Aquaculture Research

Aquaculture Research, 2015, 1–15 doi: 10.1111/are.12958

Effects of biofloc under different carbon sources and protein levels on water quality, growth performance and immune responses in black tiger shrimp *Penaeus monodon* (Fabricius, 1978)

Sujeet Kumar[1], Panantharayil S Shyne Anand[1,2], Debasis De[1], Ashutosh D Deo[1], Tapas K Ghoshal[1], Jitendra K Sundaray[1,3], Alphis G Ponniah[2], Karingalakkandy Poochirian Jithendran[2], Ramalingam Ananda Raja[1,2], Gouranga Biswas[1] & Natarajan Lalitha[2]

[1]Kakdwip Research Centre, Central Institute of Brackishwater Aquaculture (ICAR), Kakdwip, West Bengal, India

[2]Central Institute of Brackishwater Aquaculture, Chennai, India

[3]Central Institute of Freshwater Aquaculture, Bhubaneswar, India

Correspondence: S Kumar, Kakdwip Research Centre, Central Institute of Brackishwater Aquaculture, Kakdwip, South 24 Parganas, West Bengal 743347, India. E-mail: sujeetmicrobiol@gmail.com

Abstract

A 75-day 2 × 3 factorial experiment was conducted to evaluate the effect of two levels of dietary protein (32 and 40%) and two different carbon sources (rice flour–R and molasses-M), and without carbohydrate (control–C) in black tiger shrimp *Penaeus monodon* juveniles (3.37 ± 0.04 g) at 100 nos. m^{-3} in 100 L fibre reinforced plastic tanks. Biofloc volume and total suspended solid were higher in molasses added groups (32 + M and 40 + M) followed by rice flour (32 + R and 40 + R) and controls (32C and 40C). Molasses and rice flour addition significantly reduced ($P < 0.01$) the total ammonia–N compared to controls. The highest *Vibrio*, *Bacillus* and *Lactobacillus* counts were recorded in 40 + M, 32 + M and 32 + R respectively. Among the treatments, significantly higher ($P < 0.01$) final body weight was recorded in 40 + R (8.5 ± 0.3 g), 40 + M (7.8 ± 0.3 g) and 32 + R (7.5 ± 0.4 g) compared to control groups, 32C (6.1 ± 0.3 g), 40C (6.4 ± 0.3 g) and molasses added group, 32 + M (5.7 ± 0.4 g). Rice flour supplementation significantly increased ($P < 0.01$) the total haemocyte count ($\times 10^6$ cells mL^{-1}) in 32 + R (45.7 ± 3.7) and 40 + R (44.3 ± 3.1) compared to controls, 32C (27.3 ± 3.4) and 40C (25.8 ± 0.9). Similarly, higher superoxide dismutase, catalase, serum protein and glucose were recorded in the rice flour added groups, 40 + R followed by 32 + R. Among the treatments, the highest level of prophenoloxidase (OD 490 nm, 0.3 ± 0.0) and survival after challenge with *Vibrio harveyi* (55.6%) was observed in 32 + R. The study elucidates that rice flour addition produces optimum level of biofloc with better growth and immune responses compared to molasses and control. Furthermore, rice flour addition at 32% protein level could replace 40% protein feed.

Keywords: biofloc technology, black tiger shrimp, carbohydrate supplementation, growth performance, immune response, *Penaeus monodon*

Introduction

Shrimp farming is the major commercial aquaculture activity in many Asian and South American countries (FAO 2012). In developing countries like India, shrimp culture is mostly export-oriented and plays a major role in economic growth of coastal communities (Hein 2002). Despite the huge success, penaeid shrimp culture is plagued with diseases of viral and bacterial origin. Diseases such as early mortality syndrome, white spot disease, Taura syndrome etc. have been reported in many Asian countries causing catastrophic mortality with severe economic losses (Karunasagar & Otta 1998; Lightner, Redman, Pantoja, Noble & Tran 2012). Ecological sustainability of shrimp farming is another major challenge as cultured shrimp

retains only 20–30% of feed nutrients and remaining 70–80% accumulates in aquatic system (Funge-Smith & Briggs 1998). This leads to deterioration of water quality resulting in stress and disease outbreaks (Kautsky, Rönnbäck, Tedengren & Troell 2000). These issues demand sustainable shrimp farming system that have better nutrient recycling, low impact on the environment and free from disease outbreaks.

In recent years, manipulation of C:N ratio in feed has shown promising results in aquaculture (Avnimelech 2012). The C:N ratio can be manipulated by the application of various carbohydrate sources such as molasses, rice flour, tapioca powder etc. (De Schryver, Crab, Defoirdt, Boon & Verstraete 2008; Crab, Defoirdt, Bossier & Verstraete 2012). Similarly, it can be manipulated by changing the protein levels in the feed (Azim, Little & Bron 2008; Ballester, Abreu, Cavalli, Emerenciano, de Abreu & Wasielesky 2010). At high C:N ratio, heterotrophic bacteria immobilize the ammonium ions for production of biofloc. This helps to reduce toxic ammonia N in aquatic system (Avnimelech 1999). Biofloc based system found to improve growth performance and digestive enzyme activity in cultured shrimps (Xu & Pan 2012; Anand, Kohli, Kumar, Sundaray, Dam Roy, Venkateshwarlu, Sinha & Pailan 2014).

Biofloc technology is based on microbial manipulation within the aquaculture system. Biofloc particles contain beneficial bacteria like *Bacillus*, *Lactobacillus* (Anand *et al.* 2014) and bioactive compounds like carotenoids (Ju, Forster, Conquest, Dominy, Kuo & David Horgen 2008). These are known for probiotics and immunostimulant properties. Recently, Xu and Pan (2013) reported that biofloc enhanced the haemocyte count and antioxidant status of white leg shrimp, *Litopenaeus vannamei*. Similarly, biofloc improved the disease resistance in brine shrimp, *Artemia franciscana* against *Vibrio harveyi* (Crab, Lambert, Defoirdt, Bossier & Verstraete 2010). It has been reported that nature of the carbon source affects the nutritional composition of biofloc (Crab, Chielens, Wille, Bossier & Verstraete 2010). However, there is a dearth of information with respect to carbon source and protein levels in the feed over growth performance and immune response in penaeid shrimps. In this context, this study aims to investigate the interaction effects of different carbon sources and protein levels on water quality, growth performance and immune responses in black tiger shrimp *Penaeus monodon* cultured under experimental yard condition.

Materials and methods

Experimental design and set up

An on-station yard trial was conducted in triplicates as 2 × 3 factorial design. The two levels of dietary protein (32 and 40%) served as first factor and two carbohydrate sources (rice flour R and molasses-M), and without carbohydrate as control (C) as second factor. The treatments with carbohydrate supplementation are referred to as 32 + M, 32 + R, 40 + M and 40 + R, and without carbohydrate supplementation as 32C and 40C which served as controls.

The experiment was carried out for a period of 75 days during September to November, 2012 at Kakdwip Research Centre, Central Institute of Brackishwater Aquaculture, Kakdwip (21°51′ N and 88°11′ E), West Bengal, India. Experiment was conducted in 100 L fibre reinforced plastic (FRP) tanks. Tanks were filled with water from the nearby brackishwater source. The fine meshed nylon filter bag (10 μ mesh size) was used to prevent the entry of unwanted materials and suspended particles into the tanks. Healthy juvenile shrimps, *P. monodon*, tested negative for white spot syndrome virus by polymerase chain reaction, were obtained from a commercial shrimp farm (South 24 Parganas, West Bengal, India). Shrimps were acclimatized for a period of 14 days and fed with control diet (40% crude protein) two times daily, before the start of the experiment. One hundred and eighty *P. monodon* juveniles (3.37 ± 0.04 g) were randomly distributed into 18 FRP tanks at 100 nos. m^{-3} following a completely randomized design.

