), $MgCl_2$ (10 mM), NaH_2PO_4 (0.3 mM) and glucose (1.6 mM) in 100 ml distilled water, pH 7.8].

Total hemocyte count (THC) was measured based on the methods of Söderhäll (1982). Exactly 10 µl of haemolymph from each sampled individual was inoculated into a Neubauer haemocytometer. The concentration per cubic centimeter (milliliter) was calculated by counting the total number of cells in four 1-mm^2 areas using the following formula:

THC = Total cells counted × dilution factor (10) × 10^4/number of 1 − mm^2 areas counted.

Phenoloxidase activity was determined following methods of Smith and Söderhäll (1983). Ten microliter of serum was incubated with 20 µl of trypsin (2.1 mg ml^{-1}) for 15 min at 25 °C. For the control, trypsin was substituted with tris-HCl buffer (50 mM; pH 7.5). The mixtures were brought to 200 µl with 5 mM L-DOPA and further incubated for 20 min at 25 °C. The optical density of both control and treatment samples were measured spectrophotometrically at 490 nm. Total protein concentration (TPC) in shrimp serum (six animals per treatment) was determined according to the Bradford (1976) method using bovine serum albumin (BSA) as a standard.

2.7. Challenge study

At the end of experiment, 20 healthy shrimp at inter-moult stage from each treatment were challenged with a pathogenic strain of *Vibrio parahaemolyticus* (MTCC 451, IMTCC, Chandigarh, India). The shrimps were injected intramuscularly at the third segment with 1×10^4cfu/ml of pathogen. The shrimps were continuously monitored for infection and mortality during the trial. For negative control, shrimps were reared in water without pathogen. Water quality parameters were measured. The experiments were carried out in duplicate. No water was exchanged for the whole duration of the challenge trial. During the challenge trial, the shrimps were assessed for clinical signs to assess infection by the pathogen along with water quality parameter variation, shrimp survival, and cumulative numbers of dead shrimp.

2.8. Gene expression study

Total RNA from the hepatopancreas tissue of five individual samples was isolated using a Qiagen RNA isolation kit (Qiagen, USA). The presence of RNA bands was confirmed in an agarose gel. The reverse transcriptase PCR reaction was performed to convert mRNA into complementary DNA. The Prime Script 1st Strand cDNA Synthesis Kit (Bio-Rad, USA) was used for the purpose. The cDNA thus obtained was serially diluted and used for relative quantification of the target genes. Real-time PCR (Applied Biosystem's Real-Time PCR system StepOne Plus®) was used for amplification and melt-curve analysis. The designed oligonucleotides used for qPCR are listed in Table 4. The temperature cycling parameters for the two-step PCR reaction were as follow: holding stage of 10 min at 95 °C (initial denaturation), followed by 45 cycles of 15 s at 95 °C (denaturation), and 1 min at 60 °C (annealing and extension). The total reaction volume (20 µL) in each PCR tube consisted of 10 µL of 2× SYBR® Green qPCR master mix (Bio-Rad, USA), 1 µL each of forward and reverse primers (10 pmol), 1 µL of template DNA (30–60 ng) and 7 µL of PCR-grade water. A negative control without cDNA template was run with every assay to assess the overall specificity. The ß-actin gene expression was analysed as an internal control with an equal loading of RNA with primers 'actin Forward' and 'actin Reverse' (Panigrahi et al., 2007). Data were analysed using the comparative cycle threshold (CT) method (Pfaffl, 2001), where CT is defined as the cycle number at which fluorescence reaches a set threshold value. The expression level of each target gene was normalized relative to ß-actin (Table 2).

2.9. Statistical analysis

To assess the level of significance of differences among means for each parameter among the different biofloc treatments and control, one-way ANOVA was used. All the statistical analysis was done using the SPSS software package, version 17. If any significance between the treatments was observed, ANOVA was performed using Tukey's tests. The levels of significance were fixed at the 99 and 95% probability levels.

