The Effect of Substituting Fish Meal with Fermented Soybean Meal on the Growth Performance and Immune Parameters of Pacific White Shrimp (*Litopenaeus vannamei*)

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ABSTRACT

A 60-day growth trial of Pacific white shrimp (*Litopenaeus vannamei*) was performed. Fish meal (FM) was replaced with 0 %, 25%, 50%, or 100% fermented soybean meal (FSBM) to create four iso-nitrogenous experimental diets. Thirty shrimp (initial mean body weight 4.44 ± 0.03 g) were stocked into 500 L tanks, and each dietary treatment was assigned to eight replicate tanks. The shrimp survival rate was not significantly affected ($P > 0.05$) by the dietary FSBM levels. Shrimp fed the 50% FSBM diet grew the fastest, followed by the group fed 25% FSBM, zero % FSBM, and 100% FSBM. There were significant differences ($P < 0.05$) in the growth performance of the four experimental groups. The feed conversion ratio (FCR) of the shrimp fed the 50% FSBM diet was significantly lower ($P < 0.05$) than that of the shrimp fed the 25% FSBM, zero % FSBM, and 100% FSBM diets. Shrimp fed the 50% FSBM and 25% FSBM diets showed the highest immune responses, followed by the groups that were fed zero % FSBM and 100% FSBM. This study revealed that up to 50% of FM protein can be replaced by FSBM protein in shrimp diets without any negative effects on shrimp health.

Keywords: Pacific white shrimp, Fish meal, Fermented soybean meal, Growth, Immune responses

INTRODUCTION

Fish meal (FM) has been used as a primary protein source for shrimp because of its high nutritional value. Due to increasing demand, limited supply, and a dramatic increase in FM prices, suitable alternative protein sources for shrimp have been investigated (Hardy, 2010). Plant protein from soybean meal is the most common FM replacement. However, high levels of soybean protein can have a negative effect on animal growth, metabolism, and health because soybean meal contains several anti-nutritional factors, an amino acid imbalance, and a relatively low protein content compared to FM (Francis *et al*., 2001; Liu *et al*., 2010; Cheng *et al*., 2013). Further processing of soybean meal by microbial fermentation can eliminate the anti-nutritional factors and improve the nutrient status of the diet (Yamamoto *et al*., 2010). Fermented soybean meal (FSBM) has been used as an FM replacement in the diets of aquatic animals such as orange-spotted grouper, *Epinephelus coioide* (Luo *et al*., 2004), Japanese flounder, *Paralichthys olivaceus* (Kader *et al*., 2011), hybrid tilapia, *Oreochromis mossambicus* x *Oreochromis niloticus* (Zhou *et al*., 2009), black sea bream, *Acanthopagrus schlegelii* (Zhou *et al*., 2011), rainbow trout, *Oncorhychus mykiss* (Barnes *et al*., 2012), and *Macrobrachium nipponense* (Ding *et al*., 2015).

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Fermentation by *Lactobacillus* sp. has been reported to reduce the anti-nutritional factors in wheat and barley (Skrede *et al*., 2003). Replacement of unfermented cereals with lactic acid-fermented cereals in the diets of Atlantic salmon resulted in a significant increase in the overall apparent digestibility of starch, lipid, and energy (Skrede *et al*., 2002). Only a few FSBM studies have been conducted on shrimp (Meunpol *et al*., 2009; Sharawy *et al*., 2016), and only limited information is available on the effect of dietary FSBM protein on shrimp immunological status. In this study, FM was replaced with FSBM and *Lactobacillus acidophilus* in the diet of Pacific White Shrimp (*Litopenaeus vannamei*). The impact of FSBM on the growth performance and immune parameters of white shrimp fed different ratios of FM-substituted diets was examined.

MATERIALS AND METHODS

Experimental diet preparation

Four iso-nitrogenous diets were formulated to contain 38% crude protein and 7% crude lipids. The feed ingredients of the diets are shown in Table 1; the proximate compositions of the diets are shown in Table 2; the amino acid compositions of the diets are shown in Table 3. FM was replaced with FSBM in the amounts of zero %, 25%, 50%, and 100%. All materials were mixed and formulated into dry pellets with a laboratory pellet mill.

Shrimp rearing conditions

Juvenile Pacific white shrimp (mean weight 4.44 ± 0.03 g) were obtained from a commercial

Table 1. Compositions of the experimental diets (g/100 g dry weight) containing different substitution levels of fermented soybean meal (FSBM) for fish meal.

