Pakistan J. Zool., pp 1-11, 2025. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20241005150734

Evaluation of Biofloc Meal Supplementation on Growth and Physiological Responses of Pacific White Leg Shrimp, *Penaeus vannamei* in Outdoor Culture System

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Article Information
Received 05 October 2024
Revised 15 November 2024
Accepted 24 November 2024
Available online 09 January 2025 (early access)

Authors' Contribution
MN conducted the research, drafted and edited the manuscript. BA, AKP, NF, AU and PR revised the manuscript and helped the first author in conducting the study.

Key words
Biofloc, Shrimp feed, Digestive enzymes, Growth parameter, *P. vannamei*, HDPE

ABSTRACT

The evaluation of alternative protein source in shrimp feed is a priority in the aquaculture industry. Biofloc meal is a substantial protein source of shrimp feed, which has been examined by various studies under experimental conditions. The present study examined the efficacy of biofloc meal incorporated diet on growth performance, digestive enzyme activity and immune response of pacific white-leg shrimp (*Penaeus vannamei)* in 100 m^2 HDPE liner ponds. Healthy post larvae of *P. vannamei* (0.02 ± 0.00 g) were stocked and fed with control diet (without biofloc meal) and treatment (30% incorporated biofloc meal) at 10% of animal body weight, four times a day, for duration of 45 days in duplicate ponds. Among the treatments significantly increased final weight (4.98 ± 0.06 g), weight gain (4.95 ± 0.09g), specific growth rate (11.53 ±0.17 %/day) and survival rate (77 ± 0.29 %) were observed in treatment group (P<0.05). Significantly higher (P<0.05) digestive enzyme activities of protease (1.9 ± 0.22 U/mg protein), lipase (297 ± 47 U/mg protein), amylase (8.22 ±1.21 U/mg protein) and cellulase (24.8 ± 2.21 U/mg protein) were found in the experimental diet-fed group. At the end of the experiment, serum protein, prophenoloxidase and antioxidants were found to be significantly higher in experimental diet fed group. Findings of the present study revealed that the biofloc meal incorporation at 30% in shrimp diet would potentially improves the growth performance, survival, immune response and digestive activities of shrimp.

INTRODUCTION

According to the Food and Agriculture Organization, aquaculture is one of the important, food producing sector, which offers immense opportunities to alleviate starvation, malnutrition, and reduce poverty through income generation by using the source of natural resources. With the increasing human population, the demand for is escalating multiple natural resources such as water and land required for the continuous production of food. Where, aquaculture production is projected to rise from 40 million tons to 82 million tons by 2050 (FAO, 2010). However, some nutritional features, such as low palatability and digestibility, deficiency of some essential amino acids and bioactive molecules, and the existence of anti-nutritional factors may limit the use of these substitutes (Wang *et al*., 2016).

Biofloc technology has attained a great attention as a sustainable, cost effective and environmentally friendly method way that improvises the water quality and microbial protein load for aquatic species (Avnimelech and Kochba, 2009; Ekasari and Maryam, 2012; Sgnaulin *et al*., 2018; Dinda *et al*., 2020; Gao *et al*., 2019). The principle of floculation in biofloc technology was carried out by the heterotrophic bacteria and algae within the system (Avnimelech, 2006; Ahmad *et al*., 2017). In shrimp farming sectors, the production of beneficial based microbiota in biofloc culture is getting popular as it appears to enhance the water quality, disease resistance and immune response (Zhou *et al*., 2009; Crab *et al*., 2012; Martínez-Córdova *et al*., 2015).

Biofloc can be used as an alternative protein source to replace fish meal (FM), which is an expensive protein source in the feed industry. Usage of biofloc meal could lead to the reduction in the cost of feed formulation (Panigrahi *et al*., 2017, 2018). The incorporation of biofloc meal in

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0030-9923/2025/0001-0001 $ 9.00/0

aquaculture reduces the production whilst permitting the intensive culture species and it also maximizes the profits without any negative impact to the culture species and to the environment (Bauer *et al*., 2012). In addition, biofloc also serves as a high nutrient food source rich in amino acids, proteins, fatty acids and lipids in the form of different microorganisms, and it also reduces external feed supply to make it more economical. The microbial diversity of flocs provides nutritional supplementation (Panigrahi *et al*., 2017, 2018). Studies also suggest that dried biofloc can be used to replace fishmeal in shrimp feed (Hargreaves, 2006), since it contains various bioactive compounds, which includes carotenoids, chlorophyll, vitamin C and trace minerals (Ju *et al*., 2008; Crab *et al*., 2012; Vazquez *et al*., 2009).