3. Results

3.1. Water quality parameters

The water quality parameters for respective treatments and control are presented in Table 3. Temperatures in the experimental tanks ranged from 28.5–31.2 °0C and salinities from 28 to 30 ppt. Biofloc treatments showed a significant effect ($p < 0.01$) on pH reduction irrespective of protein levels in the feed when compared to control water samples. Alkalinity during the entire culture period showed a non-significant reduction ($p > 0.05$) among treatments, BFT40 (27.51 ± 4.72%), BFT32 (21.11 ± 1.10%), BFT24 (16.45 ± 2.12%).Total ammonia nitrogen (TAN) had a significant

Table 2
Primers used for quantitative real-time PCR of immune-related genes of white shrimp *P. vannamei.*

Gene	Primer sequence (5′–3′)	Accession no	Amplicon size
SOD	F-GCTGAATTGGGTGAGGAACG R-CCTCCGCTTCAACCAACTTC	AY486424	172
cMnSOD	F- GGCACAGTCAGTCCTCAGAT R- GAGAGGTGGCAAAGCATGAG	DQ298207.1	346
Hemocyanin	F- GCTTTTCGACGTCCTCATCC R- CTTGAATTTGCCAGGCGTCT	X82502	247
Peroxinectin	F-GAGTCTGAACATCCATCGCG R-TATGCCACCCACGAAGAAGT	KC708021.1	187
proPhenoloxidase	F-TTCCAGCTCTTCTTCATGCT R-TCGGGGTACTTGGCGTCCTG	AY723296.1	116
Serine protein	F-CGTCGTTAGGTTAAGTGCGTTCT R-TTTCAGCGCATTAAGACGTGTT	AY368151	61
Crustin	F-ACGAGGCAACCATGAAGG R-AACCACCACCAACACCTAC	AF430076	141
ß-actin	F-CAACCGCGAGAAGATGACAC R-TCGGTCAGGATCTTCATCAGG	GU732815.1	243

difference ($p < 0.05$) between control and biofloc treatment groups, and among the biofloc groups (BFT40, BFT32 and BFT24). Biofloc treatments corresponding to different protein levels in feed exhibited significantly ($p < 0.05$) reduced TAN levels, i.e., BFT40 (42.09 ± 5.3%), BFT32 (39.6 ± 5.8%) and BFT24 (33.4 ± 6.3%), in decreasing order compared to control. Similarly, NO_2-N and NO_3-N showed significant ($p < 0.05$) differences. Among treatments compared to the control group, the biofloc groups resulted in varying degrees of reduction in Nitrite-N, and Nitrate-N, i.e. BFT40 (34.05 ± 2.2%, 40.5 ± 9.6%), BFT32 (27.52 ± 2.9%, 36.9 ± 6.9%), BFT-24 (20.50 ± 5.0, 28.9 ± 8.9%) respectively. However, the phosphate (PO_4-P) level was higher in treatments BFT40 (43.4 ± 4.4%), BFT32 (25.2 ± 6.1%), and BFT24 (32.7 ± 2.8%) compared to control (Table 3).

3.2. Quantitative analysis of biofloc

The quantity of biofloc developed was measured by settlement in an Imhoff flask and in terms of turbidity is presented in the Table 3. All the biofloc groups with carbohydrate supplementation recorded significantly higher ($p < .05$) turbidity of 20.48 ± 2.97, 23.12 ± 3.54 and 26.98 ± 3.68 NTU in BFT24, BFT32 and BFT40, respectively, compared to 16.67 ± 2.58 NTU in the control group. Similarly, supplementation with molasses resulted in significantly higher total suspended solids (TSS) in biofloc groups BFT40 (69.3 ± 1.79%), BFT32 (63.9 ± 1.31%), and BFT24 (59.8 ± 7.43%) compared to the control group. The biofloc volume was significantly ($p < 0.05$) increased in the BFT40 (18.75 ± 3.9 ml), followed by BFT32 (16.94 ± 2.2 ml), and BFT24 (12.06 ± 2.7 ml) groups, whereas the control group (5.13 ± 1.2 ml) had the lowest value.

