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ORIGINAL RESEARCH

Evaluation of dietary soybean meal as fish meal replacer for juvenile whiteleg shrimp, *Litopenaeus vannamei* reared in biofloc system

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Abstract Different levels of dietary soybean meal (SBM) as a fish meal (FM) replacer, with and without amino acid supplementation, for whiteleg shrimp, *Litopenaeus vannamei* reared in the biofloc system was examined in eight weeks of feeding trial. Eight experimental diets consisted of a basal diet with 0% FM replacement by SBM provided in clear sea water without biofloc system (S_0SW), four diets replacing FM at 0% (S_0), 33% (S_{33}), 67% (S_{67}) and 100% (S_{100}) by SBM, and three diets replacing FM at 33% (S_{33}A), 67% (S_{67}A) and 100% (S_{100}A) by SBM supplemented with amino acids (methionine and lysine) in the seawater biofloc system. Results of water quality analyses showed significantly lower total suspended solids and nitrate for S_0SW group than all other treatments. Diets S_0 and S_{33}A resulted in higher weight gain and specific growth rate among all groups, with no significant differences with S_{33} group. In addition, whole-body protein and amino acid compositions of shrimp fed S_0SW were lower than most biofloc groups. Haemolymph parameters showed significant differences in total protein, cholesterol and triglyceride between groups S_0 and S_0SW. Also, superoxide dismutase activity showed a decreasing trend with increasing replacement level. In conclusion, based on these results, SBM could replace up to 33% of FM with or without amino acid supplementation in juvenile whiteleg shrimp diets reared in the biofloc system.

Keywords Biofloc technology · Fish meal · Soybean meal · Amino acid

Introduction

The "biofloc technology" is a sustainable technique used in minimum or zero water exchange shrimp culture systems (Avnimelech 2008; Crab et al. 2009; De Schryver et al. 2008). In this system, heterotrophic microorganisms are employed to manage chemical quality of water by converting inorganic material to organic compounds such as conversion of ammonium into bacterial biomass (Avnimelech 2006; Crab et al. 2007). With the development of microbial community, biofloc (microbial flocs) is formed containing heterogeneous mixture of organisms and organic material (Hargreaves 2006; De Schryver et al. 2008). Biofloc that is promoted in the culture water can beneficially control the quantity of ammonium and nitrite. Moreover,

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it is available as a source of food supplement for cultured shrimp (Hari et al. 2004; Arnold et al. 2009; Ballester et al. 2010), and offer essential in situ nutrients, such as protein (Emerenciano et al. 2012), lipid (Wasielesky et al. 2006), amino acids (Ju et al. 2008) and fatty acids (Izquierdo et al. 2006; Ekasari et al. 2010). Biofloc biomass in the culture system that is consumed by cultured shrimp and digested, may compensate a significant amount of protein demand, and consequently reduce the quantity of fish meal (FM) required (Burford et al. 2004; Crab et al. 2010; Hari et al. 2004; Wasielesky et al. 2006; Xu and Pan 2012; Xu et al. 2012).

Fish meal (FM) has generally been a major ingredient in shrimp diets because of its high protein quality, desirable amino acid profile, excellent palatability and digestibility, low carbohydrate content and minimum anti-nutritional factors (Zhou et al. 2004). However, FM is also overpriced, comparing to other ingredients, in formulated shrimp diets (Lee and Bai 1997; FAO 2013). Recently, the price of fish meal has been increasing due to excessive demand and static supply (FAO 2014). It has been stated that the success and sustainability of the shrimp aquaculture industry will depend partly on the reduction of FM usage in shrimp feeds (Yue et al. 2012). For this reason, many studies have aimed to replace or reduce FM inclusion in diets by less expensive alternative protein sources, such as algae (Kiron et al. 2012), bacteria (Aas et al. 2006), plants (Gatlin et al. 2007), invertebrates (Barrows and Frost 2014) and by-products (Fowler 1991). Meanwhile, soybean meal (SBM) is known to be one of the most successful replacers of FM, because of its favorable protein content and amino acid profile (McGoogan and Gatlin 1997; Kikuchi 1999), less expensive price than FM and availability (Hardy 2006). However, one of the potential problems associated with the use of SBM is the deficient levels of indispensable amino acids (viz. lysine and methionine), that is limiting their use in shrimp feed as an alternative to FM (Yue et al. 2012). In addition, SBM contains some anti-nutritional components (for example, protease inhibitors, saponins tannins, phytic acid, lectins, alkaloids and non-starch polysaccharides) that negatively influence the digestion or absorption of nutrients and cause the dysfunction of vitamins (Gatlin et al. 2007; Krogdahl et al. 2010).

Whiteleg shrimp, *Litopenaeus vannamei*, is the most globally cultured shrimp species. It is mostly cultured in China, India and Thailand and the worldwide production has increased from 1.32 million tons (mt) in 2004 to 3.28 mt in 2013 due to high market demand (FAO 2014). Considering the high level of FM (25–50%) in the diet of this species, *L. vannamei* is perhaps one of the biggest consumers of FM in the world (Olsen and Hasan 2012). Therefore, this study was undertaken to investigate the benefits of biofloc system in shrimp culture while replacing FM by SBM; to reduce the amount of FM used in the diet of *L. vannamei.* In addition, this study aimed to identify the optimum level of dietary SBM with/without amino acids (lysine and methionine) supplementation as a FM alternative and effects on growth performance, whole-body proximate, amino acids compositions and haemolymph indices of juvenile whiteleg shrimp, *L. vannamei*, reared under biofloc conditions.

