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RESEARCH PAPER

Effects of Supplementing Low-Molecular-Weight Fish Hydrolysate in High Soybean Meal Diets on Growth, Antioxidant Activity and Non-Specific Immune Response of Pacific White Shrimp (*Litopenaeus vannamei*)

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Abstract

Supplemental effects of low-molecular-weight fish hydrolysate (LWFH) was investigated in high soybean meal (HSM) diets fed to *Litopenaeus vannamei* (0.44 ± 0.03 g) for 48 days. The HSM diet, containing 15% fish meal and 47% soybean meal, was supplemented with 0, 5, 10, 15 or 20 g kg^{-1} LWFH (HSM0, HSM5, HSM10, HSM15 and HSM20). LWFH significantly ($P < 0.05$) improved growth and the highest growth was found in HSM15. Feed intake significantly increased at ≥ 10 g kg^{-1} LWFH, and the lowest feed conversion ratio was observed in HSM10 group. Shrimp survival significantly increased at ≥ 15 g kg^{-1} LWFH compared to HSM0 group. Significantly higher serum peroxidase, acid phosphatase and alkaline phosphatase activities were observed in HSM20 group and total antioxidant capacity increased at ≥ 15 g kg^{-1} LWFH. HSM10 group exhibited significantly higher phenoloxidase activity than HSM0, and superoxide dismutase activity enhanced in HSM5 and HSM10. Intestinal inflammatory genes expression assay showed the significant decrease of activating transcription factor 4 expression in HSM15 group compared to HSM0 group, and macrophage migration inhibitory factor expression decreased significantly at 5-15 g kg^{-1} LWFH. To conclude, 10-15 g kg^{-1} LWFH in HSM diet improves growth, antioxidant activity and innate immunity.

Keywords: High soybean meal diets, Low-molecular-weight fish hydrolysate, Antioxidant activity, Innate immunity, *Litopenaeus vannamei.*

Introduction

Fish meal is one of the primary protein sources in traditional and commercial shrimp feed formulations (Hernandez, Sarmiento-Pardo, & Abdo, 2004). However, high demand and limited supply have led to high prices for fish meal during the last few years (Kader & Koshio, 2012). Therefore, replacing fish meal with cost-effective alternative protein sources has become a focus. The use of plant proteins as alternative protein sources to replace fish meal in shrimp feed has been studied worldwide (Gatlin *et al*., 2007). However, negative effects on growth performance, feed intake, antioxidant activity, intestinal health and immune response have been reported in shrimp species such as *Marsupenaeus japonicus*, *Litopenaeus vannamei* and *Penaeus chinensis* fed high plant protein diets (Lim & Dominy, 1990; Bulbul *et al*., 2015a; Xie, Liu, Zeng, Niu, & Tian, 2016). These results may be due to secondary dietary amino acid composition, lower palatability, and presence of anti-nutritional components in plant protein diets (Paripatananont, Boonyaratpalin, Pengseng, & Chotipuntu, 2001; Amaya, Davis, & Rouse, 2007; Rahman *et al*., 2010; Yue *et al*., 2012b; Bulbul, Koshio, Ishikawa, Yokoyama, & Kader, 2015b; Chiu *et al*., 2015). Meanwhile, research efforts are underway to identify an appropriate blend of plant products and other alternative feed ingredients to prevent nutritional deficiencies and ensure a proper supply of essential nutrients. This may further increase the replacement level of fish meal with plant proteins without detrimental effects on animals' performance by restoring a proper balance of amino acids and increasing the palatability of the diet (Kader *et al*., 2012a; Kader *et al*., 2012b).

Fish hydrolysate is a promising core material for high plant protein diets, as it may improve growth, feed utilization and survival rate of marine animals (Aksnes, Hope, Jonsson, Bjornsson, & Albrektsen, 2006a; Aksnes, Hope, Hostmark, & Albrektsen, 2006b; Khosravi *et al*., 2015). Dietary inclusion of protein hydrolysates can improve innate immunity, change intestinal morphology and enhance the intestinal immune response (Duarte, Vinderola, Ritz, Perdigón, & Matar, 2006; Khosravi *et al*., 2015).