3.3. Microbial analysis

Carbohydrate supplementation significantly increased the total heterotrophic bacterial (THB) count in water, in the order BFT40 (82.9 ± 4.4%), BFT32 (81.5 ± 4.7), and BFT24 (69.7 ± 6.7%) compared to the control group. As culture proceeded, the increasing biomass had a significant effect ($p < 0.01$) on total microbial load, with a higher level recorded in BFT40 compared with BFT24. Similarly, carbohydrate supplementation had a significant effect on the total *Vibrio* count (TVC) in water ($p < 0.01$). TVC levels were greatly reduced in the biofloc treatment groups i.e., BFT40 (91.80 ± 0.3%), BFT32 (88.98 ± 0.84%), and BFT24 (60.82 ± 0.22%), relative to the control group. During the progress of the experiment, the shrimp growth stage and feed protein levels had a significant interaction ($p < 0.05$) effect on the total microbial content in water. The proportion of *Vibrio* count to total heterotrophic bacterial count (V/T) was lower in the biofloc groups than in the control group (Table 3). In spite of the increase in *Vibrio* load, the V/T ratio was non-significantly ($p > 0.05$) lower in the BFT groups. The V/T ratio was lower in the BPT40 rearing water (11.1 ± 1.2%), whereas in control it was higher (96.5 ± 12.8%).

3.4. Growth performance of L. vannamei

The hypothesis tested was whether reducing the protein content of

Table 3
Water quality parameters for bio-floc based shrimp culture and control groups. Control (40% protein without biofloc); BFT24 (24% protein + biofloc); BFT32 (32% protein + biofloc); BFT40 (40% protein + biofloc). THB – Total heterotrophic bacterial count; TVC – Total *Vibrio* count, and V/T ratio – *Vibrio*/Total bacterial count ratio.

Parameters	Control	BFT24	BFT32	BFT40	*P* value
pH	8.64 ± 0.52^{a}	7.82 ± 0.14^{b}	7.78 ± 0.31^{b}	7.69 ± 0.23^{b}	0.001
TAN (ppm)	0.743 ± 0.02^{a}	0.249 ± 0.05^{b}	0.295 ± 0.05^{b}	0.313 ± 0.05^{b}	0.001
NO_2 (ppm)	0.504 ± 0.02^{a}	0.098 ± 0.02^{b}	0.126 ± 0.01^{b}	0.140 ± 0.02^{b}	0.001
NO_3 (ppm)	0.294 ± 0.10^{a}	0.170 ± 0.03^{b}	0.189 ± 0.09^{b}	0.204 ± 0.05^{b}	0.001
PO_4 (ppm)	0.158 ± 0.09^{a}	0.223 ± 0.13^{a}	0.226 ± 0.09^{a}	0.231 ± 0.13^{a}	0.015
Alkalinity (ppm)	225.6 ± 11.8^{a}	188.8 ± 19.4^{b}	178.1 ± 14.3^{b}	163.0 ± 12.7^{b}	0.001
Settled floc volume (ml)	5.13 ± 1.2^{a}	12.06 ± 2.7^{ab}	16.94 ± 2.2^{b}	18.75 ± 3.9^{b}	0.001
TSS (ppm)	162.1 ± 11.5^{a}	333.0 ± 58.0^{b}	450.0 ± 48.4^{bc}	551.3 ± 38.5^{c}	0.001
Turbidity (NTU)	16.67 ± 2.58^{a}	20.48 ± 2.97^{ab}	23.12 ± 3.54^{ab}	26.98 ± 3.68^{b}	0.038
THB ($\times 10^5$ cfu/ml)	2.44 ± 0.51^{a}	14.74 ± 0.74^{c}	13.29 ± 0.63^{c}	8.04 ± 0.01^{b}	0.001
TVC ($\times 10^3$ cfu/ml)	1.22 ± 0.38^{a}	0.187 ± 0.03^{c}	0.253 ± 0.03^{c}	0.895 ± 0.02^{b}	0.001
V/T ratio (%)	96.53 ± 12.82^{a}	1.31 ± 0.02^{c}	1.89 ± 0.08^{c}	11.13 ± 0.12^{b}	0.001

*Error bars represent mean ± SE. The means with no superscript letter in common indicate significant difference ($p < 0.05$).

Table 4
Comparison of zootechnical indices among L. *vannamei* produced using various protein diets with biofloc and control shrimp. Control (40% protein without biofloc); BFT24 (24% protein + biofloc); BFT32 (32% protein + biofloc); BFT40 (40% protein + biofloc). ABW = average body weight; FCR = feed conversion ratio; PER = protein efficiency ratio.

