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Dietary biofloc supplementation in black tiger shrimp, *Penaeus monodon*: effects on immunity, antioxidant and metabolic enzyme activities

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Abstract

A 60-day indoor experiment was conducted to study the effect of dietary supplementation of biofloc on metabolic enzyme activities and immune responses in *Penaeus monodon* juveniles. Biofloc developed in indoor fibreglass-reinforced plastic (FRP) tanks (1000 L) was used as dietary supplement in *P. monodon* (2.90 ± 0.10 g) reared in 1000-L FRP tanks. Graded level of dried biofloc was included in shrimp basal diets, 0% (control, B0), 4% (B4), 8% (B8) and 12% (B12). The level of metabolic enzymes like malate dehydrogenase (MDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was not significantly different with control up to 8% dietary supplementation. A higher level of total haemocyte count (THC) was noticed in B8 ($22.16 \pm 2.17 \times 10^6$ cells mL^{-1}) and B4 ($21.11 \pm 0.56 \times 10^6$ cells mL^{-1}) compared with control, C ($14.61 \pm 2.74 \times 10^6$ cells mL^{-1}). Biofloc-supplemented groups recorded significantly higher ($P < 0.05$) serum SOD and catalase activity ($P < 0.01$) in comparison with control. The groups fed with 4% dietary biofloc supplement recorded highest relative percentage survival (RPS), 45% after challenge with *Vibrio harveyi* followed by 36% and 27% RPS in B8 and B12 groups. Based on these results, it can be concluded that supplementation of biofloc even at 4% level in the feed improves immune responses and metabolic activities in black tiger shrimp juveniles.

Keywords: biofloc, black tiger shrimp, dietary supplement, immune response, metabolic enzyme, *Penaeus monodon*

Introduction

Aquaculture is one of the fastest growing food production sectors representing 44.2% of world fish production (FAO 2016). Among the various aquaculture activities, commercial shrimp farming is highly preferred due to its better economic return over investment as shrimps are highly valued seafood commodities. However, the shrimp culture sector is suffering from disease outbreaks of viral, bacterial and parasitic origin. Pathogens such as white spot syndrome virus (WSSV), *Enterocytozoon hepatopaeni* (EHP) and *Vibrio parahaemolyticus* are threatening the sustainability of the shrimp culture (Thitamadee, Prachumwat, Srisala, Jaroenlak, Salachan, Sritunyalucksana, Flegel & Itsathitphaisarn 2016). Therefore, producers and researchers are actively looking for newer, innovative therapeutic and preventive measures to reduce the losses occurring due to diseases.

Various microbial products such as probiotics and immunostimulants have been used to improve shrimp immunity and reduce the disease incidence

(Ringø, Jose, Vecino, Wadsworth & Song 2012). Being invertebrates, shrimps lack adaptive immunity and possess only innate immune system. The latter in shrimp is comprised of circulating haemocytes, prophenoloxidase (proPA) system, oxidative enzymes like superoxide dismutase (SOD), catalase and antimicrobial peptides (Tassanakajon, Somboonwiwat, Supungul & Tang 2013). Arrays of metabolic enzymes are involved in shrimp which directly affects the physiological activities and general well-being in shrimp. Many enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) take part in amino acid metabolism within hepatopancreas, which is the invertebrate powerhouse equivalent of liver (Pan, Chien & Hunter 2003). Enzymes involved in energy production cycle like malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) reflect the physiological status and energy demand in crustaceans (Rosas, Cuzon, Gaxiola, Le Priol, Pascual, Rossignyol, Contreras, Sanchez & Van Wormhoudt 2001; Pan *et al.* 2003; Choudhury, Pal, Sahu, Kumar, Das & Mukherjee 2005; Gupta, Pal, Sahu, Dalvi, Akhtar, Jha & Baruah 2010; Anand, Kohli, Dam Roy, Sundaray, Kumar, Sinha, Venkateshwarlu & Pailan 2014a). These enzymatic parameters vary in response to various stresses and thus indicate the shrimp health status (Pan *et al.* 2003; Murray, Lall, Rajaselvam, Boutilier, Blanchard, Flight, Colombo, Mohindra & Douglas 2010).

Biofloc-based aquaculture is an evolving ecofriendly practice in shrimp culture (Avnimelech 1999; Crab, Defoirdt, Bossier & Verstraete 2012). It can be generated by supplementation of external carbon source or elevated carbon level in the feed (McIntosh 2000; Ballester, Abreu, Cavalli, Emerenciano, de Abreu & Wasielesky 2010; Anand, Kumar, Panigrahi, Ghoshal, Dayal, Biswas, Sundaray, De, Raja, Deo, Pillai & Ravichandran 2014b). Consumption of biofloc found to improve growth performance of shrimp such as *Penaeus monodon* (Hari, Kurup, Varghese, Schrama & Verdegem 2006; Kumar, Anand, De, Sundaray, Raja, Biswas, Ponniah, Ghoshal, Deo, Panigrahi & Muralidhar 2014) and *Litopenaeus vannamei* (Wasielesky, Atwood, Stokes & Browdy 2006; Xu & Pan 2012). Apart from being a source of quality proteins, bioflocs are rich source of growth promoters and bioactive compounds (Ju, Forster, Conquest & Dominy 2008) which enhance digestive enzymes (Xu & Pan 2012) and improve immune responses and disease resistance of the cultured shrimps (Ekasari, Azhar, Surawidjaja, Nuryati, Schryver & Bossier 2014; Kim, Pang, Chel Seo, Rok Cho, Samocha & Jang 2014; Xu & Pan 2014; Kumar, Anand, De, Deo, Ghoshal, Sundaray, Ponniah, Jithendran, Raja, Biswas & Lalitha 2015).