Biofloc studies showed that one of the preeminent strategies, for managing aquatic diseases through microbial interventions (Panigrahi and Azad, 2007). Biofloc has an internal antagonist activity against bacterial and viral pathogens, that can induce immunity, disease resistance and growth performance in animals (Crab *et al*., 2012; Ekasari *et al*., 2014). When the disease-causing pathogen is close to the perimeter, the biofloc system activates the immune system as a defence mechanism. Cellular and antioxidant immune response can be enhanced in the shrimp raised on the biofloc culture, because it is rich in natural microorganisms and bioactive compounds (Cardona *et al*., 2016; Ju *et al*., 2008; Xu and Pan, 2013; Panigrahi *et al*., 2018). In this study biofloc powder was harvested from the raceway ponds and their nutritional values were assessed. Research findings of this study demonstrate the growth performance, survival, immunity response and digestive enzyme activity of *P.vannamei* reared in pond system fed with biofloc meal were monitored and assessed.

MATERIALS AND METHODS

Biofloc meal production

Carbon: Nitrogen (C: N) ratio of 15:1 was maintained by supplying liquid spentwash as carbon source, in the outdoor raceway system (14 m length, 2.7 m width and 0.6 m depth) to produce biofloc and a 50 µm nylon mesh was used for the harvesting, to prepare biofloc meal (Avnimelech, 2007). The collected floc was dried at 40°C for 48 h, crushed into powder, and later, proximate composition parameters such as crude protein, crude lipid, ash, moisture, crude fibre and nitrogen free extract (NFE) of the floc were analyzed (AOAC, 1984).

Table I shows the proximate composition of feed ingredients and experimental diets used in this study.

Table I. Proximate composition of the feed ingredients and experimental diets (g/kg on dry matter basis).

	Ash	CP	Moisture	Lipid	Fiber
Biofloc	16.4	28.0	8.84	6.7	3.2
Fish meal	22	47	8.1	7.3	2.4
Soy bean meal	4.5	29	5.4	5.9	5.2
Rice flour	0.9	15	3.5	3.6	2.8
Experimental diets					
Control (C)	21.87	35.15	11.36	5.96	2.55
Treatment	20.51	35.96	10.74	5.21	3.84

C, Control; CP, crude protein.

Experimental design and animal rearing

The study followed a complete design with one control (commercial diet) and one treatment (Biofloc meal at 30%) in HDPE liner ponds 100 m^2 (duplicate) for 6 weeks. Healthy shrimp (PL 15) were procured from MK hatcheries (P) Ltd, Kancheepuram District, Tamil Nadu, India were stocked in four lined ponds, with a density of (10,000 PL/pond or 100 PL/m^2), provided with paddle wheel aerators to maintain proper aeration. For a period of 7 days shrimps were acclimatized to the pond conditions and fed with commercial feed four times in a day. Prior to the commencement of experiment, shrimp were starved for 24 h and randomly weighed (100/pond; average weight of 0.02±0.00 g) from each lined pond. The shrimp were fed with their respective treatment diets, four times in a day, at 10% of its body weight and reared for a span of 45 days. Exchange of water from the ponds was carried out twice in a week to maintain the water quality. Physico-chemical parameters of water such as dissolved oxygen, pH and temperature were measured on daily basis and ammonia (NH_4^+/NH_3)–N, nitrite (NO_2)–N, and nitrate (NO_3) – N once in a week, following the standard methods (APHA, 2005).

Growth performance

Twenty shrimps were sampled from each experimental unit, resulting in a total of 40 shrimp per treatment group (n=40). These samples were collected on a weekly basis to monitor the growth and body weight of the shrimp. Using the standard formulas described by Zokaeifar *et al*. (2012), the bio-growth parameters were calculated. The weight gain (g) was determined by subtracting the initial weight from the final weight of the shrimp. The specific growth rate (SGR) was calculated as a percentage per day, using the formula ([Ln Final body weight - Ln Initial body weight] / Days of culture) × 100. The feed conversion ratio (FCR) was obtained by dividing the total feed given by the total weight gain. Finally, the survival rate (%) was

computed as the number of shrimps harvested divided by the number of shrimps initially stocked, multiplied by 100.

Bacteriological analysis

Bacteriological analysis was conducted at regular intervals of every 15 days to assess the microbial count in both the experimental culture and the shrimp gastrointestinal tract (GIT). This analysis involved evaluating the total plate count (TPC), total *Vibrio* count (TVC), and total *Lactobacillus* count (TLC). For the bacteriological sampling, five shrimp were collected from each experimental pond. Their GIT were carefully and aseptically removed and then pooled with 0.85% saline solution. The pooled samples were used for bacteriological plating in specific agar media for each count: TSA was used for TPC, TCBS for TVC, and MRS for TLC.