Growth parameters	Treatment				Improvement (%)
	Control	BFT24	BFT32	BFT40	
Survival (%)	70.31 ± 0.81[a]	76.45 ± 0.94[b]	83.61 ± 0.94[c]	84.13 ± 0.97[c]	8.38 to 19.6↑
ABW (g)	29.91 ± 3.58[a]	36.48 ± 3.71[b]	37.18 ± 4.86[b]	38.15 ± 3.36[b]	21.9 to 27.6↑
Production (ton/ha)	28.42 ± 1.2[a]	37.69 ± 2.7 [ab]	42.0 ± 0.8[b]	43.4 ± 19.6[b]	32.6–52.6↑
FCR	2.07 ± 0.00[a]	1.88 ± 0.3 [b]	1.71 ± 0.1[b]	1.58 ± 0.2[b]	10–31↓
PER	1.21 ± 0.01[a]	2.2 ± 0.15[c]	1.83 ± 0.11[b]	1.59 ± 0.20[ab]	32–83↑

*Error bars represent mean ± SE. Means with no superscript letter in common indicates significant difference ($p < 0.05$).

diet would affect growth performance and production of shrimp reared in biofloc systems. A low-protein-based pellet diet (24% CP) was evaluated as a replacement for a conventional high-protein diet (40% CP) for L. *vannamei* under biofloc and conventional autotroph rearing conditions. The growth performances observed in the respective treatments are presented in Table 4. Supplementary feeding of different protein-level feeds significantly affected the FCR ($p < 0.01$), PER ($p < 0.01$), final weight ($p < 0.05$) and survival ($p < 0.05$), with higher values recorded in biofloc groups irrespective of protein level in the diet. Average body weight (ABW) as a measure of the final growth gain at the end of experimental period among the treatment groups is presented in Table 4. Final body weights in BFT treatments were significantly higher compared with the control. There was an improvement of 10 to 31% in FCR in the biofloc treatments (Table 4). Higher PER values were observed in treatment groups compared to control. The production values in the biofloc treatments were significantly higher compared to control. While shrimps reared in the non-biofloc control reached 25 to 30 g in 130 days following the nursery period, they gained between 36 and 40 g in the same period under biofloc treatments (Table 4). The average body weight was also higher in the biofloc-treated groups compared to the control group. The improvement in average body weight in the biofloc groups ranged from 21.98 to 27.57% over the control autotrophic group. The survival rates in the biofloc groups were significantly higher ($P < 0.05$) in BFT24; BFT32 and BFT40 compared to the control group, registering an improvement of 8.38 to 19.6%. The average productivity showed a significant improvement in the biofloc groups (32.6 to 52.6% in BFT32 to BFT40), compared to that of the control.

3.5. Immunological parameters

Total hemocyte counts were significantly ($p < 0.05$) higher in BFT40, BFT32 and BFT24 reared shrimps compared to control (Fig. 2). Phenoloxidase activity was increased in BFT groups, whereas control shrimps recorded the lowest level (Fig. 3).The expression of certain immune-related genes showed positive correlations with the dietary protein treatment. SOD mRNA transcript levels were highly up-regulated (7-fold) in the 40% protein treatment samples, and moderately upregulated (1.3-fold) in the 24% protein treatment and (1.4-fold) in the 32% protein treatment (Fig. 4). The transcript levels for the cMnSOD gene were highly up-regulated in shrimps reared in biofloc systems supplemented with 32% and 40% protein feeds (Fig. 4). The hemocyanin gene showed a similar trend in the gene expression pattern, with the 40% protein feed treatment showing maximum mRNA expression (Fig. 4). The transcripts for antimicrobial peptides like crustin showed upregulation in BFT40 (10.31-fold) and BFT32 (6.11-fold) compared to BFT24 (1.07-fold) (Fig. 4). Serine protease inhibitors (serpins) also were elevated in response to increased protein feed treatments in BFT systems; gene expression was higher in the 40% (3.17-fold) followed by 32% (3.04-fold) and 24% protein treatments (1.75-fold) respectively, compared to the non-biofloc control group (Fig. 4).Transcripts of proPhenoloxidase (proPO) and peroxinectin (PX) were highly expressed in the 32% protein feed treatment (4.67 and 3.15-fold, respectively) compared to the conventional non-biofloc group. In contrast, the expression of these transcripts was slightly lower in the shrimps fed with 40% feed (3.80 and 2.24-fold, respectively) (Fig. 4).