However, fish hydrolysates contain differently sized molecular weight compounds which may affect absorption capacity and the rate of passage of food through the gastrointestinal tract (Espe & Lied, 1999). Low-molecular-weight peptides are the main components of fish hydrolysates and play an important role in promoting growth performance in cultured marine species. Some researchers have demonstrated the functional and bioactive properties of these peptides (low molecular weight, easily absorbed, stimulate feeding, antioxidant, antihypertensive, antimicrobial, and immunomodulatory) (Byun, Lee, Park, Jeon, & Kim, 2009; Nazeer, Kumar, & Ganesh, 2012). Dietary composition affects intestinal health; thus, it can affect the expression of inflammatory factors in the intestinal mucosa, such as lipopolysaccharide-induced tumor necrosis factor-α (LITAF), macrophage migration inhibitory factor (MIF), activating transcription factor 4 (ATF4) and Ras-associated protein 6A (RAB6A). However, little is known about how dietary protein sources or fish hydrolysates affect the expression of these genes in shrimp.

The aim of this study was to evaluate the effects of supplementing low-molecular-weight fish hydrolysate (LWFH) in high soybean meal diets on growth, antioxidant activity, non-specific immune response and expression of pro-inflammatory genes in the digestive tract of Pacific white shrimp *Litopenaeus vannamei*.

Materials and Methods

Preparation of Low-Molecular-Weight Fish Hydrolysate

The LWFH was produced from whole sardine (*Sardina melanostictus*) bodies using enzymes. First, minced sardine was homogenized in a four-fold volume of distilled water and was enzymatically hydrolyzed with Nematolyt and Trypsase (3:1; Jiangsu Nanjing Pangbo Biological Engineering Co. Ltd., Nanjing Jiangsu, China). The enzymes were added at 0.4% of surimi weight, and hydrolysis was carried out at 55 °C for 8 h in a water bath (pH = 7.5). After the enzymatic treatment, the hydrolysate was maintained at 95 °C for 15 min to inactivate the enzymes. After chilling, the hydrolysate was centrifuged at 5,000 rpm and 4 °C for 40 min. The micro-molecular fish hydrolysate was obtained by filtering through a 1,000 Da StarMem-001A membrane separator (Fumei Science and Tech Co. Ltd., Xiamen, Fujian, China), followed by a 200 Da filter. The retentate was freeze-dried and used in the experimental diets. The peptide profile of the protein hydrolysate is provided in Fig. 1. The hydrolysate was freeze-dried for the peptide profile analysis. The peptide profile of the hydrolysate was determined using size exclusion chromatography and a high-performance liquid chromatography system (Aglient1200; Agilent Technologies, Palo Alto, CA, USA) equipped with TSK G2000 SWXL 300 mm × 7.8 mm chromatography column (Tosoh Bioscience LLC., King of Prussia, PA, USA) at a detection wavelength of 220 nm (Zheng, Liang, Yao, Wang, & Chang, 2013). The samples were solubilized in water containing 0.3% sodium dodecyl sulphate, centrifuged for 10 min at 10,000 rpm, decanted and filtered before applied to the column. In addition, we selected Cytochrome C (MW12500), Aprotinin (MW6533), Oxidized glutathione (MW613), amino acetic acid-amino acetic acid-amino acetic acid (MW189), and glycine (MW75) as a standard substance to make the standard curve using GPC software. The instrument condition was as follows: the flow rate of moving phase was 0.5 ml min^{-1}, pillar temperature was 30 °C, detection wavelength was 214 nm, and the sample size was 20 µl.

Experimental Diets

Formulation and proximate composition of the experimental diets are shown in Table 1. A basal diet containing 15% fish meal and 47% soybean meal was

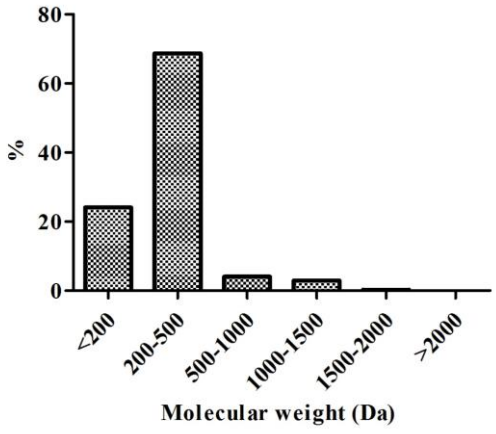

Figure 1. Peptide profile of the protein hydrolysate.