Experimental diets

Two experimental diets with 32 and 40% crude protein levels representing the C:N ratio of 10:1 and 7.5:1 respectively were prepared. The compositions of experimental diets are presented in Table 1. Ingredients like wheat flour, fish meal, soyabean, shrimp meal, guar gum and lecithin were mixed with water to make dough. The dough was steam cooked for 20 min in a pressure cooker at 69 kPa. After cooling, additives like cholesterol, butylated hydroxytoluene, oil and vitamin–mineral

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

	Protein level (%)	
Ingredients	**32**	**40**
Fish meal	320	400
Shrimp meal	120	150
Soyabean meal	131.5	246.3
Wheat flour	340	115.2
Soya oil	30	30
Cod liver oil	10	10
Lecithin	10	10
Cholesterol	1	1
Vitamin and mineral mix*	16	16
Butylated hydroxytoluene	0.5	0.5
Guar gum	20	20
Vitamic C	1	1

*Vitamin and mineral mix (Supplevite-M) supplied per kg of feed as: Vitamin A, 32 000 IU; Vitamin D3, 6400 IU; Vitamin E, 4.8 U; Vitamin B2, 12.8 mg; Vitamin B6, 6.4 mg; Vitamin K, 6.4 mg; Vitamin B12, 38.4 μg; Calcium Pantothenate, 16 mg; Nicotinamide, 64 mg; Choline Chloride, 960 mg; Ca, 4.8 mg; Mn, 176 mg; Iodine, 6.4 mg; Fe, 48 mg; Zn, 96 mg; Cu, 12.8 mg; Co, 2.88 mg.

mixture were mixed with test diets. The dough was pressed through a pelletizer with 2 mm die. The feeds were dried at 60°C till the desired moisture level (<10%) reached. It was subsequently stored at 4°C until further use.

Experimental system and feed management

The treatments such as 32 + M, 32 + R, and 32C were fed 32% protein diet, and treatments like 40 + M, 40 + R and 40C were fed 40% protein diet. The 32C and 40C represented control with no carbohydrate addition. The daily feeding started at 6% of body weight and declined gradually to 4%, at the end of the experiment (Anand *et al.* 2014). The daily ration was divided into two parts, 40% feed was given in the morning and 60% in the evening. Equal amount of feed was fed to shrimps in all the experimental tanks, twice daily at 10:00 and 18:00 hours for 75 days. Carbohydrate was added externally in the treatment groups as rice flour (32 + R and 40 + R) and beet molasses (32 + M and 40 + M) to convert the excreted nitrogen and uneaten feed into microbial protein. An average 10 g carbon is required to convert 1 g of total ammonia nitrogen (TAN), produced from excretion and uneaten feed, into bacterial biomass (Avnimelech 2012). The locally purchased wet beet molasses (23.4% moisture and 28% carbon) was added at the rate of 0.9 and 1.1 g to 32 + M and 40 + M, respectively, for each 1.0 g of feed representing addition of molasses at carbon to nitrogen ratio of 10:1. The rice flour (40% carbon) was added at the rate of 0.6 and 0.7 g to 32 + R and 40 + R, respectively, for each 1.0 g of feed representing addition of rice flour at carbon to nitrogen ratio of 10:1. This protocol was followed throughout the experimental periods. The pre-weighed molasses or rice flour was mixed in a beaker with the culture water and uniformly distributed over the tank surface after feeding at 10:00 and 18:00 hours daily (Hari, Kurup, Varghese, Schrama & Verdegem 2004). Experimental units were supplied by aerator having 120 m^3 air min^{-1} capacity. This continuously provided the aeration and agitation to each tank by two air stones, each diffusing 3.3 m^3 air $tank^{-1}$ min^{-1}. Side of the tanks was manually cleaned twice a week to remove biofilm deposit over the surface. Water exchange was done at 30% rate after 4 and 8 week of the experiment when floc volume (FV) crossed 15 mL L^{-1} in the molasses added experimental groups, 32 + M and 40 + M (Azim & Little 2008). To maintain uniformity, 30% water was also exchanged in both the control groups; 32C and 40C and rice flour added groups 32 + R and 40 + R. For rest of the period, no water was exchanged. The experimental yard was covered over the top and open from all the four sides. This allowed an average 4–6 h sunlight to descend over the experimental tanks to facilitate natural productivity. During the study period, salinity and temperature of the experimental tanks ranged from 10.3 to 15.8 g L^{-1} and 25.1 to 30.8°C respectively.

Proximate composition of experimental diets

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 Kjeldahl method after acid digestion using an auto Kjeldahl system (Kelplus, DXVA; Pelican Equipments, Chennai, 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 h in a muffle furnace. Crude fibre was estimated by sequential

digestion with H_2SO_4 and NaOH using Fibertec (Foss Tecator 2022, Hoganas, Sweden). The carbon content in the molasses and rice flour was determined by the formula of Hart, Lovis, Schulenberg and Urquhart (2007).

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

Determination of water quality parameters

The water quality parameters measured at fortnight intervals between 09:00 and 10:00 hours. Salinity, pH and dissolved oxygen (DO) were measured with conductivity probe (CDC401), pH probe (PHC281) and luminescent DO probe, respectively, using Hach multiparameter kit (HQ30D; Hach, Loveland, CO, USA). The DO reduction h^{-1} was measured in the experimental tanks on the 5th and 9th week of experiment. The air supply was turned off for 2 h after measuring the initial DO. Subsequently, DO was measured after 60 and 120 min without disturbing the water column.

Floc volume was determined by sampling 1000 mL water sample into a series of Imhoff cones (Tarson, Kolkata, India). The volume of the floc plug accumulated at the bottom of the cone was determined after 20 min (Avnimelech 2012). The total suspended solid (TSS) was determined based on the methods of Aouidi, Gannoun, Ben Othman, Ayed and Hamdi (2009) with slight modification. In brief, 50 mL water sample, collected after proper mixing, was centrifuged at 1500 *g* for 15 min in a pre-dried and pre-weighed centrifuge tube. The settled solid was dried overnight at 105°C. The difference in weight was multiplied by 20 and expressed as TSS L^{-1}.

To determine chlorophyll *a* concentration, collected floc materials were immediately transferred to centrifuge tubes containing 10 mL of 90% acetone. The tubes were sealed and stored overnight in a refrigerator. The samples were homogenized with a tissue grinder and then centrifuged for 10 min at 270 *g*. The supernatant was carefully transferred to a 3.5 mL glass cuvette. The absorbance was measured at 750, 664, 647 and 630 nm using a spectrophotometer (UV2310; Techcomp, Shanghai, China). Chlorophyll *a* concentration was calculated using the trichromatic equation given in APHA (1998).

Total alkalinity was determined by titrimetric methods. The TAN, nitrite–N, nitrate–N, and phosphate–P were analysed spectrophotometrically following the protocols of APHA (1998).

Growth performance and survival

At the end of the experiment, the final weight of the shrimps was recorded and growth performance parameters were calculated by following formulae:

$$\text{Feed conversion ratio (FCR)} = \text{feed applied/live weight gain}$$

$$\text{Protein efficiency ratio (PER)} = \text{Gain in body mass/protein applied}$$

$$\text{Specific growth rate (SGR)} = (\ln \text{final weight} - \ln \text{initial weight}) \times 100/\text{days of experiment}$$

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

Estimation of biofloc microbial community

Total heterotrophic bacteria (THB), *Vibrio*, *Bacillus* and *Lactobacillus* counts in water samples were recorded at fortnight intervals. Samples were processed as per the earlier described methods (Kumar, Anand, De, Sundaray, Raja, Biswas, Ponniah, Ghoshal, Deo, Panigrahi & Muralidhar 2014) with minor modification. In brief, 200 mL of water sample was homogenized in a kitchen blender for 30 s at high speed equivalent to 12 000 rpm to separate the flocculated microbes. Subsequently, tenfold serial dilution prepared in normal saline solution. A 0.1 mL of appropriate dilution plated on tryptone soya agar (1.0% w/v NaCl) for THB, TCBS agar for *Vibrio*, *Bacillus cereus* agar for *Bacillus* and *Lactobacillus* MRS agar for *Lactobacillus* counts. The Lactobacillus MRS agar plates were incubated in microaerophillic condition and other plates in aerobic condition at 28°C for 72 h. The colony in the range of 30–300 counted and expressed as colony forming unit (CFU mL^{-1}). Media procured from HiMedia, (Mumbai, India) was used for the above work.

Collection of haemolymph and serum preparation

After completion of the feeding experiment, nine inter-moult shrimps from each treatment group

(three from each replicate randomly) were anesthetized with clove oil (50 μL L^{-1}). The intermoult stage was determined by the setal development of the uropod using stereomicroscope (Dall, Hill, Rothlisberg & Sharples 1990). A 50 μL haemolymph was collected from the ventral sinus of each shrimp using 26 Gauge 1–mL tuberculin syringe and mixed with 450 μL cooled anticoagulant (30 mM tri-sodium citrate, 388 mM sodium chloride, 0.12 M glucose, 10 mM ethylene diamine tetra-acetic acid (EDTA), 780 mOsm/kg osmolality, and pH 7.55). To collect serum, haemolymph without anticoagulant was allowed to clot at 4°C for overnight in refrigerator. The supernatant was collected as serum after centrifugation at 600 *g* for 5 min at 4°C (5417R; Eppendorf, Hamburg, Germany). It was aliquot and stored immediately at −40°C.