Materials and methods

Experimental design

The feeding trial was carried out at the National Fisheries Research and Development Institute (NFRDI), Taean, Republic of Korea. The shrimp used in this study were produced from specific pathogen free (SPF) *L. vannamei* broodstock imported from Hawaii, USA. Prior to the start of the feeding trial, juvenile whiteleg shrimp were kept in indoor fiberglass tank (6 m^2, 5 t) containing aerated clean water for a period of 7 days at 28.3 ± 1.7 °C. In this period, shrimp were fed a commercial feed (DongA One, Busan, Republic of Korea), two time per day. At the beginning of the feeding trial, a total of 1320 juvenile whiteleg shrimp (average initial weight 0.9 ± 0.05 g) were carefully selected from the stock tank and directly distributed into 24 fiberglass tanks with a water volume of 200 L each (55 shrimp for each tank). Eight groups of shrimp were fed using each of the formulated diets. The feeding rate was 7% of body weight per day at beginning of experiment that gradually deceased to 5% at the end of experiment. After every 2 weeks, total weight in each tank was measured and daily feed was equally divided into four parts and given at 09:00, 13:00, 17:00 and 21:00 to all of the tanks. During 8 weeks of the feeding trial, biofloc was supplied from the stock tanks at the NFRDI and

aeration for all tanks was applied using air-stones that were connected to an air pump (Resun LP-60 Pond Air Pump, China), and 20% of the water was exchanged daily in all the biofloc and clear water tanks.

Throughout the experiment, dissolved oxygen, salinity, temperature (°C), and pH (YSI Model 85, YSI Incorporated, Yellow Springs, OH, USA) were measured daily in both the biofloc and clear water tanks. Water samples (500 mL) were collected from each tank at 2 weeks intervals (0, 2, 4, 6, and 8 weeks). Half of this amount of water was analyzed using a spectrophotometer for total ammonia nitrogen (TAN), nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N) using the standard methods for marine environmental analysis (MLTM 2010); the other half was filtered using vacuum pressure passing through Whatman GF/C filter paper that were pre-dried and pre-weighed. The filter paper containing suspended materials was dried at 105 °C in an oven until sustained weight, and the dried sample was weighed to 0.01 mg. The weight difference was calculated and total suspended solids (TSS) were obtained (Avnimelech and Kochba 2009).

Experimental diets

Eight experimental diets were formulated to replace FM using SBM at 0% in seawater without biofloc system (S_0SW) as a control diet. Four diets replaced 0% (S_0), 33% (S_{33}), 67% (S_{67}) and 100% (S_{100}) of FM without amino acid supplementation and three diets replaced 33% (S_{33}A), 67% (S_{67}A) and 100% (S_{100}A) of FM with supplementation of L-Methionine and L-Lysine in seawater biofloc system (Table 1). The amino acid profiles of the major feed ingredients (fish meal, soybean meal, wheat flour and wheat gluten) and diets are listed in Tables 2 and 3, respectively. For carbohydrate, lipid and non-nutritive bulk, ingredients, such as wheat flour

Table 1 Ingredients and proximate composition of the test diets (% of DM basis)

Ingredients	S_0SW	S_0	S_{33}	S_{33}A	S_{67}	S_{67}A	S_{100}	S_{100}A
Fish meal[a]	39.0	39.0	26.0	26.0	13.0	13.0	0.00	0.00
Soybean meal[b]	0.00	0.00	15.9	15.9	32.2	32.2	48.1	48.1
Wheat flour[b]	46.6	46.6	40.4	40.4	34.2	34.4	28.3	28.4
Wheat gluten meal[b]	3.60	3.60	4.60	4.50	5.40	5.00	6.30	5.70
L-Methionine[c]	0.00	0.00	0.00	0.10	0.00	0.15	0.00	0.20
L-Lysine[c]	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.30
Fish oil[d]	3.60	3.60	4.50	4.50	5.30	5.30	6.10	6.10
Lecithin[b]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Calcium phosphate[c]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Cholesterol[c]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix[e]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix[f]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Cellulose[c]	0.00	0.00	1.40	1.40	2.70	2.70	4.00	4.00
Chemical analysis (% DM)								
Protein	36.1	34.8	35.6	35.3	36.2	35.1	35.8	35.7
Lipid	8.27	8.03	8.34	8.16	7.80	8.10	8.35	7.97
Ash	10.92	10.60	10.37	10.29	9.11	8.91	7.62	7.17
Moisture	7.30	7.33	7.30	9.10	7.93	7.26	7.34	7.44
Digestible energy (kJ g^{-1})	4143	4143	4143	4143	4143	4142	4142	4142

[a] Chilean standard grade steam-dried fish meal, Suhyup feed C. Uiryeong, Republic of Korea

[b] Suhyup feed C. Uiryeong, Republic of Korea

[c] Sigma-Aldrich Korea Yongin, Republic of Korea

[d] Jeil feed Co. Hamma n, Republic of Korea

[e] Contains (as mg/kg in diets): Ascorbic acid, 300; DL-Calcium pantothenate, 150; Choline bitartrate, 3000 l Inositol, 150; Menadione, 6; Niacin, 150; Pyridoxine · HCl, 15; Riboflavin, 30; Thiamine mononitrate, 15; DL-α-Tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; Cobalamin, 0.06

[f] Contains (as mg/kg in diets): NaCl, 437.4; $MgSO_4 \cdot 7H_2O$, 1379.8; $ZnSO_4 \cdot 7H_2O$, 226.4; Fe-Citrate, 299; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; Calcium iodate, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025

