Fish and Shellfish Immunology 84 (2019) 124–129

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Dietary soybean antigen impairs growth and health through stress-induced non-specific immune responses in Pacific white shrimp, *Litopenaeus vannamei*

Tao Liu[a,b,c,1], Gaigai Zhang[a,b,c,1], Yue Feng[a,b,c], Chun Kong[a,b,c], Christian Larbi Ayisi[a,b,c], Xuxiong Huang[a,b,c], Xueming Hua[a,b,c,*]

[a] Centre for Research on Environmental Ecology and Fish Nutrition (CREEFN) of the Ministry of Agriculture, Shanghai Ocean University, Shanghai, 201306, China
[b] Key Laboratory of Freshwater Aquatic Genetic Resources, Ministry of Agriculture(Shanghai Ocean University), Shanghai, 201306, China
[c] National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai, 201306, China

ARTICLE INFO

Keywords:
Soybean meal
Fermented soybean meal
Litopenaeus vannamei
Soybean antigenic protein
Immune related gene
Growth

ABSTRACT

In order to compare the effect of substituting fish meal with fermented soybean meal and soybean meal, and confirmed whether the benefit from the two feed materials was related to the content of inclusive soybean antigen protein, two experiments were designed. In experiment 1, one of the two practical diets contained 24.9% soybean meal (SBM), the other one containing 8% fermented soybean meal and 16.95% soybean meal (FSBM); in experiment 2, two semi-purified diets were included with high antigen protein (SPD1) and low antigen protein (SPD2) approximately equal to SBM and FSBM group respectively in experiment 1. Diets were fed to *Litopenaeus vannamei* (initial weight: 7.48 ± 0.24 g) for 60 days. The results showed that in experiment 1, growth performance was not significantly different between two groups, the enzyme activity (AKP, AST, ALT, SOD and LZM) and mRNA expression levels of TLR, LZM, IMD and HSP70 were significantly higher in the SBM group; In experiment 2, weight gain and specific growth rate were significantly higher in the SPD2 group, while higher activities of AKP, ALT and LZM, lower expression levels of TLRmRNA, LZMmRNA and IMDmRNA and higher expression level of HSP70mRNA were found in SPD1 group. These results implied SBM was more likely to induce stress reaction in shrimp than FSBM, which were closely related to the antigen protein in SBM.

1. Introduction

Litopenaeus vannamei, one of the three major aquaculture species of shrimp in the world, demands high level of protein in commercial formulated feed. With shortage of fish meal in the world, we should seek for suitable protein source alternatives. Soybean meal (SBM) has been widely used plant ingredient in aqua-feed as fish meal replacement [1,2], but the negative situation of the replacement of fish meal in aquatic animal has been reported. Some scholars found it had adverse effects on the growth and immunity of aquatic animals [3,4]. Antigenic proteins, such as glycinin and β-conglycinin in soybean meal may account for this. Most antigen proteins can cause anaphylaxis in humans and animals. Numerous studies showed that soy protein antigens can cause allergic reactions early in weaned piglets and calf [5–8]. Negative influence from glycinin and β-conglycinin could be explained by breaking the intestinal tissue structure resulting in disorder of digestion and absorption, stalled growth and allergic reactions with a variety of non-specific and specific immune responses in fish body [9]. Nevertheless glycinin and β-conglycinin had different performance for different aquatic animal species, with worst impact on growth and intestinal tissue in grass carp, followed by *Clarias fuscus* and less impact on the carp [10].

Recent studies found that fermentation can significantly reduce the contents of antigenic factors (trypsin inhibitors, soybean agglutinin, phytic acid, antigen protein, etc) in soybean meal [11]. In soybean, glycinin and β-conglycinin had the strongest immunogenicity of antigenic proteins, and their content was about 65%–80% in soybean protein [12]. Investigation exposed that compared with soybean meal, globulin and β-conglycinin content in fermented soybean meal (FSBM) was reduced averagely by 57.7% and 63.2% respectively [13]. In recent

* Corresponding author. Centre for Research on Environmental Ecology and Fish Nutrition (CREEFN) of the Ministry of Agriculture, Shanghai Ocean University, Shanghai, 201306, China.
E-mail address: xmhua@shou.edu.cn (X. Hua).
[1] These authors contribute equally to this article.

https://doi.org/10.1016/j.fsi.2018.09.062
Received 8 May 2018; Received in revised form 19 September 2018; Accepted 23 September 2018
Available online 24 September 2018
1050-4648/ © 2018 Elsevier Ltd. All rights reserved.

years, fermented soybean meal has been increasingly applied to aquafeed. Zhou et al. reported that FSBM could improve the digestibility of dry matter and protein on *Penaeus* than commercial SBM [14], and some researches also indicated that appropriate inclusion of fermented soybean meal in diets did not affect growth performance of *macrobrachium nipponense* [1,15].

In our previous study, graded replacement of fish meal with fermented soybean meal was carried out, results showed that 42% crude protein with 24% of fish meal and 8% of fermented soybean meal replacing isonitrogenous fish meal did not affect the growth and physiology of *L. vannamei* immunity. But if fish meal is substituted with SBM, what the results will be, and whether there is different alternative effect between FSBM and SBM, is it actually associated with the content of soybean antigen protein in SBM? Hence, the aim of this study was to evaluate the growth performance, immune related enzymes and gene expression of the *L. vannamei* fed with or without FSBM inclusive practical diets, or different level of diets containing antigenic protein (soybean globulin and β soybean globulin) as single anti-nutrition factor, explore the possible reason for different effect from the two plant protein sources (FSBM and SBM), as to know the significance of modified soybean meal in the formulated shrimp diet.