Total haemocyte count

Haemolymph (150 μL) was collected from three randomly selected shrimp per replicate/treatment in 1350 μL cooled anticoagulant solution. After gentle mixing, haemocytes were counted in improved Neubauer bright-line chamber under 400× magnifications in phase contrast microscope (Carl Zeiss, Jena, Germany). The cells were differentiated into granulocyte and hyaline cells based upon the granular content and size of the cells (Le Moullac & Haffner 2000; Ananda Raja, Kumar, Sundaray, De, Biswas & Ghoshal 2012). Cells were expressed as total haemocyte count mL^{-1}, total granulocyte count mL^{-1} and total hyaline cells count mL^{-1}.

Estimation of immunological and biochemical parameters

Prophenoloxidase (proPO) activity was measured spectrophotometrically by recording the formation of dopachrome from L-3,4-dihydroxyphenylalanine (L-DOPA) (Hernández-López, Gollas-Galván & Vargas-Albores 1996). Briefly, 50 μL serum was incubated for 10 min at 25°C with 50 μL 0.1% trypsin in cacodylate citrate (CAC) buffer (0.45 M sodium chloride, 0.10 M tri-sodium citrate, 0.01 M sodium cacodylate, pH 7.0). Subsequently, 50 μL L-DOPA (0.3% in CAC buffer) was added and incubated for 5 min at 25°C. Then, 800 μL-CAC buffer was added and further incubated at 25°C for 3 min. The optical density (OD) was recorded at 490 nm against the blank (50 μL of L-DOPA, 50 μL 0.1% trypsin and 850 μL CAC buffer). The OD at 490 nm was expressed as proPO activity representing dopachrome formation in 50 μL of serum.

The superoxide dismutase (SOD) activity was determined according to the method of Beauchamp and Fridovich (1971) and Krishnan, Chattopadhyay, Kundu and Chaudhuri (2002). The 2.5 mL reaction mixture contained 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA in 50 mM phosphate buffer (pH 7.8) along with 0–100 μL serum. Reaction started by adding riboflavin and run for 20 min in tubes of uniform thickness under fluorescent light (20 W). The reaction was stopped by switching-off the fluorescent light and covering the tubes with black cloth. The absorbance at 560 nm was recorded in UV–VIS spectrophotometer (model UV2310; Techcomp). A non-irradiated mixture run in parallel with no colour development, served as control. The SOD unit was calculated by using the formula (Krishnan *et al.* 2002).

$$\text{SOD unit mL}^{-1} = [(V/v) - 0.973] \times \text{dilution factor}$$

V = Rate of change of OD in absence of SOD, v = Rate of change of OD in presence of SOD.

The catalase activity was determined following the method of Takahara, Hamilton, Neel, Kobara, Ogura and Nishimura (1960). In brief, 50 μL serum was added in 1.2 mL phosphate buffer (0.05 M, pH 7). The reaction was initiated by addition of 1 mL H_2O_2 substrate (30 mM in phosphate buffer). The decrease in OD at 240 nm was recorded for 3 min and expressed as μmoles of H_2O_2 decomposed/min/mg protein.

Serum protein was estimated by Lowry's method (Lowry, Rosebrough, Farr & Randall 1951) using bovine serum albumin as standard. Serum glucose was quantified by 3,5-dinitrosalicylic acid method (Miller 1959).

Challenge test

After 75 days of feeding trial, shrimps were challenged with virulent strain of *Vibrio harveyi* isolated on TCBS agar from disease affected shrimp pond. It was further characterized on *Vibrio harveyi* agar and by biochemical tests (Harris, Owens & Smith 1996). Bacterium was inoculated in tryptic soya broth (1% w/v NaCl) for 18 h at 28°C.

The culture was washed and re-suspended in NSS at 10^7 CFU mL^{-1}. Shrimp was challenged intramuscularly by 20 μL of bacterial suspension resulting in 2×10^5 CFU per shrimp. A blank control group received 20 μL of NSS without bacterium. The challenge dose was decided based on 50% mortality within 24 h in *P. monodon* juveniles. All the challenged shrimps were released back into their respective tanks and observed for mortality for 10 days. No water was exchanged during the period. The result was presented as survival post challenge over the time periods.

Statistical analysis

Water quality, microbial count, growth performance and immune response parameters were analysed by factorial ANOVA to find out the interaction effects between dietary protein levels and various carbon sources. If the main effect was significant, the ANOVA was followed by Tukey's test. Level of significance was made at 99% and 95% probability levels. Before analysis, data were checked for normality by probability plots and homogeneity of variances by Levene's test. All analyses were performed using statistical software package SAS v.9.2 program (SAS Institute, Cary, NC, USA).

Results

Nutrient composition of diet and carbon supplements

Proximate composition of the experimental diets and carbohydrate sources are presented in Table 2. The protein content was 31.8 ± 0.4 and 40.5 ± 0.3% in 32% and 40% experimental diets respectively. Molasses and rice flour used as carbon sources and contained 28% and 40% carbon and 54.9% and 81.1% nitrogen free extract respectively.

Water quality parameters

Water quality parameters of the experimental groups are presented in Table 3 and Fig. 1. Salinity and temperature during the study period ranged between 10.3 to 15.8 g L^{-1} and 25.3–30.8°C respectively. Supplementation of carbohydrate reduced the TAN by 43–57% with higher level of TAN reduction observed in 32 + R, 40 + R and 32 + M groups. The maximum TAN reduction was observed in the initial 30 days of carbohydrate addition (data not shown). However, no significant difference ($P > 0.05$) in nitrite–N and nitrate–N were noticed among the treatments. Supplementation of Carbohydrate significantly reduced ($P < 0.01$) the level of DO in treatment groups. Moreover, addition of molasses (32 + M and 40 + M) recorded the lower DO and higher levels of DO reduction per hour compared to other treatments. The chlorophyll *a* level was higher in 32C, 40C and 40 + R (each 0.3 ± 0.0 mg L^{-1}) while the lowest level was observed in 32 + M (0.1 ± 0.0 mg L^{-1}).

Biofloc was quantified in terms of FV and TSS. Addition of molasses and rice flour increased the FV by 122–143% and 69–75% respectively compared to controls, 32C and 40C. Similarly, molasses added groups recorded the higher level of TSS compared to other treatments. The highest

Table 2 Proximate composition (%) of experimental diets and carbon supplements (mean ± SD)

	Experimental diets		Carbon supplements	
Nutrients	**32% protein**	**40% protein**	**Rice flour**	**Beet molasses**
Moisture	6.2 ± 0.2	6.5 ± 0.1	7.5 ± 0.1	23.4 ± 0.1
Crude protein	31.8 ± 0.4	40.5 ± 0.3	8.1 ± 0.0	7.4 ± 0.1
Crude lipid	5.8 ± 0.6	6.0 ± 0.5	0.8 ± 0.0	1.2 ± 0.1
Crude fibre	12.1 ± 0.1	12.4 ± 0.1	1.5 ± 0.1	0.7 ± 0.0
Total ash	13.7 ± 0.2	15.2 ± 0.1	1.0 ± 0.0	12.3 ± 0.3
Acid insoluble ash	2.4 ± 0.1	3.1 ± 0.0	0.3 ± 0.0	0.6 ± 0.0
Nitrogen free extract*	30.5 ± 0.1	19.6 ± 1.1	81.1 ± 0.2	54.9 ± 0.8
Gross energy (MJ kg^{-1} diet)†	17.1 ± 0.1	17.4 ± 0.1	16.4 ± 0.0	11.8 ± 0.1

*Nitrogen free extract = 100 − (Crude protein + Crude fat + Crude fibre + Ash + Moisture).
†Gross energy (MJ kg^{-1} diet) was calculated assuming 23.7, 39.5, 17.2 and 17.2 MJ kg^{-1} of protein, lipids, fibre and nitrogen free extract respectively.

