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Biofloc meal incorporated diet improves the growth and physiological responses of *Penaeus vannamei*

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Abstract

The study was conducted to evaluate the efficacy of biofloc meal incorporated diets on growth performance and physiological responses in *Penaeus vannamei.* The feeding trial was conducted using two control diets and four experimental diets, having biofloc meal at various incorporation levels, namely control diet (commercial diet), BFT0 (biofloc meal control, biofloc meal at 0%) BFT10 (biofloc meal at 10%), BFT20 (biofloc meal at 20%), BFT30 (biofloc meal at 30%), and BFT40 (biofloc meal at 40%). Healthy post larvae of *P. vannamei* (0.03 ± 0.00 g) were stocked (50 shrimp per tank) in a glass tank and fed with the experimental diets at 10% of animal body weight, four times a day, for a duration of 60 days. Among the different treatments, significantly higher final body weight (1.30 ± 0.06 g), weight gain (1.27 ± 0.06 g), specific growth rate (6.21 ± 0.01%/day), and survival rate (62 ± 0.20%) were recorded in BFT30 diet. In contrast, shrimp reared using control diet exhibited lower specific growth rate (5.53 ± 0.02%/day) and survival (48 ± 0.14%) compared to other treatment groups. At the end of the experiment, serum protein (1.6 ± 0.02 g/dl), prophenoloxidase (16.35 ± 2.98 U/mg protein), and peroxidase (0.2 ± 0.03 U/ml) were found to be significantly higher in BFT30 diet-fed group. Similarly, the digestive enzyme activities of amylase (4.75 ± 0.87 U/mg protein) and lipase (118 ± 5.10 U/mg protein) of BFT30 reared shrimp were found to be significantly higher. In addition, the histological observation of the shrimp revealed the larger proportion of mature cells with intracellular digestion (B) and absorption (R) in BFT30 group. Overall, the study revealed that biofloc meal incorporation at 30% in shrimp diet would potentially improves the growth performance and physiological responses of shrimp.

Keywords Biofloc · Shrimp feed · Digestive enzymes · Fish physiology · Growth · White leg-shrimp

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Introduction

Aquaculture is one of the fast-growing food production sectors, globally, and its production is increasing steadily in the past few decades. Presently, the sector accounts for more than 50% of the world's food fish production; however, at the same time, the intensification practices adopted in the sector producing an enormous amount of impure effluent, comprising of uneaten feed and feces, which threatens the stability of natural ecosystem (Crab et al. 2007; FAO 2020). Many technological interventions, including recirculatory aquaculture practices and biofloc technology (BFT), have been made especially, in the recent times, to overcome those challenges which are creating a positive change in the sector. But, feed, the foremost input of aquaculture practices, is the major hindering force for the sector expansion due to shortage of feed ingredients and their price variations (Khatoon et al. 2016). Constantly, still, the sector is searching the alternative feed ingredients to make a more sustainable feed which could help to overcome the contemporary challenges in the aquafeed industry (Promthale et al. 2019).

In the past decade, BFT have been successfully adopted in the shrimp farming industry. Recently, biofloc was projected as a novel strategy for disease management, as it possesses natural probiotic effect, which additionally act as antimicrobial, antifungal, and external probiotic role (Emerenciano et al. 2013). The growth of microbial community in biofloc pond utilizes the dissolved nitrogen and converts the waste into additional feed supplement, microbial protein (Avnimelech 2009; Bauer et al. 2012). Hence, BFT can reduce the protein requirement in shrimp farming and improve the feed conversion rates (Avnimelech 1999; Bauer et al. 2012). The protein rich effluents of BFT system, loaded with beneficial microbial communities, could be used as an alternative protein source for the preparation of aquafeeds, which in turn reduce the contemporary environmental issues in shrimp farming. Biofloc meal has been projected as a potent alternative feed ingredient in shrimp diet (Kuhn et al. 2009, 2010; Bauer et al. 2012) which can partially replace the fish meal, an expensive feed ingredient in shrimp diet (Azim and Little 2008).

Biofloc meal, a dried form of biofloc, comprises of conglomerate of microorganisms, exocellular polymers, and uneaten feeds (Emerenciano et al. 2013). Recent studies suggest that the dried biofloc contains different bio-active compounds which includes proteins, carotenoids, chlorophylls, vitamin C, and other trace minerals (Tacon et al. 2002; Ju et al. 2008; Crab et al. 2012). The heterotrophic bacterial community present in the floc is suspected to have a controlling effect on pathogenic bacteria (Michaud et al. 2006; Ju et al. 2008). Additionally, the carotenoids in biofloc provide essential nutrients and bio-active physiological functions in animal tissue and stimulate the animal immune system. Previous reports suggest that the incorporation of processed biofloc meal in shrimp diets often exhibits positive responses such as improved growth, immune performance, antioxidant response, resistance against various infections, and better health condition in shrimp (Crab et al. 2012; Xu and Pan 2013; Kuhn et al. 2016; Ahmad et al. 2017; Bakhshi et al. 2018; Promthale et al. 2019). It has been demonstrated that biofloc meal obtained from fish pond effluent is a practical alteration for fishmeal and soy meal in shrimp diets (Kuhn et al. 2009).

The usage of biofloc meal, as a replacement source for fishmeal, in shrimp feed demands the understanding of nutritional value of floc meal as well as nutritional

requirement of shrimp (Promthale et al. 2019). However, the rate of inclusion and replacement varies based on the floc nutritional composition and culture species nutritional requirement. Therefore, the present study has been designed to find out the rate of inclusion level of biofloc meal in the diets for rearing *P. vannamei.*

Material and methods

The present study was carried out at the Indoor culture system of Advanced Research Farm Facilities, Tamil Nadu Dr. J. Jayalalithaa Fisheries University (TNJFU), Madhavaram, Chennai, Tamil Nadu, India.

Biofloc meal

Biofloc, produced using spentwash, as a carbon source with C:N ratio of 15:1, in the raceway system (14 m length, 2.7 m width, and 0.6 m depth) and later, it was harvested using a 50-µm nylon mesh and used for biofloc meal preparation (Avnimelech 2007). The biofloc system was maintained at 26–28 °C and pH 7.7 to 8.4. 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 fiber, and nitrogen free extract (NFE) of the floc were analyzed (AOAC 1984).

Experimental diets preparation

The study consisted of two control diets (commercial diet (C) and prepared diet without biofloc meal inclusion (BFT0)) and four experimental diets, namely BFT10 (biofloc meal at 10%), BFT20 (biofloc meal at 20%), BFT30 (biofloc meal at 30%), and BFT40 (biofloc meal at 40%). Commercial shrimp feed with 35% crude protein was used as control diet (C). Experimental diets with different incorporation levels of biofloc meal were formulated, separately, and prepared using feed ingredients (Table 1). The total crude protein percentage in all the formulated diets was 35% by adjusting the inclusion level of fishmeal with biofloc meal.