Table 2 Amino acid composition of the major feed ingredients (% of as is basis)

Amino acids[a]	Fish meal	Soybean meal	Wheat flour	Wheat gluten
EAA				
Threonine	2.96	2.11	0.29	2.09
Valine	3.25	2.52	0.43	3.13
Isoleucine	2.71	2.45	0.51	3.15
Leucine	4.94	4.10	0.70	5.38
Phenylalanine	2.62	2.70	0.49	3.98
Histidine	2.29	1.89	0.29	2.08
Lysine	4.75	3.20	0.21	1.32
Arginine	4.09	3.75	0.39	2.81
Methionine	1.41	0.58	0.14	1.04
NEAA				
Aspartic	6.63	6.36	0.43	3.23
Serine	3.20	2.75	0.49	3.76
Glutamic	9.00	10.03	3.53	29.57
Proline	2.98	2.67	1.12	9.32
Glycine	4.41	2.17	0.35	2.63
Alanine	3.93	2.27	0.29	1.97
Tyrosine	2.01	1.56	0.27	2.28
Cysteine	0.88	1.00	0.34	2.22

[a] Amino acid sample were analyzed at Feeds and Foods Nutrition Research Center, Pukyong National University. Values are means of triplicate samples

Table 3 Amino acid composition of the test diets (% of DM basis)

Amino acids[a]	S_0SW[b]	S_0	S_{33}	$S_{33}A$	S_{67}	$S_{67}A$	S_{100}	$S_{100}A$
EAA								
Threonine	1.01	1.01	1.03	1.02	1.23	1.22	1.21	1.23
Valine	1.74	1.75	1.73	1.66	1.54	1.52	1.58	1.57
Isoleucine	1.46	1.46	1.51	1.46	1.43	1.41	1.96	1.48
Leucine	2.47	2.45	2.49	2.38	2.50	2.48	2.57	2.56
Phenylalanine	1.38	1.44	1.53	1.44	1.62	1.59	1.73	1.72
Histidine	1.03	0.94	0.93	0.96	1.08	1.04	1.06	1.09
Lysine	1.88	1.80	1.75	1.77	1.51	1.71	1.52	1.70
Arginine	1.86	1.72	1.86	1.81	2.03	1.98	2.07	2.12
Methionine	0.30	0.28	0.32	0.35	0.22	0.33	0.20	0.31
NEAA								
Aspartic	2.58	2.49	2.69	2.69	3.09	3.14	3.17	3.39
Serine	0.86	0.90	0.98	1.04	1.70	1.66	1.72	1.76
Glutamic	6.87	6.98	7.24	7.14	7.95	7.60	8.30	8.22
Proline	2.30	2.36	2.51	2.48	2.41	2.26	2.29	2.48
Glycine	1.81	1.88	1.62	1.54	1.48	1.49	1.33	1.33
Alanine	1.61	1.58	1.46	1.40	1.35	1.36	1.28	1.28
Tyrosine	0.64	0.69	0.85	0.69	1.00	1.05	1.12	1.18
Cysteine	0.75	0.73	0.84	0.85	0.73	0.77	0.75	0.71

[a] Amino acid sample were analyzed at Feeds & Foods Nutrition Research Center, Pukyong National University. Values are means of triplicate samples

[b] For experimental diets refer to Table 1

and wheat gluten meal, fish oil and cellulose were used, respectively. Other nutrients were added to meet the nutritional requirements of *L. vannamei* (Hu et al. 2008), and were kept in the same levels in all diets.

Methods for preparation of diets were pursued according to the research done by Bai and Kim (1997). At first, all dry ingredients were mixed (HYVM-1214, Hanyoung Food Machinery, Republic of Korea) thoroughly and the experimental diets were prepared. After that, according to formulation table, fish oil and water were added to the diets. A screw-type pelleting machine (SFD-GT, Shinsung, Republic of Korea) was used to form the pellets by passing the dough through. After the pelletizing process, the pellets were air dried for approximately 48 h and were broken up, sieved into the proper pellet size, sealed, and stored at −20 °C until utilization.

Biofloc collection and shrimp sampling

Biofloc samples were collected at the beginning and the middle of the feeding period by passing the water through a 10-μm mesh size nylon bag. Biofloc samples were dried in an oven at 60 °C until constant weight and preserved at −20 °C for proximate composition that is shown in Table 4. At the end of the feeding trial, feeding was denied for 24 h, and the total number and weight of shrimp in each tank were determined. Growth performance parameters, such as weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER), feed conversion ratio (FCR) and survival were calculated according to the formulas described by Mohanty (1999).

$$\text{Weight gain (WG)} = (\text{final weight} - \text{initial weight}) \times 100/\text{initial weight}.$$
$$\text{Specific growth rate (SGR)} = 100 \times (\ln \text{final weight} - \ln \text{initial weight})/\text{days}.$$
$$\text{Feed conversion ratio (FCR)} = \text{total dry feed intake, g}/\text{total wet weight gain, g}$$
$$\text{Protein efficiency ratio (PER)} = \text{wet weight gain, g}/\text{protein intake, g}.$$

Ten intact shrimp per tank (30 individuals per treatment group) were randomly selected and kept at −20 °C for whole-body proximate and amino acid compositions. Five shrimp were randomly selected from each tank and about 0.3 ml of haemolymph was taken from the ventral sinus in the first pleomere using a 1-ml syringe that had a hypodermic needle with 2 mm of thickness. About 0.2 ml of an anticoagulant substance (113 mM glucose, 27.2 mM sodium citrate, 2.8 mM citric acid and 71.9 mM NaCl) was passed through each syringe. Haemolymph samples were centrifuged at 5000×*g* for 10 min and the plasma was separated and stored at −70 °C for determination of haemolymph biochemical parameters, including plasma total protein, cholesterol, triglyceride and glucose. The same shrimp were used for another set of haemolymph samples, this time anticoagulant was not used and after 30 min haemolymph was clot at room temperature. Then, using a centrifuge (at 5000×*g*) for 10 min the serum was divided and kept at −70 °C for the analysis of non-specific immune responses such as superoxide dismutase (SOD) and trypsin activities.

