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Bioflocs substituted fishmeal feed stimulates immune response and protects shrimp from *Vibrio parahaemolyticus* infection

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ABSTRACT

Fishmeal is the main source of protein in the shrimp feed industry and is normally derived from trash fish. As such, the production of fishmeal has an adverse effect on the marine environment by taking away small and juvenile fish, leading to depletion of marine species. There is a need for alternative sources of protein which will substitute fishmeal in the aquaculture industry. This study evaluated the components and nutritional efficacy of bioflocs, which were used to substitute fishmeal protein. The effect of bioflocs diets on growth performance, survival rate, and immune response in shrimp compared to normal fishmeal feed were determined. Bioflocs were harvested from the shrimp ponds (C:N ratio >12:1) at Shrimp Village, Chaiya district, Surat Thani, Thailand. The total protein in bioflocs was about 48% and the total lipid was about 5% (dried weight) and the percentages of essential amino acids (EAA) and fatty acids (EFA) in bioflocs were similar to those of fishmeal feed. Shrimp fed with the different dietary bioflocs feed regimens [% to replace fishmeal; 0% (B0), 25% (B25), 50% (B50), 75% (B75), and 100% (B100)] for 42 days revealed that all growth parameters were almost similar to those of the control shrimp (shrimp fed with normal fishmeal, B0) including final body weight, weight gain, specific growth rate, and feed conversion ratio. Remarkably, the survival rates, the levels of immune parameters, and expression of immune genes (proPO-I, PEN-4 and dicer) were significantly higher in bioflocs fed shrimp, especially in B25 and B50 shrimp. Moreover, B25 and B50 bioflocs fed shrimp showed notably increased survival rates following *Vibrio parahaemolyticus (V. parahaemolyticus)* infection. In conclusion, the present study demonstrates that shrimp survival and immunity are enhanced by biofiocs substituted fishmeal. Significantly, the bioflocs diets activated the immune response to prevent *V. parahaemolyticus* infection.

1. Introduction

Penaeid shrimp is acknowledged to be the fastest-growing aquaculture economy in the world [1,2]. Among the various nutritional components of shrimp feed, protein is the most important factor affecting the survival and growth of the shrimp. In general, the shrimp require 30–45% protein in the feed (dry weight), and in particular, *Penaeus monodon (P. monodon)* need a protein content >40%. Fishmeal is an important source of protein in commercial shrimp feed and is also costly. Moreover, a major concern is that its use by capture fisheries is causing a decrease of marine species [3]. Hence, the aquaculture industry needs to seek alternative sources of protein to substitute for fishmeal. In this regard researchers are studying alternative ingredients from plant products, for instance, corn, cottonseed, canola and soybean [4]. However, factors limiting the use of these products include low digestibility, low palatability, anti-nutritional factors, amino acid deficiency and also high cost [5].

Biofloc technology (BFT) has been widely used in shrimp ponds for enhancing water quality through the addition of extra carbon sources to the aquaculture system. An external carbon source promotes nitrogen

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uptake by bacterial growth and thereby decreases the nitrogen and phosphorus waste in the shrimp ponds [6]. The BFT therefore improves aquaculture productivity and reduce environmental impact in aquaculture units [7,8]. However, this system must also be constantly monitored due to possible pollution from nitrate accumulation [9].

Bioflocs can be taken up by aquaculture species, i.e., shrimp [10–12], red tilapia [13], nile tilapia [14,15] and artemia [16,17], and that uptake is different, depending on the species and its size and feeding traits, as well as bioflocs size and density [16–18]. Bioflocs comprise bacterial colonization, fungi, microalgae, zooplankton such as copepods, rotifers, and amphipods and also tiny nematodes gathered together [18]. These organisms serve as an alternative source of protein in shrimp feed [14]. Moreover, it has been reported that there is a decrease in the feed conversion ratio (FCR) and an increase in growth and survival rates of shrimp raised in BFT ponds [14,19]. Bioflocs residues have been proposed as an alternative source for fishmeal protein and are cost effective [14,20,21]. Recent reports suggested that dried bioflocs could be used to substitute fishmeal in shrimp pellets [6] since they contain various bioactive compounds including proteins, carotenoids, chlorophylls, vitamin C and trace minerals [22–24]. However, the use of bioflocs to substitute for fishmeal in shrimp feed necessitates an understanding of its component nutritional value, and scientific data on EAAs and EFAs content is still limited.

A recent study showed enhanced levels of immune genes, and prophenoloxidase-activating enzyme1 (PPAE1), serine proteinase1 (SP1), masquerade-like serine proteinase (mas) and ras-related nuclear protein (Ran) were found in shrimp raised in BFT ponds [19]. We hypothesized that the cell components or the metabolites of microorganisms in bioflocs might serve as immunostimulants to strengthen the shrimp innate immune system and might provide better protection against pathogens [25]. In the present study, we sought to harvest bioflocs powder from shrimp ponds, and to formulate it in shrimp feed pellets. The component and nutritional values of bioflocs were investigated, and the effects of bioflocs diets on the growth performance, survival rate and immune response of shrimp were monitored and assessed. Findings from this study demonstrate that bioflocs contain nutritional values that can be a preferential fishmeal feed replacement for the shrimp feed industry, providing for cost reduction and a sustainable aquaculture that is environment friendly.

2. Materials and methods

2.1. Bioflocs powder

Bioflocs were collected from rearing brackish water of *P. monodon* cultured in round canvas ponds (12 m diameter and 0.8 m depth) at the Shrimp Village, Chaiya district, Surat Thani, Thailand. The BFT system was cultured for 4 weeks using molasses as the carbon source with the C:N ratio >12:1. The rearing system was carried out at 26–28 °C, 25 ppt salinity and pH 7.8–8.2. There was no medicine used in the cultured ponds. The collected bioflocs were dried at 40 °C for 48 h, crushed into powder, and the nutritional components of bioflocs were then analyzed.

2.2. Proximate analysis of bioflocs powder and bioflocs supplemented diet

Proximate compositions of crude protein, crude lipid, ash, moisture, crude fiber and nitrogen free extract (NFE; carbohydrate) were analyzed according to the standard methods of AOAC (2012) [26].