Many dietary supplements are reported to act as immunostimulants through elevated antioxidant enzymes like SOD, catalase or improved physiological status of the cultured aquatic animals (Tejpal, Pal, Sahu, Kumar, Muthappa, Vidya & Rajan 2009). Similarly, few reports suggest that biofloc can be used as a dietary ingredient to enhance the growth performance of *L. vannamei* (Ju, Forster, Conquest, Dominy, 2008; Kuhn, Lawrence, Boardman, Patnaik, Marsh & Flick 2010) and *P. monodon* (Anand, Kohli, Dam Roy, Sundaray, Kumar, Sinha, Pailan & Sukham 2015). Biofloc apart from having diverse microbial, algal components and bioactive compounds (Ju, Forster, Conquest, Dominy, Kuo & David Horgen 2008) is known for a source of detritus and faecal components which in higher level can also interfere the physiological condition of the animal unless it is treated. Hence, it is imperative to optimize the inclusion levels in shrimp diets and to have understanding about its role in physiological or immunological responses in shrimps. Moreover, there is a dearth of information to support the dietary role of biofloc on antioxidant and metabolic enzyme level in shrimps. In this context, the present study aims to evaluate the effect of dietary supplementation of biofloc on immune response and metabolic enzyme activities in *P. monodon* juveniles at various inclusion levels.

Materials and methods

Experimental design moreover

Biofloc produced in outdoor tanks was used as dietary supplement in shrimp feed over a 60-day indoor growth trial. A control diet without biofloc was compared against three experimental diets with graded level of biofloc inclusion. The experiment was conducted at Kakdwip Research Centre, Central Institute of Brackishwater Aquaculture, Kakdwip (21°51′N and 88°11′E), West Bengal, India.

Production of biofloc and experimental diets

Biofloc production was carried out in three indoor circular fibreglass-reinforced plastic (FRP) tanks (1000 L; bottom area 2 m^2) in seven batches at 5-day interval as described by Anand *et al.* (2014a).

Briefly, C/N ratio was maintained at 10:1 using ammonium sulphate as nitrogen and wheat flour as carbon source to generate biofloc in the tanks. On fifth day, biofloc was allowed to settle and harvested by passing through a nylon filter bag (10 μm pore size). The collected floc was centrifuged at 2000 rpm, and flocs were dried under shade followed by drying in a hot air oven at 45°C. The dried floc was ground into fine powder (less than 200 μm) and used to make experimental diets (Table 1). The dried biofloc used in the present experiment contained 24.30 ± 0.28% crude protein, 3.53 ± 0.35% crude lipid with ash and acid-insoluble ash content, 31.98 ± 1.01 and 10.75 ± 1.06% of dried biofloc respectively (Anand *et al.* 2014a). Four isonitrogenous and isoenergetic experimental diets were formulated with graded level of biofloc at 4 (B4), 8 (B8) and 12% (B12). The crude protein content of the experimental diets ranged between 37.77 ± 0.14 and 38.05 ± 0.29% with no significant difference among the diet (Table 2).

Experimental system and feeding

Healthy juvenile *P. monodon* tested negative for white spot syndrome virus (PCR test) were obtained from a shrimp farm (South 24 Parganas, West Bengal, India). Shrimps were acclimatized for 14 days and fed with commercial diet (40% crude protein) three times daily before start of experiment. The experiment was conducted in triplicate in FRP tanks (1000 L; bottom area 2 m^2) filled with chlorine-free brackish water. Two hundred and fifty-two *P. monodon* juveniles (2.9 ± 0.10 g) were randomly distributed in the four experimental groups at 21 nos. $tank^{-1}$ following a completely randomized design (CRD).

Table 1 Composition of experimental diets on dry matter basis (g kg^{-1})

	Experimental diets			
Ingredients	**C**	**B4**	**B8**	**B12**
Fish meal	380	380	380	380
Shrimp meal	150	150	150	150
Soybean meal	207.6	194	120	166.9
Wheat flour	172.9	146.5	180.5	93.6
Dried biofloc powder	0	40	80	120
Soya oil	15	15	15	15
Cod liver oil	20	20	20	20
Lecithin	10	10	10	10
Cholesterol	1	1	1	1
Vitamin mineral mix†,‡	23	23	23	23
BHT	0.5	0.5	0.5	0.5
Guar gum	20	20	20	20
Total	1000	1000	1000	1000

†Vitamin mineral mix (Supplevite-M) (quantity kg^{-1}): vitamin A, 20 00 000 IU; vitamin D3, 400 000 IU; vitamin B2, 800 mg; vitamin E, 300 unit; vitamin K, 400 mg; vitamin B6, 400 mg; vitamin B12, 2.4 mg; calcium pantothenate, 1000 mg; nicotinamide, 4 g; choline chloride, 60 g; Mn, 10 800 mg; iodine, 400 mg; Fe, 3000 mg; Zn,6 g; Cu, 800 mg; Co, 180 mg.

