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Short communication

Comparison of dietary inclusion of commercial and fermented soybean meal on oxidative status and non-specific immune responses in white shrimp, *Litopenaeus vannamei*

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ABSTRACT

This study compared the effects of partial replacement of fish meal (FM) protein with commercial soybean meal (SBM) or *Lactobacillus* spp. fermented SBM (FSBM) on the oxidative status and non-specific immune responses in white shrimps, *Litopenaeus vannamei*. A FM-based diet was used as the control. To prepare the two experimental diets, 25% of the FM protein was replaced with SBM and FSBM. Three experimental diets were fed to three groups of shrimps (initial wt: 0.63 ± 0.01 g) in a recirculating rearing system for 12 weeks. The SBM-diet group had the highest hepatopancreatic thiobarbituric acid-reactive substance value, followed by the FSBM-diet group, and the lowest in control-diet group. The activity of hepatopancreatic superoxide dismutase was highest in the control-diet group, followed by the FSBM-diet group, and was lowest in the SBM-diet group. The total haemocyte, hyaline cell, semigranular cell, and granular cell counts were highest in the control-diet group, followed by the FSBM-diet group and the SBM-fed group. Haemolymph phenoloxidase activity was higher in the control-diet and FSBM-diet groups than in the SBM-diet group. The results indicate that replacing 25% FM protein with SBM significantly reduces non-specific immune responses and induces oxidative stress in white shrimps and that FSBM prepared using *Lactobacillus* spp. can reduce these negative effects.

1. Introduction

White shrimp, *Litopenaeus vannamei*, is a widely cultured shrimp species. The annual aquaculture production of white shrimps has increased from 8286 MT in 1980 to 3,679,606 MT in 2014, accounting for approximately 80% of the global shrimp production [1]. Fish meal (FM) continues to be the primary protein source in shrimp feed [2]. Because the availability of FM is expected to decrease and its price is expected to increase sharply, cheaper and more sustainable protein sources must be identified and used in aquafeeds. Soybean meal (SBM) is a widely used plant ingredient in the aquafeed industry. However, its use as aquafeed is limited because of antinutritional factors and insufficient levels of methionine and lysine [3].

Traditionally, the quality of SBM is improved through fermentation by using microorganisms, such as *Lactobacillus* spp., *Bacillus* spp., and *Aspergillus* spp [4]. SBM processing by fermentation ameliorates the antinutritional factors and enhances protein utilisation in animals [5]. In addition, *Lactobacillus* spp.-fermented SBM (FSBM) has been reported to exhibit higher dry matter and protein digestibility than do commercial SBM in white shrimp [4] and grouper [6].

Zhang et al. [7] reported that common carp fed a diet containing 8% β-glycinine, the main antigenic component derived from SBM, exhibits a higher level of hepatic malondialdehyde (MDA) than do carps fed a β-glycinine-free control diet. Similarly, turbots fed a diet containing SBM has been reported to exhibit reduced serum total antioxidant capacity [8]. Furthermore, the suppression of non-specific immune capacity because of high concentrations of dietary soybean proteins has been reported in rainbow trout [9], Nile tilapia [10], and turbot [8]. Lim and Dominy [11] indicated that 28% FM protein can be replaced with SBM in white shrimp aquafeeds without negative effects on growth performance. However, the effects on physiological status, such as oxidative status and immune responses, and whether these responses are affected by the inclusion of dietary SBM remain unclear. Thus, the present study compared the partial replacement (25%) of FM protein with commercial SBM and FSBM in the aquafeed for the Pacific white shrimp,

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Litopenaeus vannamei. Apart from the growth performance of the shrimp, hepatopancreatic thiobarbituric acid reactive substance (TBARS) value and superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were used as oxidative status parameters, and counts of total haemocyte count (THC), hyaline cells (HC), semigranular cells (SGC), and granular cells (GC) as well as phenoloxidase (PO) activity were used as indicators of immunity.