Digestive enzyme analysis

At the end of the feeding trial, three shrimp were randomly selected from each treatment, resulting in a total of six shrimp per treatment group. The GIT of these shrimps were carefully collected for further analysis of their digestive enzymes. The collected GIT was dissected and then homogenized using poly pestles and a 0.25 M of cold sucrose solution. The resulting homogenate was centrifuged for 10 min at 5000 g in 4ºC, and the further analysis was carried out from the collected supernatant. Activities of all the enzymes were reported in U/mg protein.

The intestinal protease activity was determined using the method described by Drapeau (1976). In this procedure, 0.1 ml of tissue homogenate was incubated with 2.5 ml of 1% casein, which was prepared in 0.01 N NaOH, and 0.05 M tris phosphate buffer (pH 7.8) at 37°C for 15 min. To terminate the reaction, 2.5 ml of 10% trichloroacetic acid (TCA) was added. The reaction mixture was then filtered, and the optical density (OD) was measured at 280 nm using a UV spectrophotometer (UV-1800, Sl. No. 11635203671 CD, Shimadzu Corporation, Japan). In this assay, 1% casein served as the substrate, and a calibration curve was constructed using tyrosine as the standard solution. One unit of protease activity was defined as the amount of enzyme required to release 1 µm/min/mg protein at 37°C.

Activity of amylase in the GIT samples were assessed using the Bernfeld (1955) technique. In the assay, 1 ml of a 1% soluble starch substrate, prepared in 0.1 M phosphate buffer with a pH of 7.0, was added to the test tube along with the 1 ml of homogenized tissue. By incubating the mixture at 37°C for 15 min the reaction was started. 3,5-dinitrosalicylic acid (DNSA) was added 2 ml to end the reaction. The tubes were then cooled by immersing them at 100°C in water bath for 5 min. Once after cooling, the distilled water was used to dilute the tube contents upto10 ml and intensity in the resulting colour was observed using a UV spectrophotometer at 560 nm. A calibration range was generated using maltose as the reference solution to quantify the number of µmol of maltose released- min/mg protein at 37 ˚C, which served as a measure of amylase activity.

The activity of lipase in the samples of GIT were assessed using a stabilized olive oil emulsion method, following (Cherry and Crandall, 1932). A mixture consisting of 2 ml of olive oil emulsion, 1 ml of tissue homogenate, and 0.1 M phosphate buffer (pH 7.0) was incubated for a period of 24 h at a temperature of 27°C. After the incubation period, the tubes were filled with 3 ml of 95% alcohol and 2 drops of phenolphthalein indicator. The mixture was then titrated against 0.05 N NaOH. The endpoint was indicated by persistent pink colour. In the control tube, activity of enzyme was inhibited by incubating at 100°C using water bath for 15 min before adding olive oil emulsion and the buffer. Lipase activity was quantified by determining the number of fatty acids released-/min/mg protein at a temperature of 27°C.

The cellulase activity of the GIT samples were assessed using the methodology outlined in the study conducted by González-Pea *et al*. (2002). To assess cellulase activity, a tissue homogenate (1 ml) was combined with1 ml of phosphate buffer (0.1 M, pH 6.8) and carboxymethyl cellulose substrate 1 ml. The mixture was then incubated at 37°C for a duration of 1 h. DNSA reagent was used as a stop reaction, and the optical density was measured using a spectrophotometer at 540 nm. Activity of cellulase was quantified based on the amount of glucose released- /min/mg protein.

Haemolymph collection and immune parameter determination

The haemolymph was extracted from the ventral sinus cavity of shrimp using a 1 ml syringe and a 22-gauge needle. After collection, the haemolymph was centrifuged at 6000×g for 10 min to separate the serum. The serum was then transferred to 1 ml vials and used for the analysis of serum protein and prophenoloxidase (PO). The estimation of total serum protein was carried out using the standard method described by Lowry *et al*. (1951).

The activity of PO was assessed using a modified version of the method described by Gollas-Galván *et al*. (1997). The measurement involved the conversion of L-dihydroxyphenylalanine (L-DOPA, Sigma) to dopachrome, which was monitored spectrophotometrically at 490 nm. The assay was performed over a period of 60 min, with readings taken at 2-min intervals. The increase in absorbance per minute (OD/min) was used to determine

the activity of PO under the given assay conditions.

Lysozyme

A turbidometric assay was conducted to determine the activity of serum lysozyme, following the method described by Binuramesh and Michael (2011) with slight modifications. In this assay, 800 µl of *Micrococcus lysodeikticus* suspension (0.3 mg/ml) in potassium phosphate buffer (66 mM, pH 6.4) was mixed with 30 µl of serum. The initial absorbance of the mixture was measured at 450 nm using a spectrophotometer. The mixture was then incubated at 30°C for 5 min, and the reduction in absorbance was once again measured at 450 nm using the spectrophotometer. The decrease in absorbance at a rate of 0.001 per min was defined as one unit of lysozyme activity.