‡Vitamin C, 1000 mg.

C, Control; B4, 4% biofloc inclusion; B8, 8% biofloc inclusion; B12, 12% biofloc inclusion.

Sample preparation for enzyme assay

After completion of the feeding experiment, 18 intermoult shrimps from each treatment groups (six from each replicate) were sacrificed for the analysis of metabolic and antioxidant parameters. The hepatopancreas and muscle of the shrimp from replicates of each treatment groups were dissected out and separately homogenized with 0.25 M chilled sucrose on wet basis (pH 7, 1:10 w/v) in a hand-held glass homogenizer in ice-cooled condition. The homogenate was centrifuged at 6000 rpm (2400 *g*) for 20 min at 4°C (Centrifuge 5417R, Eppendorf, Germany). After centrifugation, the floating top lipid layer was removed and the supernatant solution was divided as aliquots in 1.5-mL Eppendorf tubes. The samples were stored at −40°C until further analysis.

Metabolic enzymes assay

Lactate dehydrogenase (LDH) activity was assayed as per the Wroblewski and Ladue (1955) protocol using 0.1 mM NADH in 100 mM phosphate buffer (pH 7.5). Addition of 0.02 M sodium pyruvate initiated the reaction, and final OD value was recorded 340 nm at 30-s interval for 3 min. The enzyme activity was expressed as micromoles of NAD released mg $protein^{-1}$ min^{-1}. Malate dehydrogenase (MDH) activity was estimated similar to LDH activity except 0.02 M oxaloacetate was used as substrate (100 mM phosphate buffer, pH 7.5, 0.1 mM NADH) as per Ochoa (1955). Aspartate aminotransferase or glutamate oxaloacetate (GOT) was assayed using 0.2 M D, L-aspartic acid and 2 mM alpha-ketoglutarate as substrate in 0.05M phosphate buffer (pH 7.4). Briefly, the substrate and homogenate were incubated for 1 h at 37°C and reaction was stopped by the addition of 2, 4-dinitrophenyl hydrazine (DNPH) followed by

Table 2 Proximate composition (%) of experimental diets supplemented with graded level of biofloc (mean ± SD)

Nutrients	C	B4	B8	B12	Level of significance
Organic matter†	82.12 ± 0.10^{c}	81.75 ± 0.14^{bc}	81.51 ± 0.10^{ab}	81.17 ± 0.06^{a}	**
Moisture	8.10 ± 0.42^{a}	7.98 ± 0.17^{a}	7.88 ± 0.04^{a}	7.91 ± 0.15^{a}	NS
Crude protein	37.88 ± 0.03^{a}	37.92 ± 0.33^{a}	37.77 ± 0.14^{a}	38.05 ± 0.29^{a}	NS
Crude lipid (EE)	7.67 ± 0.26^{a}	7.91 ± 0.10^{a}	8.10 ± 0.15^{a}	8.02 ± 0.05^{a}	NS
Ash	17.88 ± 0.10^{a}	18.25 ± 0.14^{ab}	18.49 ± 0.10^{bc}	18.84 ± 0.06^{c}	**
Crude fiber	3.16 ± 0.08^{a}	3.37 ± 0.18^{a}	3.16 ± 0.13^{a}	3.35 ± 0.01^{a}	NS
NFE‡	25.32 ± 0.73^{a}	24.58 ± 0.93^{a}	24.62 ± 0.01^{a}	23.85 ± 0.17^{a}	NS
Gross energy§	401.23 ± 0.71^{a}	401.57 ± 0.25^{a}	401.79 ± 1.13^{a}	400.23 ± 0.52^{a}	NS

†Organic matter = 100 − ash.

‡Nitrogen-free extract (NFE) = 100 − (CP + EE + CF + ash + moisture).

§Gross energy (GE) = (CP × 5.6) + (EE × 9.44) + (CF × 4.1) + (NFE × 4.1) kcal per 100 g.

C, Control; B4, 4% biofloc inclusion; B8, 8% biofloc inclusion; B12, 12% biofloc inclusion.

Different superscripts in the same row indicate significant difference among experimental diets (Tukey's multiple range test ** = 0.01).

0.4 N NaOH addition after 20 min. A control and a standard (sodium pyruvate) were run along with the samples, and OD was recorded at 540 nm (Wootton 1964). The enzyme activity was expressed as nanomoles oxaloacetate formed per min per mg protein at 37°C. The alanine aminotransferase or glutamate pyruvate transaminase (GPT) activity was assayed similar to AST procedure where the substrate L-alanine was used instead of aspartic acid. Total protein content was analysed from the supernatant (Lowry, Rosebrough, Farr & Randall 1951) for calculating enzyme activities. All the assays were carried out using UV–VIS spectrophotometer (model UV2310; Techcomp, Beijing, china).