Ingredients	0% FSBM	25% FSBM	50% FSBM	100% FSBM
Fish meal, 60% CP (Crude Protein)	8.70	6.52	4.35	0
Fermented soybean meal	0	2.32	4.51	9.10
Shrimp & Squid meal	11.91	12.91	12.91	12.91
Tuna Liver powder	5.35	5.35	4.35	4.35
Poultry meal, 64% CP	9.04	9.04	10.35	11.15
Soybean meal	15.65	15.65	15.91	15.91
Wheat Flour	19.57	18.26	19.47	15.85
Rice Bran	1.57	1.57	0	0
Premix	2.48	2.48	2.48	2.48
Mono calcium phosphate	1.09	1.24	1.36	2.56
Vitamin C, 35%	0.09	0.09	0.09	0.09
Carbon	1.52	1.52	1.52	1.52
Calcium carbonate	1.09	1.09	1.09	1.09
Fish & Squid, soluble	4.35	4.35	4.35	4.35
Lecithin	2.39	2.39	2.39	2.39
Shrimp pellet, reprocessed	13.04	13.04	13.04	13.04
Fish pellet, reprocessed	2.17	2.17	2.17	2.17
Sum	**100.00**	**100.00**	**100.00**	**100.00**

Table 2. Chemical compositions of the experimental diets by proximate analysis.

Ingredients	0% FSBM	25% FSBM	50% FSBM	100% FSBM
Protein (%N × 6.25)	39.88	40.76	41.05	41.31
Lipid (%)	6.80	7.11	7.10	6.29
Crude Fiber (%)	3.65	3.14	3.57	3.60
Moisture (%)	8.91	9.75	9.40	9.49
Ash (%)	12.96	12.77	12.68	13.34
Calcium (%)	2.9	2.9	2.9	3.20
Phosphorus (%)	1.57	1.56	1.71	1.77
NaCl (%)	1.84	1.59	1.53	1.53

Table 3. Total amino acid (g/100 g) compositions of the experimental diets containing different substitution levels of fermented soybean meal (FSBM) for fish meal.

Free amino acid composition (g•100 g^{-1})	0% FSBM	25% FSBM	50% FSBM	100% FSBM
Essential amino acids				
Phenylalanine	1.65	1.74	1.77	1.76
Valine	1.84	1.92	1.93	1.85
Threonine	1.48	1.53	1.53	1.50
Isoleucine	1.43	1.53	1.54	1.47
Leucine	2.72	2.85	2.87	2.82
Lysine	1.94	2.03	1.99	1.92
Methionine	0.89	0.91	0.88	0.81
Tyrosine	1.45	1.51	1.48	1.50
Histidine	0.87	0.91	0.93	0.91
Cystine	0.61	0.65	0.66	0.68
Non-essential amino acids				
Aspartic acid	3.34	3.51	3.56	3.59
Serine	2.03	2.10	2.15	2.18
Arginine	2.38	2.48	2.52	2.53
Glutamic acid	6.39	6.78	6.91	6.77
Alanine	2.05	2.09	2.07	2.02
Glycine	2.59	2.65	2.67	2.61
Proline	2.38	2.55	2.45	2.55
Total	36.04	37.74	37.91	37.47

shrimp farm (Chantaburi Province, Thailand) and acclimated for seven days. During this period, they were fed a commercial feed four times daily. After acclimatization, the shrimp were randomly sampled and stocked in 32 (500 L) fiberglass tanks with a density of 30 shrimp/tank (eight replicates per treatment). Feed was applied to the shrimp four times daily to satiation for 60 days. The salinity, pH, and temperature during the acclimation and experimental periods were maintained at 25‰, pH 7.8–8.0, and 28°C, respectively. All tanks were cleaned daily by siphoning off leftover feed and feces. The water in each tank was changed every day at 10%.

The shrimp's growth performance, including weight gain, percentage of weight gain, average daily gain, and survival rate were analyzed. Feed efficiency, including feed consumption and the feed conversion ratio (FCR) were examined.

Analysis of immune parameters

Preparation of hemolymph samples

At the end of the 60-day feeding trial, 40 shrimp/treatment group were used for immunological tests. A hemolymph sample of 0.5 ml was withdrawn from the base of the third walking leg of each shrimp with a syringe containing 1.5 ml anticoagulant (K-199+5% L-cysteine).

Total hemocytes

After collecting the hemolymph, the hemocytes were counted using a hemocytometer and the number of blood cells per volume was calculated (total hemocytes per cubic millimeter).

Phagocytic activity

Phagocytic activity was determined according to Itami *et al*. (1994). A hemolymph sample (200 µl) was collected from the base of the third walking leg of each shrimp and mixed with 800 µl of sterile anticoagulant. The collected hemocytes were rinsed with shrimp saline, and the viable cell number was adjusted to 1×10^6 cells/ml. The cell suspension (200 µl) was placed onto a glass coverslip. After two minutes, the cell suspension was removed and rinsed three times with shrimp saline. Heat-killed yeast (2 ml) were added, and the suspension was incubated for two hours. After incubation, the heat-killed yeast were removed, and the suspension was rinsed five times with shrimp saline and fixed with 100% methanol. The coverslip was stained with Giemsa stain and mounted with permount.