3.6. Challenge study

To investigate the possibility of protective response in shrimp reared in biofloc systems, the shrimps were challenged with a pathogen, *V. parahaemolyticus* (MTCC 451). The results showed that the biofloc-reared shrimp with different levels of protein in feed showed cumulative percentage mortalities of 45.8 ± 2.4%,58.9 ± 1.4% and 61.1 ± 1.1% in BFT40, BFT32 and BFT24, respectively, whereas the control group showed 68.2 ± 3.3% (Fig. 1). The differences were statistically significant ($p < 0.01$) between the control and biofloc groups, showing a significant increase in the relative percent survival compared to the control group, irrespective of the protein level in feed.

4. Discussion

4.1. Water quality

The temperatures and salinities of the experimental tanks were within favourable ranges for the juvenile shrimp (Van Wyk et al., 1999; Hariati et al., 1996).Lower levels of nitrogenous metabolites were recorded in BFT treatments than in control systems in terms of TAN, NO_3-N, NO_2-N, and were higher in treatments fed with high-protein feeds. The lower value in the biofloc systems was presumably due to the efficient assimilation and oxidation of the nitrogenous waste metabolites by the heterotrophic bacterial population in the biofloc systems

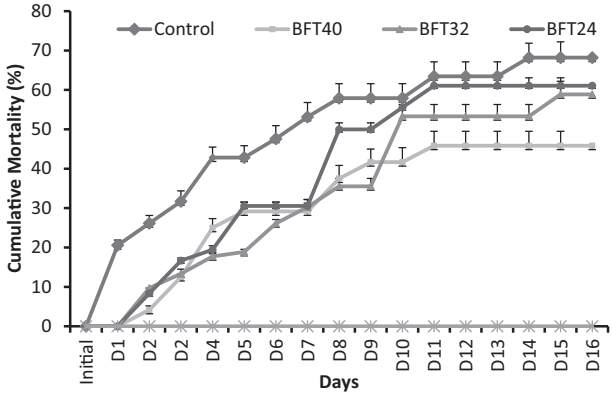

Fig. 1. Cumulative percent mortality of conventional, and biofloc, (BFT40, BFT32 and BFT24) reared groups of L. *vannamei* challenged with *V. parahaemolyticus*.

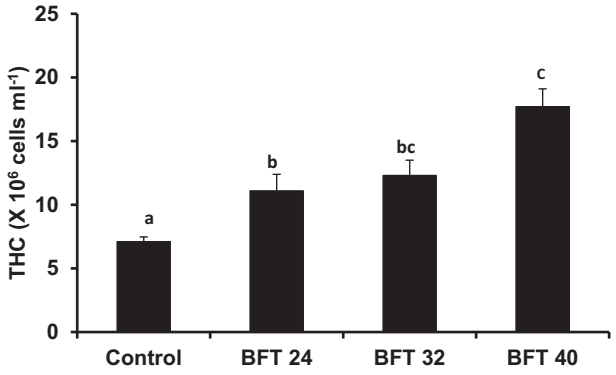

Fig. 2. Total hemocyte counts for BFT and control groups shrimp ($n = 10$). Data shown as mean ± standard deviation. Significant difference ($p < 0.05$) between groups is indicated by different letters on top of the bar.

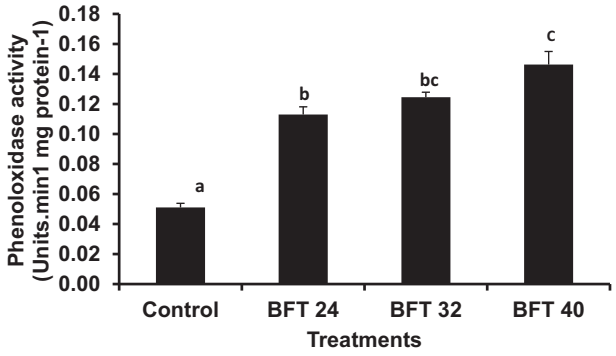

Fig. 3. Phenoloxidase activity of BFT and conventionally reared shrimp sera ($n = 20$). Data shown as mean ± standard deviation. Significant difference ($p < 0.05$) between the groups is indicated by different letters on top of the bar.