Antioxidant enzymes and serum parameters

The activity of superoxide dismutase (SOD) was assayed using the reaction mixture consisted of 0.5 mL of sample, 0.5 mL of EDTA (0.6 mM) solution in 1 mL of carbonate bicarbonate buffer (0.1 M; pH 10.2) using substrate 0.5 mL Epinephrine (1.8 mM) as per Misra and Fridovich (1972). The increase in absorbance was recorded at 480 nm at every 30 s for 3 min. The values are expressed as 50% inhibition of epinephrine auto-oxidation min^{-1} mg protein^{-1}.

The catalase assay was carried out using H_2O_2 substrate (0.03 M in phosphate buffer) following the method of Takahara, Hamilton, Neel, Kobara, Ogura and Nishimura (1960). The reaction was initiated by addition of substrate in 1.2 mL of phosphate buffer (0.05 M, pH 7) containing 0.05 mL sample. The decrease in OD at 240 nm was recorded at every 30 s for 3 min and expressed as μmoles of H_2O_2 decomposed min^{-1} mg protein^{-1}. Serum and muscle protein were estimated by Lowry's method (Lowry *et al.* 1951) using bovine serum albumin as standard.

Total haemocyte count

To measure the total haemocyte count, 50 μL of haemolymph–anticoagulant solution (1:10) was mixed with 50 μL of Rose Bengal solution (1.2% Rose Bengal in 50% ethanol) immediately after haemolymph collection. The stained haemolymph solution was counted in improved Neubauer bright-line chamber under 40× objective (Carl Zeiss, Jena, Germany). The cells were differentiated into granulocyte and agranulocyte based upon the granular content (Le Moullac 2000) and expressed as total haemocyte count mL^{-1} (THC mL^{-1}) and total granulocyte count mL^{-1} (TGC mL^{-1}).

Challenge

After 60 days of feeding experiment, 15 shrimps from each treatment group were challenged with virulent *Vibrio harveyi.* A 20 μL of *V. harveyi* suspension (10^7 cfu mL^{-1}) was injected intramuscularly to five shrimps per replicate in each treatment by 1-mL syringe. After challenge, the shrimps were released back into their respective tanks and 10 days regularly monitored for mortality. During challenge tests, no water was exchanged and the shrimp survival was measured every day. The

Table 3 Metabolic enzyme activity in hepatopancreas of *Penaeus monodon* juveniles fed with graded level of biofloc-supplemented diets

Experimental groups	LDH†	MDH‡	ALT§	AST¶
C	0.015 ± 0.002^{a}	5.64 ± 0.60^{a}	14.38 ± 0.79^{a}	15.16 ± 0.68^{a}
B4	0.011 ± 0.001^{a}	4.67 ± 0.33^{a}	13.43 ± 0.84^{a}	13.56 ± 0.79^{ab}
B8	0.013 ± 0.001^{a}	5.00 ± 0.63^{a}	13.30 ± 1.05^{a}	12.78 ± 0.58^{ab}
B12	0.005 ± 0.004^{a}	1.00 ± 0.47^{b}	7.96 ± 0.77^{b}	10.05 ± 1.47^{b}
Level of significance	NS	**	**	*

†Lactate dehydrogenase activity (LDH) expressed as micromoles of NAD released per mg protein per min.
‡Malate dehydrogenase (MDH) activity (MDH) expressed as micromoles of NAD released per mg protein per min.
§Alanine aminotransferase activity (ALT) expressed as nanomoles of pyruvate formed per mg protein per min.
¶Aspartate aminotransferase activity (AST) expressed as nanomoles of oxaloacetate formed per mg protein per min.
C, Control; B4, 4% biofloc inclusion; B8, 8% biofloc inclusion; B12, 12% biofloc inclusion.
The means with no superscript letter in common per factor indicate significant difference. If the effects were significant, ANOVA was followed by Tukey's test. *$P < 0.05$; **$P < 0.01$; NS, not significant. Values are presented as mean ± SE.

relative percentage of survival (RPS) in different treatment groups was calculated by the following formula (Burrells, Williams & Forno 2001)

$$\text{Relative percentage survival} = 100 - [(\text{mortality in test}/\text{mortality in control}) \times 100].$$

Statistical analysis

The data were statistically analysed by statistical package SPSS version 17.0 (SPSS, Chicago, IL, USA). Before all analyses, data were analysed for normality and homogeneity of variances. One-way ANOVA was used to determine the significance of each parameter among different treatments. If a main effect was significant, the ANOVA was followed by Tukey's test. Level of significance was made at 95% probability level.