2. Materials and methods

2.1. Experimental diets

Table 1 showed the formulation and proximate composition of three experimental diets. A fish meal (FM, Pesquera Diamante S.A., Lima, Peru) based diet was used as control. To prepare the two experimental diets, 25% of the FM protein was replaced with soybean meal (defatted and dehulled SBM, TTET Union Corp., Tainan, Taiwan) and *Lactobacillus* spp. fermented soybean meal (FSBM, DaBomb Protein Corp., Taiwan), respectively, as the two experiential diets. The fermented soybean meal was processed through solid state fermentation in anaerobic condition by the company. Proximate composition of SBM and FSBM were as follow: for SBM, 10.51% moisture, 47.59% crude protein, 2.67% crude lipid, 5.84% ash, 3.31% crude fiber and 30.08% nitrogen free extract (NFE); for FSBM, 7.03% moisture, 51.31% crude protein, 3.71% crude lipid, 6.23% ash, 2.48% crude fiber and 29.24% NFE. Gelatinized starch (Trust River Trading Co., Ltd., Taipei, Taiwan), fish oil (Semi-refined fish oil, Oleaginosa Victoria S.A., Lima, Peru) and soybean oil (Tai-Tang Industrial, Taipei, Taiwan) were used as the carbohydrate and lipid sources, respectively. An attractant, squid liver meal (Power Omega, Korea), was added to all diets to increase dietary palatability and acceptance. All ingredients of the experimental diet were mixed, and enough cold water was added to form stiff dough. This was then passed through a mincer with die, and the resulting strands were dried using an electrical fan at 20 °C. After drying, the diets were broken up and sieved into pellets (2.0 mm in diameter) and stored at −20 °C until used.

Table 1
Formulation and proximate composition of the experimental diets.

	Control	SBM	FSBM
Ingredient (g kg^{-1})			
Fish meal	526	300	300
Soybean meal (SBM)	0	327	0
Fermented soybean meal (FSBM)	0	0	309
Fish oil	22	33	28
Alpha-starch	260	165	170
Squid liver meal	70	70	70
Choline chloride	15	15	15
Cholesterol	–	0.5	0.5
Vitamin premix[1]	10	10	10
Mineral premix[2]	20	20	20
Methionine	0	2.92	2.92
Lysine	0	2.88	2.88
Cellulose	76.7	33.0	51.7
Proximate composition (g kg^{-1})			
Moisture	20.8	38.8	25.6
Ash	102.4	90.0	93.6
Crude protein	434.8	436.2	435.5
Ether extract	50.8	58.4	47.4

[1] Vitamin mixture supplied the following (mg g^{-1} mixture): pyridoxine HCl, 8.9; retinyl acetate, 1.68; riboflavin, 2.25; nicotinic acid, 0.72; thiamin-HCl, 1.4; menadione, 4; α-tocopheryl acetate, 10; vitamin B_{12}, 0.02; cholecalciferol (D_3), 0.01; folic acid, 0.21; choline HCl, 200; L-ascorbyl-2-monophosphate- Mg, 4; d-biotin, 0.24; Ca pantothenate, 13.9; *myo*-inositol, 350. All ingredients are diluted with α-cellulose to 1 g.

[2] Mineral mixture supplied the following (mg g^{-1} mixture): Na_2HPO_4, 215; $Ca(H_2PO_4)_2.H_2O$, 265; $CaCO_3$, 105; Ca lactate, 165; KCl, 139.5; KI, 0.23; $MgSO_4.7H_2O$, 100; ferric citrate, 10; $CuCl_2.2H_2O$, 0.62; $AlCl_3.6H_2O$, 0.24; $MnSO_4.H_2O$, 1.07; ZnSO4, 4.76; $CoCl_2.6H_2O$, 1.4. All ingredients are diluted with α-cellulose to 1 g.

2.2. Experimental procedure

Juvenile *L. vannamei* purchased from a local hatchery (Kaohsiung, Taiwan) were used in the study. Upon arrival, the shrimp were acclimated to laboratory conditions for four weeks in a 3500-L FRP tank. During acclimation period, shrimp were fed 4 times daily (at 0700, 12:00, 17:00 and 2200 h) with a commercial diet (Uni-President Enterprise Corp., Tainan, Taiwan). Shrimp used for the subsequent tests were all in the intermolt stage (stage C). The molt stage was determined by examination of the uropoda in which partial retraction of the epidermis could be distinguished [12]. At the beginning of the experiment, 25 shrimp (mean initial weight ± SD: 0.63 ± 0.01 g) were stocked in each of 9 FRP tanks (300 L). Each experimental diet was assayed in triplicate (3 experimental diets × 3 tanks). The diets were randomly assigned to groups of shrimp. Each aquarium was part of a closed recirculating system with a common reservoir of seawater at 30–32‰ salinity. The system consisted of a common filter, biofilter, protein skimmer and UV light to maintain the water quality. The water temperature of the rearing system was controlled at 29 ± 1 °C.