Antioxidant enzymes

To assess the antioxidant enzyme activity, three shrimps from each experimental tank were selected, and their hepatopancreas was collected for analysis. The hepatopancreas was dissected under chilled conditions and then mixed with a chilled 0.25 M sucrose solution. The tissue was homogenized to prepare a 5% homogenate using poly pestles. The resulting homogenate was centrifuged at 5000 rpm for 10 min at 4°C, and the supernatant was subsequently used for the antioxidant enzyme analysis.

A total of 500 ml of the collected hemolymph was added with 250 ml of DTNB solution and phosphate solution 2 ml. A blank was also created simultaneously using 200 ml of distilled water and 300 ml of precipitating solution. A spectrophotometer is used to evaluate the yellow colour's intensity at 412 nm in comparison to a blank. The samples' glutathione activity was estimated from the observed OD values (Hemmadi, 2017).

Activity of superoxide dismutase (SOD) was measured in the hepatopancreas samples using an amended version of Misra and Fridovich's (1972) technique. The experimental mixture was created by combining 50 µl of tissue homogenate with 1.5 ml of phosphate buffer at pH 7.4. Additionally, 0.5 ml of an epinephrine solution with a concentration of 0.03 M was added to the mixture. The changes in OD at 480 nm were monitored using a UV spectrophotometer for a duration of 2 min with measurements taken at 30 sec interval.

Total haemocyte count

At the conclusion of the study, haemolymph samples were obtained from two inter-moult *vannamei* per tank. This sampling method resulted in a total of four shrimps per treatment being included in the study, to determine the total haemocyte count (THC). The sampling procedure involved the following steps: A sterile 1 ml syringe with a 25 G × 13 mm needle was used to withdraw 100 µl of haemolymph from the second segment of cephalothorax in each shrimp. Before extracting the haemolymph, the syringe was prepared by filling it with a pre-cooled anticoagulant solution. This solution consisted of 10% EDTA (ethylenediaminetetraacetic acid), Na^2, and was kept at a temperature of 4°C. The collected haemolymph was mixed with 150 µl of 4% formaldehyde solution to preserve the samples. A volume of 20 µl haemolymph was kept on a haemocytometer. Using an optical microscope, the total THC was determined by counting the haemocytes in the specified area of the haemocytometer. This procedure allowed for the assessment of the total number of haemocytes present in the shrimp haemolymph.

Challenge study

A challenge study was conducted against the *Vibrio alginolyticus* strain. The study involved the following steps: A total of 30 shrimp from the treatment pond and control ponds were randomly selected and acclimatized in separate FRP tanks for 3 days. The acclimatized shrimp were then transferred to glass tanks with a capacity of 100 L, with 10 shrimp per tank. The challenge study was performed in triplicates. Inter-moult stage of shrimp was chosen for the challenge study. The shrimp were injected with 50 µl of *V. alginolyticus* suspension (1×10^7 cfu/ml), which was prepared by growing the bacteria overnight in TSB medium with 1.5% NaCl supplementation at 30°C. The bacterial suspension was suspended in NSS (0.85% NaCl) using a sterile syringe with a 22-gauge needle. During the experimental infection period, the shrimp were continuously fed with their respective treatment diets. The number of deceased shrimps in each triplicate was systematically documented over a period of 5 days following the infection. The calculation of relative percent survival (RPS, %) was carried using the formula:

RPS (%) = (1 - M1/M0) × 100 Where, M0 - mortality in the control group and M1 - mortality in the treatment group. By comparing the mortality rates between the control and treatment groups, RPS was determined. The RPS value indicates the level of protection provided by the treatment against *V. alginolyticus* infection.

Statistical analysis

The collected data on growth parameter, bacteriological analysis of culture water and digestive tract of cultured shrimp, enzymatic activities and immunological parameters were analysed in one-way analysis of variance (ANOVA) using SPSS version 16.0. Duncan's multiple range test was used for ranking the mean values of different treatment groups after post hoc comparison. The statistical significance of the test was set at $P<0.05$.

Table II. Effect of biofloc meal on the growth performance and survival of *P. vannamei.*

Treatments	Initial weight (g)	Final weight (g)	Weight gain (g)	SGR (%/day)	Survival (%)	FCR
Control	0.032±0.00^{b}	4.61 ± 0.07^{b}	4.58 ± 0.07^{b}	11.18 ± 0.13^{b}	72 ± 0.24^{b}	1.33 ± 0.03^{a}
Treatment	0.031±0.00^{a}	4.98 ± 0.06^{a}	4.95 ± 0.09^{a}	11.53 ± 0.17^{a}	77 ± 0.29^{a}	1.17 ± 0.01^{b}
P value	0.381	0.002	0.002	0.041	0.001	0.002

FCR, Feed conversion ratio; SGR(%/day), Specific growth rate (%/day). In each column, mean values with different superscripts differ significantly at (P <0.05).