Result

Metabolic enzymes

The activity of metabolic enzymes in hepatopancreas is presented in Table 3. The alanine aminotransferase activity (ALT) and aspartate aminotransferase activity (AST) in hepatopancreas did not show significant difference with control group up to 8% biofloc inclusion levels, whereas supplementation at 12% found to significantly reduce its level with respect to control. Similarly, no significant differences in lactate dehydrogenase (LDH) activity were noticed among the treatments, while hepatopancreas MDH activity was found to be significantly lower in B12 group. Enzyme in pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH) activity in shrimp hepatopancreas is presented in Fig. 1. Significant difference ($P < 0.05$) in G6PDH activity was observed in hepatopancreas with the highest activity in B4 and the lowest in control.

Total haemocyte count

Haemocyte counts are presented in Table 4. There was no significant difference in total haemocyte count, granulocyte and agranulocyte count among the treatments. The highest total haemocyte count was noticed in B8 ($22.16 \pm 2.17 \times 10^{6}$ cells mL^{-1}) followed by B4 ($21.11 \pm 0.56 \times 10^{6}$ cells mL^{-1})

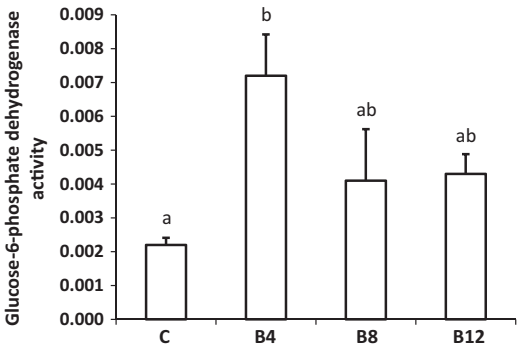

Figure 1 Glucose-6-phosphate dehydrogenase activity in hepatopancreas of *Penaeus monodon* juveniles fed with biofloc-supplemented diets. Means (mean ± SE) with no superscript letter in common indicate significant difference ($P < 0.05$). Activity is expressed as unit per mg protein per min. C, Control; B4, 4% biofloc inclusion; B8, 8% biofloc inclusion; B12, 12% biofloc inclusion.

Table 4 Haemocyte count in *Penaeus monodon* juvenile fed with graded level of biofloc-supplemented diets

Haemocyte count	C	B4	B8	B12	Level of significance
Total haemocyte count ($\times 10^6$ cells mL^{-1})	14.61 ± 2.74[a]	21.11 ± 0.56[a]	22.16 ± 2.17[a]	17.15 ± 2.891[a]	NS
Total granulocyte count ($\times 10^6$ cells mL^{-1})	8.26 ± 0.82[a]	11.12 ± 0.96[a]	11.11 ± 2.78[a]	10.00 ± 29[a]	NS
Total agranulocyte count ($\times 10^6$ cells mL^{-1})	6.34 ± 2.09[a]	9.96 ± 0.44[a]	11.04 ± 3.47[a]	7.14 ± 1.49[a]	NS

C, Control; B4, 4% biofloc inclusion; B8, 8% biofloc inclusion; B12, 12% biofloc inclusion.
The means with no superscript letter in common per factor indicate significant difference. If the effects were significant, ANOVA was followed by Tukey's test. NS, not significant. Values are presented as mean ± SE.

Table 5 Serum and muscle protein level in *Penaeus monodon* juveniles fed with graded level of biofloc-supplemented diets

Treatment	C	B4	B8	B12	Level of significance
Serum protein (mg mL^{-1})	5.68 ± 0.3[a]	7.94 ± 0.32[b]	7.41 ± 0.7[ab]	6.35 ± 0.51[ab]	*
Muscle protein (mg g^{-1}	16.66 ± 0.40[a]	20.11 ± 3.80[a]	18.53 ± 1.14[a]	17.73 ± 4.10[a]	NS

C, Control; B4, 4% biofloc inclusion; B8, 8% biofloc inclusion; B12, 12% biofloc inclusion.
The means with no superscript letter in common per factor indicate significant difference. If the effects were significant, ANOVA was followed by Tukey's test. *$P < 0.05$; NS, not significant; values are presented as mean ± SE and expressed as mg protein mL serum^{-1}.

and the lowest in control (14.61 ± 2.74 $\times 10^6$ cells mL^{-1}). Among granulocyte, the highest number was recorded in B4 (11.12 ± 0.96 $\times 10^6$ cells mL^{-1}). Similarly, the highest number of agranulocyte was noticed in B8 (11.04 ± 3.47 $\times 10^6$ cells mL^{-1}) followed by B4 (9.96 ± 0.44 $\times$ 10^6 cells mL^{-1}).

Muscle and serum protein

Serum and muscle protein level in *P. monodon* juveniles fed with graded level of biofloc-supplemented diets are presented in Table 5. There was significant difference ($P < 0.05$) in serum protein level with the highest mean value (7.94 ± 0.32 mg protein mL^{-1}) observed in B4 which was statistically higher ($P < 0.05$) compared with control. However, no significant difference in muscle protein was recorded among the treatments, although treatment groups recorded comparatively higher muscle protein level compared with control.