2. Materials and methods

2.1. Experimental diets

Experiment1: In order to compared the effect of fermented soybean meal and soybean meal on *L. vannamei*, two isonitrogenous (approximately 42% crude protein) and isocaloric value (17 MJ kg^{-1}) diets were formulated, which contained different levels of fermented soybean meal (FSBM) and soybean meal (SBM), flour and fish oil were used as the carbohydrate and lipid sources, respectively. Fermented soybean meal was provided by Shanghai Yuanyao Co. Ltd. The basic nutrition was as follows: crude ash < 7%; crude fiber < 7.5%; crude protein 49%. Experiment 2: In order to explore whether the alternative effect of fermented soybean meal and soybean meal on *L. vannamei* was related to soybean antigenic protein (soybean globulin and soybean β-globulin), we prepared two semi-purified diets (SPD1 and SPD2) in which soybean antigenic protein was the only anti-nutritional factor. Casein, gelatin and fish oil were used as the main protein and lipid sources, respectively. Because of the high cost of the purified antigen protein with advanced extraction technology, the soybean antigen in semi-purified diets was obtained from the isolated soybean protein, and the main antigen protein was determined by the enzyme linked immunoassay. There was approximately 180 mg g^{-1} globulin and 140 mg g^{-1} β-globulin in soybean meal, while about 10 mg g^{-1} globulin and 3 mg g^{-1} β-globulin in fermented soybean meal with other possibly contained anti-nutritional factors. In the isolated soy protein, globulin (179.74 mg g^{-1}) and β-globulin (138.18 mg g^{-1}) was the sole type of anti-nutrient without other anti-nutritional factors. Total antigenic protein content in SPD1 was approximate with SBM group of the experiment1, in SPD2 approximate with FSBM group of the experiment1 (Table 1). Determination of feed crude protein was conducted by Kjeldahl method, crude fat by Soxhlet extraction.

Table 1
Formulation and proximate composition of the experimental diets.

Experiment1	SBM	FSBM	Experiment2	SPD1	SPD2
Ingredients (%)			Ingredients (%)		
Fish meal	24.20	24.20	Fish meal	8.90	8.90
Fermented soybean meal	0.00	8.00	Casein	9.88	13.68
Soybean meal	24.90	16.95	Soybean protein isolate	24.27	19.81
Meat bone meal	2.99	2.99	Gelatin	2.47	3.42
Peanut meal	13.59	12.96	α-starch	26.49	28.31
Flour	19.02	19.62	Phagostimulant	0.40	0.40
Squid visceral ointment	3.98	3.99	Squid visceral ointment	4.00	4.00
Brewers yeast	4.98	4.99	Brewers yeast	4.99	4.99
Lecithin powder	2.19	2.19	Lecithin powder	2.00	2.00
Fish oil	2.19	2.19	Fish oil	5.89	5.89
Vitamin premix[a]	0.80	0.80	Vitamin premix[a]	0.80	0.80
Mineral premix[b]	0.70	0.70	Mineral premix[b]	0.70	0.70
Monocalcium phosphate	0.30	0.30	Monocalcium phosphate	0.30	0.30
Crystalline lysine	0.09	0.07	Carboxymethyl cellulose	5.26	5.26
Crystalline methionine	0.05	0.04	Microcrystalline cellulose	2.95	3.79
Crystalline threonine	0.02	0.01	Crystalline lysine	0.03	0.00
Proximate composition (%)[c]			Crystalline methionine	0.04	0.00
CP(crude protein)	42.08	42.09	Crystalline arginine	0.61	0.70
EE(ether extract)	6.81	6.77	Crystalline threonine	0.02	0.01
Globulin	4.48	3.13	Proximate composition (%)[c]		
β-conglycinin	3.24	2.23	CP(crude protein)	42.09	42.09
Gross energy (MJ kg^{-1})	17.1	17.1	EE(ether extract)	6.77	6.77
			Globulin	4.28	3.03
			β-conglycinin	3.29	2.33
			Gross energy (MJ kg^{-1})	17.1	17.1

[a] Contained the following per kg of vitamin premix: VA 8000000 IU, VD 2000000 IU, VE 50 g, VK 10 g, VB_1 5 g, VB_2 15 g, VB_6 8 g, VB_{12} 0.02 g, nicotinamide 40 g, calcium D-pantothenate 25 g, folic acid 2.5 g, biotin 0.08 g, inositol 100 g.

[b] Contained the following per kg of mineral premix: Ca 10.5 g, K 90 g, Mg 12 g, Fe 1.0 g, Cu3.0 g, Zn 10 g, M n 3.8 g, Co 0.8 g, Se 20 mg.

[c] Determination of feed crude protein was conducted by Kjeldahl method, crude fat by Soxhlet extraction, antigen protein by the enzyme linked immunoassay, and gross energy was calculated based on contents of crude protein, crude fat and carbohydrate.

2.2. Feeding management

All ingredients of four diets were sieved through an 80 μm mesh after grinding. All dry ingredients were homogenized and mixed thoroughly with fish oil and distilled water using progressive enlargement method. The mincer was used to create spaghetti-like strings (1.2 mm in diameter), then the diets were dried at 50 °C, broken up and sieved into proper pellet size. Prior to feeding, experimental diets were stored at − 20 °C.