2.3. Determination of amino acid and fatty acid profiles

The amino acid profiles were analyzed by analytical reversed-phase high performance liquid chromatography (HPLC) according to Guo et al. (2007) [27]. Fatty acid profiles were analyzed using gas chromatography (GC) according to AOAC (2012) [26]. Essential amino acid ratio (A/E) and essential amino acid index (EAAI) in both dried bipoflocs and feed were estimated according to Penaflorida (1989) [28] as follows:

Essential amino acid ratio (A/E) = aa_1/AA_1

Essential amino acid index (EAAI) = $\sqrt[10]{aa1/AA1 \times aa2/AA2 \times \ldots \times aa10/AA10}$

Where aa is the amount of an EAA in the dried bioflocs or fishmeal and AA is the required amount of the same EAA for the shrimp [2]. 1 through 10 represents each of the EAAs. Values for A/E and EAAI higher than 1.00 were considered as 1.00.

2.4. Effects of bioflocs diets on growth performance, survival rate and immune response in shrimp

2.4.1. Shrimp and bioflocs feed

Healthy juvenile *P. monodon,* averaged weight 10–20 g, were obtained from Shrimp Village, Chaiya district, Surat Thani, Thailand. The feeding trial was conducted according to current animal welfare regulations: Protocol No. MUSC61-006-408 from Faculty of Science, Mahidol University. Shrimp were kept in bio-filter laboratory tanks containing seawater (20 ppt salinity) at 26 °C, fed with general commercial feed, and were acclimatized for 7 days. Four bioflocs diet regimens were prepared with bioflocs incorporated into fishmeal at 25, 50, 75 and 100% (% dry mass: w/w) namely B25, B50, B75 and B100, respectively. The composition of each bioflocs diet formula is shown in Table 1. The total protein fraction in all diets was 37%, which is a suitable nutritional value for *P. monodon* sized 15–20 g [2]. The fishmeal diet with no bioflocs was used as the normal control diet (B0). Four hundred and fifty shrimp were equally distributed into 15 tanks (30 per tank) and groups of shrimp (3 tanks per group) were fed a diet with either fishmeal as a sole protein source (B0 shrimp) or a diet of fishmeal substituted with bioflocs at 25, 50, 75 and 100% w/w (B25, B50, B75 and B100 shrimp, respectively). The shrimp were fed twice daily with the bioflocs feed meal (3% of body weight per day) at 8 a.m. and 4 p.m. for 42 days.

2.4.2. The growth performance and survival rate of shrimp

The growth performance of the shrimp was determined using final body weight, weight gain, specific growth rate (SGR), FCR, and survival rate following the 42day feeding treatment (n = 90). The parameters were calculated based on the following formulas [22]:

Final weight (%) = 100 x (Final body weight / Initial body weight)

Weight gain (%) = 100 x [(Final body weight) - (Initial body weight)] / Initial body weight

Specific growth rate (% /day) = 100 x [Ln (Final body weight) - Ln (Initial body weight)] / Experiment duration (days)

Feed conversion ratio = Total dry weight of feed offered / Total shrimp weight gained

Survival rate (%) = 100 x (Final shrimp count / Initial shrimp count)

2.4.3. Immune parameters analysis

Ten shrimp from each diet group were analyzed for immune

Table 1
Ingredient composition of bioflocs substituted diets (B0 to B100) (% dry mass: w/w) by proximate analysis, including crude protein, crude lipid, ash, moisture, crude fiber and nitrogen free extract [NFE]; carbohydrate. Data are presented as a mean of triplicate independent experiments (mean ± SD).

Ingredients (%)	B0	B25	B50	B75	B100
Fishmeal	60.0	45.0	30.0	15.0	0.0
Bioflocs meal	0	15.0	30.0	45.0	60.0
Soybean meal	13.6	14.6	15.5	16.5	17.4
Wheat gluten	3.5	3.5	3.5	3.5	3.5
Squid oil	2.0	2.0	2.0	2.0	2.0
Soybean lecithin	1.5	1.5	1.5	1.5	1.5
Cholesterol	0.5	0.5	0.5	0.5	0.5
Wheat flour	12.0	12.0	12.0	12.0	12.0
Cellulose	5.2	4.2	3.3	2.3	1.4
Mineral premix	1.0	1.0	1.0	1.0	1.0
Vitamin premix	0.7	0.7	0.7	0.7	0.7
Proximate composition (% dry mass: w/w)	**B0**	**B25**	**B50**	**B75**	**B100**
Crude protein (CP)	37.49 ± 0.02	37.47 ± 0.18	37.47 ± 0.13	37.45 ± 0.32	37.44 ± 0.69
Crude lipid (EE)	7.73 ± 0.11	8.65 ± 0.19	8.62 ± 0.11	9.23 ± 0.33	10.15 ± 0.07
Ash	13.23 ± 0.10	14.98 ± 0.50	15.48 ± 0.30	16.43 ± 0.78	16.84 ± 0.33
Moisture	9.91 ± 0.00	7.90 ± 0.01	7.89 ± 0.01	11.86 ± 0.01	10.85 ± 0.01
Crude fiber (CF)	2.50 ± 0.01	2.77 ± 0.00	2.85 ± 0.00	3.72 ± 0.00	3.67 ± 0.01
Nitrogen free extract (NFE)[a]	29.14 ± 1.40	28.23 ± 0.88	27.69 ± 1.44	21.31 ± 0.69	21.05 ± 0.75

[a] NFE = 100-(CP + EE + CF + ash + moisture).

parameters, including total haemocyte count (THC), differential haemocyte count, phagocytic activity and phenoloxidase (PO) activity at day 0, 7, 21 and 42, and transcripts of immune related genes at day 0 and 42.

2.4.3.1. Total and differential haemocyte count. THC and differential haemocyte count were performed according to the methods described previously [29]. Haemolymph was withdrawn (100 µl) from the ventral sinus of each shrimp into a 1 ml syringe, fixed with an equal volume of 10% formalin in 0.45 M NaCl (ratio = 1:1), and incubated at room temperature for 10 min. An aliquot of 10 µl was transferred to a haemocytometer and incubated at room temperature for 5 min before counting. THC was expressed as the number of cells/mm^3. For differential haemocyte count, an aliquot of 20 µl was mixed with 20 µl of Rose Bengal solution, incubated for 20 min at room temperature, then smeared on glass slide, counterstained with haematoxylin solution. Number of semi-granular cells, granular cells, and hyaline cells on a slide were counted under a light microscope (Olympus BX53, Olympus, Tokyo, Japan).