Table 3 Water quality parameters (mean ± SE) at varied protein levels and carbohydrate sources (2 × 3 factorial ANOVA)

	Protein (32%)			Protein (40%)			Interaction effects*		
Parameters	**Control (32C)†**	**Rice flour (32 + R)**	**Molasses (32 + M)**	**Control (40C)**	**Rice flour (40 + R)**	**Molasses (40 + M)**	**P**	**C**	**P × C**
DO (mg L^{-1})	7.5 ± 0.0^{ab} (7.2–7.8)	7.1 ± 0.1^{c} (6.0–7.8)	7.0 ± 0.1^{c} (5.7–7.6)	7.8 ± 0.1^{a} (7.4–8.4)	7.3 ± 0.1^{bc} (6.7–7.7)	7.0 ± 0.1^{c} (6.3–7.6)	*	**	NS
DO reduction per hour (mg L^{-1})	0.4 ± 0.1^{c} (0.2–0.5)	0.7 ± 0.1^{ab} (0.5–0.9)	1.0 ± 0.1^{a} (0.6–1.3)	0.3 ± 0.0^{c} (0.2–0.4)	0.6 ± 0.1^{bc} (0.4–0.7)	1.0 ± 0.1^{a} (0.7–1.3)	NS	**	NS
Total ammonia–N (mg L^{-1})	0.6 ± 0.1^{ab} (0.2–1.5)	0.3 ± 0.0^{b} (0.1–0.7)	0.3 ± 0.0^{b} (0.1–0.6)	0.7 ± 0.1^{a} (0.1–1.7)	0.3 ± 0.1^{b} (0.1–0.7)	0.4 ± 0.0^{ab} (0.1–0.9)	NS	**	NS
Nitrite–N (mg L^{-1})	0.6 ± 0.1 (0.1–0.8)	0.6 ± 0.1 (0.1–0.8)	0.6 ± 0.1 (0.1–0.9)	0.7 ± 0.1 (0.1–0.8)	0.5 ± 0.1 (0.1–0.8)	0.6 ± 0.1 (0.1–0.9)	NS	NS	NS
Nitrate–N (mg L^{-1})	2.9 ± 0.4 (0.2–4.4)	2.7 ± 0.4 (0.1–4.0)	2.6 ± 0.4 (0.2–4.0)	3.1 ± 0.4 (0.1–4.5)	2.6 ± 0.4 (0.2–4.0)	2.7 ± 0.3 (0.1–3.7)	NS	NS	NS
Phosphate–P (mg L^{-1})	0.5 ± 0.0^{ab} (0.2–0.8)	0.4 ± 0.0^{bc} (0.2–0.5)	0.4 ± 0.0^{bc} (0.1–0.7)	0.6 ± 0.1^{a} (0.2–1.0)	0.4 ± 0.0^{bc} (0.2–0.7)	0.4 ± 0.0^{c} (0.1–0.5)	NS	**	NS
TSS (mg L^{-1})	161.0 ± 19.4^{b} (28–298)	272.7 ± 36.9^{ab} (32–560)	358.2 ± 54.0^{a} (26–894)	160.8 ± 21.7^{b} (30–344)	281.0 ± 37.1^{ab} (22–536)	391.2 ± 53.0^{a} (32–758)	NS	**	NS
Biofloc volume (mL L^{-1})	2.8 ± 0.5^{b} (0–6)	5.7 ± 1.0^{ab} (0–13)	8.5 ± 1.6^{a} (0–21)	2.7 ± 0.4^{b} (0–5.2)	6.5 ± 1.2^{ab} (0–15)	8.7 ± 1.5^{a} (0–19.3)	NS	**	NS
Chlorophyll *a* (mg L^{-1})	0.3 ± 0.0^{ab} (0.1–0.4)	0.2 ± 0.0^{ab} (0.1–0.4)	0.2 ± 0.0^{b} (0.1–0.2)	0.3 ± 0.0^{a} (0.1–0.4)	0.3 ± 0.0^{a} (0.1–0.5)	0.1 ± 0.0^{b} (0.1–0.2)	NS	**	NS

*Means in the same row having different superscript differ significantly. *$P < 0.05$; **$P < 0.01$; NS, not significant. P, protein sources (32% and 40% protein feed); C, Carbon sources (rice flour, molasses and without carbohydrate supplemented control).

†32C, Control group fed 32% protein diet with no carbohydrate supplementation; 40C, Control group fed 40% protein diet with no carbohydrate supplementation; 32 + R and 40 + R, rice flour added groups; 32 + M and 40 + M, molasses added groups.

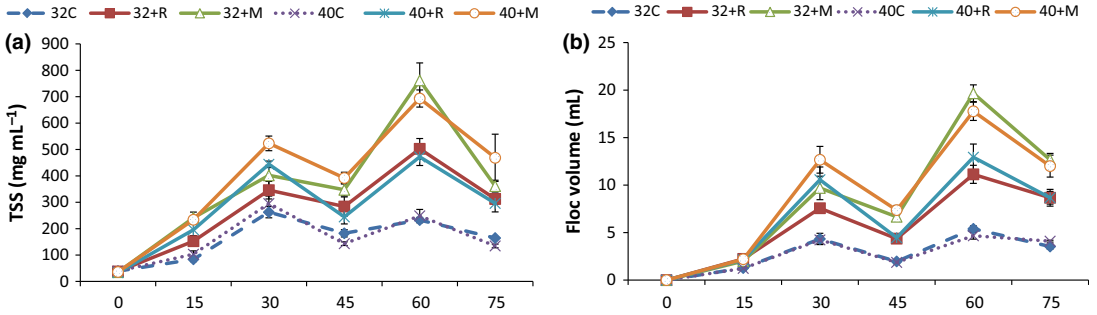

Figure 1 Total suspended solid (TSS) (a) and Floc volume (b) during experimental periods in biofloc system at different protein levels and carbon sources. Data represent mean ± SE of three replicates.

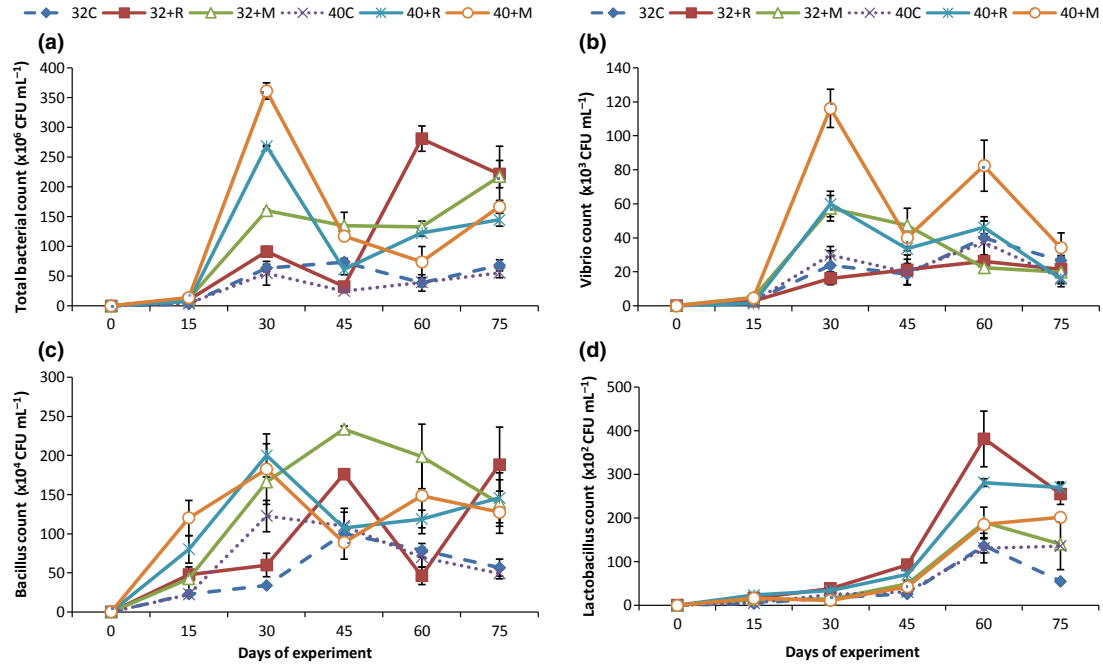

Figure 2 Microbial dynamics in water of biofloc groups at different protein levels and carbon sources. (a) Total microbial count ($\times 10^6$ CFU mL^{-1}), (b) *Vibrio* count ($\times 10^3$ CFU mL^{-1}), (c) *Bacillus* count ($\times 10^4$ CFU mL^{-1}), (d) *Lactobacillus* count ($\times 10^2$ CFU mL^{-1}).

value of TSS (894 mg L^{-1}) and FV (21 mL) was recorded in 32 + M group during the 8th week of experimental period.