The tested diets, shrimp whole-body and amino acid compositions from each dietary treatment, and biofloc samples were analyzed according to the standard methods of AOAC (2005). Moisture content of samples was estimated by drying oven at 135 °C for 2 h to constant weight. Crude protein was determined using the Kjeldahl method (N× 6.25) after acid digestion. Soxhlet extraction was used to evaluate crude lipid using the

Table 4 Proximate composition (% dry weight basis) of the outdoor biofloc-based culture pond water at the beginning and the middle of the feeding trial

Parameters[a]	Beginning	Middle	Pooled SEM[b]
Crude protein (%)	26.9	27.4	0.07
Crude lipid (%)	0.34	0.37	0.02
Crude Ash (%)	47.6	48.6	0.11
Moisture (%)	86.3	86.7	0.09

[a] Biofloc samples were analyzed at Feeds & Foods Nutrition Research Center, Pukyong National University. Values are means of triplicate samples

[b] Pooled standard error of means: $SD/\sqrt{n}$

Soxtec system 1046 (Tacator AB, Hoganas, Sweden), and a muffle furnace was used to determine ash of dried samples by combustion at 550 °C for 6 h. Ninhydrin method (Sykam Amino Acid Analyzer S433; Sykam, Eresing, Germany) was used to analyze amino acids.

The concentrations of triglyceride, total protein, cholesterol, and glucose levels of plasma were determined by a chemical analyzer Fuji DRI-CHEM 3500i (Fuji Photo Film Ltd., Tokyo, Japan). SOD Assay Kit (Sigma-Aldrich, 19160) was used to measure the activity of SOD by inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) and xanthine oxidase according to instructions of manufacturer. Each assay was observed at absorbance of 450 nm after 20 min of reaction at 37 °C. The inhibition percent was assigned by mg protein and the values were known as SOD activity. The trypsin activity of shrimp was measured in the serum using a commercial kit (BioVision, CA, USA).

Statistical analysis

One-way ANOVA was used to analyze data for the effects of the dietary treatments. When significant differences were found, a least significant difference (LSD) test used to identify differences among experimental groups. The significance level of $P < 0.05$ was used to compare differences. SAS version 9.0 (SAS Institute, Cary, NC, USA) application was used for statistical analyses.

Results

Water quality parameters

The results for water quality parameters and biofloc development in term of total suspended solids (TSS) are shown in Table 5. The measured water quality in all experimental groups remained within recommended levels for shrimp culture. TSS levels almost stabilized in the biofloc tanks with average level of around 202.4 mg L^{-1} throughout the experimental period, but with significant differences with the clear water tanks. There were also significant differences in the amount of NO_3-N between all biofloc tanks and clear water tanks ($P < 0.05$).

Growth performance

At the end of the feeding trial, WG and SGR of shrimp fed S_0 and $S_{33}A$ diets were significantly higher than those of shrimp fed the other diets with no significant differences with S_{33} group (Table 6). FCR gradually increased among biofloc groups by addition of dietary SBM with significant differences between S_0 group and S_{67A}, S_{100} and S_{100A} groups. There were also significant differences in FCR between S_0SW and S_0 groups. PER of shrimp fed S_0 diet were significantly higher than those of shrimp fed S_{67}, $S_{67}A$, S_{100}, $S_{100}A$ and S_0SW

Table 5 Average water quality parameters of different experimental diets for 8 weeks

Parameters	S_0SW[a]	S_0	S_{33}	$S_{33}A$	S_{67}	$S_{67}A$	S_{100}	$S_{100}A$	Pooled SEM[b]
Salinity (g L^{-1})	31.8	32.2	32.0	32.0	32.2	32.1	32.1	32.1	0.01
DO (mg L^{-1})	5.9	5.8	5.8	5.8	5.8	5.7	5.8	5.8	0.02
pH	7.9	7.6	7.6	7.6	7.6	7.6	7.6	7.3	0.01
TAN (mg L^{-1})	0.56	0.44	0.32	0.38	0.31	0.33	0.35	0.42	0.08
NO_2^--N (mg L^{-1})	0.64	0.49	0.66	0.65	0.57	0.51	0.58	0.59	0.02
NO_3^--N (mg L^{-1})	18.7^v	3.08^u	3.13^u	3.16^u	3.12^u	3.23^u	3.34^u	3.35^u	8.00
TSS (mg L^{-1})	36.4^v	206^u	191^u	192^u	196^u	209^u	215^u	208^u	7.36

Values in same row with different superscript are significantly different at $P < 0.05$

DO dissolved oxygen, *TAN* total ammonia nitrogen, *TSS* total suspended solids

[a] For experimental diets refer to Table 1

[b] Pooled standard error of means: SD/$\sqrt{n}$

Table 6 Growth performance of juvenile whiteleg shrimp fed different experimental diets for 8 weeks