Table 1 Formulation and proximate composition of the experimental diets (g kg^{-1})

Ingredients	HSM0	HSM5	HSM10	HSM15	HSM20
Fish meal[1]	150	150	150	150	150
Soybean meal[2]	470	470	470	470	470
LWFH[3]	0	5	10	15	20
Squid visceral paste	20	20	20	20	20
Shrimp meal	50	50	50	50	50
Wheat flour	223	218	213	208	203
Fish oil	22	22	22	22	22
Soybean oil	10	10	10	10	10
Lecithin	10	10	10	10	10
Choline chloride	5	5	5	5	5
Monocalcium phosphate	15	15	15	15	15
Premix[4]	10	10	10	10	10
Sodium alginate	10	10	10	10	10
Amino acid mixture[5]	5	5	5	5	5
Proximate composition (%)					
Moisture	135	118	114	113	123
Crude protein	445	437	457	454	456
Crude lipid	75.6	75.5	75.5	75.4	75.4
Gross energy (kJ/g)	189	198	198	199	198

[1]Fish meal, obtained from Tecnologica de Alimentos S.A, Peru, crude protein 665, crude lipid 86 (g kg^{-1} dry matter).
[2]Soybean obtained from Tecnologica de Alimentos S.A, Peru, crude protein 489, crude lipid 10 (g kg^{-1} dry matter).
[3]Low-molecular-weight fish hydrolysate
[4]Mineral premix (5 g kg^{-1}), Vitamin premix (2 g kg^{-1}), Mold inhibitor (1.5 g kg^{-1}) and Ethoxyquin (0.5 g kg^{-1}); Mineral premix and Vitamin premix was prepared according to Ye *et al*. (2012).
[5]Provided as percentage of total: lysine, 50; methionine, 30; threonine, 20. All amino acids are coated amino acids.

prepared and used as a high soybean meal (HSM) diet. Then, the basal diet was supplemented with 0, 5, 10, 15 or 20 g kg^{-1} LWFH to make five experimental diets designated as HSM0, HSM5, HSM10, HSM15, and HSM20. The dry ingredients were ground using a hammer mill and then passed through a 180 µm mesh. The filtered ingredients were thoroughly mixed with lipids before water was added to produce a mash. The dough was pelleted through a 1.5-mm diameter twin-screw granulator (CLFM4×1TS; SCUT, Guangdong, China). The diets were air-dried at room temperature for 24 h, packed in double plastic bags, and stored at −20 °C until use. The amino acid composition of the experimental diets is shown in Table 2. Amino acid composition of the experimental diets was analyzed according to the method of GB/T18246-2000 detected by Pony Testing Co. Ltd (Beijing, China).

Experimental Shrimp and Feeding Trial

L. vannamei were supplied by a hatchery at Pokphand Group Co. Ltd. in Zhangzhou (Fujian, China). Prior to starting the experiment, all shrimp were kept in indoor tanks and fed a commercial diet in a temporary rearing tank for 2 weeks to acclimate them to the experimental conditions. At the end of the acclimation period, 30 shrimp of similar size (mean weight, 0.44 ± 0.03 g) were distributed randomly into each of 20 fiberglass cylindrical tanks (150 L). Each diet was assigned randomly to quadruplicate tanks. These tanks were part of a recirculating water system that included a settling tank, an air pump, and a water pump. The shrimp were hand fed to apparent satiation three times daily (08:00, 14:00, and 19:00) for 48 days. Each aquarium had a water flow velocity of 5 L/min. During the rearing period, water temperature fluctuated from 28 to 31 °C, pH was 7.8 ± 0.3, salinity was 20–23‰, and the dissolved oxygen concentration was 7.0 ± 0.2 mg l^{-1}. Photoperiod was maintained on a 12:12 light:dark schedule. All rearing tanks were provided with continuous aeration.

Sample Collection

At the end of the feeding trial, shrimp were fasted for 24 h before harvest. All the shrimps from each tank were weighed individually on a microbalance, and the final number of shrimp in each tank was recorded to determine growth parameters and survival (Gao *et al*., 2017). Hemolymph of 15 shrimp from each aquarium was withdrawn from the pericardial cavity using a 1 mL syringe and then kept at 4 °C overnight. Serum was obtained by centrifugation at 4000 rpm and 4 °C for 10 min and stored at −80 °C for the antioxidant enzymes activity analyses. The intestinal tract was removed from the same 6 shrimp, frozen immediately in liquid nitrogen, and stored at −80 °C for inflammatory genes expression assay (Huang, Wang, Zhang, & Song, 2017).

Analytical Methods

Proximate Analysis of the Experimental Diets

Proximate composition of experimental diets

Table 2 Amino acid composition of the experimental diets (g kg^{-1} dry matter)