2.4.3.2. Phagocytic activity of haemocytes. Phagocytic activity of haemocytes was measured using latex beads according to the method described previously [30] with a slight modification. Briefly, haemolymph (100 µl) was drawn into a 1 ml syringe containing 100 µl of anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, and 10 mM EDTA-Na_2, at a pH of 7.55 with osmolality adjusted to 780 mOsm/kg using 0.115 M glucose), mixed and incubated for 10 min at room temperature. The mixture was transferred into a microfuge tube, centrifuged at 10,000 rpm for 5 min at 4 °C, washed twice with PBS, and 200 µl of PBS added. The haemocyte suspension (200 µl, 3 x 10^4 cells) was mixed with 200 µl of latex beads (10^8/ml, particle diameter 1.094 Å), a volume of 1:1. The mixture was incubated at room temperature for 30 min and 10 µl of solution dropped on a clean glass slide and air-dried. The cells were fixed with 4% formaldehyde, 10 µl (1:1) for 5 min, and stained with Giemsa stain. The phagocytic haemocytes were observed under a light microscope (Olympus BX53, Olympus, Tokyo, Japan). Phagocytic activity was determined from the number of bead ingested cells per 200 cells and is expressed as follows; % Phagocytic activity = [Number of bead ingested cells/Number of cells observed] x 100.

2.4.3.3. Phenoloxidase activity of haemocytes. PO activity was determined according to the method described previously [31]. Haemolymph (300 µl) was drawn into a 1 ml syringe containing 300 µl of anticoagulation, mixed and centrifuged at 10,000 rpm for 10 min at 4 °C. The pellet was washed twice with 500 µl of cacodylate buffer (CAC), (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, 0.26 M magnesium chloride, pH 7.0) and resuspended in chilled CAC buffer. The haemocyte suspension was sonicated in an ice bath and centrifuged at 10,000 rpm for 10 min at 4 °C, and the haemocyte lysate (HLS) collected. PO activity was determined using L-DOPA and trypsin as substrate and elicitor, respectively [32]. The reaction mixture containing 75 µl (1 mg/ml) trypsin; 150 µl (2 mg/ml) L-DOPA, 75 µl HLS fluid sample, and 150 µl of CAC buffer was placed into a 96-well plate. PO activity was determined by following the formation of dopachrome at 490 nm kinetically every 15 s for 5 min in a spectrophotometer. The blank contained 225 µl of CAC buffer, 75 µl of trypsin and 150 µl of L-DOPA. A unit of PO activity was defined as the amount of enzyme required to produce an absorbance of 0.001/min (change in optical density/min).

2.4.3.4. Transcripts of immune-related genes. The immune-related gene transcripts were determined prior to the start of the diet (day 0) and at day 42 of diet administration. Haemolymph (500 µl) was drawn from the ventral sinus of each shrimp into a 1 ml syringe containing an equal volume of anticoagulant solution. The haemolymph mixture of 10 shrimp in each diet group was pooled and gently mixed in a sterile microfuge tube and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was discarded, and Trizol solution (400 µl) was added, mixed and stored at −20 °C.

Table 2
RT-qPCR primer sets and conditions used to determine the expression of immune-related genes.

Primers	Sequences (5' to 3')	Annealing temp/cycles
proPO-I-F	GAGAGGCTGAACCGAGACTGA	55.7°C, 1 min, 40 cycles
proPO-I-R	AAGAAAACGGCCCCCAATT	
PEN-4-F	ATGCTACGGAATTCCCTCCT	57°C, 45 s, 45 cycles
PEN-4-R	ATCCTTGCAACGCATAGACC	
Dicer-F	CCGGAGATAGAACGGTTCAGTG	60°C, 1 min, 35 cycles
Dicer-R	CGATAATTCCTCCCAACACCTG	
EF-1α-F	GAAGTAGCCGCCCTGGTTG	57°C, 30 s, 28 cycles
EF-1α-R	CGGTTAGCCTTGGGGTTGAG	

Table 3
The proximate composition (% dry mass: w/w) of bioflocs powder by proximate analysis showing the crude protein, crude lipid, ash, moisture, crude fiber and nitrogen free extract (NFE); carbohydrate. Data are presented as a mean of triplicate independent experiments (mean ± SD).

Nutrients	Proximate composition (% dry mass: w/w)
Crude protein (CP)	47.94 ± 1.15
Crude lipid (EE)	5.02 ± 0.01
Ash	1.41 ± 0.01
Moisture	10.10 ± 0.07
Crude fiber (CF)	5.73 ± 1.28
Nitrogen free extract (NFE)[a]	29.81 ± 2.47

[a] NFE = 100-(CP + EE + CF + ash + moisture).

Table 4
EAA profiles of bioflocs powder and fishmeal by HPLC analysis expressed as A/E ratios and EAAI. Data are presented as a mean of triplicate independent experiments (mean ± SD).

Essential amino acids (EAAs)	A/E	
	Dried bioflocs	Fishmeal [Soares et al (2004)]
Arginine (Arg)	0.60	0.72
Histidine (His)	1.00	1.00
Isoleucine (Ile)	1.00	1.00
Leucine (Leu)	0.71	1.00
Lysine (Lys)	1.00	1.00
Methionine (Met)	1.00	1.00
Phenylalanine (Phe)	1.00	0.80
Threonin (Thr)	1.00	1.00
Tryptophan (Try)	1.00	0.88
Valine (Val)	1.00	1.00
EAAI	0.92	0.93

RNA was extracted from the haemocytes in 200 µl of TRI reagent according to the manufacturer's protocol (Sigma Aldrich, USA). The concentration and quality of RNA was determined by measuring the absorbance at 260/280 nm using a NanoDrop 2000 spectropho-tometer (Thermo Scientifc, USA). cDNA was transcribed from RNA (1 µg) using the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) containing Revert Aid reverse transcriptase (200 U/µl), RiboLock RNase inhibitor (20 U/µl), Oligo (dT) 18 primer (100 µM), dNTP mix (10 mM), and 5x reaction buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM MgCl2, 50 mM DTT) at 42 °C for 1 h. The expressions of immune-related genes for the prophenoloxidase-I (proPO-I), anti-microbial peptide (PEN-4) and antiviral immunity (dicer) were determined using quantitative reverse transcription PCR (RT-qPCR) with specific primer sets and conditions as shown in Table 2. The shrimp EF-1α gene was amplified as an internal control. The experiments were performed in triplicate.