Antioxidant enzymes

Activity of superoxide dismutase in serum and muscle is given in Fig. 2. The SOD activity in serum showed significant difference ($P < 0.05$) among the treatments, while in muscle it did not differ significantly ($P > 0.05$). As for serum, significantly higher ($P < 0.05$) SOD activity was observed in B12

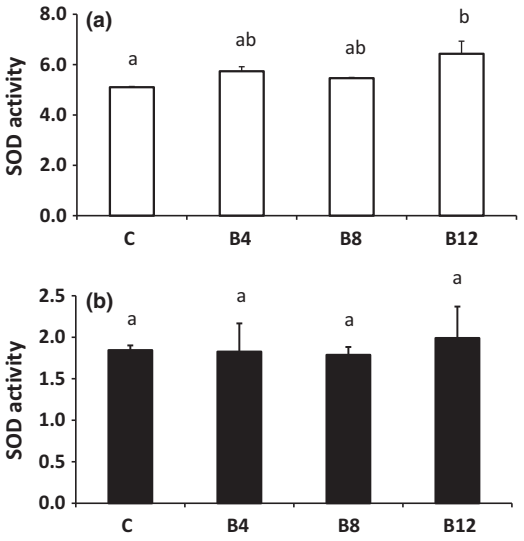

Figure 2 Superoxide dismutase activity in the serum (A) and muscle (B) of *Penaeus monodon* juveniles fed with biofloc-supplemented diets. Error bars represent mean ± standard error. The means with no superscript letter in common per factor indicate significant difference ($P < 0.01$). One unit of superoxide dismutase activity is equivalent to amount of protein to give 50% inhibition of epinephrine auto-oxidation. C, Control; B4, 4% biofloc inclusion; B8, 8% biofloc inclusion; B12, 12% biofloc inclusion.

(6.43 ± 1.10 U mg^{-1} protein) which statistically differed from control (5.10 ± 0.34 U mg^{-1} protein), while no significant differences were noticed

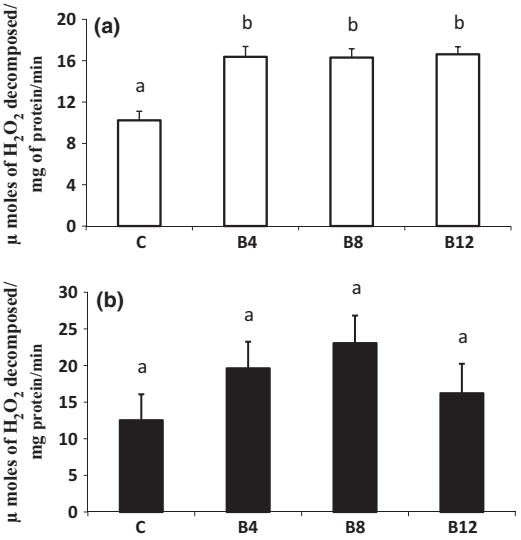

Figure 3 Specific activity of catalase (μ moles of H_2O_2 decomposed per min per mg protein) in serum (a) and muscle (b) of *Penaeus monodon* fed with experimental diets having different levels of biofloc inclusion level. Error bars: mean ± standard error. The means with no superscript letter in common per factor indicate significant difference ($P < 0.01$). C, Control; B4, 4% biofloc inclusion; B8, 8% biofloc inclusion; B12, 12% biofloc inclusion.

between B4 and B8. In case of muscle, SOD activity did not differ significantly among the experimental groups with highest activity noticed in B12.

Serum catalase activity (Fig. 3) showed the highest activity in B12 which was 60.5% significantly higher ($P < 0.01$) compared with control. Similarly, there was 60% and 59.4% significant increase ($P < 0.01$) in serum catalase activity in B4 and B8, respectively, compared with control. As for muscle, the highest catalase activity was observed in shrimp fed with 8% (B8) and 4% (B4) biofloc-incorporated diet, respectively, but did not differ significantly with B12 and control.

Relative percentage survival (RPS)

Relative percentage survival and cumulative mortality in the different treatment groups are presented in Fig. 4. After challenge with *V. harveyi,* the cumulative mortality in the control group was 73%, while the treatment group supplemented with 4% level of biofloc (B4) recorded the lowest level of mortality (40%) and the highest relative percentage survival 45% (RPS). Similarly, B8 had better relative percentage survival compared with control and B12.

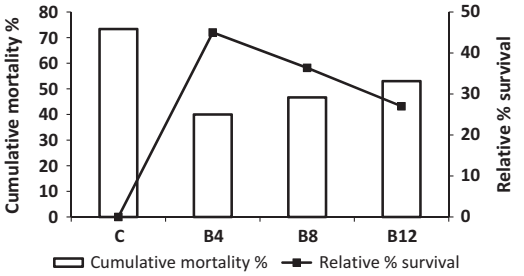

Figure 4 Cumulative mortality percentage and relative percentage survival in experimental groups fed with graded level of biofloc-supplemented diets. C, Control; B4, 4% biofloc inclusion; B8, 8% biofloc inclusion; B12, 12% biofloc inclusion.