Experimental shrimps from local farms were sent to storage tanks with freshwater at Shanghai Ocean University for two-week acclimatization (Shrimp before the purchase has been completed low salinity acclimation). During the acclimatization, shrimps were fed 4 times a day to apparent satiation with basal feed (30%FM). Then total of 960 healthy shrimp (initial average weight 7.48 ± 0.24 g) post 24 h starvation were selected for 60-day feeding trial in 4 indoors cement tanks with 4 net cages (1.90m*1.25m*1.00m) per tank (4 groups and replicates, 60 shrimp per cage). The diets were randomly assigned to four groups of shrimp with the feed ratio 8% of their body weight to apparent satiation, 4 times per day at 5:30, 10:00, 16:00 and 21:30, and 60–70% of total feeding was in the morning and evening. Feeding rate was adjusted according to the growth on weekly basis. During the test period, water temperature was about 24.0–30.7 °C, NH_4^+-N < 0.3 mg L^{-1}, pH = 8.0 ± 0.2, DO > 6.5 mg L^{-1}. The water with 0.15–0.18‰ salinity originated from nearby river by gauze filtration, 1/3 of the water was replaced every 2 days.

2.3. Sample collection and analysis

At the end of the feeding trial, shrimp were starved for 24 h, final

Table 2
Primers used in this study.

Name	Sequence
β-actin-F	CTG TTC CAG CCC TCA TTC C
β-actin -R	TGT CCA CGT CGC ACT TCAT
LZM mRNA-F	GTT CCG ATC TGA TGT CCG
LZM mRNA -R	TGC TGT TGT AAG CCA CCC
HSP70-F	GAG ACC GCT GAG GCT TAC
HSP70-R	GCA CAT TCA GAC CCG AGA T
TLRmRNA-F	TGC CAA GCA GTG ATG TGA
TLRmRNA -R	GCG GGA AGG AAG TGA TGT
IMDmRNA-F	GCG GCT CTG CGG TTC ACA T
IMDmRNA -R	CTC GAC CTT GTC TCG TTC CTC

weight, weight gain, specific growth rates and survival rate were calculated. After the final weight was noted, 10 shrimps from each cage were randomly sampled; hemolymph and hepatopancreas were collected and stored in a refrigerator at −20 °C for evaluation of the enzyme activity determined by commercial kit (Nanjing Jiancheng Institute). In addition, gills and hepatopancreas of 3 shrimps from each cage were collected into the liquid nitrogen container and stored −80 °C. The gill tissue was used to determine mRNA expression of toll-like receptors (TLR), immune deficiency (IMD), lysozyme (LZM) while the hepatopancreas was used to determine mRNA expression of heat shock protein70 (HSP70). Total RNA of gills and hepatopancreas were extracted using Trizol reagent kit (TaKaRa) following the manufacturer's instruction. The gene primers were designed based on biotechnology information of US National Center reported on the Web site for *L. vannamei* TLR gene (GenBank: DQ923424.1), LZM gene (GenBank: AYl70126.2), HSP70 gene (GenBank: AY645906.1) and IMD gene (GenBank: FJ592176.1), and β-actin Primer was designed as a reference (Table 2). All primers were synthesized by Shanghai Ding Jing biotechnology Co. Ltd. Fluorescence quantitative PCR methods was used for detection, the first strand cDNA synthesis in RT was performed using Pri-meScript™ RT-PCR Kit (TaKaRa, Japan), and Real-time PCR by SYBR Green I chimeric fluorescent response. The amplifications were performed in a 96-well plate in a reaction volume of 20 μl containing 10 μl of Goldstar PCR Master Mix (2×), 1.2 μl (each) of the forward and reverse primers, 2 μl of template (1 μg cDNA), 0.4 μl ROX, and 6.4 μl of H_2O. Amplification was performed using the following protocol: 95 °C for 15 min followed by 40 cycles of 95 °C for10 s, 55 °C for 32 s, 72 °C for 32 s. A melting curve detection at 95 °C was performed to confirm a single PCR product amplified. Each sample was amplified in triplicate and each replicate well set a reference gene β-actin. The relative mRNA expression level of genes was normalized to *β*-actin by 2-△△Ct method [16], and then those of the group FSBM and SPD2 were set to 1, regarded as the control in experiment 1 and experiment 2 respectively.

2.4. Statistical analysis

The equations used are shown below:

Weight gain (WG, %) = 100*(final body weight - initial body weight) / initial body weight

Survival rate (SR, %) = (final shrimp number / initial shrimp number)*100

Special growth rate (SGR, %/d) = 100*[ln (mean final weight) – ln (mean initial weight)] / 60 days

All data were expressed with mean ± standard deviation, and differences in mean values were analyzed with independent samples t-experiment, Levene experiment of variance in spss software (Ver17.0). Differences with a $P < 0.05$ were considered statistically significant.

3. Result

3.1. Growth performance

Results of growth performance of white shrimp fed different diets for 60 days are presented in Table 3. There was no significant difference in the survival rate, weight gain and specific growth rates between FSBM-diet and SBM-diet group ($P > 0.05$). However, the SPD2-diet were significantly higher than SPD1-diet group in terms of weight gain and specific growth rate ($P < 0.05$). From the point of corresponding antigen protein content in the two experiments, the rate of weight gain and specific growth rate did not show the same trend with exception of the survival rate.

3.2. Immune health and antioxidant index

In experiment1, the health indexes about alanine aminotransferase (ALT), aspartate aminotransferase (AST) activity in haemolymph of SBM-diet group was significantly higher than FSBM-diet group ($P < 0.05$); while in experiment 2, significantly higher ALT activity in SPD1-diet groups than SPD2-diet($P < 0.05$), and almost the same value of AST activity in the two groups (Table 4).

LZM and alkline phosphatase (AKP) activity in SBM-diet group and SPD1-diet group was significantly higher than FSBM-diet group and SPD2-diet group ($P < 0.05$). In experiment 1, T-SOD activities in SBM-diet group were higher than FSBM-diet group, but in experiment 2, the T-SOD activities in SPD2-diet group was significantly higher than SPD1-diet group, and there was no significant difference in the malondialdehyde (MDA) content (Table 4).