Microbial load

The mean of total bacterial count (TBC), *Vibrio*, *Bacillus* and *Lactobacillus* counts are presented in Fig. 2 and Table 4. Overall, carbohydrate addition significantly increased ($P < 0.01$) the bacterial load compared to control. Among carbohydrate added groups, molasses addition at 40% protein level (40 + M) recorded the highest level of TBC and *Vibrio* counts. Comparatively higher level of *Lactobacillus* count was recorded in rice flour added groups (32 + R and 40 + R) compared to other treatments. Furthermore, the highest level of *Lactobacillus* count was observed in 32 + R group which was 241.6% higher compared to control 32C. The highest *Vibrio* population was observed on the 30th day while *Bacillus* and *Lactobacillus* counts peaked on the 45th and 60th days respectively. Among the measured bacterial population, *Bacillus* was the most dominant bacterial group

Table 4 Bacterial count (mean ± SE) in biofloc system at varied protein levels and carbohydrate sources (2 × 3 factorial ANOVA)

	Protein 32%			Protein 40%			Interaction effects†		
Parameters*	Control (32C)‡	Rice flour (32 + R)	Molasses (32 + M)	Control (40C)	Rice flour (40 + R)	Molasses (40 + M)	P	C	P×C
Total bacteria ($\times 10^6$)	51.3 ± 8.6^{ab}	136.0 ± 33.8^{ab}	127.7 ± 25.6^{ab}	37.8 ± 7.1^{b}	112.9 ± 26.6^{ab}	148.5 ± 36.1^{a}	NS	**	NS
Vibrio ($\times 10^3$)	22.8 ± 4.2^{b}	18.0 ± 2.9^{b}	27.8 ± 6.4^{ab}	20.9 ± 4.4^{b}	27.5 ± 6.5^{ab}	53.6 ± 12.4^{a}	NS	**	NS
Bacillus ($\times 10^4$)	58.2 ± 9.0^{b}	111.4 ± 23.9^{ab}	141.7 ± 23.6^{a}	72.5 ± 12.4^{ab}	120.8 ± 17.6^{ab}	133.0 ± 15.0^{a}	NS	**	NS
Lactobacillus ($\times 10^2$)	48.5 ± 14.5^{a}	165.7 ± 44.3^{a}	79.4 ± 25.2^{a}	72.1 ± 18.8^{a}	135.8 ± 36.0^{a}	101.4 ± 27.4^{a}	NS	**	NS

*All the bacterial counts have been presented as CFU mL^{-1}.

†Means in the same row having different superscript differ significantly. **$P < 0.01$; NS, not significant. P, protein sources (32% and 40% protein feed); C, carbon sources (rice flour, molasses and without carbohydrate supplemented control).

‡32C, Control group fed 32% protein diet with no carbohydrate supplementation; 40C, Control group fed 40% protein diet with no carbohydrate supplementation; 32 + R and 40 + R, rice flour added groups; 32 + M and 40 + M, molasses added groups.

Table 5 Growth performance parameters (mean ± SE) in biofloc system at varied protein levels and carbon sources (2 × 3 factorial ANOVA)

	Protein 32%			Protein 40%			Interaction effects*		
Parameters	Control (32C)†	Rice flour (32 + R)	Molasses (32 + M)	Control (40C)	Rice flour (40 + R)	Molasses (40 + M)	P	C	P × C
Final weight (g)‡	6.1 ± 0.3^{bc}	7.5 ± 0.4^{ab}	5.7 ± 0.4^{c}	6.4 ± 0.3^{bc}	8.5 ± 0.3^{a}	7.8 ± 0.3^{a}	NS	**	NS
Feed conversion ratio	3.5 ± 0.3^{a}	2.2 ± 0.2^{b}	3.6 ± 0.2^{a}	3.1 ± 0.1^{a}	1.8 ± 0.1^{b}	2.3 ± 0.1^{b}	**	**	*
Protein efficiency ratio	0.9 ± 0.1^{c}	1.5 ± 0.1^{a}	0.9 ± 0.1^{c}	0.8 ± 0.0^{c}	1.4 ± 0.1^{ab}	1.1 ± 0.0^{bc}	NS	**	NS
Specific growth rate	0.8 ± 0.0^{c}	1.1 ± 0.1^{ab}	0.7 ± 0.1^{c}	0.9 ± 0.1^{bc}	1.3 ± 0.1^{a}	1.2 ± 0.1^{ab}	**	**	*
Survival %	63.3 ± 3.3^{b}	83.3 ± 3.3^{a}	66.7 ± 3.3^{b}	66.7 ± 3.3^{b}	86.7 ± 3.3^{a}	73.3 ± 3.3^{ab}	NS	**	NS

*Means in the same row having different superscript differ significantly. *$P < 0.05$; **$P < 0.01$; NS, not significant P, protein sources (32% and 40%); C, carbon sources (rice flour, molasses and without carbohydrate supplemented control).

†32C, Control group fed 32% protein diet with no carbohydrate supplementation; 40C, Control group fed 40% protein diet with no carbohydrate supplementation; 32 + R and 40 + R, rice flour added groups; 32 + M and 40 + M, molasses added groups.

‡Initial weight of the animal was 3.37 ± 0.04 g.

(58.2–141.7 × 10^4 CFU mL^{-1}) throughout the experimental periods.

Growth performance

Growth performance parameters among different treatments are presented in Table 5. The significantly higher ($P < 0.01$) final body weights, 8.5 ± 0.3, 7.8 ± 0.3 and 7.5 ± 0.4 g were noticed in 40 + R, 40 + M and 32 + R, respectively, compared to control groups, 32C (6.1 ± 0.3 g) and 40C (6.4 ± 0.3 g). Moreover, 32 + R did not differ significantly ($P > 0.05$) with 40 + R and 40 + M. However, molasses addition at 32% protein level (32 + M) did not significantly improve growth (5.6 ± 0.4 g) compared to control. Protein levels and carbohydrate supplementation had significant interaction ($P < 0.05$) on the specific growth rate (SGR) and feed conversion ratio (FCR). Overall, growth rate and FCR were significantly better ($P < 0.01$) at 40% compared to 32% protein levels. On the contrary, 32 + R had better growth rate, FCR, protein efficiency ratio (PER) and survival compared to control fed with 40% protein diet. Among the carbohydrate added treatments, rice flour added groups, 32 + R and

40 + R recorded the higher SGR, PER and lower FCR compared to other groups. Similarly, better survival was recorded ($P < 0.05$) in rice flour added groups, 40 + R (86.7) and 32 + R (83.3%), compared to other treatments.

Total and differential haemocyte count

Total haemocyte, granulocyte and hyaline cell counts were higher in carbohydrate added groups compared to controls, 32C and 40C (Fig. 3). Rice flour supplementation significantly increased ($P < 0.01$) the total haemocyte count ($\times 10^6$ cells mL^{-1}) in 32 + R (45.7 ± 3.7) and 40 + R (44.3 ± 3.1) compared to controls, 32C (27.3 ± 3.4) and 40C (25.8 ± 0.9). Similarly, higher granulocyte and hyaline cell counts were observed in 32 + R and 40 + R compared to molasses added groups and controls (32C and 40C). Although, molasses supplementation improved the haemocyte count in the treatments, 32 + M and 40 + M, it did not differ significantly with the controls (32C and 40C).

Immunological and biochemical parameters

Immunological parameters like proPO activity and antioxidant enzymes such as SOD and catalase are presented in Fig. 4. The protein level in the diet

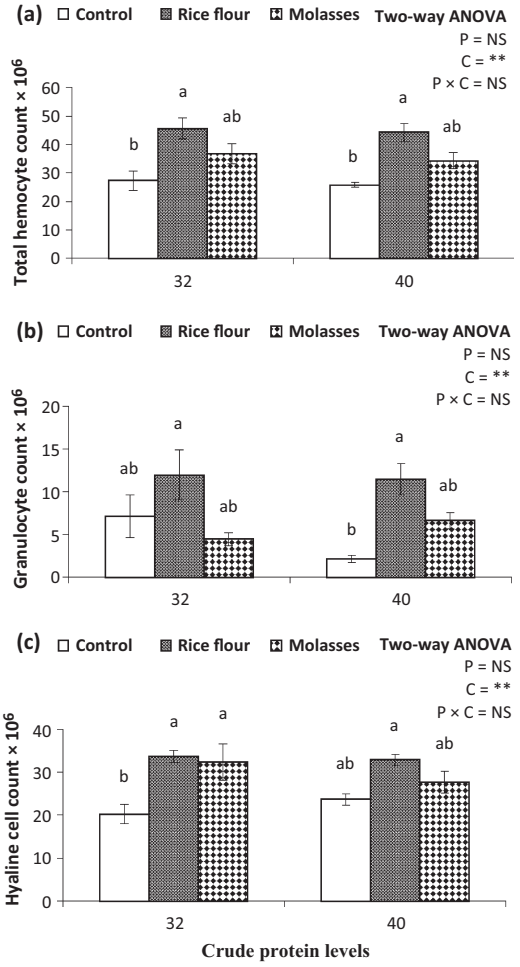

Figure 3 Total haemocyte count (a), Granulocyte count (b) and Hyaline cell count (c) in *P. monodon* juveniles reared in biofloc system at different protein levels and carbon sources. Data represent mean ± SE of three replicates. Column with different superscript differ significantly ($P < 0.05$) as determined by Tukey's test. P, protein; C, carbon source; NS, not significant.