Experimental animal rearing

Healthy shrimp (PL 15 stage) were procured from Aqua Nova hatcheries (P) LTD, Kancheepuram District, Tamil Nadu, India, and conditioned at Fiber Reinforced Plastic tank (2000 L capacity) for 7 days and fed with commercial diet (35%). Nine hundred shrimp (average weight 0.03 ± 0.00 g) were randomly stocked (50 PL/tank) in 18 glass tanks (100 L capacity) which represents triplication of each treatment group. The shrimp were fed with their respective treatment diets, four times in a day, at 10% of its body weight

Table 1 Ingredients composition used for preparing the biofloc meal incorporated experimental diets and proximate composition of experimental diets

Ingredients (g/kg)	BFT0	BFT10	BFT20	BFT30	BFT40
Biofloc meal	0	100	200	300	400
Fish meal	500	450	400	350	300
Soybean meal	120	140	160	180	200
Rice flour	320	250	180	110	40
Binder (cmc)	10	10	10	10	10
Trace mineral	5	5	5	5	5
Vitamin C	10	10	10	10	10
Vitamin premix	5	5	5	5	5
Fish oil	10	10	10	10	10
Soylecithin (70%)	20	20	20	20	20
Total	1,000	1,000	1,000	1,000	1,000
Proximate composition of the feed ingredients (g/kg on dry matter basis)					
Ingredients	**Ash**	**CP**	**Moisture**	**Lipid**	**Fiber**
Biofloc	19.5	28.0	8.84	6.4	3.7
Fish meal	28.8	48	9.2	8.1	2.7
Soy bean meal	4.5	30	4.0	7.1	5.2
Rice flour	0.5	14	3.8	5.3	1.8
Proximate composition of the experimental diets (g/kg on dry matter basis)					
C	21.69	35.33	12.12	6.38	2.63
BFT0	22.77	35.44	10.65	4.81	3.79
BFT10	23.41	35.79	11.69	4.24	3.16
BFT20	23.79	34.96	12.78	4.79	3.91
BFT30	21.18	35.61	12.66	5.38	3.56
BFT40	23.17	35.46	11.39	5.17	3.84

C control, *BFT0* biofloc meal at 0%, *BFT10* biofloc meal at 10%, *BFT20* biofloc meal at 20% *BFT30* biofloc meal at 30%, *BFT40* biofloc meal at 40%, *CMC* carboxymethyl cellulose, *CP* crude protein

and reared for a span of 60 days. Water exchange, at the rate of 50%, 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

Sampling was carried out once in a week, and each time, 10 shrimp from each tank ($n = 30$/treatment) were collected for body weight measurement. The growth indicators like weight gain, specific growth rate (SGR), feed conversion ratio (FCR), and survival rate were calculated using standard formulas (Zokaeifar et al. 2012). Weight gain (g) = Final weight (g) – Initial weight (g), Specific growth rate (%/day) = ([Ln Final body weight—Ln Initial body weight]/ Culture days) × 100, Feed conversion ratio (FCR) = Total feed given (g)/ total weight gain (g), Survival rate (%) = (Number of shrimp harvested / Number of shrimp stocked) × 100.

Bacteriological analysis

On monthly intervals, culture water, shrimp gastrointestinal tract (GIT) bacteriology count was assessed, where total plate (TPC), total *Vibrio* count (TVC) and total *Lactobacillus* count (TLC) were evaluated. At the end of each week, aseptically, GIT of shrimp was collected, randomly, from two shrimp at each tank ($n = 6$/treatment) which was then macerated with 0.85% saline for bacteriological plating of TPC, TVC, and TLC using tryptone soy agar (TSA, Hi-Media), thiosulphate-citrate-bile salt sucrose agar (TCBS, Hi-Media), and de Man, Rogosa, and Sharpe agar (MRS, Hi-Media), respectively.

Digestive enzyme analysis

Three shrimp from each glass tank ($n = 9$/treatment) were collected at the end of the feeding trial and their GIT was collected for digestive enzyme analysis. Followed by the evacuation of ingested food items, the dissected GIT was homogenized (5% homogenate), using poly pestles, by mixing them with chilled sucrose solution (0.25 M). After centrifugation, at 5000 × g for 10 min at 4 °C, the homogenate supernatant was collected and utilized for further analysis. The activities of all enzyme were expressed in specific activity (U/ mg protein).

Following the method of (Bernfeld 1955) using 1% of soluble starch as a substrate, GIT samples' amylase activity was analyzed. By adding 1 ml of starch (prepared in 0.1 M phosphate buffer, pH 7.0) solution to the 1 ml of tissue homogenate, in a test tube, the reaction was initiated and the mixture was incubated for 15 min at 37 °C. The reaction was stopped by the addition of 2 ml of 3, 5 dinitrosalicylic acid (DNSA). After keeping the tubes in water bath (100 °C), for 5 min, the tubes were cooled. Finally, using distilled water, volume of the tube was made up to 10 ml and the developed color intensity was recorded in a UV spectrophotometer at 560 nm. Maltose was used as a standard solution for calibration curve preparation. One unit of amylase activity was defined as number of micromoles of maltose released/min/mg of protein at 37 °C.

The GIT samples' lipase activity was analyzed according to (Cherry and Crandall 1932) using a stabilized emulsion of olive oil. Tissue homogenate (1 ml) was mixed with distilled water (3 ml), phosphate buffer (0.1 M, pH 7.0) (0.5 ml), and olive oil emulsion (2 ml) and it was incubated for 24 h at 27 °C. After incubation, 95% of alcohol (3 ml) and phenolphthalein indicator (two drops) were added to the tubes and it was titrated against alkali (0.05 N NaOH). The end point is the appearance of a permanent pink color. Meantime, in control tube, prior to the addition of buffer and olive oil emulsion, enzyme activity was inactivated by incubating them in a water bath (100 °C) for 15 min. A porcine type pancreatic lipase was used for standard curve preparation. The number of fatty acid released/min/mg of protein at 27 °C was expressed as one unit of lipase activity.

Cellulase activity of the GIT samples was examined by following the method of (del C González-Peña et al. 2002). To the 1 ml of tissue homogenate, 1 ml of carboxymethyl cellulose, as a substrate, and 1 ml of phosphate buffer (0.1 M, pH 6.8) were added and it was kept in 37 °C for 1 h. After incubation, 0.5 ml of stop solution (DNSA reagent) was added and the final OD value was recorded at 540 nm using a spectrophotometer. The amount of glucose released/min/mg protein was expressed as one unit of cellulase activity.

Hemolymph collection and immune parameter determination

The 1-ml syringe with 22-gauge needle was used to collect the hemolymph from ventral sinus cavity of shrimp (two shrimp from each tank: $n = 6$/treatment). From the collected hemolymph, serum was separated by centrifuging them at 6000 × g for 10 min. Then, the serum was transferred to 1-ml vials and used for the serum protein and prophenoloxidase (PO) analysis. The total serum protein was estimated by following the standard method (Lowry et al. 1951).