The shrimp were fed to 8% of their wet weight 4 times per day at 0700, 12:00, 17:00 and 2200 h. The uneaten feed were collected by a siphon immediately, then dried and weighed for recording the feed intake during the feeding period. Shrimp were weighed once every two weeks and half of rearing water was exchanged. Any dead shrimp were removed and not replaced during the experiment. The shrimp were fed the test diets for a 12-week period.

2.3. Growth performance and assay methods

At the end of the feeding trial, shrimp were bulk weighed and growth performance including final weight, weight gain (WG), feed efficiency (FE) and survival rate were calculated. The equations used are shown below:

$$WG = (\text{final body weight} - \text{initial body weight}) \times /\text{initial body weight} \times 100$$

$$FE = (\text{final body weight} - \text{initial body weight})/\text{feed intake}$$

$$\text{Survival} = 100 \times (\text{final shrimp number}/\text{initial shrimp number})$$

The immune parameters of the shrimp following feeding with different diets were determined. Haemolymph (100 μL) was withdrawn from the ventral sinus of each shrimp into a 1-mL sterile syringe (25-gauge) containing 0.9 mL anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, and 10 mM EDTA, at a pH of 7.55 and with the osmolality adjusted with glucose to 780 mOsm/kg) at a ratio of 1:9, and then placed on ice and used for the analysis [13]. They were divided into two parts. A drop of 100 μL anticoagulant-haemolymph mixture was placed on a Buker haemocytometer to measure the total haemocyte count (THC), hyaline cell (HC), semigranular cell (SGC) and granular cell (GC) counts using an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems, Wetzlar, Germany). All haemocytes in both the top and bottom fields (1 × 1 mm) of the haemocytometer were manually counted [10].

Phenoloxidase (PO) activity of haemolymph was measured spectrophotometrically by recording the formation of dopachrome produced following the procedures of a previous study [14]. The L-

3,4-dihydroxy- phenylalanine (Sigma Chemical) and trypsin (Sigma Chemical) were served as a substrate and an elicitor, respectively. The measurement was described previously [15]. The optical density of PO activity was expressed as dopachrome formation in 50 μL of haemolymph.

After the final weight was noted, hepatopancreas sample was collected from 5 ice-anesthetized shrimp selected randomly per aquarium. Hepatopancreatic thiobarbituric acid reactive substance (TBARS) values were determined by the method of Uchiyama and Mihara [16]. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were determined by commercial kit (Randox Crumlin, UK).

2.4. Statistical analysis

Each experimental diet was fed to three groups of shrimp according to a completely randomized design. The results were analyzed by a one-way analysis of variance (ANOVA). When the ANOVA identified differences among the groups, multiple comparisons were made among the means using Student Newman-Keuls (SNK) test. Statistical significance was determined by setting the aggregate type I error at $P < 0.05$.

3. Results

Results of growth performance of white shrimp fed different diets for 12 weeks were presented in Table 2. Final weight, weight gain, feed efficiency and survival were not significantly different among all dietary treatments.

The SBM-diet group had the highest hepatopancreatic thiobarbituric acid-reactive substance value, followed by the FSBM-diet group, and the lowest in control-diet group. (Table 3). The activity of hepatopancreatic superoxide dismutase was highest in the control-diet group, followed by the FSBM-diet group, and was lowest in the SBM-diet group. Glutathione peroxidase (GPx) activity in hepatopancreas was higher in shrimp fed the SBM- and FSBM-diet groups than that in shrimp fed the control-diet group.