3.3. Gene expression

The relative expression levels of immune related genes are shown in Fig. 1. In experiment 1, the relative genes expression levels of TLR, LZM, IMD in gill and HSP70 in hepatopancreas were significantly higher in SBM-diet group than FSBM-diet group. In experiment 2, there was reverse trend that TLR, LZM and IMD gene expression were higher in SPD2-diet group than SPD1-diet group in terms of same level of antigen protein as experiment 1, although the mRNA expression of TLR,

Table 3
Growth performance of shrimp fed different diets for 60 days.

	Experiment1		Experiment2	
	SBM	FSBM	SPD1	SPD2
Initial weight (g fish $^{-1}$)	7.55 ± 0.32^a	7.30 ± 0.4^a	7.55 ± 0.13^a	7.53 ± 0.04^a
Final weight (g fish $^{-1}$)	21.79 ± 1.48^a	19.98 ± 0.94^a	17.63 ± 0.41^a	18.67 ± 0.35^a
Survival rate (%)	89.46 ± 2.53^a	92.78 ± 4.81^a	87.79 ± 8.39^a	89.46 ± 2.53^a
weight gain (%)	188.50 ± 7.69^a	174.63 ± 24.0^a	133.67 ± 9.25^a	148.00 ± 4.11^b
specific growth rates (%/d)	1.77 ± 0.0^a	1.68 ± 0.1^a	1.41 ± 0.07^a	1.51 ± 0.03^b

Data were expressed as mean ± SD (n = 4). T-test was conducted in the same experimental group. Values with different superscripts in the same column were significantly different ($P < 0.05$).

Table 4
Health, immune and antioxidant index of shrimp with different diets for 60-day feeding.

	Experiment1		Experiment2	
	SBM	FSBM	SPD1	SPD2
AST (in haemolymph) (U/L)	20.97 ± 1.32^{b}	10.38 ± 0.20^{a}	13.59 ± 0.11^{a}	13.28 ± 1.06^{a}
ALT (in haemolymph) (U/L)	49.87 ± 2.42^{b}	36.04 ± 2.29^{a}	52.44 ± 2.00^{b}	36.42 ± 2.35^{a}
ALP (in haemolymph) (U/L)	38.55 ± 1.66^{b}	24.21 ± 0.92^{a}	31.21 ± 0.83^{b}	26.45 ± 1.18^{a}
LZM (in hepatopancreas) (U/mg prot)	108.60 ± 0.83^{b}	70.64 ± 3.28^{a}	115.41 ± 2.96^{b}	104.15 ± 2.90^{a}
T-SOD (in hepatopancreas) (U/mg prot)	105.33 ± 3.11^{b}	59.29 ± 0.15^{a}	94.25 ± 2.59^{a}	155.09 ± 9.12^{b}
MDA (in hepatopancreas) (nmol/mg prot)	5.85 ± 0.47^{a}	5.84 ± 0.04^{a}	4.99 ± 0.25^{a}	5.44 ± 0.09^{a}

Data were expressed as mean ± SD (n = 4). T-test was conducted in the same experimental group. Values with different superscripts in the same column were significantly different ($P < 0.05$).

LZM in gill was no significant differences between SPD1 and SPD2; HSP70mRNA in hepatopancreas was significantly higher in SPD1 with higher level antigen protein than SPD2 with lower level antigen protein.

4. Discussion

Previous studies indicated that it was possible to partially replace fish meal with soybean meal without affecting growth performance of shrimp and fish [1,4,17]. As fish meal alternative, fermentation soybean meal has better performance than soybean meal on the growth performance of aquatic animals, because of the improved nutritional quality and reduced anti-nutritional factors (soy antigens, antivitamins, phytic acid, phytoestrogens, saponins, and lectins) [15,17]. Lin and Luo [18]evaluated the effects of replacing fish meal with different levels of soybean meal in practical diet for tilapia, the results showed that the growth was affected by dietary SBM level, while the growth rates had no difference between the FM and 25% FSBM group. These studies confirmed fermented soybean meal have better results in growth performance compared to soybean meal on aquatic animals. But in our present study, the results were beyond our expectation that there were no significant difference in survival, weight gain and specific growth rate of *L. vannamei* between FSBM and SBM. However, Wang et al. [15] found that replacing fish meal with 45% SBM, 60% SBM, and 60% FSBM could significantly reduce growth performance and feed utilization in juvenile turbot fed diets. This also showed that replacing fish meal with soybean meal and fermented soybean meal appropriately will not affect growth performance of aquatic animals, but growth performance would be affected when there was a high level alternative. Some scholar deemed the better performance of FSBM in aqua-feed was associated with anti-nutritional factor reduction [19]. Experiment 2 was conducted in order to verify the influence of antigen protein in soybean meal on *L. vannamei*. The result indicated that there was no significant difference in survival for higher antigen protein diet (SPD1) and lower antigen protein diet (SPD2), but the SPD1 group exhibited poorer weight gain than SPD2 group. Although the experiment 2 had the same antigen protein content as experiment 1, weight gain and specific growth rate didn't show the similar trend between the two experiments with exception of the survival rate. Practical diets group, compared with semi-purified diets group, contained much higher level