Parameters	S_0SW[a]	S_0	S_{33}	$S_{33}A$	S_{67}	$S_{67}A$	S_{100}	$S_{100}A$	Pooled SEM[b]
WG (%)[c]	539^{y}	901^{w}	834^{wx}	891^{w}	773^{x}	800^{x}	540^{y}	544^{y}	28.2
SGR (% day^{-1})[d]	3.49^{z}	4.34^{w}	4.21^{wxy}	4.33^{w}	4.09^{y}	4.14^{xy}	3.50^{z}	3.51^{z}	0.07
FCR[e]	1.46^{x}	1.06^{z}	1.09^{yz}	1.08^{yz}	1.17^{yz}	1.18^{y}	1.59^{w}	1.54^{wx}	1.07
PER[f]	1.91^{z}	2.71^{w}	2.58^{wx}	2.61^{wx}	2.38^{y}	2.43^{yz}	1.76^{z}	1.83^{z}	0.04
Survival rate (%)	75.8^{x}	84.2^{wx}	89.1^{w}	89.7^{w}	89.7^{w}	84.8^{w}	86.7^{w}	91.5^{w}	1.12

Values in same row with different superscript are significantly different at $P < 0.05$

[a] For experimental diets refer to Table 1

[b] Pooled standard error of means: $SD/\sqrt{n}$

[c] Weight gain (WG) = (final weight − initial weight) × 100/initial weight

[d] Specific growth rate (SGR) = 100 × (ln final weight − ln initial weight)/days

[e] Feed conversion ratio (FCR) = total dry feed intake, g/total wet weight gain, g

[f] Protein efficiency ratio (PER) = wet weight gain, g/protein intake, g

diets. Survival of shrimp fed S_0SW diet was significantly lower ($P < 0.05$) than those of shrimp fed the other diets, except the S_0 group.

Whole-body proximate composition

Crude protein and crude lipid contents of shrimp fed S_0SW diet were significantly lower and higher, respectively, than shrimp fed the other diets (Table 7). Crude ash content of shrimp fed S_{100} and $S_{100}A$ diets was significantly higher than those of shrimp fed the other diets. Moisture percentage of shrimp fed $S_{100}A$ diet was significantly higher ($P < 0.05$) than the shrimp fed other diets among biofloc groups.

Whole-body amino acid composition

The whole-body amino acid composition of shrimp is shown in Table 8. The total essential amino acids of S_0SW group were significantly lower than all other groups that were cultured in biofloc system except for the S_0 group ($P < 0.05$). Meanwhile, the whole-body non-essential amino acids of shrimp fed the S_0SW and S_0 diets were significantly lower than the shrimp fed on diets $S_{33}A$, S_{67}, $S_{67}A$, S_{100} and $S_{100}A$ ($P < 0.05$).

Haemolymph parameters

Plasma protein values of shrimp fed S_0 and S_{33} diets were significantly higher than those of shrimp fed $S_{100}A$ and S_0SW diets (Table 9). Moreover, plasma glucose value of shrimp fed S_{100} and $S_{100}A$ diets was significantly higher than those of shrimp fed S_{33} diet. Significantly higher ($P < 0.05$) plasma cholesterol and triglyceride values were found in shrimp fed S_0SW diet compared to the other diets.

Table 7 Whole-body proximate composition (% dry weight basis) of juvenile whiteleg shrimp fed different experimental diets for 8 weeks

Composition	S_0SW[a]	S_0	S_{33}	$S_{33}A$	S_{67}	$S_{67}A$	S_{100}	$S_{100}A$	Pooled SEM[b]
Crude protein (%)	77.1^{v}	80.1^{u}	80.2^{u}	80.2^{u}	79.6^{u}	79.5^{u}	80.3^{u}	79.5^{u}	2.44
Crude lipid (%)	4.10^{u}	2.70^{wxy}	2.98^{wx}	3.50^{v}	3.10^{vw}	2.59^{xyz}	2.38^{yz}	2.22^{z}	0.11
Crude Ash (%)	10.8^{wxy}	10.7^{xy}	11.5^{w}	11.0^{wx}	10.0^{y}	12.8^{v}	14.1^{u}	14.0^{u}	0.28
Moisture (%)	77.6^{uv}	75.2^{y}	75.7^{x}	75.8^{x}	77.3^{v}	76.6^{w}	77.3^{v}	77.7^{u}	0.15

Values in same row with different superscript are significantly different at $P < 0.05$

[a] For experimental diets refer to Table 1

[b] Pooled standard error of means: $SD/\sqrt{n}$

Table 8 Whole-body amino acid composition (mg 100 mg^{-1}) of juvenile whiteleg shrimp fed different experimental diets for 8 weeks