Total haemocyte count (THC), hyaline cells (HC), semigranular cells (SGC) and granular cells (GC) counts were highest in the control-diet group, followed by the FSBM-diet group and the SBM-fed group. (Table 4). Haemolymph phenoloxidase activity was higher in the control-diet and FSBM-diet groups than in the SBM-diet group.

Table 2
Growth performance of white shrimp fed different diets for 12 weeks.

	Control	SBM	FSBM
Initial weight (g)	0.64 ± 0.00	0.62 ± 0.01	0.62 ± 0.01
Final weight (g)	5.94 ± 0.19	5.55 ± 0.42	5.39 ± 0.19
Weight gain (%)	827 ± 28	794 ± 54	770 ± 35
Feed efficiency	0.65 ± 0.01	0.61 ± 0.02	0.60 ± 0.02
Survival (%)	92.00 ± 4.00	92.00 ± 6.93	89.00 ± 6.93

Data are expressed as mean ± SD (n = 3).

4. Discussion

A previous study indicated that 28% FM protein can be replaced with SBM in white shrimp aquafeeds [11]. Thus, we prepared an aquafeed in which 25% FM protein was replaced with commercial SBM and FSBM and compared the effects of the two ingredients on growth, oxidative status, and immune responses of white shrimp. Our results indicated that weight gain and feed efficiency of shrimp fed diets in which 25% FM protein was replaced with the two ingredients were not significantly different from the those of shrimp in the control group (Table 3), suggesting that the inclusion of 25% SBM or FSBM does not affect the growth performance of shrimp. SBM inclusion in diets has been demonstrated to induce oxidative stress [7,8] and to reduce immune responses [8–11]. Our previous studies reported that the defects of SBM can be improved through *Lactobacillus* spp. fermentation, including less antinutritional factors and hydrolyzed soy protein [4,6]. It is interesting whether the use of FSBM reduces oxidative stress and improves the immune responses for white shrimp compared with use of SBM. Therefore, these two ingredients needed to be evaluated using other parameters, such as oxidative status and immune responses, other than growth performance of white shrimp.

Malondialdehyde (MDA) is a secondary oxidation product of polyunsaturated fatty acids. The reactive substance TBARS is formed by the condensation of one molecule of MDA with two thiobarbituric acid (TBA) molecules [16]. TBARS analysis is one of the most commonly used methods for tissue peroxidation [17]. Hepatopancreatic oxidative stress appears to be induced by the inclusion of SBM inclusion the white shrimp diet (Table 3). Phytic acid derived from SBM has been demonstrated to interfere with the microelements utilisation [18,19]. Two microelements, namely copper (Cu) and zinc (Zn), are involved in the antioxidant enzyme system of Cu/Zn superoxide dismutase [20]. Our results also indicated that shrimps fed diets containing SBM exhibit lower SOD activity than do shrimps fed the control (entirely FM) diet. Moreover, common carps fed a diet containing 8% β-glycinine, the main antigenic component derived from SBM, exhibited higher hepatic MDA than did carp fed β-glycinine-free control diet [7]. This may be another cause of oxidative stress in the shrimps fed a diet including SBM. Our previous work demonstrated that antinutritional factors,

Table 4
Total haemocyte count (THC), hyaline cells (HC), semigranular cells (SGC), granular cells (GC) counts and phenol oxidase (PO) activity of white shrimp fed different diets for 12 weeks.[1]

	Control	SBM	FSBM
THC ($\times 10^4$ mL^{-1})	3.68 ± 0.25^c	2.10 ± 0.13^a	2.87 ± 0.19^b
HC ($\times 10^4$ mL^{-1})	1.88 ± 0.14^c	1.28 ± 0.09^a	1.57 ± 0.16^b
SGC ($\times 10^4$ mL^{-1})	1.23 ± 0.10^c	0.65 ± 0.05^a	0.93 ± 0.09^b
GC ($\times 10^4$ mL^{-1})	0.57 ± 0.01^c	0.18 ± 0.0.5^a	0.38 ± 0.07^b
PO (O.D. 490 nm)[2]	1.49 ± 0.21^b	1.09 ± 0.02^a	1.45 ± 0.06^b

[1] data are expressed as mean ± SD (n = 3), means in the same row sharing a same superscript letter are not significantly different determined by Student Newman-Keuls test.
[2] The PO activity was measured as dopachrome formation in 50 μL.