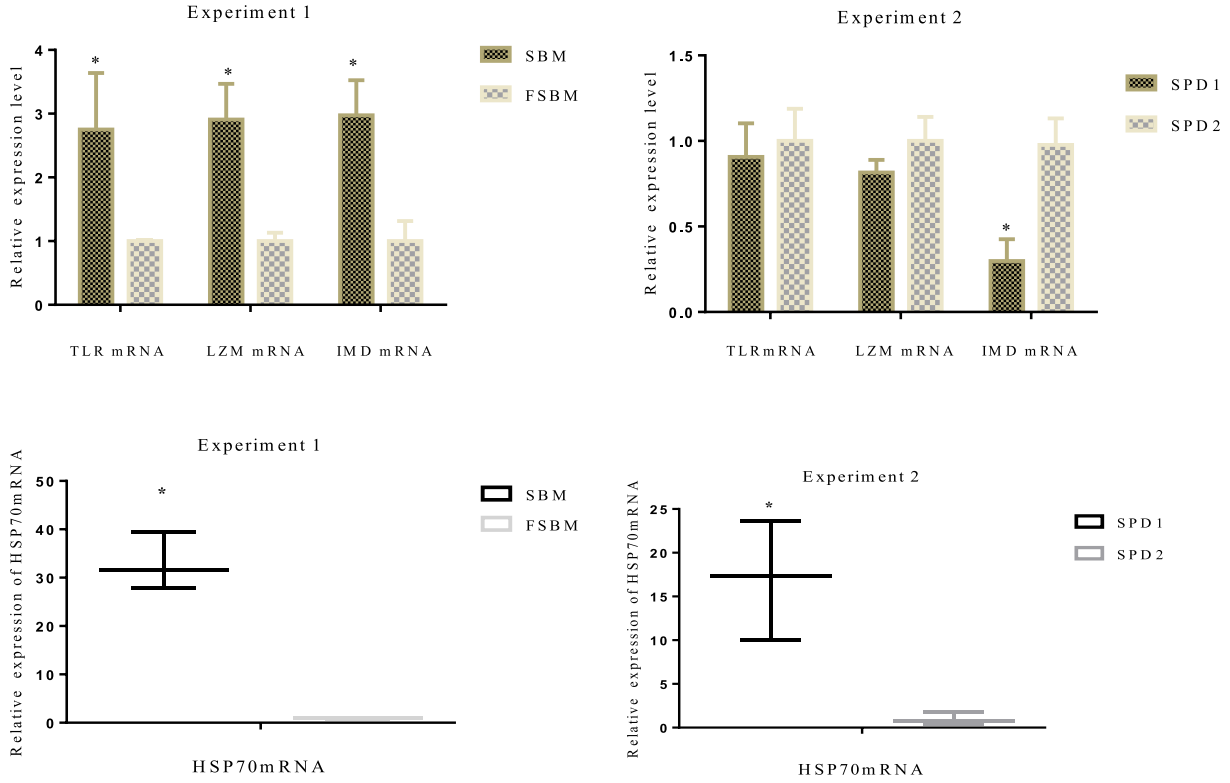

Fig. 1. The transcription expression profile of TLR, LZM, IMD in gill, and HSP70 in hepatopancreas of *Litopenaeus vannamei* with different diets measured by real-time quantitative PCR.

of fish meal content, functional nutrients in more fish meal may be comparative for the growth compared to the harmful effects of antigen protein. Moreover, interaction among anti-nutritional factors may weaken negative effect of antigen protein, and increase the tolerance of *L. vannamei* to soybean antigen protein. When casein and isolated soybean protein were served as the main sources of protein and antigen protein respectively in semi-purified diet groups, very little fish meal inclusion could not balance antigrowth function of antigen protein, so different contents of antigen protein caused different growth performance for *L. vannamei*. Above facts showed that high level of soybean antigen protein was one factor resulting in reduced growth rate in *L. vannamei*. Similar findings were observed on juvenile Jian carp and Pacific white shrimp respectively [3,20].

It has been widely accepted that crustacean immune system differs from vertebrates, and shrimp which has no special immunity, mainly depends on phagocytosis of immunocytes, hydrolytic enzymes, oxidant enzymes and oxygen reactive species to kill phagocyted organisms [21]. As other animals, ALT and AST in crustaceans are important enzymes in the metabolism of amino acids, and often used to characterize the severity of liver injury. Cheng et al. found that when the cell membrane was damaged, the important metabolic enzymes, such as AST and ALT were released from the cells the activity of AST and ALT in serum were increasing [22]. Zhang et al. also found the activities of AST and ALT in medium were enhanced when the β-conglycinin content increased [3]. In the present study, AST and ALT activity in hemolymph of shrimp in SBM-diet group and SPD1 group were significantly higher than FSBM-diet and SPD2 group. We may think that soybean meal is more likely to cause hepatopancreas damage of shrimp than fermented soybean meal. In addition, the results of experiment 2 also showed that the high soybean antigen protein content of the diet had high risk for hepatopancreas health. Generally, it is more difficult to promptly evaluate immunity at different body physiological status when external environment changes. To further confirm the effects of soybean meal and soybean antigen protein on the health of the *L. vannamei*, we also analyzed the molecular level which was less affected by environment changes. HSP70 gene is a kind of protein which is very sensitive to environmental stimulation, hepatopancreas as the main tissue of prawn responding to environmental stress and pathogen stimulation, showed significantly higher expression of HSP70 gene than other tissues when exposed to environmental stimulation. Wu et al. successfully amplified the HSP70 gene of *L. vannamei* [23], In this study, it was found that the relative expression of HSP70 gene in the soybean meal group in experiment 1 was significantly higher than that in the fermented soybean meal group, and the same trend also appeared in experiment 2. The high antigen protein group was significantly higher than the low antigen protein group. It is indicated that high antigen protein can cause the stress stimulation of shrimp hepatopancreas. Taking the results of AST and ALT together, it could explain that fermented soybean meal was more beneficial to the health of shrimp hepatopancreas than soybean meal, which was closely related to the content of antigen protein.