(Avnimelech et al., 1989, 1992, 1994; Avnimelech, 1998, 1999; Ebeling et al., 2006; Xu et al., 2016). There was a significant reduction in the alkalinity in the biofloc treatment as compared to control, which was due to heightened nitrification of ammonia in the biofloc treatment groups (Ebeling et al., 2006; Loyless and Malone, 1997). The phosphate concentration in the biofloc treatments was higher than in the control group, which is attributed to the abundance of microbial floc in the system, which became deposited in the bottom of the tank and released phosphorus through decay by the action of heterotrophic bacteria (Xu et al., 2016; Elnady et al., 2010). There were significant increases of turbidity, TSS and biofloc volume in the biofloc treatments, particularly so in BFT40 as compared to the control treatment due to the development and formation of biofloc. These results are presumably due to reconversion of unutilised feed and other nitrogenous waste matter through assimilation by heterotrophic bacteria (Ebeling et al., 2006; Schneider et al., 2005).

The total heterotrophic bacterial counts were significantly higher for the biofloc treatment groups compared to the control, which is consistent to results of earlier studies, as bacterial count in these systems ranged from 10^7 to 10^8 cells mL^{-1} in zero-exchange intensive ponds (Avnimelech, 2012), 3.9×10^8 cells mL^{-1}in raceway-based intensive RAS shrimp tank (Otoshi et al., 2006) and 3.35 to 5.42×10^7 cells mL^{-1}in intensive shrimp ponds (Burford et al., 2003). We also found a consistent decrease in the *Vibrio* count in all the treatments compared to control, the lowest in the BFT40 treatments. The *Vibrio* reduction in the BFT groups showed probiotic effects of biofloc in the culture system in the abundance of heterotrophic bacteria (Jha and Naik, 2009; Panigrahi et al., 2017, 2018).

4.2. Production parameters

Zoo technical performance metrics and survival of shrimp reared in biofloc systems were higher than in the control group. Earlier studies have demonstrated beneficial effects of biofloc on shrimp growth performance (Ballester et al., 2010; Haslun et al., 2012; Ray et al., 2011; Wasielesky et al., 2006; Xu and Pan, 2012; Zhao et al., 2012). Further, the results of current study show that the low-protein based pellet diet containing 32% crude protein are optimal relative to the high protein feed given the trade-off of immunostimulation and cost. Several immune responses are better in high protein (40% CP) for L. *vannamei* under biofloc conditions. This may bedue to in-situ recycling of protein from biofloc which acts as a supplemented protein and micronutrient source for the shrimps. The result of the study is convergent with those of earlier reports by Ballester et al. (2010), Arnold et al. (2009), and Asaduzzaman et al. (2010), who reported that dietary protein levels can be reduced without impairing shrimp growth performance in a biofloc system. Similarly, improved FCR (1.58 to 1.88) was noted in the biofloc treatment even at high-density rearing in the present experiment, similar to results obtained by Ballester et al. (2010) and Xu and Pan (2014). In the current experiment, the BFT32 treatment showed the minimum FCR, which not only could reduce the cost of production, but also could reduce the ammonia concentration in the culture system (Hopkins et al., 1995). Even a low protein level of 24% under the biofloc system could perform better in terms of higher biomass production and zootechnical performance of shrimp compared to control. The biofloc system which converts nitrogenous metabolites and other waste material to microbial protein by heterotrophic bacteria, helps to reduce feed requirement and production cost increases protein conversion efficiency of biofloc-reared shrimp when compared to the autotrophic system (control). The shrimp in the BFT32 treatment showed a moderately higher protein efficiency ratio than in the other biofloc treatments which is helpful for reducing the requirement for crude protein in the diet. The PER result of the current experiment was similar to the results of Xu and Pan (2012).

4.3. Immune response

Expression of eight selected non-specific immune genes, SOD, serine protease, antimicrobial peptides like crustin, cMnSOD, hemocyanin, proPhenoloxidase (proPO), and peroxinectin (PX), along with the housekeeping gene β-actin (Han and Zhang, 2007, Lee et al., 2002),were quantified in the present study. Our results show higher expression levels of the selected genes in the biofloc treatments than in the control, which suggests that biofloc can enhance the immune status of the shrimps through presentation of the microbial cell wall of microbes in the biofloc, consisting of peptidoglycans (PG), lipopolysaccharides (LPS) and β-1, 3-glucans, which were known to have a potential to trigger the immune response in shrimps by activating the major non-specific defence mechanism, the proPhenoloxidase (proPO) defence system in crustaceans (Labbe and Little, 2009; Rao et al., 2010; Panigrahi et al., 2018).