2.5. Bacterial challenge assay

Three hundred and sixty juvenile *P. monodon* (10–20 g) were equally distributed into 12 tanks, 3 tanks for each of 4 experimental groups. Group I comprised shrimp fed with normal diet as a normal control (B0-). Group II comprised shrimp fed with normal diet and challenged with *V. parahaemolyticus* (3HP strain) as an infected control shrimp (B0–V). Groups III and IV comprised shrimp fed with B25 and B50 bioflocs diets and challenged with *V. parahaemolyticus* (B25–V and B50–V, respectively). Shrimp were fed daily with diets corresponding to each group for 7 days prior to intramuscular injection with *V. parahaemolyticus* (1 x 10^9 colony-forming units; CFU/ml, 100 µl). The shrimp mortality rate was observed every day for 14 days, and shrimp (3 shrimp from each tank) were collected for histopathological examination at day 1 and 6 after bacterial injection. The hepatopancreas (size 0.5 × 0.5 cm) was removed by using sterile forceps and scissors, fixed in Davidson's fixative for 24 h, and processed for routine histological examination. Tissues were stained with haematoxylin and eosin (H&E), and observed under a light microscope (Olympus BX53, Olympus, Tokyo, Japan).

2.6. Statistical analysis

Data were presented as mean ± SD of triplicate samples, and analyzed by one-way ANOVA followed by Tukey's multiple comparison for comparing more than three groups. The statistically significant difference was required at p-value less than 0.05.

2.7. Key Resourse table

Resource	Source	Identifier
Bacteria		
Vibrio parahaemolyticus	CENTEX SHRIMP, Thailand	N/A
Chemical		
Cacodylate buffer	Sigma®, USA	
Calcium chloride	EMSURE®, Merch, Germany	
DTT	Sigma®, USA	
EDTA-Na2	Sigma®, USA	
Eosin	Sigma®, USA	
Formaldehyde	Sigma®, USA	
Formalin	Sigma®, USA	
Haematoxylin	Sigma®, USA	
HCl	EMSURE®, Merch, Germany	
KCl	EMSURE®, Merch, Germany	
L-DOPA	Sigma®, USA	
Magnesium chloride	EMSURE®, Merch, Germany	
NaCl	EMSURE®, Merch, Germany	
Rose Bengal	Sigma®, USA	
Sodium cacodylate	EMSURE®, Merch, Germany	
Tris	Sigma®, USA	
Trisodium citrate	EMSURE®, Merch, Germany	

Table 5
Fatty acid profiles of bioflocs powder and fishmeal by GC analysis: comparison of PUFA, SFA (g/100 g lipid), and ω6/ω3 PUFA, PUFA/SFA, DHA/DPA ratios. Data are presented as a mean of triplicate independent experiments (mean ± SD).

Polyunsaturated fatty acid (PUFA) (g/100 g lipid)	Dried bioflocs	Fishmeal [Wasielesky et al (2006)]
Linoleic acid (LA-ω6)	0.19	0.06
α-Linolenic acid (ALA-ω3)	0.10	0.02
Docosahexaenoic acid (DHA –ω3)	0.30	0.16
Eicosapentaenoic acid (EPA –ω3)	0.25	0.17
Saturated fatty acid (SFA) (g/100 g lipid)		
Myristic acid	0.07	0.06
Pentadecanoic acid	0.05	0.01
Palmitic acid	0.34	0.25
Heptadecanoic acid	0.07	0.02
Stearic acid	0.35	0.07
Arachidic acid	0.02	0.01
Behenic acid	0.03	0.01
Ratio		
ω6/ω3 PUFA	0.30	0.20
PUFA/SFA	0.90	0.95
DHA/EPA	1.00	0.94

3. Results

3.1. The proximate compositions of bioflocs powder and experimental diets

The proximate composition (% dry mass, w/w) of bioflocs powder comprised 47.94 ± 1.15% crude protein and 5.02 ± 0.01% crude lipid, and for total ash, moisture, crude fiber and NFE (carbohydrate) the percent content was 1.41 ± 0.01%, 10.10 ± 0.07%, 5.73 ± 1.28%, and 29.81 ± 2.47%, respectively (Table 3). No significant differences in the crude protein content was found among all bioflocs diet regimens (25%–100% substituted fishmeal). The crude lipid, ash, and crude fiber contents showed tendency related with the bioflocs content while the carbohydrate content showed an inverse relationship (Table 1). The average crude protein content was 37.46% in all the experimental diets.

3.2. The amino acid and fatty acid profiles of bioflocs powder

The EAA profiles of bioflocs powder are shown in Table 4. The A/E ratio of arginine and leucine of bioflocs powder was slightly lower than that of fishmeal previously reported [33]. While, the A/E ratio of phenylalanine and tryptophan of bioflocs powder was slightly higher than that of fishmeal. However, the EAAI showed no significant differences between bioflocs powder and fishmeal.

The fatty acid profiles of bioflocs powder are shown in Table 5. The linoleic acid (LA), docosahexaenoic acid (DHA) and stearic acid content of bioflocs powder were significantly higher than those of fishmeal previously reported [34]. Nevertheless, there was no significant differences in ω6/ω3 polyunsaturated (PUFA) and PUFA/saturated fatty acid (SFA) and DHA/eicosapentaenoic acid (EPA) ratios between bioflocs powder and fishmeal.

3.3. The effect of bioflocs diets on growth and survival rate of shrimp

The growth performances of juvenile *P. monodon* after cultured for 42 days are shown in Table 6. The initial weights of both bioflocs and control (B0) groups were similar, averaged weight of 16.90 g. After 42 days of feeding, no significant differences on final body weight, weight gain, SGR or FCR values were found between the two groups. Interestingly, the B25 and B50 bioflocs diets improved survival rates to over 90% compared to B0 bioflocs 78% survival rate. The B75 and B100 diets also improved survival rates to over 80%, but the results were not significant from B0.