LZM is one of the most important non-specific immune factors in organisms. It does not only catalyze the body tissue to hydrolyze bacterial cell walls as a result of dissolution, but also induce and regulate the synthesis and secretion of other immune factors [24,25], and ALP is an important part of lysosomal enzymes of crustaceans [26]. They are both important immune indices of shrimp. In this study, the two immune indexes were higher in soybean meal group and high antigen protein group than fermentation soybean meal group and low antigen protein group. The high activities of lysozyme and alkaline phosphatase usually indicates the high immunity in aquatic animals, so the present result seems to show that soybean meal easy boosts the immunity of shrimp compared to fermented soybean meal. However soybean meal and high antigen protein can cause hepatopancreas damage in shrimp, which can make shrimp more susceptible to be infected by bacteria. Consequently the shrimp tries to keep the organism healthy via releasing more immune-related enzymes. Previous research showed that LZM transcription in head kidney, spleen and ovaries were significantly improved by Edward's infection [27]. The vast majority of Toll-like receptors (TLR) on the cell membrane were a kind of the most important pathogen associated molecular model (Pathogen-associated molecular pattern, PAMP) recognition receptors. TLR pathway mechanisms play an important role in immunity primarily through against gram positive bacteria and fungi [28–31]. In recent years, more attention has been given to TLR genes in crustaceans [32,33]. IMD pathway firstly found in drosophila, was one of the important pathways of natural immunity, and participated in the regulation of body immune against the virus [34]. Same as drosophila, IMD of the blood cells in *L. vannamei* executed vital immune function. Wang et al. reported that immune deficiency homolog (LvIMD) transcription levels were upregulated by WSSV [35]. It has been found that the above immune genes are expressed in all tissues of prawn, there were no tissue specificity. So we chose the gill as sample tissue, which is directly in contact with the environmental water. In this study, SBM-diet group's LZMmRNA expression levels was significantly higher than FSBM-diet one, which indicated that the soybean meal would alter organisms' susceptibility to pathogeny both in transcription level and protein level with enhanced LZM activity. Meanwhile, the IMDmRNA and TLRmRNA expression levels in gill of shrimp were significantly higher in SBM-diet group than FSBM. However, in experiment 2, we found that the gene expression of TLR, IMD, and LZM in SPD1 (high antigen protein group) were lower than that of SPD2 (low antigen protein group). The contradiction between two experiments inferred that antigen protein was an important factor for transcription expression regulation of immune related genes in shrimps, and further work should be needed for exploring complex dose-effect responsible for down-regulation or up-regulation of genes expression.

As an important antioxidant enzyme in the free radical scavenging system, SOD plays a vital role in scavenging the superoxide anion in body [36]. In experiment 1 of present study, SOD activities in SBM-diet group was significant higher than FSBM-diet group, but in higher antigen protein semi-purification diet (SPD1) group in experiment 2, SOD activity was significant lower than SPD2. Findings from this study are not in agreement with those reported that FSBM increased SOD activity and enhanced antioxidative ability of shrimp *L. vannamei* or *Pseudobagrus fulvidraco* [2,37]. But Chen et al. found that shrimp hepatopancreas SOD activity was decreased with the increase of fermented soybean meal [38]. Ding et al. reported that there was no significant differences in SOD activity of *Macrobrachium nipponense* fed fermented soybean meal and fish meal [1]. Too much oxygen free radicals not only cause oxidative stress to the body, but also partly play some bactericidal function. These different results manifested the body would be capable to balance the antioxidant and bactericidal function, accordingly, SOD and LZM, which also has antibacterial properties in the body, would be adjusted each other according to healthy need to keep dynamic change at different physiological status. In this study, there was no significant difference in the content of malondialdehyde (MDA) in each group, which showed that SOD played a role in protecting the body from oxidative stress. We also inferred that in practical SBM diets, higher level of SOD for long time would lessen protein deposition and consequently result in reduced growth. Even so, further study would be needed to affirm whether to have a dose dependent impact on shrimp antioxidant capacity with soybean antigenic proteins.

In conclusion, our results obtained in the condition of present experiments indicated that SBM and FSBM had no significant different effect on growth performance of *L. vannamei* when dietary fishmeal inclusion was 24.2%. In later studies, we can increase the substitution level to compare the differences between the two plant protein sources. In addition, it was found that fermented soybean meal was more beneficial to the health of *L. vannamei* under the given equal dietary protein level. Soybean meal compared with fermented soybean meal could significantly increase the expression of partial immune gene of *L. vannamei*, and HSP70 gene, indicating that the fermented soybean meal

could significantly reduce the stress reaction within the body of the shrimp, and the antigen protein in soybean meal (Globulin and β globulin) was the main factor leading to this difference, so it is necessary to reduce the antigen protein in the utilization of plant protein feed in the future.

Acknowledgements

The study was funded by project (No.14320502000) from Shanghai Science and Technology Committee. The authors thank Shanghai Yuanyao Biotechnology Co. Ltd for assistance with fermented soybean meal supply and Shanghai TopGen BioPharm Co. Ltd for guidance of determination of gene expression.

References

[1] Z. Ding, Y. Zhang, J. Ye, Z. Du, Y. Kong, An evaluation of replacing fish meal with fermented soybean meal in the diet of *Macrobrachium nipponense*: growth, non-specific immunity, and resistance to *Aeromonas hydrophila*, Fish Shellfish Immunol. 44 (2015) 295–301 http://doi.org/10.1016/j.fsi.2015.02.024.