Amino acids	S_0SW[a]	S_0	S_{33}	$S_{33}A$	S_{67}	$S_{67}A$	S_{100}	$S_{100}A$	Pooled SEM[b]
Threonine	2.60^{w}	2.62^{w}	2.63^{vw}	2.70^{uv}	2.73^{u}	2.73^{u}	2.72^{u}	2.71^{uv}	0.01
Valine	3.11^{wxy}	3.11^{xy}	3.07^{y}	3.24^{uv}	3.30^{u}	3.21^{uvw}	3.20^{uvwx}	3.11^{wxy}	0.02
Isoleucine	2.69^{wx}	2.70^{x}	2.64^{x}	2.82^{uv}	2.89^{u}	2.81^{uvw}	2.81^{uvw}	2.74^{vwx}	0.02
Leucine	4.66^{w}	4.78^{vw}	4.75^{vw}	4.96^{u}	4.98^{u}	4.96^{u}	4.96^{u}	4.87^{uv}	0.03
Phenylalanine	2.97	2.99	3.00	3.10	3.12	3.06	2.47	3.04	0.06
Histidine	4.24^{vw}	4.29^{vw}	4.59^{v}	4.09^{w}	4.10^{w}	4.33^{vw}	5.16^{u}	4.58^{v}	0.07
Lysine	4.68^{w}	4.95^{vw}	4.93^{v}	5.19^{u}	5.03^{uv}	5.00^{uv}	5.21^{u}	4.96^{v}	0.03
Arginine	4.50^{x}	5.29^{w}	5.23^{w}	5.32^{w}	5.40^{vw}	5.26^{w}	5.81^{u}	5.57^{v}	0.06
Methionine	0.91^{wx}	0.80^{x}	1.12^{vw}	1.41^{u}	1.45^{u}	1.42^{u}	1.45^{u}	1.32^{uv}	0.05
Essential	30.4^{y}	31.5^{xy}	32.0^{wx}	32.8^{vw}	33.0^{uv}	32.8^{vw}	33.8^{u}	32.9^{uv}	0.24
Aspartic	7.69^{uv}	7.64^{v}	7.86^{uv}	8.11^{u}	8.11^{u}	8.05^{uv}	7.88^{uv}	8.05^{uv}	0.05
Serine	2.66^{x}	2.75^{wx}	2.77^{vw}	2.79^{vw}	2.79^{uvw}	2.82^{uv}	2.83^{uv}	2.86^{u}	0.01
Glutamic	10.5^{vw}	10.5^{w}	10.3^{w}	10.8^{uv}	10.9^{u}	10.9^{u}	11.0^{u}	10.7^{uvw}	0.06
Proline	4.59	4.76	4.78	5.09	4.87	5.04	4.68	4.59	0.06
Glycine	5.09^{x}	5.42^{wx}	5.52^{vw}	5.54^{vw}	5.77^{v}	5.70^{v}	6.20^{u}	6.10^{u}	0.07
Alanine	4.02^{y}	3.98^{xy}	4.06^{wx}	4.14^{vwx}	4.25^{uv}	4.18^{uvw}	4.24^{uv}	4.27^{u}	0.03
Tyrosine	2.21	2.13	2.18	2.11	2.10	2.12	2.16	2.15	0.01
Cysteine	1.27^{vw}	1.25^{w}	1.37^{vw}	1.51^{u}	1.53^{u}	1.50^{u}	1.54^{u}	1.42^{uv}	0.03
Non-essential	38.0^{w}	38.6^{w}	38.9^{vw}	40.1^{uv}	40.4^{u}	40.3^{u}	40.5^{u}	40.1^{uv}	0.25
Total	68.4^{w}	70.1^{vw}	70.8^{v}	72.9^{u}	73.4^{u}	73.1^{u}	74.3^{u}	73.0^{u}	0.47

Values in same row with different superscript are significantly different at $P < 0.05$

[a] For experimental diets refer to Table 1

[b] Pooled standard error of means: $SD/\sqrt{n}$

Table 9 Haemolymph parameters of juvenile whiteleg shrimp fed different experimental diets for 8 weeks

Parameters	S_0SW[a]	S_0	S_{33}	$S_{33}A$	S_{67}	$S_{67}A$	S_{100}	$S_{100}A$	Pooled SEM[b]
Total protein (g dL^{-1})	4.67^{v}	8.40^{u}	8.47^{u}	6.73^{uv}	7.13^{uv}	5.60^{uv}	6.80^{uv}	4.80^{v}	0.33
Glucose (mg dL^{-1})	1518^{uv}	1437^{uv}	1412^{v}	1476^{uv}	1466^{uv}	1498^{uv}	1542^{u}	1547^{u}	13.2
Cholesterol (mg dL^{-1})	38.0^{u}	19.3^{vw}	20.7^{v}	18.7^{vwx}	19.3^{vw}	16.0^{vwx}	10.7^{wx}	10.0^{x}	1.58
Triglyceride (mg dL^{-1})	81.3^{u}	52.0^{vw}	57.3^{v}	48.0^{vw}	48.0^{vw}	40.7^{vw}	37.3^{vw}	32.7^{w}	3.10

Values in same row with different superscript are significantly different at $P < 0.05$

[a] For experimental diets refer to Table 1

[b] Pooled standard error of means: $SD/\sqrt{n}$

Non-specific immune responses

The results for non-specific immune response activity showed significantly higher serum superoxide dismutase (SOD) in shrimp fed S_0 diet compared to those of shrimp fed the other diets, except for shrimp fed $S_{33}A$ and S_0SW diets (Fig. 1a). Significantly higher levels of trypsin activity was obtained in shrimp fed S_0 diet compared to those of shrimp fed S_{100} and $S_{100}A$ diets ($P < 0.05$; Fig. 1b).