The mRNA expression levels of L. *vannamei* immune response genes was highly upregulated in the 40% protein-treated biofloc systems, but moderate in the 24 and 32% protein-treated biofloc systems, indicating that a high protein diet may be crucial for achieving enhanced immunity in this shrimp. Superoxide dismutase (SOD) is an enzyme which alternatively catalyzes superoxide anions (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (Fridovich, 1995), and it plays an important role in antioxidant defence pathways. SOD is frequently used as a biomarker for measuring response to reactive oxygen species in L. *vannamei* (Campa-Cordova et al., 2002; Rodrıguez and Le Moullac, 2000). In this study, SOD mRNA transcript levels were highly upregulated in the 40% protein treatment samples, and moderately upregulated in the 24% and 32% protein treatment samples. The expression of this gene showed positive correlation with the protein

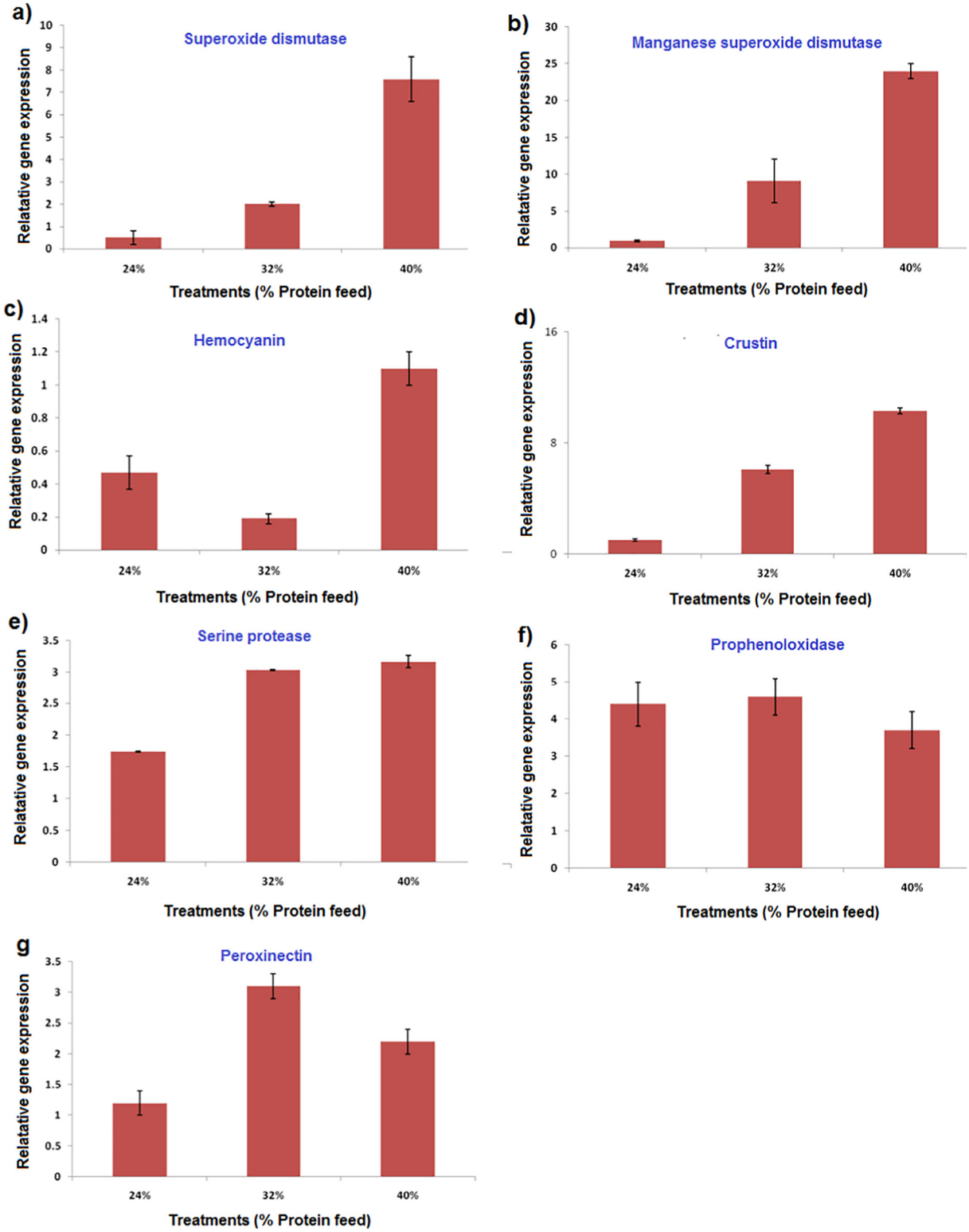

Fig. 4. Comparative mRNA expression levels for the:) SOD gene, b) MnSOD, c) hemocyanin, d) crustin, e) serine protease, f) proPhenoloxidase, and g) peroxidase genes in L. *vannamei* reared in biofloc systems supplemented with varying concentrations of protein feeds, relative to that in control shrimp as determined by real-time PCR (rt-PCR). Five individual shrimps were analysed from the control and each of the biofloc groups. A relative expression value of 1.0 indicates no change in response to treatment. Data are means ± SD of gene expression in the tissues of biofloc groups compared to that in the respective tissue of the control group performed in triplicate PCR. Values marked within asterisk are statistically significant at $p < 0.05$ as determined by one-way ANOVA followed by Tukey's test.

treatments. The MnSODs comprise a major type of SOD for crustaceans; among two types of MnSODs (i.e., cMnSOD and mMnSOD), the cytosolic MnSODs (cMnSOD) also have been reported in crabs, lobsters, and shrimp (Campa-Cardova et al., 2009; Rodrıguez and Le Moullac, 2000; Zhao et al., 2014).A similar transcriptional profile was observed for the cMnSOD gene, which also showed positive correlation with the protein treatments, exhibiting dynamic responses to different protein treatments.

Further, qPCR analysis of expression of the shrimp gene showed a similar trend; with the 40% protein feed treatment showing maximum mRNA expression. The hemocyanin gene plays a vital role by transporting oxygen in several invertebrates (Jaenicke and Decker, 2004; Bowden, 2017). Additionally, hemocyanin also functions as a phenoloxidase under certain conditions and effectively participates in the immune response (Decker and Tuczek, 2000; Decker et al., 2001; Jaenicke and Decker, 2004).