The prophenoloxidase activity was measured by following the method of (Gollas-Galván et al. 1997) with slight modifications. The formation of dopachrome from L-diydroxyphenylalanine (L-DOPA, Sigma) was measured spectrophotometrically at 490 nm for 60 min at 2-min intervals. The activity was determined by the increase of OD/min under the assay conditions.

Lysozyme

A turbidometric assay following the method of (Binuramesh and Michael 2011) with slight modifications was used for determination of serum lysozyme. Briefly, 800 µl *Micrococcus lysodeikticus* (0.3 mg/ml), suspended in potassium phosphate buffer (66 mM, pH 6.4), was mixed with 30 µl serum and the initial OD was read at 450 nm. After incubating the mixture at 30 °C for 5 min, the reduction in absorbance was again measured at 450 nm in a spectrophotometer. The reduction in absorbance at 0.001/min was defined as one unit of lysozyme activity.

Antioxidant enzymes

To the collected hemolymph (500 µl), 2 ml of phosphate solution and 250 µl of 5, 5'-Dithiobis (2 nitro benzoic acid DTNB) solution were added. Simultaneously, a blank was prepared using 200 µl of distilled water and 300 µl of precipitating solution. The intensity of the yellow color was measured in a spectrophotometer at 412 nm against the blank. From the measured OD values, glutathione activity of the samples was calculated (Hemmadi 2017).

In the hepatopancreas samples, superoxide dismutase (SOD) activity was carried out by following the method of (Misra and Fridovich 1972) with slight modifications. With 50 µl of tissue homogenate, 0.5 ml of epinephrine (0.03 M) and 1.5 ml of phosphate buffer (pH 7.4) were added and mixed well. The changes in OD values were recorded at 480 nm using UV spectrophotometer, for 2 min at 30-s intervals. The amount of protein required to give 50% inhibition of epinephrine auto-oxidation was expressed as one unit of SOD activity.

Histological analysis

The hepatopancreas and intestine of *P. vannamei* from various treatments were dissected, rinsed in normal saline, and fixed using 10% formalin buffer for 24 h. After fixation, the tissues were dehydrated in a series of alcohol with different concentrations (70%, 80%, 90%, and 100%), embedded in paraffin, sectioned at 5 mm, and later stained

with hematoxylin–eosin (H&E) (AlYahya et al. 2018). The slides were prepared in the Department of Pathology, Madras Veterinary College, Chennai, Tamil Nadu, India. Finally, the slides were micro-photographed under 100 × optical lens using Lawrence and mayo microscope.

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 analyzed 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 $P < 0.05$.

Results

Growth performance and water quality parameters

The study found a significant difference ($P < 0.05$) in the final weight, weight gain, and specific growth rate among the different treatment groups (Table 2). Significantly higher and lower growth performance in terms of final weight (1.30 ± 0.06 g and 0.82 ± 0.05 g), weight gain (1.27 ± 0.06 g and 0.80 ± 0.02 g), and specific growth rate (6.21 ± 0.01%/day and 5.53 ± 0.20%/day) were recorded in BFT30 and control diets, respectively. Also, significantly higher survival rate was observed in BFT30 (62.0 ± 0.20%). Likewise, the shrimp fed with BFT30 diet exhibited better FCR (1.38 ± 0.01). On the other side, the control group showed poor feed utilization in terms of high FCR (1.63 ± 0.03). All the water quality parameters were within the optimal range: DO (5.5 ± 0.5 mg/l), temperature (30 ± 1.0 °C), pH (7.8 – 8.5), ammonia-N (< 0.1 ± 0.01 mg/l), nitrite-N (< 0.07 ± 0.002 mg/l), and nitrate–N (< 3.0 ± 1.0 mg/l) throughout the experimental period.

Table 2 Effect of different levels of biofloc meal incorporated diets on growth performance and survival of *P. vannamei*

Treatments	Initial weight (g)	Final weight (g)	Weight gain (g)	SGR (%/day)	Survival (%)	FCR
C	0.033 ± 0.00	0.82 ± 0.05^{c}	0.80 ± 0.02^{c}	5.53 ± 0.02^{b}	48 ± 0.14^{d}	1.63 ± 0.03^{ab}
BFT0	0.033 ± 0.00	0.85 ± 0.03^{c}	0.81 ± 0.03^{c}	5.50 ± 0.20^{b}	53 ± 0.05^{c}	1.62 ± 0.02^{a}
BFT10	0.031 ± 0.00	0.86 ± 0.01^{bc}	0.82 ± 0.03^{c}	5.61 ± 0.11^{b}	55 ± 0.05^{bc}	1.52 ± 0.02^{cd}
BFT20	0.032 ± 0.00	1.02 ± 0.08^{b}	0.99 ± 0.08^{b}	5.81 ± 0.16^{ab}	59 ± 0.15^{ab}	1.55 ± 0.03^{bc}
BFT30	0.032 ± 0.00	1.30 ± 0.06^{a}	1.27 ± 0.06^{a}	6.21 ± 0.01^{a}	62 ± 0.20^{a}	1.38 ± 0.01^{e}
BFT40	0.033 ± 0.00	0.99 ± 0.01^{bc}	0.96 ± 0.01^{bc}	5.84 ± 0.01^{ab}	60 ± 0.08^{a}	1.44 ± 0.03^{de}
P-value	.381	.003	.003	.030	.001	.002

FCR feed conversion ratio, *SGR*(*%/day*) specific growth rate (%/day)

In each column, mean values with different superscripts differ significantly at ($P < 0.05$)

Table 3 Bacterial microbiota in the culture water (cfu/ml) of *P. vannamei* fed with various levels of biofloc meal incorporated diets