AA/ΣAA	Diets				
	HSM0	HSM5	HSM10	HSM15	HSM20
EAA[1]					
Valine	49	47.9	48.1	44.3	43.5
Methionine	31	35	35.6	31.3	29.8
Isoleucine	31.5	35.9	29.3	34.2	32.9
Leucine	52.8	56.9	53.8	55.8	53.2
Phenylalanine	28.3	26.3	22.8	23.2	26.9
Histidine	24.2	25.5	26.9	26.4	27.7
Lysine	77	80.5	89.4	93.5	84.3
Arginine	65.7	66	68.8	66.5	60.6
Threonine	38.5	39.7	40.8	40.1	41.6
Tryptophan	11	8.5	11.1	9.6	8.7
ΣEAA	409.2	421.7	426.6	425.2	408.9
NEAA[2]					
Tyrosine	45.2	47.6	41.8	45.9	46.6
Proline	121.9	114.1	111.1	120.2	123
Aspartic acid	92.1	92	91.3	89.7	93.8
Serine	39.6	38.9	38.6	39.5	41.1
Glutamic acid	175	177.3	173.6	176.5	175.2
Glycine	48.5	45.7	49.2	44.3	46.4
Alanine	44.7	43.5	47	39.8	47.2
Cystine	24	19.4	20.7	18.4	18.4
ΣNEAA	590.8	578.3	573.4	574.8	591.1

[1] EAA: essential amino acids.
[2] NEAA: nonessential amino acids.

was analyzed in triplicates according to the standard methods of the Association of Official Chemists (AOAC, 1995). The diet samples were oven dried at 105 °C to constant weight to determine moisture content. Crude protein content was determined by the Kjeldahl method (N × 6.25) using an Auto Kjeldahl System (FOSS Kjeltec 8400, Switzerland). Lipid content was analyzed according to the Soxhlet diethyl ether extraction method. Gross energy was determined by an adiabatic bomb calorimeter (Parr 6300, USA).

Serum Antioxidant and Non-Specific Immune Indices

Serum enzymes activity was measured with commercial assay kits (Nanjing Jiancheng Institute, Nanjing, China) according to the manufacturer's instructions. Peroxidase (POD) activity was measured following the reduction of hydrogen peroxide at 420 nm, and determined according to the change in absorbance. Phenoloxidase (PO) activity was determined using a biotin double-antibody sandwich enzyme-linked immunosorbent assay kit following the manufacturer's instructions. Total antioxidant capacity (T-AOC) was the amount of activity in 1 ml min^{-1} serum for the absorbance value to increase 0.01 units at 37 °C. Briefly, total superoxide dismutase (T-SOD) activity was measured by the ability of the sample to inhibit superoxide anions generated by xanthine and the xanthine oxidase reaction system. One activity unit was defined as the amount of enzyme necessary to inhibit 50% of the color formation measured at 550 nm. T-SOD activity was expressed as unit per milligram hepatic protein. Acid phosphatase (ACP) and alkaline phosphatase (AKP) activities were measured following disodium phenyl phosphate decomposition. One unit was defined as the amount of enzyme in 1 g of tissue that produced 1 mg phenol every 15 min at 37 °C (Zheng *et al*., 2013).

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (qPCR) Analysis

An approximately 80 mg intestinal tract sample was used to extract total RNA with Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The tissue was homogenized in Trizol Reagent using a fully automatic grinding mill (Tissuelyser-24; Shanghai Jingxing Science and Technology Co., Ltd., Shanghai, China). The mixture was precipitated with isopropanol, washed twice with 75% ethanol, and the RNA pellet was dissolved in bacteria-free water. RNA purity and concentration were measured using an ND-2000 spectrophotometer (NanoDrop 2000; NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was confirmed by 1.5% agarose gel electrophoresis of 1 μg RNA stained with ethidium bromide in 1× TAE buffer. The gels were then subjected to ultraviolet light and photographed in a GS-800 Ultraviolet Transilluminator (UVP, Upland, CA, USA). A 2-μg RNA sample was synthesized to cDNA using the Thermo Scientific RevertAid First-Strand Synthesis System for RT-PCR (Invitrogen)

with Oligo $(dT)_{18}$ primers according to the manufacturer's protocol. The reaction was incubated using a Peltier Thermal Cycler 200 (MJ Research, Watertown, MA, USA). cDNA integrity was confirmed by 1.5% agarose gel electrophoresis of 1 µg cDNA stained with ethidium bromide in 1× TAE buffer. The gels were then subjected to ultraviolet light and photographed in a GS-800 Ultraviolet Transilluminator.

The PCR reactions were performed in a total volume of 20 µl, containing 1 µl of each primer (10 µM), 9 µl of the diluted first-strand cDNA product, and 10 µl AceQ® qPCR SYBR® Master Mix (Nanjing Jiancheng Institute). The primer sequences for the reference gene (β-actin), LITAF, ATF4, MIF, and RAB6A genes were designed based on published *L.vannamei* cDNA sequences on Gen Bank and are listed in Table 3. The real-time PCR program was 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, annealing for 15 s at 60 °C, then 60 °C for 60 s. A melting curve analysis was performed to confirm that only one PCR product was present in each reaction (Gao *et al*., 2016).