Discussion

Dietary supplements incorporated in shrimp diet have direct influence on the health and physiological activities. It has been reported that when fish and shrimps were fed with dietary supplements like tryptophan (Tejpal *et al.* 2009), nutraceutical (Andrews 2008), pyridoxine (Akhtar, Pal, Sahu, Alexander, Gupta, Choudhary, Jha & Rajan 2010) or periphyton supplements (Anand *et al.* 2015) have direct influences on the health and level of metabolic enzymes. The metabolic enzymes like LDH, enzyme in carbohydrate metabolism and MDH, Kreb's cycle enzyme reflect the physiological state and energy demand in crustaceans (Rosas *et al.* 2001). Their level increases under different types of stresses like oxygen shortage (Murray *et al.* 2010) and high-density transportation (Chatterjee, Pal, Das, Mohammed, Sarma, Venkateshwarlu & Mukherjee 2006). In the present study, no significant differences in LDH and MDH activities were noticed among the experimental groups upto 8% biofloc inclusion level, whereas the MDH level in B12 group was significantly lower compared with other treatments. In general, there is a negative correlation between growth rates and LDH and MDH activity in fish (Koedijk, Le Francois, Blier, Foss, Folkvord, Ditlecadet, Lamarre, Stefansson & Imsland 2010). The lower level of these enzymes was recorded when fish and shrimps were fed with dietary supplements like tryptophan (Tejpal *et al.* 2009), pyridoxine (Akhtar *et al.* 2010) or periphyton (Anand *et al.* 2015). As overall, no significant differences were noticed in LDH and MDH activity in hepatopancreas of treatment groups compared with control indicating that dietary supplementation of biofloc helps to maintain shrimps

in less stressed condition and reduced energy demand in shrimps reared in treatment groups.

The AST and ALT are the most important aminotransferases which take part in amino acid metabolism. Their level in hepatopancreas is a direct indicator of shrimp health (Pan *et al.* 2003). According to the literature, its level increases with hepatopancreatic damage or during stress like transportation and DO depletion (Mohankumar & Ramasamy 2006). Biofloc apart from being a source of nutrient-rich autotrophic and heterotrophic microbial components is also a source of detritus and faecal components. Later is rich in ash contents which at higher level can interfere the physiological condition of the shrimp. In the present study, no significant differences in ALT or AST metabolic activities were noticed among the treatments like B4, B8 and control. On contrary, the B12 group recorded significantly lower level of AST and ALT compared with control. The earlier studies report the positive effects of immunostimulants or nutraceuticals in lowering the level of AST or ALT in liver of fish or hepatopancreas of shrimp (Pan *et al.* 2003; Choudhury *et al.* 2005; Gupta *et al.* 2010; Anand *et al.* 2015). Also, our earlier studies revealed that dietary supplementation of biofloc at higher level (12%) did not show significant differences in growth performance with control (Anand *et al.* 2014a), indicating that higher level of biofloc inclusion did not have negative effects on physiological mechanism of shrimp juveniles. Many of the earlier studies (Nakano, Kanmuri, Sato & Takeuchi 1999) reported that dietary supplements in aquafeed reduced the level of AST and ALT in liver and increased defensive potential against different kinds of stresses generated in the rearing system. Further studies are needed to understand and characterize the compound in the biofloc which allows shrimps to combat the different types of stresses generated in the shrimp culture ponds.

The enzyme glucose-6-phosphate dehydrogenase (G6PDH) is involved in synthesis of fatty acids, cholesterol and sphingolipids (McWhinnie & Kirchenberg 1962). It is a lipogenic enzyme, and its high activity in liver shows better efficiency to convert carbohydrate into lipid (Verri, Mandal, Zilli, Bossa, Mandal, Ingrosso, Zonno, Viella & Storelli Aheam 2001). Also, enzyme activity is expected to increase with increased phagocytosis indicating better oxidative status of the animal. In the present study, higher level of G6PDH was noticed in hepatopancreas of treatment groups compared with control. Even though no parallel report is available in penaeid shrimp to support this finding, the higher activity of G6PDH activity was reported in fish fed with immunostimulant (Gupta *et al.* 2010) and pyridoxine (Akhtar *et al.* 2010)-supplemented diets compared with control. Further, significantly higher G6PDH activity recorded in B4 group can be correlated with the better growth performance (Anand *et al.* 2014a) and physiological condition in this group.

It is well established that microbial products and their cell wall components elicit non-specific defence mechanism of crustaceans (Chang, Chen, Su & Liao 2000; Karunasagar, Karunasagar & Umesha 2004). Innate immune systems in shrimp comprised of circulating haemocytes, prophenoloxidase (proPA) system, oxidative enzymes like SOD and catalase other antimicrobial peptides (Tassanakajon *et al.* 2013). Shrimp haemocytes take part in phagocytosis and killing of pathogens, apart from storing many immune molecules such as proPA in their granules (Sakai 1999; Ekasari *et al.* 2014). Supplementation of probiotics (Wang 2007) and microalgal products (Montero-Rocha, McIntosh, Sanchez-Merino & Flores 2006) in shrimp feed as dietary supplements was found to enhance the total haemocyte count in shrimps.