3.4. The effect of bioflocs diets on immune response in shrimp

3.4.1. Total and differential haemocyte count

The THC of all bioflocs-fed groups was significantly higher than that of the control group at all time points (Fig. 1A). While THC levels of the control group remained constant at 4.69 x 10^8 cells, those of bioflocs groups gradually increased until day 21 before stabilizing. B25 shrimp had the highest THC at every time point, peaking at 12.69 x 10^8 cells on day 42, followed by B50, B75 and B100 shrimp with THC of 10.45 x 10^8, 9.11 x 10^8 and 8.03 x 10^8 cells, respectively. There were no significant differences between the fishmeal and bioflocs-fed groups in percentages of granular cells, semi-granular cells and hyaline cells (data not shown).

3.4.2. Phagocytic activity of haemocytes

By day 7, the phagocytic activities of B25, B50, B75 and B100 shrimp had increased markedly 20.17, 18.50, 18.50 and 19.00%, respectively, from control, whose activity remained stable at around 15.00% (Fig. 1B). The activity of bioflocs-fed groups increased until day 42, with the exception of B50 whose activity decreased slightly from 20.33% on day 21 to 19.67% on day 42. Shrimp with B75 and B100 diets showed activity levels similar to B50, with them peaking at 19.50% and 20.33%, respectively. The B25 group showed the highest activity at all time points, peaking at 23.50% on the final time point.

Table 6
Growth performance and survival rate of *P. Monodon* after 42 days feeding with bioflocs substituted diets (B25, B50, B75, B100) compared to control diet (B0). Shrimp fed with the bioflocs substituted diets showed all growth performance parameters not different from fishmeal diet. Data are presented as a mean of triplicate independent experiments (mean ± SD). *indicates value significantly different from the control ($p < 0.05$).

Parameters	B0	B25	B50	B75	B100
Initial weight (g)	16.91 ± 0.08	16.89 ± 0.19	16.93 ± 0.11	16.90 ± 0.32	16.91 ± 0.09
Final weight (g)	24.73 ± 0.11	24.65 ± 0.19	24.62 ± 0.11	24.23 ± 0.33	24.15 ± 0.07
Weight gain (%)	46.23 ± 0.10	45.98 ± 0.50	45.48 ± 0.30	43.43 ± 0.78	42.84 ± 0.33
SGR[a] (% day^{-1})	0.91 ± 0.00	0.90 ± 0.01	0.89 ± 0.01	0.86 ± 0.01	0.85 ± 0.01
FCR[b]	1.50 ± 0.01	1.52 ± 0.00	1.52 ± 0.01	1.60 ± 0.00	1.62 ± 0.01
Survival rate (%)	78.43 ± 3.40	94.12 ± 5.88*	90.20 ± 3.40*	82.35 ± 0.00	84.31 ± 3.40

SGR[a] = Specific growth rate; FCR[b] = Feed conversion ratio

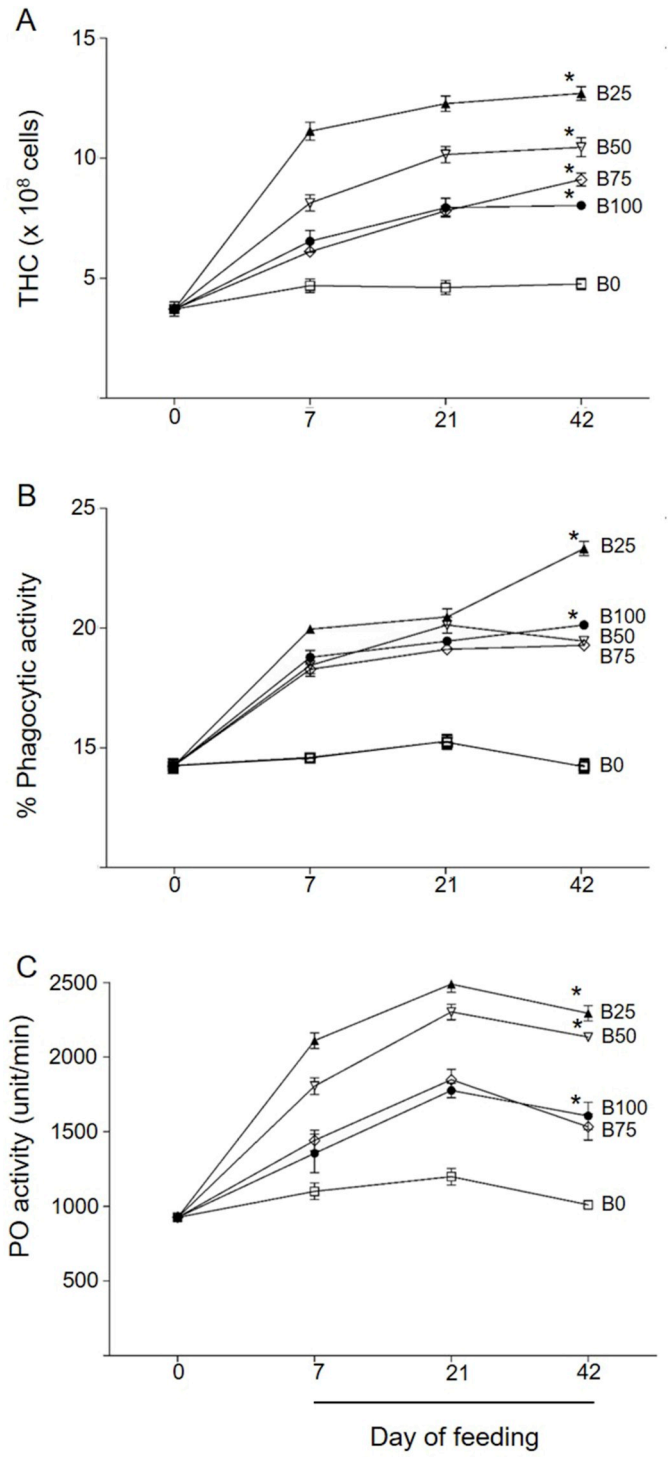

Fig. 1. Immune parameters of shrimp *P. monodon* fed with bioflocs substituted diets (B25, B50, B75, B100) compared to control diet (B0) on day 7, 21 and 42 of bioflocs administration. (A) The THC, (B) phagocytic activity, and (C) PO activity of *P. monodon* were enhanced in all bioflocs substituted diets groups. Data are presented as percent of control (mean ± SD of triplicate independent experiments). *indicates value significantly different from the control diet (B0) ($p < 0.05$).