Time (week)	Treatments	TPC (cfu/ml)	TVC (cfu/ml)	TLC (cfu/ml)
0^{th} day	**C**	$3.64 \pm 0.11 \times 10^{4}$	$4.86 \pm 0.51 \times 10^{3}$	$1.98 \pm 0.79 \times 10^{2}$
	BFT0	$3.71 \pm 0.39 \times 10^{4}$	$5.10 \pm 0.15 \times 10^{3}$	$2.04 \pm 0.14 \times 10^{2}$
	BFT10	$3.55 \pm 0.36 \times 10^{4}$	$5.17 \pm 1.22 \times 10^{3}$	$1.85 \pm 0.60 \times 10^{2}$
	BFT20	$3.53 \pm 0.56 \times 10^{4}$	$4.65 \pm 1.03 \times 10^{3}$	$1.92 \pm 0.37 \times 10^{2}$
	BFT30	$3.57 \pm 0.27 \times 10^{4}$	$4.62 \pm 0.32 \times 10^{3}$	$1.98 \pm 0.51 \times 10^{2}$
	BFT40	$3.21 \pm 0.33 \times 10^{4}$	$4.97 \pm 0.41 \times 10^{3}$	$2.04 \pm 0.37 \times 10^{2}$
	***P*-value**	.287	.162	.124
30^{th} day	**C**	$5.60 \pm 0.10 \times 10^{4(ab)}$	$1.23 \pm 0.09 \times 10^{3(e)}$	$6.26 \pm 0.28 \times 10^{2(a)}$
	BFT0	$1.29 \pm 0.15 \times 10^{4(e)}$	$1.71 \pm 0.07 \times 10^{3(d)}$	$3.17 \pm 0.33 \times 10^{2(cd)}$
	BFT10	$2.75 \pm 0.09 \times 10^{4(d)}$	$2.07 \pm 0.04 \times 10^{3(cd)}$	$2.40 \pm 0.28 \times 10^{2(d)}$
	BFT20	$4.15 \pm 0.25 \times 10^{4(c)}$	$2.56 \pm 0.14 \times 10^{3(b)}$	$5.88 \pm 0 .70 \times 10^{2(ab)}$
	BFT30	$6.30 \pm 0.60 \times 10^{4(a)}$	$2.95 \pm 0.15 \times 10^{3(a)}$	$3.52 \pm 0.15 \times 10^{2(cd)}$
	BFT40	$4.65 \pm 0.25 \times 10^{4(bc)}$	$2.23 \pm 0.11 \times 10^{3(bc)}$	$4.64 \pm 0.50 \times 10^{2(bc)}$
	***P*-value**	.001	.000	.003
60^{th} day	**C**	$2.85 \pm 0.05 \times 10^{5(ab)}$	$4.73 \pm 0.17 \times 10^{4(a)}$	$3.17 \pm 0.30 \times 10^{3(bcd)}$
	BFT0	$1.65 \pm 0.05 \times 10^{5(d)}$	$2.26 \pm 0.11 \times 10^{4(e)}$	$2.66 \pm 0.21 \times 10^{3(cd)}$
	BFT10	$1.35 \pm 0.15 \times 10^{5(d)}$	$3.31 \pm 0.16 \times 10^{4(bc)}$	$2.49 \pm 0.15 \times 10^{3(d)}$
	BFT20	$2.20 \pm 0.11 \times 10^{5(c)}$	$2.97 \pm 0.25 \times 10^{4(cd)}$	$3.37 \pm 0.21 \times 10^{3(bc)}$
	BFT30	$2.71 \pm 0.20 \times 10^{5(b)}$	$2.54 \pm 0.17 \times 10^{4(de)}$	$4.79 \pm 0.34 \times 10^{3(a)}$
	BFT40	$3.24 \pm 0.10 \times 10^{5(a)}$	$3.72 \pm 0.26 \times 10^{4(b)}$	$3.71 \pm 0.03 \times 10^{3(b)}$
	***P*-value**	.000	.001	.004

TPC (*cfu/ml*) total plate count (cfu/ml), *TVC* (*cfu/ml*) total *vibrio* count (cfu/ml), *TLC* (*cfu/ml*) total *lactobacillus* count (cfu/ml)

In each column, mean values with different superscripts differ significantly at ($P < 0.05$)

Bacteriological analysis

The present study found a significant difference ($P < 0.05$) (Table 3) in the total plate count of culture water among the different treatments diets reared shrimp and at the end of the experiment, significantly higher ($3.24 \pm 0.10 \times 10^{5}$) TPC was recorded in BFT40 culture water. In total *Vibrio* count, a significant difference ($P < 0.05$) was noted in 30^{th} and 60^{th} day of culture water. At the end of experiment, significantly higher *Vibrio* count was noticed in the control group ($4.73 \pm 0.17 \times 10^{4}$ cfu/ml). Differences in the *Lactobacillus* count were observed throughout the experiment. Shrimp fed BFT30 diet showed significantly higher ($4.79 \pm 0.34 \times 10^{3}$ cfu/ml) *Lactobacillus* count in culture water at the end of experiment.

In the GIT of shrimp, significant difference ($P < 0.05$) in total plate count and *Vibrio* count were observed among the different treatments (Table 4). Initially, higher total plate count was observed in the BFT40 treatment ($5.04 \pm 0.10 \times 10^{4}$ cfu/g) whereas the count has been found to be higher in the BFT30 group ($5.08 \pm 0.21 \times 10^{6}$ cfu/g) at the end of the feeding trial. Significantly higher *Vibrio* count ($4.83 \pm 0.28 \times 10^{5}$ cfu/g) was recorded in the control group compared to the treatments fed with biofloc incorporated diets at the final sampling. Total *Lactobacillus* count ($P > 0.05$) was differed among the

Table 4 Microbial community in the gastro intestinal tract (cfu/g) of *P. vannamei* fed with various levels of biofloc meal incorporated diets

	TPC		TVC		TLC	
Treatments	30^{th} day (cfu/g)	60^{th} day (cfu/g)	30^{th} day (cfu/g)	60^{th} day (cfu/g)	30^{th} day (cfu/g)	60^{th} day (cfu/g)
BFT0	$1.73 \pm 0.20 \times 10^{4(d)}$	$2.21 \pm 0.26 \times 10^{6(c)}$	$1.22 \pm 0.02 \times 10^{3(c)}$	$1.65 \pm 0.31 \times 10^{5(c)}$	$1.84 \pm 0.06 \times 10^{2(c)}$	$2.74 \pm 0.24 \times 10^{4(c)}$
BFT10	$2.54 \pm 0.15 \times 10^{4(cd)}$	$1.89 \pm 0.20 \times 10^{6(c)}$	$2.64 \pm 0.04 \times 10^{3(b)}$	$2.87 \pm 0.09 \times 10^{5(b)}$	$2.33 \pm 0.30 \times 10^{2(c)}$	$3.47 \pm 0.21 \times 10^{4(c)}$
BFT20	$2.82 \pm 0.20 \times 10^{4(bcd)}$	$4.55 \pm 0.22 \times 10^{6(ab)}$	$3.94 \pm 0.04 \times 10^{3(bc)}$	$3.56 \pm 0.42 \times 10^{5(b)}$	$3.56 \pm 0.81 \times 10^{2(b)}$	$4.87 \pm 0.28 \times 10^{4(b)}$
BFT30	$4.89 \pm 0.31 \times 10^{4(ab)}$	$5.08 \pm 0.21 \times 10^{6(a)}$	$3.48 \pm 0.06 \times 10^{3(b)}$	$3.16 \pm 0.48 \times 10^{5(b)}$	$4.98 \pm 1.09 \times 10^{2(a)}$	$6.56 \pm 0.21 \times 10^{4(a)}$
BFT40	$5.04 \pm 0.10 \times 10^{4(a)}$	$4.60 \pm 0.30 \times 10^{6(ab)}$	$4.38 \pm 0.07 \times 10^{3(ab)}$	$4.06 \pm 0.28 \times 10^{5(ab)}$	$3.84 \pm 0.10 \times 10^{2(b)}$	$5.34 \pm 0.36 \times 10^{4(ab)}$
C	$4.21 \pm 0.24 \times 10^{4(abc)}$	$4.05 \pm 0.20 \times 10^{6(b)}$	$5.34 \pm 0.22 \times 10^{3(a)}$	$4.83 \pm 0.28 \times 10^{5(a)}$	$3.25 \pm 0.24 \times 10^{2(bc)}$	$4.54 \pm 0.07 \times 10^{4(b)}$
P-value	.011	.001	.001	.001	.031	.001

TPC (*cfu/g*) total plate count, *TVC* (*cfu/g*) total *Vibrio* count, *TLC* total *Lactobacillus* count(cfu/g)

In each column, mean values with different superscripts differ significantly at ($P < 0.05$)

treatments and significantly higher total *Lactobacillus* count was recorded in BFT30 group ($6.56 \pm 0.21 \times 10^4$ cfu/g) at the end of the feeding trial.