Two hundred hemocytes were counted. The phagocytotic activity, defined as the percentage of phagocytic cells, was expressed as:

$$\text{Percentage phagocytosis} = \frac{\text{phagocytotic hemocytes}}{\text{Total hemocytes}} \times 100$$

Phenoloxidase activity assay

This method was modified from Supamattaya *et al*. (2000). After the hemolymph was withdrawn, the hemocytes were washed three times with shrimp saline (1,000 rpm at 4°C for 10 min). A hemocyte lysate supernatant (HLS) was prepared from the hemocytes in a cacodylate buffer pH 7.4 using a sonicator at an amplitude of 30 for 5 seconds. The suspension was centrifuged at 10,000 rpm at 4°C for 20 min, and the supernatant was collected as HLS. Then, 200 µl of 0.25% trypsin in cacodylate buffer was mixed with the 200 ml HLS, followed by 200 µl of L-dihydroxyphenylalanine (L-DOPA) at 4 mg/ml as the substrate. The enzyme activity was measured as the absorbance of dopachrome at a 490 nm wavelength. The amount of protein in the HLS was determined using the method established by Lowry *et al*. (1951). The phenoloxidase activity was calculated as the increase in optical density (OD) per minute per mg protein, as expressed in this equation:

1 unit of phenoloxidase = ΔOD490/min/mg protein

Superoxide dismutase activity assay

Superoxide dismutase activity was determined using a RANSOD kit (Randox, USA). This assay is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-

(4-nitrophenol)-5-phenyltetrazolium chloride (INT) and the superoxide radical, which is measured in a spectrophotometer at 505 nm.

Bactericidal activity

Serum was separated from each blood sample and diluted with 2.6% NaCl at 1:2, 1:4, 1:8, 1:16, and 1:32. The bactericidal activity was determined for 0.5 ml of each serum dilution and 0.5 ml of NaCl as the control. *Vibrio parahaemolyticus*, in a suspension of 0.1 ml (5.59×10^6 CFU/ml), was added to each serum dilution and the control. The solutions were incubated at room temperature for three hours before the number of bacteria were estimated using a spread-plate technique. The dilutions that decreased the *V. parahaemolyticus* concentration by up to 50% were compared with the control.

Statistical analysis

The datasets from each of the treatment groups were statistically compared using one-way ANOVA and Duncan's New Multiple Range. All research was conducted at the Aquaculture Business Research Center, Kasetsart University, Bangkok, Thailand.

RESULTS AND DISCUSSION

There was no significant difference in shrimp survival among the feeding groups during the trial. In terms of growth performance, shrimp fed the 50% FSBM diet had the highest weight gain and the highest average daily gain (ADG), followed by the groups fed the 25% FSBM diet, the zero % FSBM diet, and the 100% FSBM diet. There were significant differences ($P < 0.05$) in average weight gain and ADG among the four experimental groups. The lowest FCR was observed for the shrimp that were fed 50% FSBM. Shrimp that were fed the zero % FSBM diet showed comparable FCR and feed efficiency to shrimp that were fed the 25% FSBM diet. The FCR was significantly higher ($P < 0.05$) for the shrimp that were fed the 100% FSBM diet, as presented in Table 4.

There have been reports that FM could be substituted with terrestrial protein sources in diets of various fish and crustaceans (Webster and Lim, 2002; Tidwell *et al*., 1993 and Lim, 1996). However, the replacement of marine protein sources in the diets of *L. vannamei* have showed variable results. Our study revealed that different levels of dietary FSBM did not affect the survival of *L. vannamei* under experimental conditions.

Table 4. Growth performance and feed utilization of white shrimp that were fed different proportions of fish meal and fermented soybean meal (FSBM) for 60 days.