RESULTS

Effect of biofloc meal on growth performance and water quality

The present study revealed a significant difference (P < 0.05) in various growth parameters between the control and treatment groups. The treatment group demonstrated significantly higher values for final weight (4.98 ± 0.06 g), weight gain (4.95 ± 0.09 g), and specific growth rate (11.53 ± 0.17 %/day) compared to the control group. Conversely, the control group displayed lower values for these parameters, with a final weight of 4.61 ± 0.07 g, weight gain of 4.58 ± 0.07 g, and specific growth rate of 11.18 ± 0.13 %/day. Furthermore, the treatment group exhibited a significantly higher survival rate of 77.0 ± 3.81% compared to the control group. Additionally, the shrimp fed with the treatment diet demonstrated better feed conversion ratio (FCR) of 1.17 ± 0.01, indicating improved feed utilization. In contrast, the control group exhibited poorer feed utilization, as indicated by a higher FCR of 1.33 ± 0.03 (Table II).

Throughout the experimental period, all measured water quality parameters remained within the optimal range. Dissolved oxygen (DO) levels were maintained at 5.5 ± 0.5 mg/l, temperature at 30 ± 1.0ºC, pH in the range of 7.8 to 8.5, ammonia-N levels below 0.1 ± 0.01 mg/l, nitrite-N levels below 0.07 ± 0.002 mg/l, and nitrate-N levels below 3.0 ± 1.0 mg/l. These favourable water conditions likely contributed to the observed growth and survival performance of the shrimp in both the control and treatment groups.

Effect of biofloc on bacterial microbiole

The study revealed a significant difference (*P<0.05*) in the total plate count (TPC) of the culture water between the shrimp reared in the treatment diet and the control group. At the end of the experiment, the treatment group exhibited a significantly higher TPC of 3.54±0.10×10^{7} cfu/ml in the culture water. Regarding the *Vibrio* count, a significant difference (*P<0.05*) was observed at the end of the experiment, with the control group showing a significantly higher *Vibrio* count of 2.06±0.08×10^{6} cfu/ml. Throughout the experiment, significant differences in the *Lactobacillus* count were noted. At the end of the experiment, the shrimp fed with the treatment diet displayed a significantly higher *Lactobacillus* count of 3.93±0.22×10^{6} cfu/ml in the culture water (Table III).

Table III. Bacterial microbiota in the culture water (cfu/ml) of *P. vannamei* fed with treatment and control diet.

Time (days)/ Treatments	TPC (cfu/ml)	TVC (cfu/ml)	TLC (cfu/ml)
Day 0			
Control	2.24±0.12×10^{4b}	1.55±0.42×10^{3b}	1.82±0.04×10^{2a}
Treatment	3.18±0.85×10^{4a}	1.11±0.10×10^{3a}	2.26±0.14×10^{2b}
P-value	.000	.058	.005
Day 15			
Control	2.80±0.62×10^{4b}	1.27±0.14×10^{3a}	2.79±0.15×10^{2b}
Treatment	4.90±0.20×10^{4a}	1.59±0.04×10^{3b}	2.56±0.11×10^{2a}
P-value	.000	.064	.212
Day 30			
Control	5.75±0.05×10^{6b}	4.31±0.20×10^{6b}	8.18±0.03×10^{4a}
Treatment	8.10±0.70×10^{6a}	5.36±0.05×10^{6a}	9.16±0.07×10^{4b}
P-value	.000	.000	.148
Day 45			
Control	2.61±0.10×10^{7b}	2.77±0.10×10^{6a}	3.41±0.23×10^{6b}
Treatment	3.54±0.10×10^{7a}	2.06±0.08×10^{6b}	3.93±0.22×10^{6a}
P-value	.001	.000	.019

TPC, Total plate count; TVC, Total *vibrio* count; TLC, total *Lactobacillus* count. In each column, mean values with different superscripts differ significantly at (P <0.05).

The GIT of the shrimp showed a significant difference (*P<0.05*) in the TPC and *Vibrio* count between the control and treatment groups. Initially, a higher TPC was observed in the treatment group at 2.61±0.10×10^{5} cfu/g, and at the end of the feeding trial, a significantly higher *Vibrio* count of 1.83±0.10×10^{4} cfu/g was recorded in the control group compared to the treatment diet. Regarding the TLC, although there was no significant difference *(P>0.05)* observed among the treatments, a significantly higher

TLC of 3.41±0.23×10^3 cfu/g was recorded in the treatment group at the end of the feeding trial (Table IV).