Discussion

During this experiment, water quality parameters in biofloc system were in favorable condition and had no significant effect on survival of whiteleg shrimp. However, significant differences were observed in the

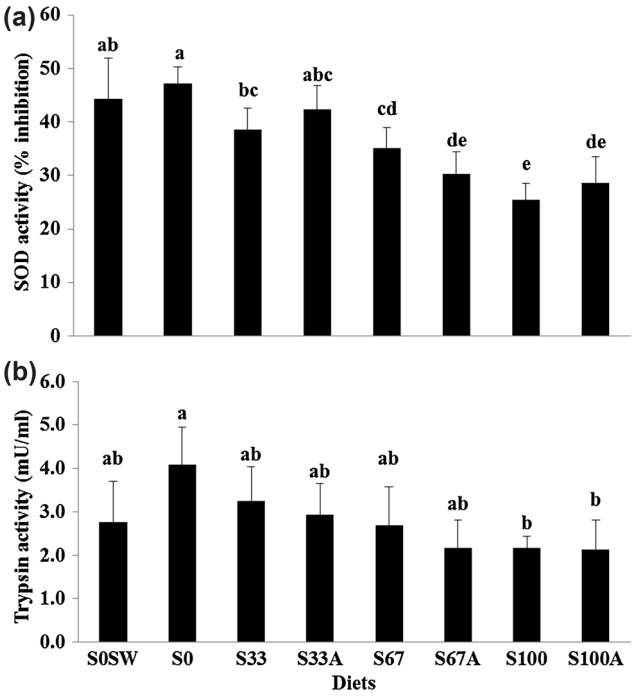

Fig. 1 Serum superoxide dismutase (**a**) and trypsin (**b**) activities of juvenile whiteleg shrimp fed different experimental diets for 8 weeks. Values are mean ± SD of three replicate tanks per sampling time in each group

amount of total suspended solids (TSS) and nitrate (NO_3^-) between clear water and biofloc system. Changes in the amount of TSS in culture water, over time, shows the development of the system. Hence, TSS is considered to be an indicator for quantitative evaluation of culture system (De Schryver et al. 2008). It has been reported that the optimal amount of TSS for shrimp culture is approximately between 200 and 400 mg L^{-1} (Plínio et al. 2015; Xu et al. 2016) and further increases in TSS level could cause gill irritation of organisms and biological oxygen demand (BOD) creates more stresses in shrimp (Beveridge et al. 1991; Hargreaves 2006; Brune et al. 2003; Ray et al. 2010), whereas lower amounts of TSS could cause growth reduction of shrimp (Samocha et al. 2004a; Ekasari et al. 2016). Proper management of TSS level could be useful for both shrimp and biofloc system (Cohen et al. 2005; Ebeling et al. 2006; De Schryver et al. 2008; Ray et al. 2010). In our experiment, nitrate, nitrite and TAN were in safe levels for whiteleg shrimp culture that is in accordance with findings of Van Wyk and Scarpa (1999). Nitrate is the final compound in the nitrification process, although it is not highly toxic for shrimp, it is recommended to be kept lower than 100 mg L^{-1} in culture water. Higher amounts of nitrate could cause stress for shrimp and reduce the growth (Plínio et al. 2015; Samocha et al. 2004a, b). These results suggest that by application of biofloc system for shrimp culture the water quality parameters could be effectively controlled in a more favorable trend (Xu et al. 2016).

The dietary protein of feed in the present study was formulated to contain 35–36% crude protein based on the requirement of whiteleg shrimp (Xia et al. 2010; Shahkar et al. 2014). In addition, the reference diet (control) contained 39% of fish meal (FM), because commercial shrimp feeds usually contain 25–50% of FM (Dersjant-Li 2002; Tacon and Barg 1998). The results illustrated for WG, SGR, FCR, PER and survival rate clearly indicate the benefits of shrimp culture in biofloc system in comparison to clear water. This is in agreement with previous studies that have proven growth and immunity enhancement of shrimp that were cultured in biofloc system (Kim et al. 2014). The observation for growth performance also showed that up to 33% of the FM in a practical diet for whiteleg shrimp, reared in biofloc system, could be effectively substituted by the soybean meal (SBM). Previously, numerous studies have been conducted to evaluate the suitability of various feed ingredients as alternative protein sources for FM; as partial or even total replacement of FM by plant ingredients in the diet of *L. vannamei* has been reported by several authors (Amaya et al. 2007; Suárez et al. 2009; Olmos et al. 2011; Ye et al. 2011; Liu et al. 2012; Yue et al. 2012; Gu et al. 2013; Sá et al. 2013; Sookying et al. 2013; Kuhn et al. 2016; Xie et al. 2016). In the present study, application of

methionine and lysine in the diet did not significantly influence the growth performance. Probably the EAA requirements of shrimp may have been met without the additional amino acids and excess amino acids in the diet did not result in higher growth. This might be related to the nutritional quality of bioflocs, as they are known to have considerable amount of protein, lipid, carbohydrate and ash content as an aquatic feed (Crab et al. 2010). In contrast to our results, Yue et al. (2012) found that using lysine and methionine in the diet of *L. vannamei* more amounts of FM could be replaced by SBM, although it should be considered that in their experiment shrimp were not cultured in biofloc system. According to our results, the higher growth performance of shrimp that were cultured in biofloc in comparison to the clear water group is in agreement with previous findings (Ray et al. 2011; Irshad et al. 2016). Valle et al. (2015), replaced fish meal with biofloc flour and protein hydrolysate in the diet of *L. vannamei* and indicated that biofloc flour is a potential ingredient that can be used as substitute for fishmeal. In our study, whole-body proximate composition was not affected much by the FM substitution with SBM. Although, higher whole-body protein level in shrimp that were cultured in biofloc system, in comparison to shrimp that were cultured in clear water, indicates the positive influence of biofloc in increasing the whole-body protein content. Similar observation has been reported in a study on whiteleg shrimp by Xu et al. (2012). Khatoona et al. (2016), reported that 50% biofloc in the diet of *L. vannamei* could increase the whole-body protein content, compared to the diets without biofloc inclusion. This shows the positive contribution of bioflocs in increasing the whole-body protein level in shrimp.