3.4.3. Phenoloxidase activity

PO activity of all bioflocs-fed groups showed a marked increase from control by day 7 and continued to increase until day 21, after which activity gradually declined but still remained significantly higher than control (Fig. 1C). At every time point, PO activity in B25-fed shrimp had a two-fold increase over that of control (which fluctuated at 926.67 unit/min) and peaked at 2,488.47 unit/min on day 21. Peak PO activities for B50, B75, and B100 groups were all on day 21 as well, at 2,302.67, 1,848.87 and 1,776.60 unit/min, respectively.

3.4.4. Expression of immune-related genes in shrimp

Immune-related gene expression was quantified by RT-qPCR on day 42 (Fig. 2). In B25, B50 and B75 shrimp, expressions of all three genes (proPO-I, PEN-4, dicer) increased compared to control shrimp, with B25 > B50 > B75. Interestingly, the expression of PEN-4 had a 45-fold increase in B25 relative to the control (Fig. 2A), while expression of proPO-I and dicer in B25 increased 4 and 5-fold, respectively (Fig. 2B and C). For B100 shrimp, only the expression of propPO-I gene was significantly higher than the control (2-fold increase) (Fig. 2B).

3.5. Challenge assay

At day 1 after *V. parahaemolyticus* injection, infected control shrimp (B0–V) and the B25–V and B50–V shrimp began to die while normal control (B0-) shrimp remained viable. At day 1 the mortality rate for B0–V was 67.55 ± 1.27%, and for the bioflocs groups B25–V and B50–V shrimp the mortality rate was 33.33 ± 0.01% and 34.16 ± 1.18%, respectively. On day 4 after bacterial injection the mortality rate for B0–V shrimp was 100% and for B25–V and B50–V shrimp it was 42.65 ± 3.31% and 44.32 ± 3.78%, respectively. On day 14, the mortality rate for B25–V and B50–V shrimp was 50.97 ± 3.30% and 52.64 ± 3.76%, respectively. The mortality rate for B0- shrimp remained at 0% for the entire experimental period (14 days) (Fig. 3).

3.6. Histopathological change

The control (B0-) shrimp presented a normal hepatopancreatic tubule with normal features of the B, F and R cells surrounding the hepatopancreatic tubule lumen (Fig. 4A–B). The shrimp infected with the *V. parahaemolyticus* (B0–V) presented histopathological signs of the bacterial infection which included hepatopancreatic collapse, disorganization of the hepatopancreatic tubules, sloughing of hepatopancreatic tubule epithelial cells, decreased number of vacuoles in the R and B cells, appearance of necrotic cells inside the lumen with severe haemocyte infiltration, and tissue melanization (Fig. 4C–D). The bioflocs fed shrimp, B25–V and B50–V presented a near normal appearance of the hepatopancreatic tubule where both cells and structures were similar to the normal control (B0-) shrimp (Fig. 4E–H).

4. Discussion and conclusion

Fishmeal is the main source of protein in shrimp feed pellets and contributes to the valuable nutritional requirements of aquaculture feed. Nowadays, fishmeal is a limited commodity and its availability varies because of reductions in fish stocks related to factors such as climatic phenomena, overexploitation and decline of ocean fisheries stocks, and thus has become a costly ingredient in fish and crustacean feed formulation. Moreover, the overexploitation produces a negative impact on the ocean environment. Therefore, there is an urgent need to seek for a sustainable alternative to fishmeal in the shrimp feed industry. The present study sought to evaluate the components and