Digestive enzyme activity

A significant difference ($P < 0.05$) in amylase and lipase activities was noted among the shrimp groups fed with biofloc incorporated diets (Fig. 1 and Fig. 2). Among these, significantly higher and lower values of amylase and lipase activities were recorded in BFT30 (4.75 ± 0.87 U/mg protein and 120 ± 4.38 U/mg protein) and control groups (3.86 ± 0.48 U/mg protein and 51 ± 10.42 U/mg protein), respectively. Significantly higher cellulase activity was observed in the BFT20 group (34.14 ± 4.40 U/mg protein) and the lower activity was observed in the BFT0 group (22.39 ± 3.38 U/mg protein) (Fig. 3).

Immunological parameters

There was a significant difference in serum protein levels among the different treatment groups. The BFT30 group showed significantly higher value (1.6 ± 0.02 U/mg protein) of total serum protein (Fig. 4). The prophenoloxidase (PO) was significantly higher in BFT30 (8.93 ± 0.46 U/mg protein) followed by BFT10 (9.60 ± 0.61 U/mg protein) compared to other treatment groups. PO activity in BFT30 shrimp had two-fold increase than the control group (Fig. 5). The significant difference in lysozyme activity was found among the different treatment groups. The significantly higher lysozyme activity was observed in the BFT30 (43 ± 0.65 U/ml) (Fig. 6).

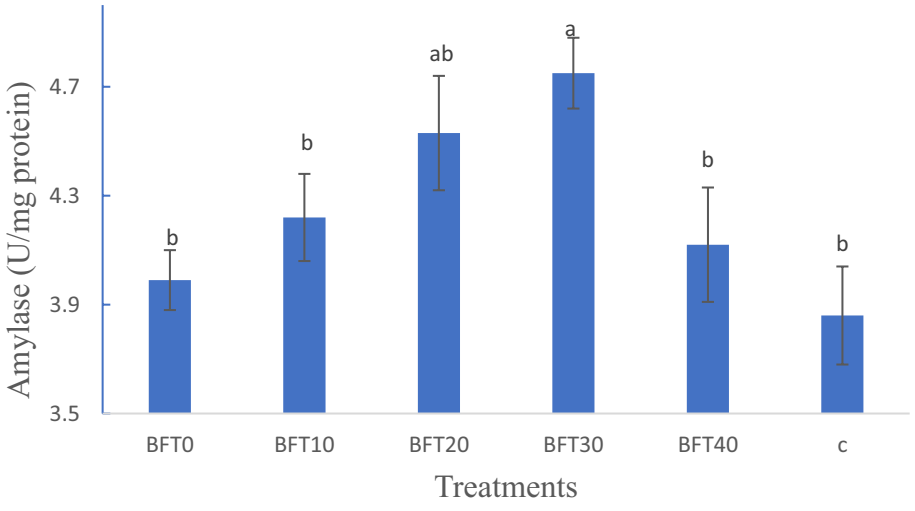

Fig. 1 Specific activity of GIT amylase (U/mg protein) of shrimp fed with different levels of biofloc meal incorporated diets. Bars with different superscripts differ significantly at $P < 0.05$

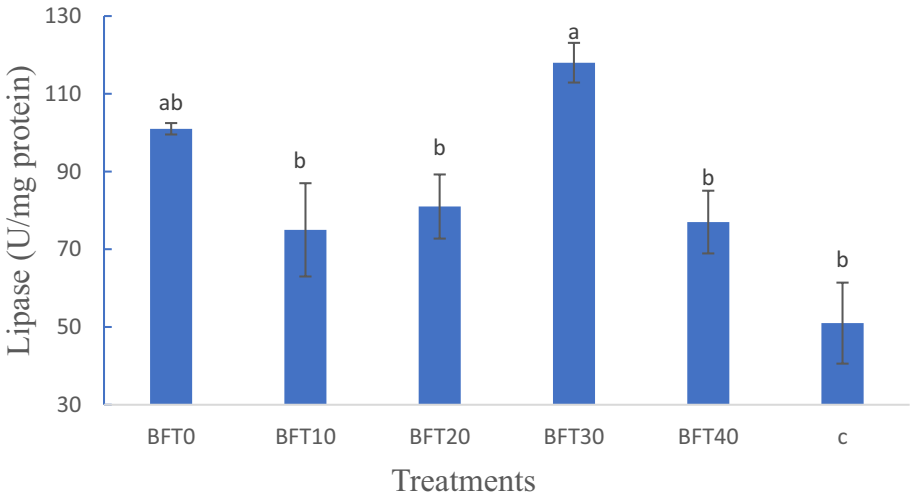

Fig. 2 Specific activity of GIT lipase (U/mg protein) of shrimp fed with different levels of biofloc meal incorporated diets. Bars with different superscripts differ significantly at $P < 0.05$

Antioxidant enzymes

There was no significant difference in the SOD activities of different treatments reared shrimp (Fig. 7). However, higher SOD was recorded in control (38.09 ± 0.06 U/mg protein) followed by BFT40 (37.05 ± 0.06 U/mg protein) and lower superoxide dismutase activity was observed in BFT30 (36.13 ± 0.16 U/mg protein). Likewise, the glutathione did not show any significant difference ($P < 0.05$) among the treatments (Fig. 8).

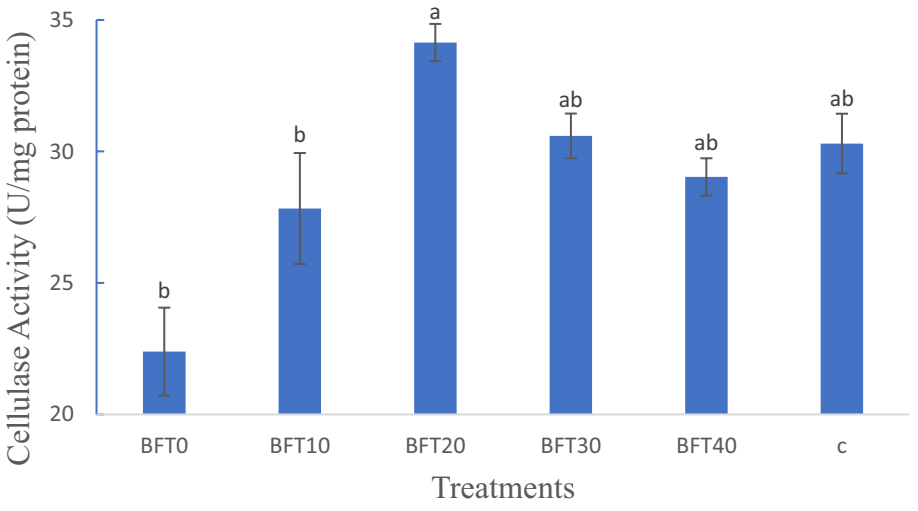

Fig. 3 Specific activity of GIT cellulase (U/mg protein) of shrimp fed with different levels of biofloc meal incorporated diets. Bars with different superscripts differ significantly at $P < 0.05$