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS ver. 17.0 software (SPSS Inc., Chicago, IL, USA). When ANOVA detected a difference among groups, Duncan's multiple range test was used to identify the difference in the means. A *P*-value < 0.05 was considered significant. Data are presented as mean ± standard error.

Results

Growth Performance

Shrimp growth performance and feed utilization fed the experimental diets are shown in Table 4. FBW and WG were significantly ($P<0.05$) increased by the increment of LWFH level up to 15 g kg^{-1}, and thereafter decreased significantly. The survival rate of shrimp fed the experimental diets was 65.52–80.06% and the highest survival rate was observed in the group fed the HSM15 diet, which was significantly higher than that of shrimps fed HSM0, HSM5 and HSM10 diets ($P<0.05$). Significantly higher feed intake was observed in groups fed ≥ 10 g kg^{-1} LWFH compared to HSM0 group. Feed conversion ratio was significantly decreased in HSM10 group in comparison to the group fed the basal diet.

Serum Antioxidant Activity and Non-Specific Immune Indices

The serum antioxidant enzymes activity and innate immune parameters are shown in Table 5. The results showed significant enhancement of POD activity in HSM20 group compared to HSM0 and HSM5 groups ($P<0.05$). T-AOC activity increased in response to increasing dietary LWFH level and significantly higher activity was detected in HSM15 and HSM20 groups compared to the other groups ($P<0.05$). Also, significantly higher PO activity was observed in the group fed HSM10 group, while increasing supplementation level of LWFH to 20 g kg^{-1} resulted in a significant decrease of PO activity. The groups fed 5 and 10 g kg^{-1} LWFH revealed

Table 3 Sequence of the primers used for q-PCR in this study

Target gene	No.sequence	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)
LITAF[1]	JN180640.1	GCAGTCAACGCACATGATCT	TTGTATTTGCCCAGGAAAGC	60
ATF4[2]	JX908828.1	AGAACCTGCTTCCCCTGTTT	TAGCATCTGCTGGTGACAGG	60
MIF[3]	KC513658.1	TGGCAAGTTAGGGGTTGAAG	TCCCAATATCTGGTGGAAGG	60
RAB6A[4]	JX073679.1	CTCCAGCTCTGGGATACTGC	TGCTTTTCGTTCACCTTCCT	60
β-actin	AF300705.2	GCTAACCGCGAGAAGATGAC	CAGGGCATATCCCTCGTAGA	60

[1]LITAF: Lipopolysaccharide-induced tumor necrosis factor-α
[2]ATF4: Activating transcription factor 4
[3]MIF: Macrophage migration inhibitory factor
[4]RAB6A: Ras associated protein 6A

Table 4 Growth performance and feed utilization of Pacific white shrimp (0.44 ± 0.03 g) fed the experimental diets for 48 days

	HSM0	HSM5	HSM10	HSM15	HSM20
FBW[1]	4.53±0.2^{c}	5.08±0.1^{b}	5.46±0.1^{ab}	5.83±0.2^{a}	5.26±0.2^{b}
WG[2]	934±40.5^{c}	1063±40.6^{b}	1152±27.8^{ab}	1236±44.0^{a}	1101±43.6^{b}
FI[3]	0.97±0.03^{b}	1.02±0.04^{ab}	1.08±0.02^{a}	1.08±0.02^{a}	1.11±0.02^{a}
FCR[4]	1.23±0.03^{a}	1.20±0.05^{ab}	1.07±0.05^{b}	1.17±0.06^{ab}	1.15±0.03^{ab}
Survival rate (%)	65.5±2.1^{c}	67.0±3.2^{c}	71.5±3.1^{bc}	80.1±1.7^{a}	75.0±1.0^{ab}

Values are mean of quadruplicate groups and presented as mean ± SE. Values in the same row having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.
[1]Final body weight (g) = total shrimp weight (g) / the number of shrimp at the end of experiment.
[2]Weight gain (%) = [(final body weight − initial body weight) / initial body weight × 100].
[3]Feed intake (% $days^{-1}$) = W / [(N_0 + N_t) / 2], W is the total feed weight (dry weight, g) shrimp ingested during the experimental period.
[4]Feed conversion ratio = dry feed fed / wet weight gain

significantly higher T-SOD activity than those fed the basal diet. Significantly higher ACP and AKP activities were detected in the group received HSM20 diet compared to those fed HSM0 and HSM5 diets.