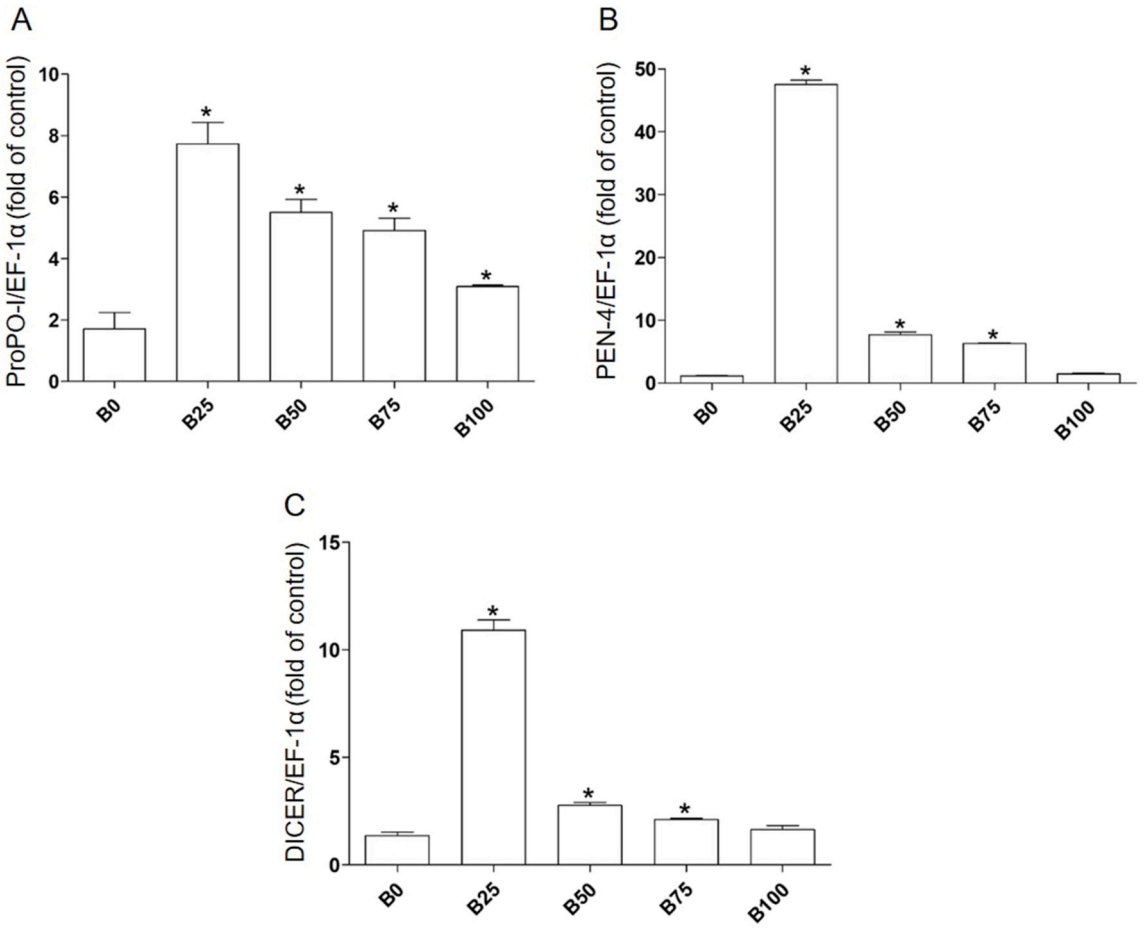

Fig. 2. The expressions of immune-related genes of *P. monodon* after 42 days feeding with bioflocs substituted diets (B25, B50, B75, B100) compared to control diet (B0). RT-qPCR analysis showing the expressions of (A) proPO-I, (B) PEN-4 and (C) dicer genes were upregulated in shrimp fed with the B25, B50, B75 bioflocs substituted diets. Data are presented as percent of control (mean ± SD of triplicate independent experiments). *indicates value significantly different from the control diet (B0) ($p < 0.05$).

nutritional value of bioflocs harvested from the shrimp ponds, as a potential food substitute (bioflocs substituted fishmeal), and provided data to support the beneficial effect of bioflocs diet on growth performance, survival rate, and immune response in shrimp.

According to the formulation of the aquaculture feed in FAO (2012) [2], juvenile penaeid shrimp feed pellets should contain high protein (>400 g/kg), adequate lipid (<100 g/kg) and low ash contents (<160 g/kg). Apparently, the amount of crude protein in bioflocs depends on the inorganic particles and microorganisms, bacterial communication and zooplankton in the different bioflocs systems [35,36], while the level of lipid in bioflocs is related to the amount of microalgae and zooplankton [37]. Reports from different bioflocs systems indicated that bioflocs contain crude protein content ranging from 230.9 to 420 g/kg [18,38–41]. In our study, the bioflocs crude protein and the crude lipid contents were higher than those reported in previous studies [38,42,43]. The similar EAAI and an equivalent EAA content to those of fishmeal suggest that our bioflocs is a feasible shrimp feed substitute [2]. In addition, the percentages of LA and DHA in our bioflocs were higher than those of fishmeal. This compositional and nutritional content in the bioflocs is in an acceptable nutritional requirement for shrimp growth suggesting that it could substitute fishmeal in shrimp feed pellets. Ash is an inorganic substance which consists of an acid-soluble part (various minerals) and an acid-insoluble part (contaminants such as silica). If the level of minerals and contaminants in ash is very high, it will negatively affect the environment and malnourishment of shrimp or aquatic animals as suggested by Shearer et al. (1992) [44]. They found that the decrease in salmon growth was correlated with the amount of ash corresponding to the food. The ash content in our bioflocs (14.10 g/kg) was notably less than previously reported (31.60–48.50 g/kg) [42,45] indicating the advantage of using bioflocs in the shrimp culture system since it would not cause negative effects on shrimp and the environment.

The feeding trial using different formulations of bioflocs with fishmeal (bioflocs substituted fishmeal) in the feed pellets demonstrated that shrimp fed with bioflocs substituted fishmeal showed a positive weight gain, SGR, survival rate and decreased FCR similar to shrimp fed with fishmeal diet [10]. A similar study by Kuhn et al. (2010) [46] showed that increasing the percentage of bioflocs in fishmeal feed pellets did not produce a higher growth rate. It has even been suggested that higher amounts of bioflocs, and hence microbial products, might decrease the feed palatability and digestibility [47]. However, another study using BFT culture of shrimp *Litopenaeus vannamei (L. vannamei)* [48] revealed that shrimp significantly increased digestive enzyme activities together with improved growth performance. It was inferred from this study that a bioflocs-related increase in growth of shrimp was due to microbial elements or probiotic microorganisms like *Bacillus* and *Lactobacillus* in the bioflocs which could stimulate the production of endogenous enzymes in the shrimp hepatopancreas [36,45]). Such disparities may simply mean that shrimp growth response depends on the specific composition and microbial nature of the bioflocs systems studied.

Interestingly, we found that higher shrimp survival rates were associated with the enhanced immune activities in the shrimp fed with the bioflocs. An increase in THC was most pronounced in shrimp fed

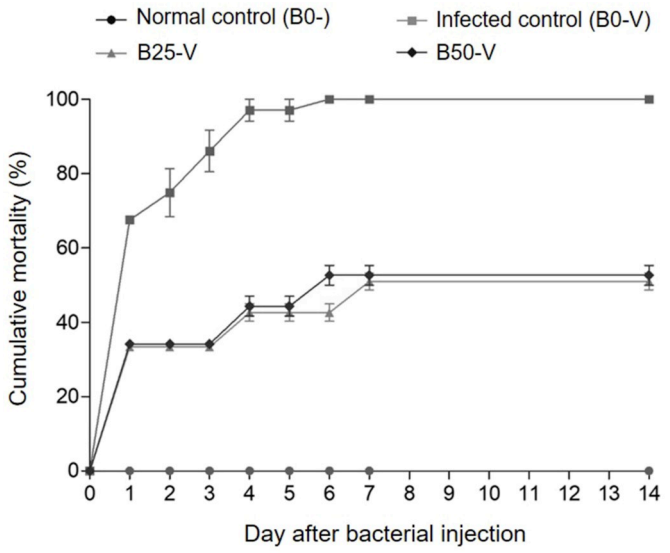

Fig. 3. Cumulative mortality of shrimp at day 14 post *V. parahaemolyticus* (3HP strain) injection. Shrimp fed with the B25 and B50 bioflocs substituted diets showed decreased mortality rate to about 50% of infected control shrimp on day 14. B0-, normal shrimp (uninfected); B0–V, infected control shrimp; B25–V and B50–V, infected bioflocs fed shrimp. Data are presented as mean ± SD from three independent experiments. *indicates values significantly different from B0 shrimp ($p < 0.05$).