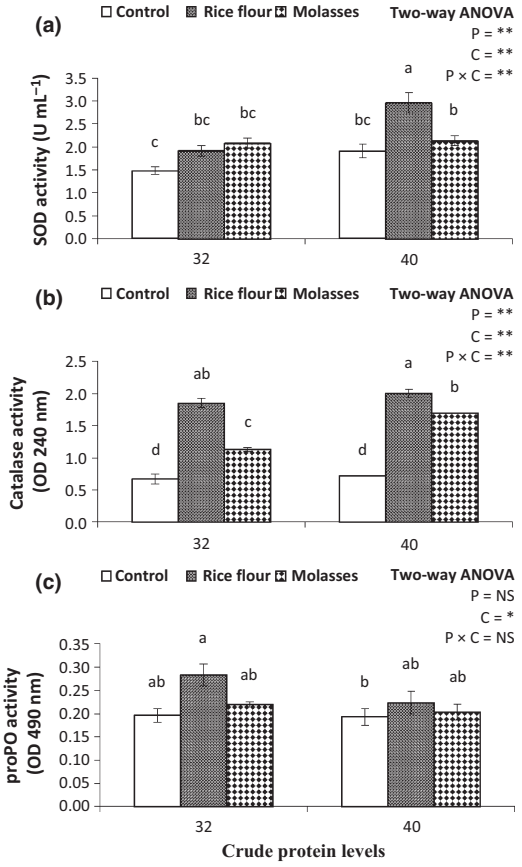

Figure 4 Superoxide dismutase (a), Catalase (b) and Prophenoloxidase activity (c) in serum of *P. monodon* juveniles reared in biofloc system at different protein levels and carbon sources. Data represent mean ± SE of three replicates. Column with different superscript differ significantly as determined by Tukey's test. P, protein; C, carbon source; NS, not significant.

had significant effect ($P < 0.01$) on SOD and catalase activity with higher level recorded at 40% compared to 32% protein diet. Among the treatments, the highest serum proPO activity (OD 490 nm) was observed in 32 + R (0.3 ± 0.0). The catalase activity was significantly higher ($P < 0.01$) in shrimps reared under rice flour supplementation (32 + R and 40 + R) compared to control groups, 32C and 40C. Similarly, the highest SOD activity was recorded in rice flour supplemented group, 40 + R (3.0 ± 0.2 U mL^{-1}). Although immunological parameters like proPO activity and SOD were comparatively higher in molasses added groups, 32 + M and 40 + M, they did not differ significantly with their respective controls 32C and 40C.

Serum biochemical parameters like protein and glucose levels are presented in Table 6. The protein level in the feed significantly influenced ($P < 0.01$) the serum protein and glucose levels with higher values recorded at 40% compared to 32% protein diet. The serum protein levels (mg mL^{-1}) were significantly higher ($P < 0.01$) in 40 + R (18.04 ± 0.46), 32 + R (13.97 ± 0.25) and 40 + M (13.56 ± 0.37) groups compared to other treatments. The similar trend was observed for serum glucose with the highest level recorded in rice flour added group followed by molasses and control.

Survival post challenge

The survival among different treatment groups after challenge with *V. harveyi* is presented in Fig. 5. A significantly higher ($P < 0.05$) survival was recorded in 32 + R (55.5) and 40 + R (44.4%) compared to controls 32C (11.1%) and 40C (0) while molasses supplemented group had survival between 11.1% and 22.2%.

Discussion

In this study, experimental diets contained 32% and 40% protein equivalent to C:N ratio 10:1 and 7.5:1 (Avnimelech 1999; Hari *et al.* 2004). Manipulation of C:N ratio by reducing the protein level in the feed (Azim *et al.* 2008) or addition of external carbohydrate helps in biofloc production (Burford, Thompson, McIntosh, Bauman & Pearson 2004; Hari *et al.* 2004). In this study molasses and rice flour were used as carbon sources at the C:N ratio 10:1 to augment microbial growth.

The recorded water quality parameters across the treatments were within the acceptable ranges for brackishwater shrimp culture (Chen 1985). In this study, carbohydrate addition significantly reduced the TAN level in the water column. This is in line with the earlier findings, where the addition of carbohydrate reported to reduce the TAN concentration in tilapia (Azim & Little 2008), *L. vannamei* (Wasielesky, Atwood, Stokes & Browdy 2006) and *P. monodon* culture (Hari *et al.* 2004; Kumar *et al.* 2014). It has been reported that bacteria utilizes TAN and added carbon for the production of microbial floc within the culture system (Avnimelech 1999; Azim & Little 2008). The reduced TAN levels, overall, helped to maintain better water quality in carbohydrate added groups.

Floc volume and TSS are the true indicators of biofloc formation (Avnimelech 2012). In the present experiment, carbohydrate added groups recorded significantly higher FV and TSS compared

Table 6 Serum biochemical parameters (mean ± SE) in biofloc system at varied protein levels and carbon sources (2 × 3 factorial ANOVA)

	Protein 32%			Protein 40%			Interaction effects*		
Parameters	Control (32C)†	Rice flour (32 + R)	Molasses (32 + M)	Control (40C)	Rice flour (40 + R)	Molasses (40 + M)	P	C	P × C
Protein (mg L^{-1})	5.98 ± 0.27^{d}	13.97 ± 0.25^{b}	10.62 ± 0.54^{c}	10.43 ± 0.30^{c}	18.04 ± 0.46^{a}	13.56 ± 0.37^{b}	**	**	NS
Glucose (mg L^{-1})	1.79 ± 0.02^{c}	2.06 ± 0.02^{b}	1.87 ± 0.02^{c}	1.90 ± 0.02^{c}	2.48 ± 0.03^{a}	2.14 ± 0.04^{b}	**	**	**

*Means in the same row having different superscript differ significantly. **$P < 0.01$; NS, not significant. P, protein sources (32% and 40% protein feed); C, carbon sources (rice flour, molasses and without carbohydrate supplemented control).

†32C, Control group fed 32% protein diet with no carbohydrate supplementation; 40C, Control group fed 40% protein diet with no carbohydrate supplementation; 32 + R and 40 + R, rice flour added groups; 32 + M and 40 + M, molasses added groups.

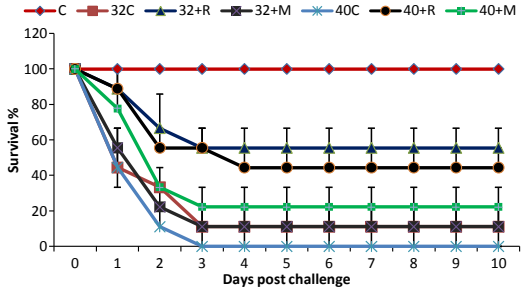

Figure 5 Survival of *P. monodon* over 10–day period after challenge with *Vibrio harveyi*. The different experimental groups were reared under biofloc system at different protein levels and carbon sources. Values represent mean ± SE. The C represents blank control injected with 20 µL normal saline solution. The 32C and 40C represents control groups fed with 32% and 40% protein diet with no carbohydrate supplementation. The 32 + R and 40 + R represents rice flour added groups while 32 + M and 40 + M was supplemented with molasses.

to control. Furthermore, the FV and TSS levels were higher in molasses added groups (32 + M and 40 + M) compared to rice flour (32 + R and 40 + R). Widely used carbon sources in aquaculture are wheat flour, maize, tapioca powder, molasses etc. (Hari *et al.* 2004; De Schryver *et al.* 2008). Microbes are capable to utilize the diverse range of carbon sources originating from the agricultural products (Thomsen 2005). However, type of carbon sources seems to affect the biofloc production rate as molasses containing sucrose, a disaccharide, was more effective compared to rice flour having starch, a polysaccharide (Zhou, Robards, Helliwell & Blanchard 2002). This indicates that nature of carbohydrate affects the quantum of biofloc production with higher level of production by application of simple sugar.

Aquatic animals need sufficient DO level for their growth and protection against diseases. The optimum DO level for shrimp is 5 mg L^{-1}, and the concentration below 3.7 mg L^{-1} is considered critical (Chen 1985). In spite of providing the round the clock aeration, supplementation of carbohydrate reduced the DO levels with the highest rate of DO reduction h^{-1} recorded in molasses added groups. A biofloc system demands oxygen for microbial respiration apart from the metabolic need of shrimp (De Schryver *et al.* 2008). The higher microbial load in the molasses added group compared to rice flour and control might have resulted in lower DO level and faster rate of DO reduction h^{-1}. Moreover, molasses added groups recorded lower chlorophyll *a* level compared to other treatments. This indicates the dominance of heterotrophs over autotrophs and may have influenced the DO level.