LITAF, ATF4, MIF, and RAB6A mRNA Expression Levels

LITAF, ATF4, MIF, and RAB6A mRNA expression levels are shown in Table 6. The highest expression level of LITAF was detected in HSM20 group which significantly differed from that of the groups fed HSM10 and HSM15 diets. ATF4 mRNA expression was significantly lower in the HSM15 group than that in the HSM0 group. MIF mRNA expression was significantly decreased in groups fed 5-15 g kg^{-1} dietary LWFH in comparison to those fed HSM0 and HSM20 diets. No significant differences were observed in RAB6A expression among dietary treatments ($P>0.05$); however, it seemed to be upregulated as LWFH supplementation level was increased.

Discussions

Previous research on the biological functions of dietary fish hydrolysates focusing on cultured fish species revealed their promoting effects on growth and feed utilization (Cahu, Infante, Quazuguel, & Gall, 1999; Aksnes *et al*., 2006a; Aksnes *et al*., 2006b; Zheng, Liang, Yao, Wang, & Chang, 2012; Zheng *et al*., 2013; Cai *et al*., 2015; Khosravi *et al*., 2015). Niu *et al*. (2014) reported that adding fish protein hydrolysate increases growth rate and feed efficiency of *L. vannamei*. Similar results were also observed in rainbow trout (Aksnes *et al*., 2006a). Results of the present study confirmed the previous findings suggesting that a certain amount of dietary LWFH promotes growth performance and feed efficiency in *L. vannamei*. Protein hydrolysates may improve animal growth and feed utilization because free amino acids and low molecular weight compounds released during hydrolysis may act as feed attractants, promoting FI and WG (Berge & Storebakken, 1996; Carvalho, Sá, Oliva-Teles, & Bergot, 2004; Grey, Forster, Dominy, Ako, & Giesen, 2009; Chotikachinda, Tantikitti, Benjakul, Rustad, & Kumarnsit, 2013; Ho, Li-Chan, Skura, Higgs, & Dosanjh, 2014). A feed-promoting effect was also observed in the present study, as FI was increased when the LWFH level was increased in the experimental diets. Another reason may be that the experimental fish hydrolysates produced in the present study mainly contained low molecular weight

Table 5 Serum non-specific immune responses and antioxidant enzymes activity of Pacific white shrimp fed the experimental diets for 48 days

	HSM0	HSM5	HSM10	HSM15	HSM20
POD[1]	35.3±0.4^{b}	35.4±0.3^{b}	36.8±0.7^{ab}	36.8±0.4^{ab}	38.1±0.9^{a}
PO[2]	5.18±0.12^{b}	5.07±0.02^{b}	5.42±0.07^{a}	5.28±0.05^{ab}	4.77±0.08^{c}
T-AOC[3]	5.49±0.4^{b}	6.25±0.2^{b}	6.25±0.2^{b}	7.52±0.4^{a}	7.89±0.4^{a}
T-SOD[4]	333.7±8.6^{c}	350.7±2.8^{ab}	356.9±4.8^{a}	348.9±5.4^{abc}	336.0±2.2^{bc}
AKP[5]	6.09±0.6^{b}	6.37±0.9^{b}	8.72±1.6^{ab}	8.24±0.4^{ab}	9.49±0.5^{a}
ACP[6]	15.95±2.1^{b}	18.66±1.8^{b}	20.675±2.3^{ab}	20.57±0.5^{ab}	22.84±1.7^{a}

Values are mean of quadruplicate groups and presented as mean ± SE. Values in the same row having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.
[1]Peroxidase activity (U ml^{-1})
[2]Phenol Oxidase activity (UL^{-1})
[3]Total Antioxidant Capacity (U ml^{-1})
[4]Total Superoxide Dismutase activity (U$mg prot^{-1}$)
[5]Alkaline Phosphatase activity (U$g prot^{-1}$)
[6]Acid Phosphatase activity (U$g prot^{-1}$)

Table 6 Relative expression level of LITAF, ATF4, MIF and RAB6A genes in Pacific white shrimp fed the experimental diets for 48 days

Index	HSM0	HSM5	HSM10	HSM15	HSM20
LITAF[1]	1.00±0.25^{ab}	0.98±0.19^{ab}	0.55±0.14^{b}	0.85±0.03^{b}	1.46±0.04^{a}
ATF4[2]	1.00±0.08^{a}	0.90±0.10^{ab}	0.59±0.21^{ab}	0.43±0.03^{b}	0.71±0.26^{ab}
MIF[3]	1.00±0.05^{a}	0.52±0.14^{b}	0.38±0.10^{b}	0.46±0.10^{b}	1.05±0.11^{a}
RAB6A[4]	1.00±0.15	0.93±0.11	1.06±0.18	1.10±0.22	1.13±0.21