Table IV. Microbial community in the intestinal tract (cfu/z) of *P. vannamei* fed with treatment and control diet.

Time (days)/ Treatments	TPC (cfu/g)	TVC (cfu/g)	TLC (cfu/g)
Day 0			
Control	2.05±0.11×10^{4b}	1.21±0.10×10^{3a}	1.22±0.05×10^{2b}
Treatment	2.60±0.06×10^{4a}	1.29±0.05×10^{3b}	1.57±0.03×10^{2a}
P-value	.016	.458	.065
Day 15			
Control	3.55±0.28×10^{4b}	1.27±0.15×10^{3a}	2.49±0.01×10^{2b}
Treatment	4.11±0.10×10^{4a}	1.28±0.05×10^{3b}	2.70±0.08×10^{2a}
P-value	.000	.078	.192
Day 30			
Control	6.70±0.10×10^{4b}	4.49±0.37×10^{3a}	1.12±0.09×10^{3b}
Treatment	7.10±0.10×10^{4a}	3.81±0.16×10^{3b}	1.48±0.06×10^{3a}
P-value	0.036	0.017	0.046
Day 45			
Control	1.50±0.20×10^{5b}	1.83±0.05×10^{4a}	2.48±0.30×10^{3b}
Treatment	2.61±0.10×1^{05a}	2.77±0.10×1^{04b}	3.41±0.23×1^{03a}
P-value	.003	.000	.037

For abbreviations and statistical details see Table III.

Effect of biofloc meal on digestive enzymes

Protease, amylase and lipase activities showed significant difference (P<0.05) in the shrimp fed with treatment diet (Fig. 1). Among these, significantly higher and lower values of protease, amylase and lipase activities were recorded in treatment diet (1.99 ± 0.22 U/mg protein, 8.22 ± 1.21 U/mg protein and 292 ± 47 U/mg protein), respectively. Significantly higher cellulase activity was observed in the treatment group (24.08±2.21 U/mg protein).

Effect of biofloc meal on immunological parameters

A significant difference was observed in serum protein levels between the control and treatment groups. The treatment group exhibited a significantly higher value of total serum protein, measuring at 3.4 ± 0.05 U/mg protein (Table V). In terms of PO activity, the treatment group displayed significantly higher levels of PO (16.21 ± 0.51 U/mg protein) compared to the control group. The PO activity in the treatment group was approximately higher than that of the control group (Table V).Additionally, a significant difference in lysozyme activity was observed between the different treatment groups. The treatment group exhibited significantly higher lysozyme activity, measuring at 67 ± 0.79 U/ml (Table V).

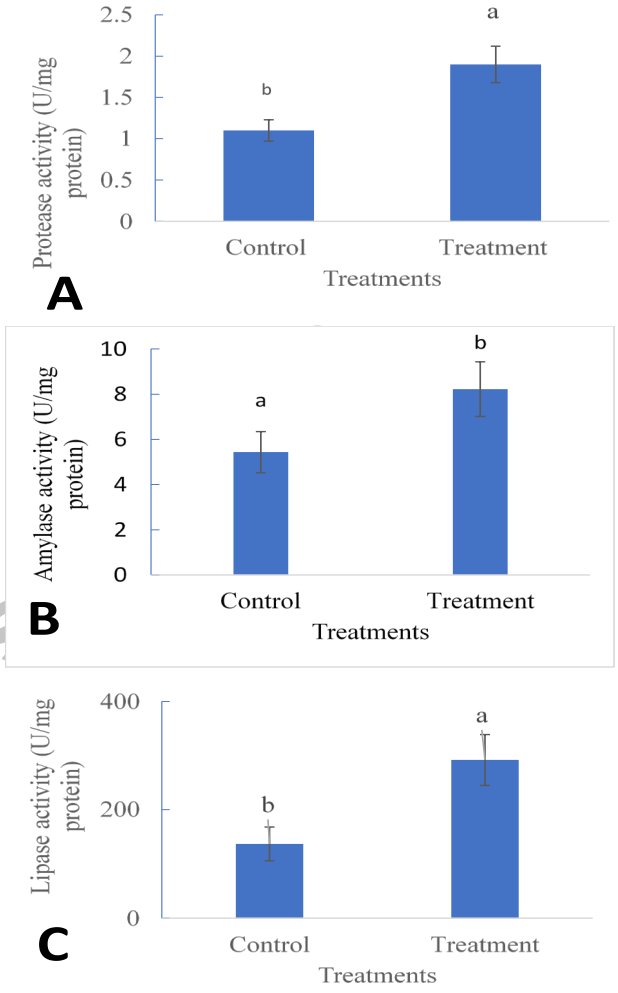

Fig. 1. Specific activities (U/mg protein) of GIT protease, amylase, lipase and cellulase of shrimp fed with different levels of biofloc meal incorporated diets. Bars with different superscripts differ significantly at P <0.05.