with bioflocs substituted at 25 and 50%, which data were consistent with the earlier studies on shrimp reared in bioflocs systems [12]. The circulating haemocytes of shrimp are vital in immunity, performing functions such as phagocytosis, encapsulation, and storage and release of the proPO system [12]. Phenoloxidase is an enzyme of the shrimp defense mechanism that causes melanization and inactivation of foreign cells thus preventing their spread throughout the body. This enzyme is particularly stimulated by components of the microbial cell wall including lipopolysaccharides (LPS) and β-1,3-glucans, which trigger a proPO activating system [12,49]. We further examined the effect of bioflocs on PO activity and found that the expression of proPO-I, which regulates the PO activation system, was significantly higher in shrimp fed with the bioflocs substituted fishmeal at 25, 50 and 75% compared to control. In addition, the expression levels of both PEN-4 and dicer were also significantly higher than control in a similar trend to the proPO-I. However, our findings differ from those of Xu and Pan (2014) [50] who reported that anti-microbial peptide and antiviral immunity were not significantly altered by bioflocs compared to control. It has been reported that bacteria in the bioflocs, such as *Bacillus,* have been shown to modify the physiological and immunological status of the shrimp gastrointestinal tract with subsequent changes in the endogenous microbiota [51] and humoral defense factor production (antimicrobial and antiviral peptides). We propose that the enhanced expression of PEN-4 and dicer by our bioflocs might be the result of different probiotics in bioflocs. A further study of bacterial identification in order to understand the microbes involved in enhanced immunity is, therefore, essential for future BFT practice.

Acute hepatopancreatic necrosis disease (AHPND) (or early mortality syndrome) is a pathological shrimp disease that challenges the global shrimp farming industry. AHPND is caused by the pathogenic *V. parahaemolyticus* [52]. Studies show that there is a lower prevalence of AHPND infection associated with farms that operate with bioflocs. Our data support these previous findings that shrimp fed with bioflocs were less susceptible to *V. parahaemolyticus* infection than their fishmeal counterparts as we show that the mortality rates of B25–V and B50–V shrimp were decreased compared to B0–V, and AHPND histopathology was not evident. We propose that organisms found in bioflocs may

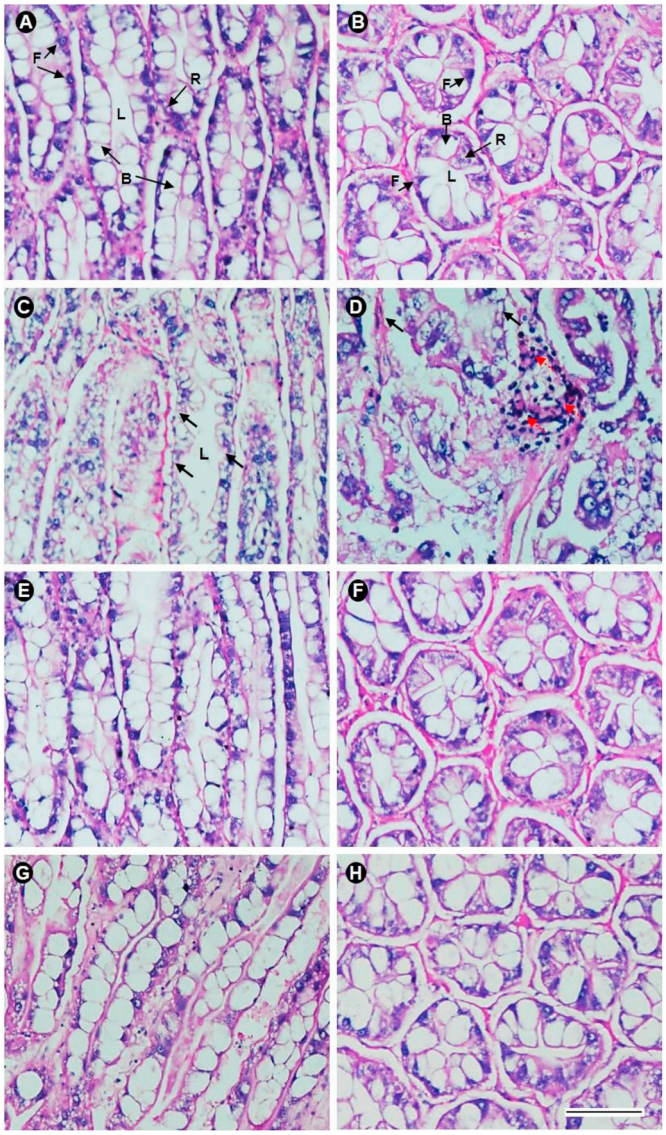

Fig. 4. Photomicrographs showing the hepatopancreatic tissues of shrimp after *V. papahaemolyticus* (3HP strain) injection. (A) Longitudinal and (B) transverse sections of normal shrimp (B0-) showing the normal hepatopancreatic tubule lumens (L) surrounded by the B (blister-like), F (fibrillar) and R (resorptive) cells. (C) Longitudinal and (D) transverse sections of B0–V showing collapse of hepatopancreatic epithelium, sloughing of tubular epithelial cells, flattened tubules after cell sloughing (arrows), and haemocyte infiltration (dash arrows). (E) Longitudinal and (F) transverse sections of B25–V, (G) longitudinal and (H) transverse sections of B50–V showing near normal structural features of the hepatopancreatic tubules and cells. Scale bar = 100 μm.

provide improved protection against pathogens through their metabolites which act to enhance the shrimp innate immune system [16,25].

In conclusion, the present study has demonstrated that our bioflocs contains sufficient nutritional constituents which are vital for shrimp growth. The bioflocs diet may provide a promising alternative protein source to fishmeal, improve shrimp immune activity, and provide the capacity to control *V. parahaemolyticus*, which would have a significant impact on both the feedmill industry and the environment.

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