Mean values and standard error (± SE) are present for each parameter. The values of the expression of the target genes are presented as relative to control (set to 1). Data were normalized by β-actin.
Values are mean of quadruplicate groups and presented as mean ± SE. Values in the same row having different superscript letters are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.
[1]Lipopolysaccharide-induced tumor necrosis factor-α
[2]Activating transcription factor 4
[3]Macrophage migration inhibitory factor
[4]Ras associa*18: 881-889 (2018)*ed protein 6A

compounds (essential amino acids, such as lysine and methionine; nucleotides, anserine and taurine) that stimulate production of insulin-like growth factors I and II and growth hormone, which enhance growth performance (Hevrøy *et al*., 2007; Espe, Hevrøy, Liaset, Lemme, & El-Mowafi, 2008; Martínez-Alvarez, Chamorro, & Brenes, 2015). In this study further increment of LWFH level from 15 to 20 g kg^{-1} resulted in significant decrease of growth performance suggesting that dietary LWFH level should be controlled strictly and that an excessive dietary LWFH level is detrimental to shrimp. This may be because high quantities of free amino acids in feed can change absorption rates in the gastrointestinal tract, which induce premature absorption of particular free essential amino acids in relation to absorption of amino acids presented in polypeptide chains (Martínez-Alvarez *et al*., 2015). Notably, high free amino acid and di- and tri-peptide concentrations in feed may be rapidly absorbed by enterocytes and metabolized rather than being used for protein synthesis and growth, which could saturate intestinal transporters, resulting in imbalanced amino acid absorption and reduced retention of dietary protein (Cahu, Infante, Quazuguel, & Gall, 1999; Aragao *et al*., 2004; Niu *et al*., 2014).

In the present study, the survival rate of shrimp was increased with increasing dietary LWFH supplementation level up to 15 g kg^{-1}, and slightly decreased thereafter. A previous study on Japanese flounder (*Paralichthys olivaceus*) also showed that increasing the level of size-fractionated fish hydrolysate in high plant protein diet leads to higher survival rate (Zheng *et al*., 2012). However, overall the survival rate of shrimp (65.52–80.06%) in the present study was relatively low in comparison to the previous studies (75.0%–98.9%) (Rahman *et al*., 2010; Yue *et al*., 2012b). This may be due to differences in dietary composition. In the present study, a very high level of soybean meal (47%) was used as dietary protein source which may negatively affect shrimp health and survival rate resulting from imbalanced amino acids, lower palatability and presence of anti-nutritional and toxic factors, and indigestible carbohydrates (Bulbul *et al*., 2015a). The improvement of shrimp survival rate by adding LWFH to the soybean meal based diets can be due to the improved nutritional quality.

Enzymatic hydrolysis of proteins can produce biologically active peptides with immunostimulating and antibacterial properties (Kotzamanis, Gisbert, Gatesoupe, Infante, & Cahu, 2007; Kim & Wijesekara, 2010). The proPO system is acknowledged to be the most important immune system in crustaceans (Iwanaga & Lee, 2005). The terminal enzyme of the proPO system, PO activity is involved in crucial immune responses of invertebrate animals (Soderhall & Cerenius, 1998). T-SOD is one of the main antioxidant enzymes which can detoxify from superoxide radical by dismutation and H_2O_2 formation, while POD is one enzyme involved in the cellular detoxification of H_2O_2 (Dorr, Pacini, Abete, Prearo, & Elia, 2008; Regalado & García-Almendarez, 2004). T-AOC is a comprehensive index that is used to measure the function status of the organic antioxidant system, which represents and reflects the organic antioxidant enzyme system and non-enzymatic system to external stimulation of compensatory ability and the state of the metabolism of free radicals (Tan, Dian-Yi, Yan, & Liang, 2005). In our study, serum POD activity and T-AOC were enhanced by increasing LWFH level and the highest activities were found at 20 g kg^{-1} LWFH. Also, the shrimp fed HSM10 diet exhibited the highest PO and T-SOD activities. Therefore, including a moderate level of LWFH in the diet is necessary to enhance the non-specific immune response and antioxidant activity in shrimp. Several researchers have reported improved immune response of fish following protein hydrolysates administration. Including approximately 50 g kg^{-1} krill hydrolysate, shrimp hydrolysate or tilapia hydrolysate in diets for red seabream (*Pagrus major*) improved innate immunity and disease resistance (Bui, Khosravi, Fournier, Herault, & Lee, 2014). Liang, Wang, Chang, & Mai (2005) found that addition of 150 g kg^{-1} fish protein hydrolysate (hydrolyzing pollock, *Theragra chaloogramma* with formic acid and protease) stimulates the non-specific immune response in sea bass (*Lateolabrax japonicus*). Tang, Wu, Zhao, & Pan (2008) also certified that with dietary supplementation of 100 g kg^{-1} FPH (hydrolyzing pollock, *Theragra chalcogramma* with Flavourzyme and Alcalase), immunity of large yellow croaker (*Larimichthys crocear*) can be upregulated. The immune enhancement effect of fish protein hydrolysates may be related to the small and medium-sized peptides (500–3,000 Da) (Gildberg, Johansen, & Bøgwald, 1995). Bøgwald, Dalmo, Leifson, Stenberg, & Gildberg (1996) and Gildberg *et al*. (1995) demonstrated that the non-specific defense system of fish can be stimulated by small and medium-sized peptides from fish protein hydrolysate. AKP and ACP are marker enzymes of lysosome macrophages in the immune system of shrimp and are important indices of immune function and health. In the present study, supplementing 20 g LWFH/kg diet significantly enhanced AKP and ACP activities indicating enhanced immune response. However, Zheng *et al*. (2013) reported no significant changes in AKP and ACP activities of turbot (*Scophthalmus maximus* L.) following FPH administration.