As a plant protein source, SBM is widely used as a partial or complete substitute for animal protein (Hertrampf and Pascual 2000). Several nutritionists have investigated the feasibility of SBM in aquafeeds, and the results are contradictory, because the levels of SBM that can be used without causing growth reduction are highly species-specific and influenced by culture systems (Yue et al. 2012). For instance, Markey (2007) reported that 58% of SBM is being used in grow out culture of whiteleg shrimp. Lim and Dominy (1990) demonstrated that 40% level of a marine mixed protein could be replaced by solvent extracted SBM, whereas higher levels exerted reduced growth performance of *L. vannamei*. It was also reported that 80% FM could be replaced by co-extruded soybean and poultry by-product meal supplemented with egg in indoor recirculating water system (Davis and Arnold 2000). Likewise, other researchers suggested different inclusion levels of FM in the diet of whiteleg shrimp culture in different conditions (Samocha et al. 2004a; Browdy et al. 2006; Patnaik et al. 2006; Amaya et al. 2007). This is while in marine shrimp diets, higher levels of dietary SBM usually resulted in lower growth (Akiyama 1990; Floreto et al. 2000). Likewise, Yue et al. (2012) suggested that FM inclusion can be reduced to approximately 200 g kg^{-1} diet of whiteleg shrimp when SBM and peanut meal are included instead. Reduced growth performance and increased FCR in *L. vannamei* fed different diets that gradually replace FM by SBM could be because of anti-nutritional factors, such as saponins, phytoestrogens, allergens, phytate (myo-inositol-1,2,3,4,5,6-hexakisphosphate), protease inhibitors, trypsin inhibitors, lectins and antivitamins (Francis et al. 2001). For example, trypsin inhibitors and lectins prevent the proper activity of digestive enzymes (Gemede and Ratta 2014). The other limitation in application of SBM in fish diet is the improper amino acid composition that does not meet the nutritional requirements of fish (Floreto et al. 2000). In addition, n-3 lipid composition of SBM is much lower comparing to FM, and thus high inclusion levels of SBM in the diet may result in reduced growth performance (Sharawya et al. 2016).

According to the results obtained for whole-body amino acid composition of shrimp in this study, higher essential and non-essential amino acids were observed in biofloc cultured shrimp comparing to clear water cultured shrimp. In addition, higher amounts of FM seem to have negative effect of the amino acid composition. This might be partly related to the higher digestibility of amino acids in FM comparing to SBM (Liu et al. 2013; Yang et al. 2009). Similar results were observed by Xie et al. (2016), while replacing FM by soy protein concentrate and soybean meal based protein blend for juvenile white shrimp. Moreover, more investigations are required for clarifying the mechanism.

Haemolymph metabolites in crustaceans represent the physiological, nutritional and immunological stress indicators (Becerra-Dorame et al. 2012). In this study, plasma protein was higher in lower substitute level of SBM which means high FM containing diet resulted in high total protein content in haemolymph of whiteleg shrimp and this was according to the results obtained by Rosas et al. (2000). Furthermore, in the present study, plasma cholesterol and triglyceride levels have decreasing trend among the dietary treatments. In other studies, same decreasing trend of cholesterol was found in rainbow trout, *Oncorhynchus mykiss* (Kaushik et al. 1995) and gilthead sea bream, *Sparus aurata* (Venou et al. 2006) due to inclusion of SBM in the diet. This might have happened because of the hypocholesterolemic effect of SBM (Davis and Morris 1997; Yue et al.

2012). The plasma glucose levels in whiteleg shrimp increased with increasing substitute level of SBM among the experimental diets. These results are probably related to higher inclusion level of plant protein (SBM) in the diet, which is definitely followed by the increment of carbohydrate source in the diet.

Trypsin is a protease enzyme that is excreted by the pancreas and plays a great role in the digestion of proteins (Erlanger et al. 1961). The results of the present study showed that the 100% replacement level of FM, with no regard to the inclusion of amino acids, had negative effects on the activity of trypsin. This might be related to the trypsin inhibitory properties of SBM (Francis et al. 2001). There are several natural trypsin inhibitors, also known as serine protease inhibitors, and SBM is one of the most important ones. SBM controls the activation and catabolism of proteins by the inhibition of serine proteases (Silverman et al. 2001). Thus, at 100% replacement level, SBM will act as trypsin inhibitor for juvenile whiteleg shrimp. Previously, it was shown that biofloc contain several extracellular enzymes such as protease and amylase (Yu et al. 2009). The extracellular enzymes that are synthesized by the microorganisms attached to the biofloc would release to the digestive tract of the host organism after being ingested. These enzymes would take part along with the total enzymes activities of the body. In addition, the bioactive compounds present in the biofloc may possibly improve digestive enzyme activities of the shrimp (Xu et al. 2012). Observation with the trypsin activity suggested an improved efficiency of whiteleg shrimp to digest SBM diet reared in biofloc system as compared to those shrimp reared in clear water fed diets with 100% FM. The superoxide dismutase (SOD) is an antioxidant enzyme that functions by removing damaging reactive oxygen species (ROS) from the cell. The mechanism is by catalyzing the segregation of the superoxide (O_2-) radicals to ordinary molecules such as oxygen or hydrogen peroxide (Fattman et al. 2003; Lin et al. 2010). In the present study, SOD activity was reduced when the FM substitution level exceeded 33% of diet. The results of this study demonstrated that shrimp fed diets with up to 33% replacement level were in better physiological and health condition compared to higher inclusion rates. In conclusion, this study showed that up to 33% FM in the diets of juvenile whiteleg shrimp reared in biofloc system could be replaced by SBM with or without supplementation of methionine and lysine.

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