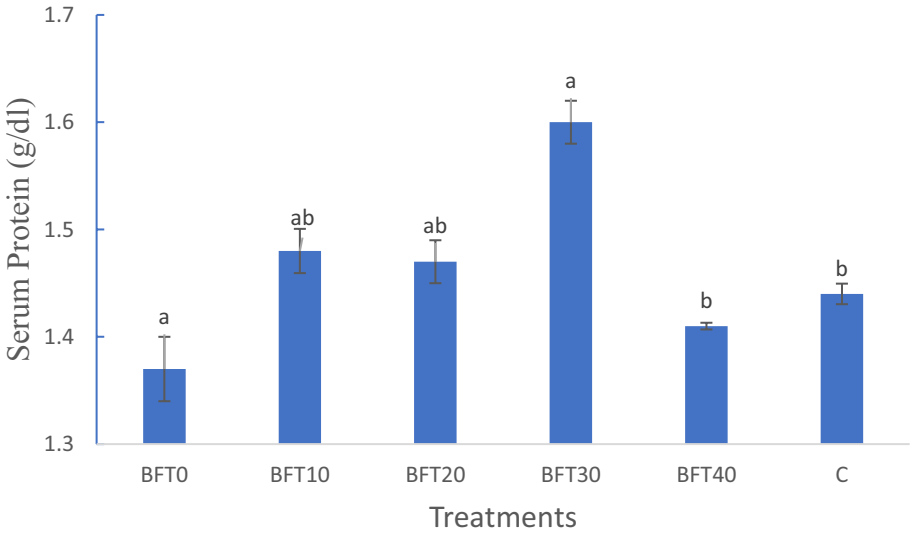

Fig. 4 Specific activity of serum protein (g/dl) of shrimp fed with different levels of biofloc meal incorporated diets. Bars with different superscripts differ significantly at $P < 0.05$

Histological analysis

The study found significantly higher numbers of tubules in the proximal portion of the BFT30 group reared animals with larger proportion of cells with intracellular digestion (B) and absorption (R). The R and B cells in BFT30 group were found predominantly on the tubules of intestinal tissues of the animals fed. In other treatments, B cells were regular in

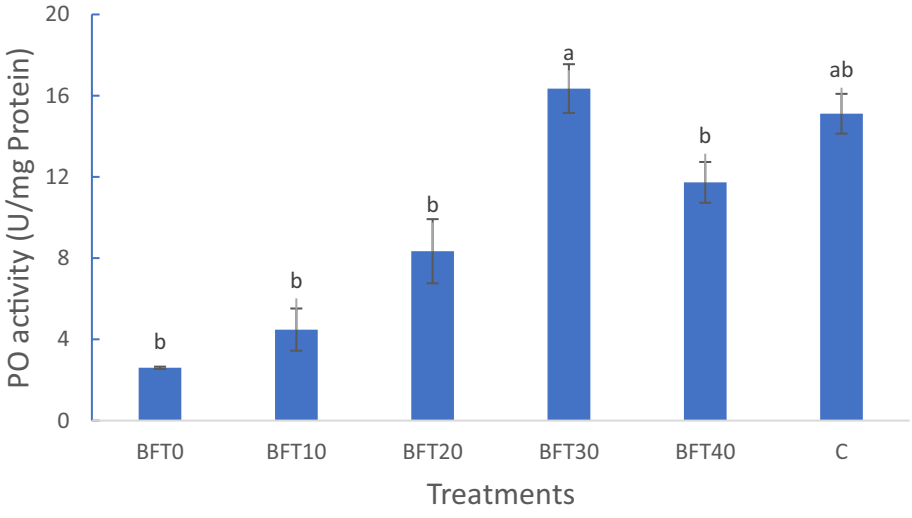

Fig. 5 Specific activity of prophenoloxidase (U/mg protein) from shrimp fed with different levels of biofloc meal incorporated diets. Bars with different superscripts differ significantly at $P < 0.05$

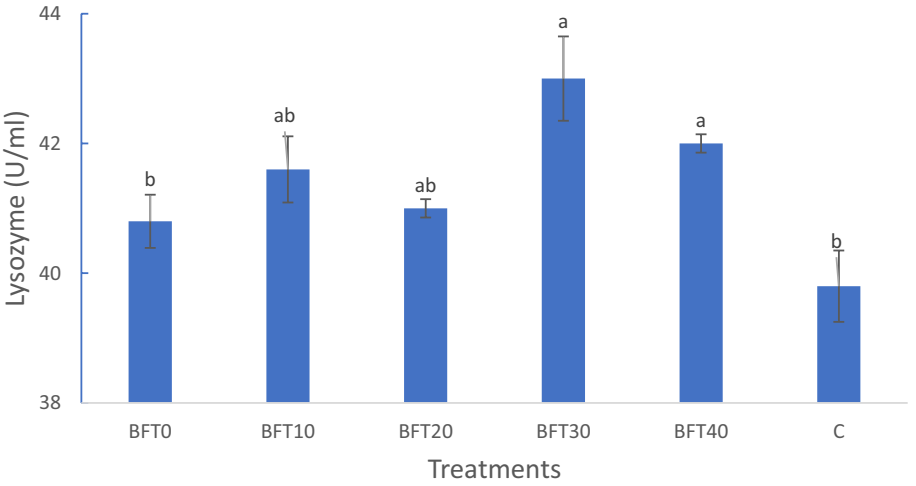

Fig. 6 Specific activity of lysozyme (U/ml) from shrimp fed with different levels of biofloc meal incorporated diets. Bars with different superscripts differ significantly at $P < 0.05$

the proximal and middle regions, whereas R cells had reduced proportions reduced towards the blind end of the tubules (Fig. 9).