Table 3
Hepatopancreatic thiobarbituric acid reactive substance (TBARS) value, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities of white shrimp feed different diets for 12 weeks.[1]

	Control	SBM	FSBM
TBARS value (nmole MDA g^{-1} tissue)	268.79 ± 17.26^a	626.91 ± 10.13^c	512.03 ± 21.87^b
SOD activity (unit g^{-1} protein)	44.12 ± 3.89^c	20.47 ± 1.72^a	26.44 ± 1.88^b
GPx activity (unit g^{-1} protein)	214.14 ± 36.93^a	349.17 ± 63.56^b	352.40 ± 61.95^b

[1] data are expressed as mean ± SD (n = 3), means in the same row sharing a same superscript letter are not significantly different determined by Student Newman-Keuls test.

such as β-glycinin and β-conglycinin, can be properly degraded through *Lactobacillus* spp. fermentation [21]. This finding supports our suggestion that SBM fermented using *Lactobacillus* spp. can reduce the adverse effects of SBM on the oxidative status of white shrimps. However, the oxidative status in shrimps fed the diet containing FSBM was higher than that of shrimps fed the (entirely FM) control diet. The oxidative stress may be caused by other undegraded antinutritional factors in the SBM. Additional studies on this phenomenon are required.

Unexpectedly, glutathione peroxidase (GPx) activity was higher in shrimps fed diets containing SBM or FSBM than in shrimp fed the control diet. GPx catalyses reactions necessary for the conversion of hydrogen peroxide and fatty acid hydroperoxides into water and fatty acid alcohols by using reduced glutathione, thereby protecting cell membranes against oxidative damage [22]. The increase in GPx activity may be associated with a compensative effect of high oxidative status but may not be enhanced by SBM or FSBM supplementation in the diet of shrimp. The GPx activity depends on the selenium (Se) status in animals [22]. Organic Se is a common form of Se in natural foods. It is involved in amino acid metabolism and is also incorporated into some proteins [23,24]. It might prevent the Se from the chelation with phytic acid derived from the two soy protein sources in digestion tract. Thus, GPx activity did not decrease in shrimps fed SBM- or FSBM-containing diets.

Haemocytes are generally classified into three morphologically distinct cell types, namely HC, SGC, and GC [25]. Haemocytes are involved in the production of melanin via the PO system and is stored and produced by both SGC and GC [26]. The HC and SGC are involved in phagocytosis [27,28]. The haemocyte count and PO activity are commonly used as non-specific immune indicators in shrimp studies [29]. Shrimps fed a diet with SBM substituting FM exhibited the lowest haemocyte count and PO activity. It was closely associated with the oxidative status of white shrimps. Our previous studies have demonstrated that oxidative status is adversely associated with the immune status in grouper [30–32]. This association may explain the reduced immune responses of the shrimp in our study.

Our study demonstrated that fermentation of SBM using *Lactobacillus* spp. inhibits the immunosuppression caused by SBM in white shrimp. A previous study indicated that SBM protein was well degraded through *Lactobacillus* spp. fermentation and the small peptide content (M.W. < 10 kDa) was 16.35% of the total peptides in the FSBM [4]. Bioactive peptides derived from soybean have been well documented in research on human nutrition [33]. The bioactive peptides may have several beneficial health benefits, such as antihypertensive, antioxidative, antiobesity, immunomodulatory, antidiabetic, hypocholesterolemic, and anticancer activities. The bioactives may therefore be responsible for the immune-stimulatory ability of FSBM. Furthermore, the lactic acid concentration in the FSBM used in the present study was approximately 6% [6]. Organic acid supplementation has been demonstrated to enhance nutrient utilisation and to modulate the intestinal morphology and microflora [34]. The overall enhanced of nutritional value of FSBM may show a stronger effect on improving the oxidative status and immune responses of white shrimp than does SBM.

In conclusion, our results indicate that replacing 25% FM protein with SBM significantly reduces non-specific immune responses and induces oxidative stress in white shrimps and that FSBM prepared using *Lactobacillus* spp. can reduce these negative effects.

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