The transcripts of antimicrobial peptides like crustin and serine protease inhibitors (serpins) also were elevated in response to feed treatments with higher protein levels, showing more upregulation among the BFT groups. The crustin gene is among the most important antimicrobial peptides (AMP) found in shrimp (Han and Zhang, 2007, Tassanakajon et al., 2018) and plays a key role in their innate immunity against various pathogens. This gene was found to be strongly expressed in all the biofloc groups, with higher protein-fed groups showing higher expression levels. The proPhenoloxidase genes involved in the proPO system were analysed for further understanding the effect of treatment on innate immune response in shrimps (Fagutao et al., 2012; Sritunyalucksana et al., 2001). Our results revealed that the transcripts of proPhenoloxidase (proPO) and peroxinectin (PX) were highly expressed in the 32% protein feed treatment. In contrast, the expression of these transcripts was slightly lower in the shrimps fed with 40% feed. The proPO and PX genes are involved in activation of the proPhenoloxidase (proPO) cascade, thus playing an essential role in the shrimp innate immunity (Panigrahi et al., 2018), while transglutaminase is an enzyme involved in a conserved defence mechanism in shrimps (Huang et al., 2004; Fagutao et al., 2012). The mRNA profile of certain immune genes, namely proPO, exhibited differential expressional patterns generating variable outputs, showing upregulation in biofloc groups, a result that we demonstrated in our earlier study (Panigrahi et al., 2018).

The protective response of shrimps reared in biofloc and/or periphyton-based farming under different protein-level feeds was determined through a challenge trial against the bacterial pathogen, *V. parahaemolyticus*. The biofloc systems supplemented with 40% protein feed showed less mortality compared to the conventional system, indicating that high-protein feed may directly enhance the immune system of the shrimps reared in biofloc systems.

5. Conclusion

Our results revealed that protein content in pelletized feed can be reduced when shrimps are raised in a biofloc-based environment. BFT has emerged as source of supplemental protein for compounded diets, originating from its diverse microbiota. In a biofloc system, the optimal dietary protein level could be reduced to 32% or lower without greatly compromising weight gain, feed efficiency ratio (FER), protein efficiency ratio (PER), survival, growth and immune performance of shrimp.

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