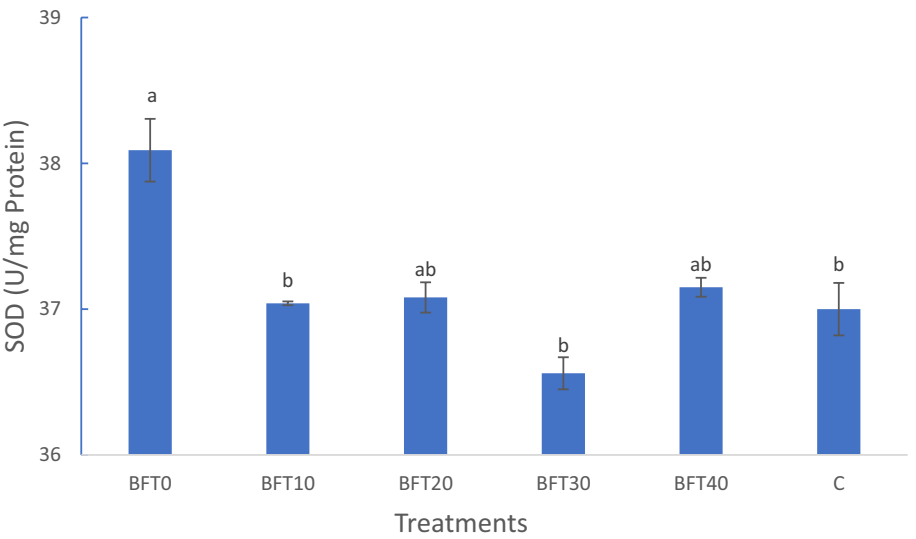

Fig. 7 Specific activity of SOD (U/mg protein) of shrimp fed with different levels of biofloc meal incorporated diets. Bars with different superscripts differ significantly at $P < 0.05$

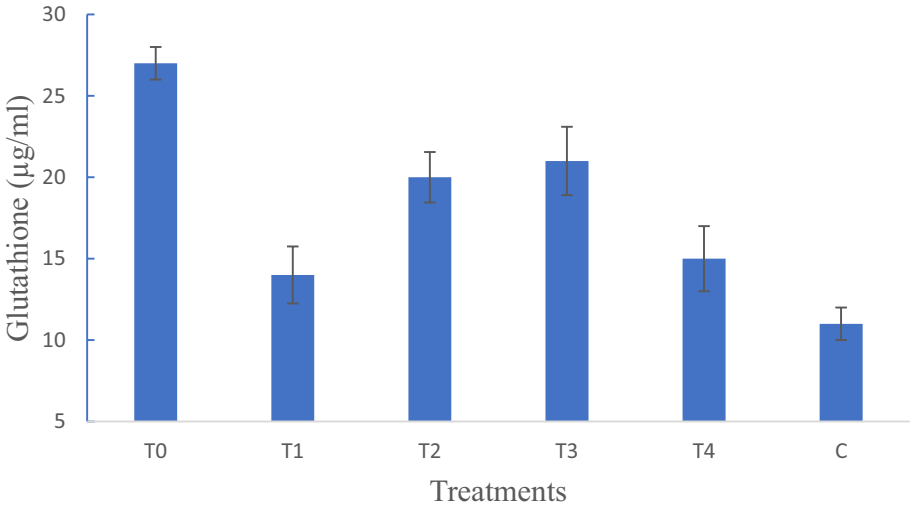

Fig. 8 Specific activity of glutathione (µg/ml) from shrimp fed with different levels of biofloc meal incorporated diets

Discussion

Fishmeal, a major source of protein, is a main feed ingredient which fulfills nutritional requirements of fish under captive conditions through the pellet feed, especially in shrimp and carnivorous fish farming. Fishmeal is a limited source of feed ingredient and its availability varies based on the production of capture fisheries which in turn threatened by the contemporary environmental changes. According to (FAO 2012), the formulated shrimp feed should contain high protein (> 400 g/kg), adequate lipid (< 100 g/kg), and low ash (< 160 g/kg) contents. On the other hand, biofloc meal contains lipid in the range of 230.9 to 420 g/kg and protein in the range of 30 to 45% and its composition is influenced by the microalgae and zooplankton diversity (Wasielesky et al. 2006; Avnimelech 2009; Bauer et al. 2012; Valle et al. 2015). Similarly, crude protein content in the biofloc relies on the bacterial communication, microorganisms, inorganic particles, and zooplanktons (Promthale et al. 2019). Moreover, similarity in nutritional composition and EAA (essential amino acids) of fish meal and biofloc, suggesting that, biofloc could replace fishmeal in shrimp feed (FAO 2012). Therefore, the urge for sustainable alternative source toss replace the fish meal paved a way to conduct various studies on replacement of fishmeal using biofloc meal, especially in shrimp diets.

The study found significantly increased weight gain, SGR, survival rate, and better FCR in BFT30 diet fed group. Study on *L. vannamei* showed that shrimp fed with biofloc meal, at various inclusion level from 0 to 30%, had resulted in positive impact over the growth and survival (Kuhn et al. 2010). Further, they found that inclusion level of biofloc meal over 30% in fish pellet did not enhance growth of the pacific white shrimp, *L. vannamei*. Likewise, it has been recommended that fishmeal in *L. vannamei* diet can be replaced up to 30% by biofloc meal (Bauer et al. 2012). Previous studies suggest that higher amount of biofloc and microbial products in fish feed might decrease the feed palatability and digestibility of *P. vannamei* (Promthale et al. 2019). However, studies on the biofloc culture of