Application of carbohydrate improves growth rate in *P. monodon* (Hari *et al.* 2004; Anand, Kumar, Panigrahi, Ghoshal, Dayal, Biswas, Sundaray, De, Raja, Deo, Pillai & Ravichandran 2013) and *L. vannamei* (Xu & Pan 2012). Enhanced growth performance of shrimps reared in treatments like 32 + R, 40 + R and 40 + M are in consonance with the previous studies. Biofloc forms a quality natural food for the cultured shrimp (Burford *et al.* 2004) apart from being a source of bioactive compounds and growth promoters (Ju *et al.* 2008). Moreover, digestive enzyme secretions from many probiotic bacteria like *Bacillus*, *Lactobacillus* in the carbohydrate added groups might have improved the shrimp growth performance (Ringo, Jose, Vecino, Wadsworth & Song 2012; Anand *et al.* 2014). Carbohydrate added groups also recorded increased serum protein and glucose level. This indicates that microbial communities present in biofloc enhances the digestive and assimilative ability of shrimp resulted in better growth and survival in biofloc groups (Anand *et al.* 2014).

Protein level in the feed is the single most cost influencing factor in the shrimp. In the present experiment, treatment group with 32% protein and rice flour addition (32 + R) recorded better growth and survival compared to both the control groups fed with 32% and 40% protein diet. This supports the earlier findings that feed with lower protein level with biofloc could replace the higher protein diet (Hari *et al.* 2004; Ballester *et al.* 2010). Though 40 + R and 40 + M groups recorded comparatively higher growth rate in comparison to 32 + R but the difference was not significant. This suggests 32 + R could be a better option in comparison to biofloc system at 40% protein level. On the contrary, the treatment 32 + M did not show significant growth improvement compared to control groups. Earlier, Samocha, Patnaik, Speed, Ali, Burger, Almeida, Ayub, Harisanto, Horowitz and Brock (2007) reported that molasses addition at 30% protein level did not influence growth over control. Moreover, the group 32 + M recorded alarming level of biofloc with FV and TSS as high as 21 mL L^{-1} and 894 mg L^{-1} respectively. It has been reported that

higher level of suspended particles reduces visibility, which lead to lower food intake and poor growth performance (Azim & Little 2008; Ray, Lewis, Browdy & Leffler 2010). In the present experiment, decision for water exchange was taken based on the FV measurement at weekly intervals. This allowed reaching the FV at alarming level of 21 mL^{-1} in molasses added groups. On the basis of the present experimental data, we suggest to measure FV every day with periodical removal of unutilized floc particles. Earlier, combination of wheat bran and molasses has been reported to give a better growth in pink shrimp *Farfantepenaeus paulensis* (Ballester *et al.* 2010) and *F. brasiliensis* (Emerenciano, Ballester, Cavalli & Wasielesky 2012). The present results suggest that rice flour had steady rate compared to rapid biofloc production by molasses. Therefore, the combination of rice flour or wheat flour with molasses should be tested to further optimize the biofloc system.

Shrimps like other crustaceans lack adaptive immune system and rely entirely on the non-specific immune mechanism (Bachère 2000). The circulating haemocytes and proPO activity plays key role in defence mechanism of crustaceans (Rodrıguez & Le Moullac 2000). On the other hand, antioxidant enzymes like SOD and catalase protect the cells from oxidative stress during host pathogen interaction (Holmblad & Söderhäll 1999). In this study, carbohydrate supplemented groups such as 32 + R, 40 + R and 40 + M elicited a better immune response in terms of haemocyte, granulocyte count, and proPO activity and antioxidant enzymes such as SOD and catalase compared with controls (32C and 40C). Increased level of haemocyte and antioxidant status had been noticed after dietary supplementation of probiotics (Li, Tan & Mai 2009) and immunostimulant like β-glucan in shrimp (Lopez, Cuzon, Gaxiola, Taboada, Valenzuela, Pascual, Sanchez & Rosas 2003). Recently, Xu and Pan (2013) reported increased haemocyte count and antioxidant status in *L. vannamei* in biofloc based system. Ju *et al.* (2008) reported that bioflocs contains many bioactive compounds such as carotenoids, chlorophylls, phytosterols, bromophenols and amino sugars which could exert immuno-stimulatory effect on shrimp. It is also possible that presence of beneficial bacteria such as *Bacillus* spp. and *Lactobacillus* in the ingested biofloc might have improved their colonization in the gastrointestinal tract leading to better digestive enzyme activity and immune mechanism (Xu & Pan 2013). This necessitates the further characterization of beneficial microbial communities in biofloc and to investigate their role in shrimp immune mechanism.

In the present experiment, immunological parameters like haemocyte count and proPO activity did not differ significantly between the groups fed with 32 and 40% protein diet. Moreover, the group 32 + R recorded the highest level of proPO activity and survival after challenge with *V. harveyi*. Recently, Xu & Pan (2014) observed no significant difference in *L. vannamei* immunological parameters among the diet with 20–35% protein level with biofloc. This reflects the tremendous potential of biofloc in replacing the dietary protein level along with improving the shrimp growth and immune responses. The experiment revealed that nature of carbohydrate source applied for biofloc production affects the immune responses. For example, treatment with rice flour addition like 32 + R and 40 + R exhibited better immune response compared to molasses added groups (32 + M and 40 + M). It seems that higher level of suspended particles and lower DO in molasses added groups led to lower immune response compared to rice flour based biofloc system (Le Moullac & Haffner 2000). This further suggests that molasses as carbon source needs cautious application with routine monitoring on the level of biofloc production and shrimp health.

Conclusion

Present study confirms the importance of biofloc in controlling toxic ammonia nitrogen, and enhancing the growth and immune response in black tiger shrimp, *P. monodon*. Both, molasses and rice flour addition significantly reduced the total ammonia–N compared to controls. The nature of carbon sources influenced the amount of biofloc generation as molasses was more effective compared to rice flour. However, better growth and immune responses were observed in rice flour added groups compared to molasses. Moreover, rice flour added group at 32% protein had better growth and immune response compared to control at 40% protein diet. Further research is required to understand the responses of probiotic bacteria like *Bacillus* and *Lactobacillus* in biofloc system. Optimum utilization of suspended particles and their efficient control in biofloc based system is also a matter of further research.

Acknowledgments

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 of Kakdwip Research Centre of CIBA are also duly acknowledged.

References

Anand P.S.S., Kumar S., Panigrahi A., Ghoshal T.K., Dayal J.S., Biswas G., Sundaray J.K., De D., Raja R.A., Deo A.D., Pillai S.M. & Ravichandran P. (2013) Effects of C: N ratio and substrate integration on periphyton biomass, microbial dynamics and growth of *Penaeus monodon* juveniles. *Aquaculture International* **21**, 511–524.

Anand P.S.S., Kohli M.P.S., Kumar S., Sundaray J.K., Dam Roy S., Venkateshwarlu G., Sinha A. & Pailan G.H. (2014) Effect of dietary supplementation of biofloc on growth performance and digestive enzyme activities in *Penaeus monodon*. *Aquaculture* **418**, 108–115.

Ananda Raja R., Kumar S., Sundaray J.K., De D., Biswas G. & Ghoshal T.K. (2012) Hematological parameters in relation to sex, morphometric characters and incidence of white spot syndrome virus in tiger shrimp *Penaeus monodon Fabricius* (1798) from Sunderban, West Bengal. *Indian Journal of Fisheries* **59**, 169–174.

AOAC (1995) *Official Methods of Analysis* (ed. by K. Helrich), pp. 1094. Association of Official Analytical Chemists, Virginia, Washington DC, USA.

Aouidi F., Gannoun H., Ben Othman N., Ayed L. & Hamdi M. (2009) Improvement of fermentative decolorization of olive mill wastewater by *Lactobacillus paracasei* by cheese whey's addition Process. *Biochemistry* **44**, 597–601.

APHA (1998) *Standard Methods for the Examination of Water and Wastewater* (ed. by L.S. Clesceri, A.E. Greenberg & A.D. Eaton). American Public Health Association, American Water Works Association and Water Environment Federation, United Book Press, Washington DC, USA.

Avnimelech Y. (1999) Carbon/nitrogen ratio as a control element in aquaculture systems. *Aquaculture* **176**, 227–235.

Avnimelech Y. (2012) *Biofloc Technology–A Practical Guide Book* (2nd edn), pp. 272. The World Aquaculture Society, Baton Rouge, LA, USA.

Azim M.E. & Little D.C. (2008) The biofloc technology (BFT) in indoor tanks: water quality, biofloc composition, and growth and welfare of Nile tilapia (*Oreochromis niloticus*). *Aquaculture* **283**, 29–35.