Effect of biofloc meal diet on Antioxidant enzymes and haemocyte count

There was no significant difference in the SOD activities of different treatments reared shrimp. However, higher SOD was recorded in control (41.17± 0.09 U/ mg protein). Likewise, the glutathione did not show any significant difference (P<0.05) among the treatment and control groups (Table V). Significant difference was observed between the control and treatments. Higher level of haemocyte count was observed in the treatment diet (36 ± 1.8 ×10^{6}cells/ml) (Fig. 2) compared to the control diet.

Table V. Effect of biofloc meal on immunological and antioxidant parameters *P. vannamei.*

Treatments	Serum protein (g/dL)	Prophenoloxidase (U/mg protein)	SOD (U/mg protein)	Glutathione (U/mg protein)	Lysozyme (U/mg protein)
Control	2.7± 0.07[b]	11.5 ± 0.37[b]	41.17 ± 0.09[a]	16.51± 0.22[a]	53 ± 0.47[b]
Treatment	3.4 ± 0.05[a]	16.21 ± 0.51[a]	34.2 ± 0.12[b]	14.16± 0.17[b]	67 ± 0.79[a]
P value	0.038	0.079	0.154	0.647	0.040

SOD, superoxide dismutase. Note: In each column, mean values with different superscripts differ significantly at (P <0.05).

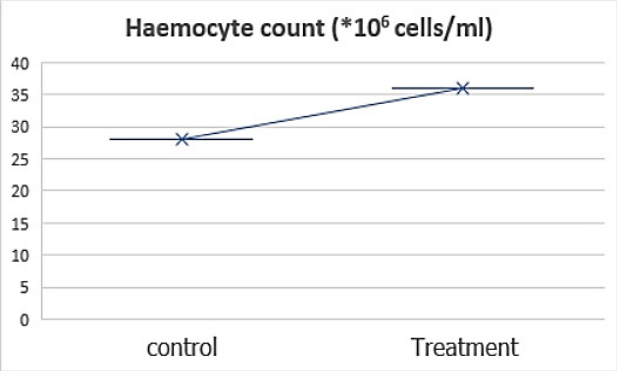

Fig. 2. Effect of biofloc meal diet on haemocyte count (*10^6 cells/ml) of shrimp.

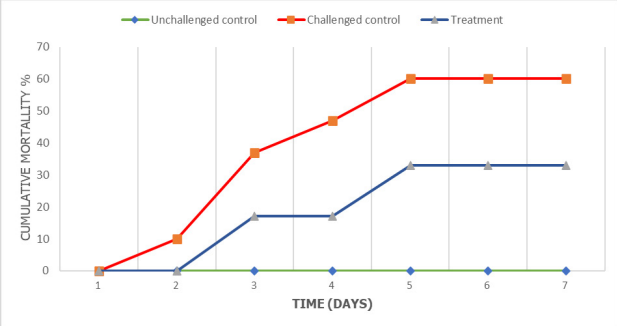

Fig. 3. Effect of biofloc meal diet on cumulative mortality of vannamei group challenged with *V. alginolyticus.*

The cumulative mortality (%) in the treatment group diet was significantly lower (33 ± 2.57%) than the control group (60 ± 4.79%) (Fig. 3). There was no mortality after 5 days of post infection in both the control and treatment group. No mortality was observed in unchallenged control group.

Histopathological analysis

The treatment group cultured animals had considerably more tubules in the proximal region and a higher percentage of cells that were capable of intracellular digestion (B) and absorption (R). The R and B cells in the treatment group (Fig. 4) were primarily located on the tubules of the animals digestive tissues after feeding. In control, R cells exhibited reduced proportions at the blind end of the tubules, but B cells were normal in the proximal and middle sections.

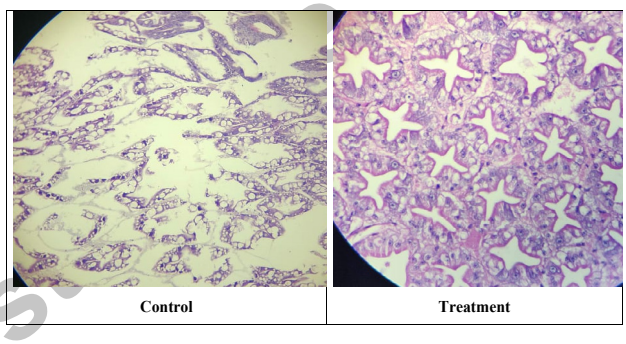

Fig. 4. Effect of biofloc meal diet on histological structure of hepatopancreas of shrimps.