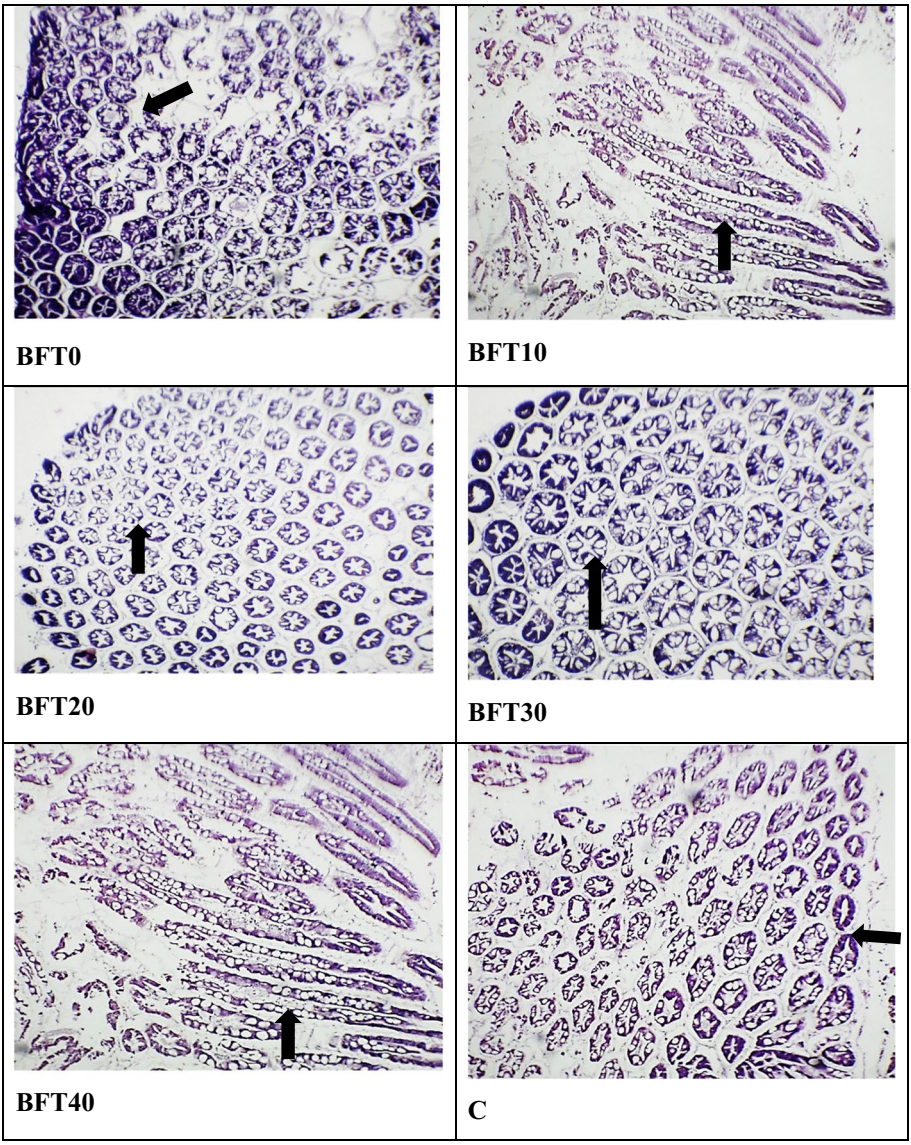

Fig. 9 Microphotographs of hepatopancreas under 100 × magnification; C, T0: Normal structure of tubules, lumen and cells, BFT10: Change in structure of tubule and cell elongation, BFT20: Mild disintegration of lumen with increased B and R cells, BFT30: Increased B cells with subapical vacuoles occupying most of the cytoplasm, BFT40: Change in structure of tubule and cell elongation

L. vannamei revealed that the shrimp displayed significantly improved digestive enzyme activities along with the enhanced growth performance (Promthale et al. 2019). The presence of microbial elements or probiotic strains *Bacillus* and *Lactobacillus* and their

supplementation of endogenous enzymes could be the possible reason for enhanced growth of shrimp reared in biofloc meal incorporated diet treatment groups (Tamilarasu et al. 2021).

The counts of heterotrophic bacteria were significantly higher in the biofloc treatment groups compared to the control, which is similar to the result of previous studies, where bacteria count was from 10^6 to 10^7 cfu/ml in zero exchange water intensive ponds (Otoshi et al. 2006; Avnimelech 2012) and 3.35 to 5.42×10^7 cfu/ml in intensive shrimp ponds (Burford et al. 2004). The study also revealed decreased counts of *Vibrio* in all the treatment groups, especially significant reduction in the BFT40 treatment compared to the control, which may be due to the presence of extracellular substance and antimicrobial peptides, from the load of beneficial bacteria in the biofloc feed. Research findings revealed that shrimp fed with biofloc meal were less susceptible to *V. parahaemolyticus* (Promthale et al. 2019). The dietary supplementation of probiotic strains, isolated from the biofloc system, also improved the growth, digestion, and survival against pathogenic bacteria (Tamilarasu et al. 2021).

The present study revealed that specific activities of lipase and amylase in the gut of cultured shrimp improved significantly with 30% inclusion of biofloc meal. The production and activity of digestive enzyme are endogenously managed by the digestive system and physiology rhythms of shrimp (Hernandez-Cortes et al. 1999). Previous studies of biofloc culture had found positive effect on digestive enzyme (especially amylase) activities in the GIT of cultured shrimp (Xu and Pan 2012; Xu et al. 2012). When feed enters into stomach of the shrimp, it releases the microbial protease and amylase, produced by the microorganisms present in the biofloc meal, which enhance the digestive enzyme activities in the stomach of cultured shrimp (Xu et al. 2012; Cavalcanti Nery et al. 2019). The activity of lipase showed significant increase in the gut of cultured shrimp fed with biofloc supplemented diet. Increase of dietary lipids in BFT30 shrimp feed might have enhanced lipolytic activity of that group at the end of the culture.

Interestingly, the study found higher survival of shrimp, with the enhanced immune activities, in the BFT30 group. The enzyme prophenoloxidase acts as the defense mechanism in the shrimp that causes melanization and inactivation of foreign cells which evade the pathogen infection in shrimp. This enzyme is stimulated by the lipopolysaccharides (LPS) and β-1,3-glucans that triggers the prophenoloxidase 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 biofloc incorporated meal when compared to the control group. It is reported that the *Bacillus* bacteria present in biofloc will modify the immunological and physiological status of the shrimp gastrointestinal tract which subsequently adjust the endogenous microbiota and inhibits the colonization of pathogenic bacteria in shrimp GIT (Zhao et al. 2012).

Invertebrates, especially shrimp, use innate immune responses, whereas the vertebrates, while encountering a foreign substance, it activates the acquired immune responses (Tseng et al. 2009). Immune status of the fish is indicated by the concentration of the serum protein. Higher concentration directly relates with stronger immune response. Serum proteins (albumin and globulin) are novel components which are essential for efficient functioning of the immune system (Yengkokpam et al. 2016). The present study showed significant difference in total serum protein among the different treatment groups. Similarly, increased serum protein in shrimp was reported when fed with probiotic bacteria compared to control due to the enriched bacterial load in feed (Ferreira et al. 2015).

Hepatopancreas is one of the health indicators in shrimp which can be used to identify the shrimp health condition. It is the main organ for food absorption, transport, secretion of

digestive enzymes and storage of lipids, glycogen, and few minerals. In the control group, hepatopancreas was normal along proximal and apical regions. Higher numbers of the tubules in the proximal portion of BFT30 group indicated that there is a greater quantity of materials ingested by the animals, which would justify intracellular digestion (B) and absorption (R) in large proportion of cells. The increase of B cells facilitates the synthesis and excretion of digestive enzymes, which enable *P. vannamei* to derive more energy from the ingested food by mobilizing the nutrients in hepatopancreas tubules.

Overall, the results demonstrated that inclusion of biofloc meal, up to 30% level, has positive effect in the shrimp health in terms of enhanced growth performance, boosted immunity, and improvised digestive enzyme activity. However, the inappropriate incorporation of biofloc meal, either below 30% or above 30%, in shrimp diet resulted in poor growth and feed utilization. Therefore, incorporation of biofloc meal in shrimp diet, at optimal level (30%), provides a promising substitutive protein source to fishmeal, which in turn would produce positive impact on feed mill industry and environment.

Author contribution M. Nethaji conducted this research and has drafted the manuscript. B. Ahilan aided in conducting this research. A. Kathirvel Pandiyan has reviewed and corrected this paper. N. Felix assisted in feed preparation and drafting of this paper. A. Uma assisted in laboratory works and results interpretation. TLS. Samuel mosses assisted in sampling, data collection, and lab works. R. Somu Sunder Lingam contributed in data analysis and assisted in manuscript preparation.

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Data availability Sufficient data has been provided in the form of tables and figures.

Declarations

Ethics approval Please note that the manuscript does not need an ethical approval. You are therefore kindly requested to consider the manuscript.

Conflict of interest The authors declare no competing interests.

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