Bioflocs being a rich source of heterotrophic beneficial bacteria, it can act as a potential source of immunostimulants (Crab *et al.* 2012; Xu & Pan 2014; Kumar *et al.* 2015). Dietary supplementation of biofloc in the present study led to non-significantly higher total haemocyte count in treatments compared with control suggesting better immune status in these treatments which is accordance with earlier findings (Ekasari *et al.* 2014; Xu & Pan 2014) where they reported significantly higher total haemocyte counts in shrimps grown in *in situ* developed biofloc system. From this, it can be inferred that supplementation of biofloc contains immunostimulatory compounds which can elicit cellular defence mechanism and innate immune responses in shrimp.

During respiratory burst, shrimps synthesize reactive oxygen radicals (ROS) such as superoxide, peroxide, hydroxyl ion to kill the pathogens, but protect themselves from the lethal effect of ROS by secreting oxidative enzymes like SOD and catalase (Tassanakajon *et al.* 2013). The SOD and catalase are the main antioxidant enzymes related to immunity in crustacean (Holmblad & Soderhall 1999; Mohankumar & Ramasamy 2006). In the

present study, significantly higher level of serum catalase and SOD activity was noticed in shrimp fed with biofloc-incorporated diet (B12) compared with control. This is supported by earlier reports which claim significantly higher levels of catalase and SOD activity in haemolymph of shrimp fed with β-1,3 glucan (Chang, Su, Chen & Liao 2003), vitamin E, β-carotene (Pacheco, Ascencio, Zarain, Gomez & Campa 2011), yeast (Yang, Wu, Jian & Zhang 2010) and periphyton (Anand *et al.* 2015)-incorporated diets. Moreover, the recent study reveals higher level of SOD activity in shrimps grown in biofloc-based culture system (Kim *et al.* 2014; Xu & Pan 2014). Hence, the present study supports that dietary supplementation of bioflocs elicits antioxidant components in the shrimps and enhances the immune responses. However, variation in level of catalase and SOD activity in shrimps fed with graded level of biofloc indicates the lack of proportionate increase in level of antioxidant enzymes with respect to dietary inclusion level and suggests the need to verify the compounds which have inherent limit on immunostimulatory action.

Our earlier studies on supplementation of biofloc in shrimp diet and its growth performance revealed that at higher inclusion level, above 8% does not found to enhance the growth performance of shrimps (Anand *et al.* 2014b). Biofloc apart from being a complex of many nutrients like autotrophic and heterotrophic components, it is also a source of detritus and faecal components with higher ash content which in higher level may interfere the physiological condition of the animal unless it is treated. Kuhn *et al.* (2010) used biofloc generated from a bioreactor as a feed ingredient and recorded significantly higher growth rate at 10 and 15% and non-significant difference at 21% and 30% dietary inclusion level of biofloc in *L. vannamei*. Ash content in his study was up to 19%, whereas biofloc used in our studies had up to 36% with 10% acid-insoluble ashes. This was mainly due to difference in the biofloc production system. Wang (2007) who reported increase in dietary supplementation of probiotics showed no significant increase in growth or survival. These studies suggest that raw supplementation at higher level can reduce the feed palatability or increase the FCR unless and until it is treated. However, non-significant difference in the metabolic MDH, LDH, AST or ALT parameters with control indicates that the inclusion level does not have any negative impact on shrimps physiological state. This also throws light to further refine the methods of biofloc production which can remove the chance of presence of any antinutritional components or limit the level of acid-insoluble ashes in the floc. Supplementation of bacterial consortium like cellulolytic bacteria which can improve the digestibility factor of shrimp can also lead to its better nutrient availability which needs further study.

Relative percentage survival analysis indicated that the use of bioflocs as dietary supplement significantly improves the disease resistance of shrimp against *V. harveyi*. In addition, shrimp fed with 4% and 8% bioflocs dietary supplementation showed higher immune response with better resistance to *Vibrio* challenge compared with control. Recently, Ekasari *et al.* (2014) reported disease resistance of shrimp fed biofloc grown on different carbon sources against infectious myonecrosis virus. This is comparable to the other findings about better disease resistance in *L. vannamei* (Nonwachai, Purivirojkul, Limsuwan, Chuchird, Velasco & Dhar 2010) and *P. monodon* (Anand *et al.* 2014a) upon challenge with *V. harveyi* when fed with algal meal- and periphyton meal-incorporated diet compared with control. It indicates the potential role of biofloc in eliciting non-specific immune responses of shrimp, and it reduces their susceptibility to bacterial infection.

Conclusion

The present findings elucidate the potential of biofloc as a dietary immunostimulant in *P. monodon*. The study demonstrates that dietary supplementation of biofloc at 4–8% level has an immunomodulatory effect on tiger shrimp and enhances the physiological condition of cultured shrimp. Future studies are required to characterize the immunomodulatory components in biofloc and production of biofloc supplements using manipulated beneficial microbial communities which can improve the digestibility of the ingredients at higher inclusion levels.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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