[2] Y.H. Lin, J.J. Mui, Comparison of dietary inclusion of commercial and fermented soybean meal on oxidative status and non-specific immune responses in white shrimp, *Litopenaeus vannamei*, Fish Shellfish Immunol. 63 (2017) 208–212 http://doi.org/10.1016/j.aquaculture.2016.11.032.

[3] J.-X. Zhang, L.-Y. Guo, L. Feng, W.-D. Jiang, S.-Y. Kuang, Y. Liu, et al., Soybean beta-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish, PLoS One 8 (2013), http://doi.org/10.1371/journal.pone.0058115.

[4] F. Huang, L. Wang, C-x Zhang, K. Song, Replacement of fishmeal with soybean meal and mineral supplements in diets of *Litopenaeus vannamei* reared in low-salinity water, Aquaculture 473 (2017) 172–180 http://doi.org/10.1016/j.aquaculture.2017.02.011.

[5] D. Dreau, C. Larre, J.P. Lalles, Semi-quantitative purification and assessment of purity of three soybean proteins-glycinin, beta-conglycinin and alpha-conglycinin by SDS-PAGE electrophoresis, densitometry and immunoblottig, J. Food Sci. Technol. 31 (1994) 489–493.

[6] J.P. Lalles, D. Dreau, H. Salmon, R. Toullec, Identification of soyabean allergens and immune mechanisms of dietary sensitivities in preruminant calves, Res. Vet. Sci. 60 (1996) 111–116 http://doi.org/10.1016/S0034-5288(96)90003-X.

[7] D.F. Li, J.L. Nelssen, P.G. Reddy, F. Blecha, J.D. Hancock, G.L. Allee, et al., Transient hypersensitivity to soybean meal in the early-weaned pig, J. Anim. Sci. 68 (1990) 1790–1799 http://doi.org/10.2527/1990.6861790x.

[8] S.M. Adeyemo, A.A. Onilude, Enzymatic reduction of anti-nutritional factors in fermenting soybeans by *Lactobacillus plantarum* isolates from fermenting cereals, Niger. Food J. 31 (2013) 84–90 http://doi.org/10.1016/S0189-7241(15)30080-1.

[9] L.-F. Wu, Z.-W. Sun, G.-X. Qin, D.-M. Zhang, H.-H. Wang, D. Zhu, Effects of dietary Glycinin and β-conglycinin on growth performance and intestinal tissue of fishes with different food habits, J. NW A&F Univ. (Nat Sci Ed). (2011) 59–66 [in Chinese with English abstract] http://dx.chinadoi.cn/10.13207/j.cnki.jnwafu.2011.02.034.

[10] G. Francis, H.P.S. Makkar, K. Becker, Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish, Aquaculture 199 (2001) 197–227 http://doi.org/10.1016/S0044-8486(01)00526-9.

[11] A. Krogdahl, A.M. Bakke-McKellep, G. Baeverfjord, Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar L.*), Aquacult. Nutr. 9 (2003) 361–371.

[12] M. Hirabayashi, T. Matsui, H. Yano, T. Nakajima, Fermentation of soybean meal with *Aspergillus usamii* reduces phosphorus excretion in chicks, Poultry Sci. 77 (1998) 552–556 http://10.1093/ps/77.4.552.

[13] Y.-J. Yang, Y.-S. Yao, Y.-C. Qin, J. Qiu, J.-G. Li, J. Li, et al., Investigation and analysis of main AFN in soybean meal and fermented soybean meal, Sci. Agric. Sin. 49 (2016) 573–580 [in Chinese with English abstract] http://dx.chinadoi.cn/10.3864/j.issn.0578-1752.2016.03.015.

[14] L.-C. Zhuo, K. Liu, Y.-H. Lin, Apparent digestibility of soybean meal and *Lactobacillus spp*. fermented soybean meal in diets of grouper, Epinephelus coioides, Aquacult. Res. 47 (2016) 1009–1012 http://doi.org/10.1111/are.12543.

[15] L. Wang, H. Zhou, R. He, W. Xu, K. Mai, G. He, Effects of soybean meal fermentation by Lactobacillus plantarum P8 on growth, immune responses, and intestinal morphology in juvenile turbot (*Scophthalmus maximus L.*), Aquaculture 464 (2016) 87–94 http://doi.org/10.1016/j.aquaculture.2016.06.026.

[16] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method, Methods 25 (2001) 402–408 http://doi.org/10.1006/meth.2001.1262.

[17] Y.-H. Lin, M.-Y. Cheng, Effects of dietary organic acid supplementation on the growth, nutrient digestibility and intestinal histology of the giant grouper *Epinephelus lanceolatus* fed a diet with soybean meal, Aquaculture 469 (2017) 106–111 http://doi.org/10.1016/j.aquaculture.2016.11.032.

[18] S. Lin, L. Luo, Effects of different levels of soybean meal inclusion in replacement for fish meal on growth, digestive enzymes and transaminase activities in practical diets for juvenile tilapia, *Oreochromis niloticus x O. aureus*, Anim. Feed Sci. Technol. 168 (2011) 80–87 http://doi.org/10.1016/j.anifeedsci.2011.03.012.

[19] Y.-L. Shiu, S.-L. Wong, W.-C. Guei, Y.-C. Shin, C.-H. Liu, Increase in the plant protein ratio in the diet of white shrimp, *Litopenaeus vannamei* (Boone), using *Bacillus subtilis* E20-fermented soybean meal as a replacement, Aquacult. Res. 46 (2015) 382–394 http://doi.org/10.1111/are.12186.