Azim M.E., Little D.C. & Bron J.E. (2008) Microbial protein production in activated suspension tanks manipulating C: N ratio in feed and the implications for fish culture. *Bioresource Technology* **99**, 3590–3599.

Bachère E. (2000) Shrimp immunity and disease control. *Aquaculture* **191**, 3–11.

Ballester E.L.C., Abreu P.C., Cavalli R.O., Emerenciano M., de Abreu L. & Wasielesky J.W. (2010) Effect of practical diets with different protein levels on the performance of *Farfantepenaeus paulensis* juveniles nursed in a zero exchange suspended microbial flocs intensive system. *Aquaculture Nutrition* **16**, 163–172.

Beauchamp C. & Fridovich I. (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44**, 276–287.

Burford M.A., Thompson P.J., McIntosh R.P., Bauman R.H. & Pearson D.C. (2004) The contribution of flocculated material to shrimp (*Litopenaeus vannamei*) nutrition in a high-intensity, zero-exchange system. *Aquaculture* **232**, 525–537.

Chen H.C. (1985) Water quality criteria for farming the grass shrimp, *Penaeus monodon*. First international conference on the culture of penaeid prawns/shrimps. Aquaculture Department. SEAFDEC, pp. 165.

Crab R., Lambert A., Defoirdt T., Bossier P. & Verstraete W. (2010) The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi*. *Journal of Applied Microbiology* **109**, 1643–1649.

Crab R., Chielens B., Wille M., Bossier P. & Verstraete W. (2010) The effect of different carbon sources on the nutritional value of bioflocs, a feed for *Macrobrachium rosenbergii* postlarvae. *Aquaculture Research* **41**, 559–567.

Crab R., Defoirdt T., Bossier P. & Verstraete W. (2012) Biofloc technology in aquaculture: beneficial effects and future challenges. *Aquaculture* **356**, 351–356.

Dall W., Hill B.J., Rothlisberg P.C. & Sharples D.J. (1990) The biology of the penaeidae. In: *Advances in Marine Biology*, **Vol. 27** (ed. by J.H.S. Blaxter & A.J. Southward), pp. 213–240. Academic Press, San Diego, CA, USA.

De Schryver P., Crab R., Defoirdt T., Boon N. & Verstraete W. (2008) The basics of bio–flocs technology: the added value for aquaculture. *Aquaculture* **277**, 125–137.

Emerenciano M., Ballester E.L., Cavalli R.O. & Wasielesky W. (2012) Biofloc technology application as a food source in a limited water exchange nursery system for pink shrimp *Farfantepenaeus brasiliensis* (Latreille 1817). *Aquaculture Research* **43**, 447–457.

FAO (2012) *The State of World Fisheries and Aquaculture*. FAO, Rome, Italy.

Funge-Smith S.J. & Briggs M.R. (1998) Nutrient budgets in intensive shrimp ponds: implications for sustainability. *Aquaculture* **164**, 117–133.

Hari B., Kurup B.M., Varghese J.T., Schrama J.W. & Verdegem M.C.J. (2004) Effects of carbohydrate addition on production in extensive shrimp culture systems. *Aquaculture* **241**, 179–194.

Harris L., Owens L. & Smith S. (1996) A selective and differential medium for *Vibrio harveyi*. *Applied and Environmental Microbiology* **62**, 3548–3550.

Hart J.P., Lovis W.A., Schulenberg J.K. & Urquhart G.R. (2007) Paleodietary implications from stable carbon isotope analysis of experimental cooking residues. *Journal of Archaeological Science* **34**, 804–813.

Hein L. (2002) Toward improved environmental and social management of Indian shrimp farming. *Environmental Management* **29**, 349–359.

Hernández-López J., Gollas-Galván T. & Vargas-Albores F. (1996) Activation of the prophenoloxidase system of the brown shrimp *Penaeus californiensis* (Holmes). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* **113**, 61–66.

Holmblad T. & Söderhäll K. (1999) Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. *Aquaculture* **172**, 111–123.

Ju Z.Y., Forster I.P., Conquest L., Dominy W., Kuo W.C. & David Horgen F. (2008) Determination of microbial community structures of shrimp floc cultures by biomarkers and analysis of floc amino acid profiles. *Aquaculture Research* **39**, 118–133.

Karunasagar I. & Otta S.K. (1998) Disease problems affecting cultured penaeid shrimp in India. *Fish Pathology* **33**, 413–419.

Kautsky N., Rönnbäck P., Tedengren M. & Troell M. (2000) Ecosystem perspectives on management of disease in shrimp pond farming. *Aquaculture* **191**, 145–161.

Krishnan N., Chattopadhyay S., Kundu J.K. & Chaudhuri A. (2002) Superoxide dismutase activity in haemocytes and haemolymph of *Bombyx mori* following bacterial infection. *Current Science* **83**, 321–325.

Kumar S., Anand P.S.S., De D., Sundaray J.K., Raja R.A., Biswas G., Ponniah A.G., Ghoshal T.K., Deo A.D., Panigrahi A. & Muralidhar M. (2014) Effects of carbohydrate supplementation on water quality, microbial dynamics and growth performance of giant tiger prawn (*Penaeus monodon*). *Aquaculture International* **22**, 901–912.

Le Moullac G. & Haffner P. (2000) Environmental factors affecting immune responses in Crustacea. *Aquaculture* **191**, 121–131.

Li J., Tan B. & Mai K. (2009) Dietary probiotic *Bacillus* OJ and oligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). *Aquaculture* **291**, 35–40.

Lightner D.V., Redman R.M., Pantoja C.R., Noble B.I. & Tran L. (2012) Early mortality syndrome affects shrimp in Asia. *Global Aquaculture Advocate Magazine* **40**, (Jan/Feb).

Lopez N., Cuzon G., Gaxiola G., Taboada G., Valenzuela M., Pascual C., Sanchez A. & Rosas C. (2003) Physiological, nutritional and immunological role of dietary [beta] 1-3 glucan and ascorbic acid 2-monophosphate in *Litopenaeus vannamei* juveniles. *Aquaculture* **224**, 223–243.

Lowry O.H., Rosebrough N.J., Farr A.L. & Randall R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

Miller G.L. (1959) Modified DNS method for reducing sugars. *Analytical Chemistry* **31**, 426–428.

Ray A.J., Lewis B.L., Browdy C.L. & Leffler J.W. (2010) Suspended solids removal to improve shrimp (*Litopenaeus vannamei*) production and an evaluation of a plant-based feed in minimal-exchange, super intensive culture systems. *Aquaculture* **299**, 89–98.

Ringo E., Jose R.E.O., Vecino L.G., Wadsworth S. & Song S. (2012) Use of immunostimulants and nucleotides in aquaculture: a review. *Journal of Marine Science Research Development* **2**, 1–22.

Rodrıguez J. & Le Moullac G. (2000) State of the art of immunological tools and health control of penaeid shrimp. *Aquaculture* **191**, 109–119.

Samocha T.M., Patnaik S., Speed M., Ali A.M., Burger J.M., Almeida R.V., Ayub Z., Harisanto M., Horowitz A. & Brock D.L. (2007) Use of molasses as carbon source in limited discharge nursery and grow–out systems for *Litopenaeus vannamei*. *Aquaculture Engineering* **36**, 184–191.

Takahara S., Hamilton H.B., Neel J.V., Kobara T.Y., Ogura Y. & Nishimura E.T. (1960) Hypocatalasemia: a new genetic carrier state. *Journal of Clinical Investigation* **39**, 610–619.

Thomsen M.H. (2005) Complex media from processing of agricultural crops for microbial fermentation. *Applied Microbiology and Biotechnology* **68**, 598–606.

Wasielesky W. Jr, Atwood H., Stokes A. & Browdy C.L. (2006) Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei*. *Aquaculture* **258**, 396–403.

Xu W.J. & Pan L.Q. (2012) Effects of bioflocs on growth performance, digestive enzyme activity and body composition of juvenile *Litopenaeus vannamei* in zero-water exchange tanks manipulating C/N ratio in feed. *Aquaculture* **357**, 147–152.

Xu W.J. & Pan L.Q. (2013) Enhancement of immune response and antioxidant status of *Litopenaeus vannamei* juvenile in biofloc-based culture tanks manipulating high C/N ratio of feed input. *Aquaculture* **412**, 117–124.

Xu W.J. & Pan L.Q. (2014) Evaluation of dietary protein level on selected parameters of immune and antioxidant systems, and growth performance of juvenile Litopenaeus vannamei reared in zero-water exchange biofloc-based culture tanks. *Aquaculture* **426**, 181–188.

Zhou Z., Robards K., Helliwell S. & Blanchard C. (2002) Composition and functional properties of rice. *International Journal of Food Science & Technology* **37**, 849–868.