The innate immune system of invertebrates is an important defense against infectious agents (Hoebe, Janssen, & Beutler, 2004; Iwanaga & Lee, 2005). LITAF functions as a transcription factor regulating expression of the tumor necrosis factor-α gene and various inflammatory cytokines in response to stimulation by lipopolysaccharide (Jin *et al*., 2012). Bushell *et al*. (2011) reported that LITAF mRNA expression and protein levels are higher in tissues

with inflammatory bowel disease compared with those in normal tissues, strongly supporting the participation of LITAF in intestinal inflammation. ATF4 is a potent stress-responsive gene thought to play a protective role by regulating cellular adaptation to the integrated stress response. Overexpression of ATF4 is frequent in a wide variety of tumors to protect tumor cells against multiple stressors (Zhu *et al*., 2012). MIF is an inflammatory multifunctional cytokine in vertebrates and plays a significant role as a regulator of innate and adaptive immunity (Baugh & Richard, 2002; Calandra & Roger, 2003). Inada *et al*. (2013) suggested that MIF is important in innate immunity of Kuruma shrimp, which was the first report on the homology of a shrimp cytokine gene to vertebrate MIF. Several studies have indicated that members of the RAB family of small GTPases participate in the regulation of numerous signal transduction pathways that strongly affect cell proliferation, cell nutrition, innate immune response and compartmental fragmentation during mitosis and apoptosis through their effectors (Bucci & Chiariello, 2006). Yue *et al*. (2012a) cloned the *L. vannamei* RAB6A gene and predicted that it may take part in cell endocytosis and the antiviral immune reaction. LITAF, MIF, ATF4, and RAB6A are *L. vannamei* inflammatory factors and their expression is affected by the healthy, nutritional conditions. Our results showed that dietary inclusion of 10 – 15 g kg^{-1} LWFH significantly decreased LITAF, MIF, and ATF4 expression levels compared with those of shrimp fed the HSM0 diet, while further increment of LWFH up to 20 g kg^{-1} increased expression of these factors. This may have occurred because soybean meal contains various compounds such as lectins, saponins, and allergens that can cause histological changes in the fish intestine (Buttle *et al*., 2001; Bakke-McKellep *et al*., 2007; Knudsen, Jutfelt, Sundell, Koppe, & Frokiaer, 2008). These results suggest that including a moderate amount of LWFH in high soybean meal diets regulates the expression of particular inflammatory factors to strengthen innate immunity and maintain homeostasis in shrimp. The small peptides in LWFH play a key role in immune regulation, including antioxidant capacity, anti-microbial activity, and antimicrobial and tumor cell inhibitory activities (Luna-Vital, Mojica, Mejía, Mendoza, & Loarca-Piña., 2015). Oxidoreductases inhibit the enzymatic activity of MIF (Yin, Shen, Hu, & Wu, 2012). Therefore, the changes in MIF gene expression caused by dietary peptides may be due to changes in oxidoreductase activities, such as PO and T-SOD. No significant differences were observed in RAB6A gene expression levels among the dietary treatments at the end of our experiment. A similar result was observed in healthy Prawns (Yue *et al*., 2012a). Our results indicate that the relative levels of LITAF, MIF and ATF4 expression can be used as indices to evaluate the intestinal immune response in shrimp fed high plant protein diets.

In conclusion, the present results indicated that supplementation of LWFH in high soybean meal diets can improve growth, feed utilization, antioxidant activity and innate immunity and that the optimum inclusion level seems to be 10-15 g kg^{-1} of diet.

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