[20] J. Gamboa-Delgado, L. Le Vay, Natural stable isotopes as indicators of the relative contribution of soy protein and fish meal to tissue growth in Pacific white shrimp (*Litopenaeus vannamei*) fed compound diets, Aquaculture 291 (2009) 115–123 http://doi.org/10.1016/j.aquaculture.2009.03.012.

[21] Z.-P. Ma, F.-S. Zhang, J.-K. Tong, Overview and graph theory of the inmune system of crustacean, Agric. Sci. Technol. 39 (2011) 291–294 [in Chinese with English abstract] http://dx.chinadoi.cn/10.16175/j.cnki.1009-4229.2010.08.047.

[22] W.T. Cheng, C.H. Liu, C.M. Kuo, J.C. Chen, Dietary administration of sodium alginate enhances the immune ability of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*, Fish Shellfish Immunol. 18 (2005) 1–12 http://doi.org/10.1016/j.fsi.2004.03.002.

[23] R. Wu, S.-T. Xie, Y. Sun, Y.-X.-Q. Wan, Q.-Z. Zhang, High level prokaryotic expression of heat shock protein 70 in *Litopenaeus vannamei*, J. Fish. Sci. China 13 (2006) 305–309 [in Chinese with English abstract] http://dx.chinadoi.cn/10.3321/j.issn:1005-8737.2006.02.022.

[24] H.G. Boman, I. Faye, G.H. Gudmundsson, J.Y. Lee, D.A. Lidholm, Cell-free immunity in *Cecropia*. A model system for antibacterial proteins, Eur. J. Biochem. 201 (1991) 23–31 http://doi.org/10.1111/j.1432-1033.1991.tb16252.x.

[25] K. Mori, T. Nakanishi, T. Suzuki, I. Oohara, [Defense mechanisms in invertebrates and fish]. Tanpakushitsu kakusan koso Protein, nucleic acid, enzyme, 34 (1989) 214–223.

[26] E.G. Giannini, R. Testa, V. Savarino, Liver enzyme alteration: a guide for clinicians, Can. Med. Assoc. J. 172 (2005) 367–379 http://doi.org/10.1503/cmaj.1040752.

[27] J. Hikima, I. Hirono, T. Aoki, Characterization and expression of c-type lysozyme cDNA from Japanese flounder (Paralichthys olivaceus), Mol. Mar. Biol. Biotechnol. 6 (1997) 339–344.

[28] P.-H. Wang, Z.-H. Gu, X.-D. Huang, B.-D. Liu, X-x Deng, H.-S. Ai, et al., An immune deficiency homolog from the white shrimp, *Litopenaeus vannamei*, activates antimicrobial peptide genes, Mol. Immunol. 46 (2009) 1897–1904 http://doi.org/10.1016/j.molimm.2009.01.005.

[29] S. Akira, K. Takeda, Toll-like receptor signalling, Nat. Rev. Immunol. 4 (2004) 499–511 http://doi.org/10.1038/nri1391.

[30] G. Liu, L. Zhang, Y. Zhao, Modulation of immune responses through direct activation of Toll-like receptors to T cells, Clin. Exp. Immunol. 160 (2010) 168–175 http://doi.org/10.1111/j.1365-2249.2010.04091.x.

[31] C. Uribe, H. Folch, R. Enriquez, G. Moran, Innate and adaptive immunity in teleost fish: a review, Vet. Med. 56 (2011) 486–503.

[32] C. Yang, J. Zhang, F. Li, H. Ma, Q. Zhang, T.A.J. Priya, et al., A toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to Vibrio anguillarum infection, Fish Shellfish Immunol. 24 (2008) 564–574 http://doi.org/10.1016/j.fsi.2007.12.012.

[33] T. Mekata, T. Kono, T. Yoshida, M. Sakai, T. Itami, Identification of cDNA encoding Toll receptor, MjToll gene from kuruma shrimp, *Marsupenaeus japonicus*, Fish Shellfish Immunol. 24 (2008) 122–133 http://doi.org/10.1016/j.fsi.2007.10.00.

[34] Z. Huang, M.B. Kingsolver, V. Avadhanula, R.W. Hardy, An antiviral role for antimicrobial peptides during the arthropod response to *alphavirus* replication, J. Virol. 87 (2013) 4272–4280 http://10.1128/JVI.03360-12.

[35] P.-H. Wang, J.-P. Liang, Z.-H. Gu, D.-H. Wan, S.-P. Weng, X.-Q. Yu, et al., Molecular cloning, characterization and expression analysis of two novel Tolls (LvToll2 and LvToll3) and three putative Spatz le-like Toll ligands (LvSpz1-3) from *Litopenaeus vannamei*, Dev. Comp. Immunol. 36 (2012) 359–371 http://doi.org/10.1016/j.dci.2011.07.007.

[36] Y. Abe, G. Ishikawa, H. Satoh, K. Azumi, H. Yokosawa, Primary structure and function of superoxide dismutase from the ascidian *Halocynthia roretzi*, Comp. Biochem. Physiol B Comp. Biochem. Mol. Biol. 122 (1999) 321–326 http://doi.org/10.1016/S0305-0491(99)00022-X.

[37] S. Peng, Application of Fermented Soybean Meal and Fermented Peanut Meal in Diets of White Shrimp, *Litopenaeus vannamei* Shanghai Ocean University, 2015 (in Chinese with English abstract).

[38] X.-M. Chen, X.-M. Hua, W.-X. Zhu, Y.-D. He, C. Shui, Z. Wu, et al., Effects of soybean allergic proteins on growth, digestion and non-specific immune of *Litopenaeus vannamei*, Chin. J. Anim. Nutr. (2015) 2115–2127 [in Chinese with English abstract] http://dx.chinadoi.cn/10.3969/j.issn.1006-267x.