DISCUSSION

The increasing food demand among the growing population have become the major constraints on the global scale. Several studies have been reported to satisfy the growing demand for the animal protein, intensive aquaculture is one of the hopeful alternatives for replacing fish meal. Various studies have been reported in recent years to replace fish meal with biofloc meal as an alternative protein source (Bauer *et al*., 2012; Dantas *et al*., 2016; Kuhn *et al*., 2010). Biofloc is considered as a modern aquaculture farming system for many years, by indorsing environment, effluent discharges, feed management, biosecurity and inflating production.

The biofloc meal serves as high nutrients food source for the culture animals. Similarly, crude protein content in the biofloc relies on the bacterial communication, microorganisms, inorganic particles and zooplanktons. The nutritional profiling and essential amino acids (EAA) of fish meal and biofloc meal, revealsthat, biofloc could replace fishmeal in shrimp feed (FAO, 2012). Therefore, the requirement for sustainable alternative source to replace fishmeal had layed a path to conduct various studies over replacement of fishmeal using biofloc meal. The present

study is to evaluate the proximate profiling of the biofloc meal harvested from the raceway tanks was an alternative feed protein source (biofloc substituted fishmeal), and data was provided to support the positive effects of biofloc diet on growth, survival and immune response in shrimp.

The study found significantly increased weight gain, SGR, survival rate and better FCR in treatment group (Kuhn *et al*., 2010) reported that studies on various inclusion of biofloc meal had resulted positive impact on the survival and growth performance of *L. vannamei* fed with biofloc meal Kanost *et al*. (2008) reported that usage of biofloc meal as feed, for *L. vannamei* had significantly inflated the digestive enzyme activities, and together improved the growth performance in shrimp. The increase in the growth of shrimps was due to the role of probiotic microorganisms like *Bacillus* and *Lactobacillus* present in the biofloc, that excites, the production of endogenous enzymes in the shrimp hepatopancreas.

The significantly higher heterotrophical bacterial count were found in the treatment group compared to the control, related to the results, previous studies of bacterial count in the biofloc system ranged from 10^7-10^9 cells/ml in zero-exchange intensive ponds. 3.9×10^8 cells/ml in intensive RAS shrimp tank which is raceway-based (Otoshi *et al*., 2006) and 3.35 to 5.42×10^8cells/ml in intensive shrimp ponds (Burford *et al*., 2003). The study also resulted in the decreased *vibrio* count in the treatment group compared with the control diet. The reduction of *vibrio* counts in the treatment showed the probiotics effects of heterotrophical microbial diversity in the biofloc culture system (Jha and Naik, 2009; Panigrahi *et al*., 2017, 2018).

Studies reported that the bioflocs are rich in sources of bioactive compounds like amino sugars, chlorophylls antimicrobial compounds (Ju *et al*., 2008; Crab *et al*., 2010). Biofloc meal plays an important role in digestive enzyme profile in shrimp (Gamboa-Delgado *et al*., 2003). In the dietary supplementation of biofloc significantly improved ($p > 0.05$) the specific activity of digestive enzymes like amylase, cellulase, protease and lipase in the treatment groups compared with control. Significantly higher digestive enzyme activities and better growth performance in *L. vannamei* was reported in the biofloc based system (Xu and Pan, 2012). Digestive enzyme activity has been enhanced in fed with probiotics, microalgae and periphyton supplemented diets (Anand *et al*., 2013b; Lara-Flores *et al*., 2003; Ziaei-Nejad *et al*., 2006). The presence of microbial components in the biofloc supplemented diet might have stimulated the production of endogenous enzymes by the shrimp hepatopancreas compared with control.

Higher rate of survival in shrimp, and enhanced immune activities, were found in the treatment group. The shrimp haemocytes are dynamic in immunity, which performs functions such as, phagocytosis, encapsulation, storage and release of the PO system. ProPo is the known defence mechanism in shrimp, that causes melanization and inactivation of foreign bodies to prevent their spread throughout the body. Lipopolysaccharides (LPS) and β-1,3- 392 glucans are the enzymes that stimulate the microbial cell wall, which trigger a proPO activating system (Kanost and Gorman, 2008; Ekasari *et al*., 2014). The present study found that the prophenoloxidase activity was significantly higher in shrimp fed with treatment group when compared to the control group. It is reported that the immunological and physiological status of the shrimp gastrointestinal tract, was modified by the *Bacillus* bacteria present in biofloc, which subsequently adjust the endogenous microbiota and inhibits the colonization of pathogenic bacteria in shrimp GIT (Zhao *et al*., 2012).

DECLARATIONS

Funding

This research has been supported by grants from National Fisheries Developmental Board (NFDB) (project code: 13-101-14).

Statement of conflict of interest

The authors have declared no conflict of interest.

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