Growth performance	0% FSBM	25% FSBM	50% FSBM	100% FSBM
Number of shrimp (ind./tank)	30	30	30	30
Initial weight (g/ind.)	4.45 ± 0.97^a	4.40 ± 1.03^a	4.44 ± 1.25^a	4.48 ± 1.43^a
Final weight (g/ind.)	9.72 ± 0.77^c	10.64 ± 1.05^b	11.52 ± 1.03^a	9.22 ± 0.76^d
Weight gain (g/ind.)	5.27 ± 0.40^c	6.24 ± 0.22^b	7.08 ± 0.34^a	4.74 ± 0.26^d
Percent weight gain (%)	118.37 ± 9.07^c	141.76 ± 5.02^b	159.8 ± 7.68^a	105.75 ± 5.73^d
Average daily gain (g/ind./d)	0.09 ± 0.01^c	0.10 ± 0.01^b	0.12 ± 0.01^a	0.08 ± 0.01^d
Specific growth rate (%/d)	1.30 ± 0.07^c	1.47 ± 0.03^b	1.59 ± 0.05^a	1.20 ± 0.05^d
Survival rate (%)	93.75 ± 2.14^a	90.42 ± 2.78^a	91.25 ± 3.53^a	89.17 ± 2.35^a
Feed intake (g/ind./day)	7.31 ± 0.16^c	8.49 ± 0.18^a	8.04 ± 0.41^b	7.15 ± 0.11^c
Feed efficiency (%)	0.72 ± 0.06^b	0.73 ± 0.02^b	0.88 ± 0.08^a	0.66 ± 0.04^c
Feed conversion ratio	1.39 ± 0.11^b	1.36 ± 0.04^b	1.14 ± 0.10^c	1.51 ± 0.09^a

Data represent mean ± standard deviation. Means in the same row with different superscript letters are significantly different from each other ($P < 0.05$)

These results indicate that FSBM can replace 50% of FM without any negative effects on shrimp health. Shrimp fed 50% FSBM had improved growth, feed intake, feed efficiency and FCR (Table 4). Because there was no difference in the amino-acid content among each experimental group (Table 3), this growth improvement may be attributed to the greater lipid digestibility of FSBM (Refstie *et al*., 2005) and the reduction of anti-nutritional factors during fermentation (Cheng *et al*., 2013).

However, there was a reduction in the growth of the shrimp that were fed a diet of 100% FSBM. This decrease could be attributed to the presence of non-digestible oligosaccharides, lower protein digestibility, or nutritional imbalance (Sharawy *et al*., 2016). Previous reports have indicated that the complete replacement of marine protein sources in the practical diets of *L. vannamei* was unsuccessful (Tan *et al*., 2005; Lim, 1996; Davis and Arnold, 2000 and Forster *et al*., 2003). Gomes *et al*. (1993) suggested that the poor growth performance of animals that were fed high levels of FSBM was related to a reduction in the voluntary feed intake, which consequently lowered the intake of essential dietary nutrients and digestible energy. These reports corroborate with our results. Shrimp that were fed 100% FSBM had the lowest feed intake and percent feed efficiency and the highest feed conversion ratio compared to the other groups (Table 4).

Immune status

The immune parameters of the studied shrimp are presented in Table 5. Shrimp that were fed 50% FSBM had the highest total hemocyte count, followed by the shrimp that were fed 25% FSBM, zero % FSBM, and 100% FSBM. There were significant differences ($P < 0.05$) among the four experimental groups. The shrimp fed 50% FSBM had the highest percent phagocytosis, followed by the 25% FSBM group. The percent phagocytosis for these groups was significantly different ($P < 0.05$) from that of the groups that were fed zero % FSBM and 100% FSBM. There was a significant difference ($P < 0.05$) in percent phagocytosis between shrimp that were fed zero % FSBM and 100% FSBM. Shrimp that were fed 50% FSBM had the highest phenol oxidase activity, followed by the 25% FSBM and zero % FSBM groups. The phenol oxidase activity of the shrimp from these groups was significantly different ($P < 0.05$) from that of the 100% FSBM group. Shrimp that were fed 50% FSBM had the highest superoxide dismutase activity, followed by the groups that were fed 25% FSBM and zero % FSBM. The superoxide dismutase activity of the shrimp from these groups was significantly different ($P < 0.05$) from the group that was fed 100% FSBM. Moreover, the shrimp that were fed zero %, 25%, and 50% FSBM had bactericidal activity at a serum dilution of 1:8, higher than the shrimp fed 100% FSBM, which had a serum dilution of 1:4.

Table 5. Immunity parameters of the Pacific white shrimp after 60 days of feeding with different proportions of fish meal and fermented soybean meal (FSBM).

Immune parameters	**0% FSBM**	**25% FSBM**	**50% FSBM**	**100% FSBM**
Total hemocyte count (THC) ($\times 10^6$ cell/ml)	57.75 ± 6.38^{b}	59.06 ± 3.62^{b}	71.44 ± 10.63^{a}	27.84 ± 4.16^{c}
Phagocytosis (%)	65.38 ± 6.72^{b}	76.50 ± 6.50^{a}	77.63 ± 6.41^{a}	57.50 ± 9.52^{c}
Phenol oxidase activity (min/mg protein)	266.60 ± 1.17^{b}	272.24 ± 3.07^{ab}	274.90 ± 3.27^{a}	252.96 ± 5.23^{c}
Superoxide dismutase (SOD unit/ml)	31.00 ± 1.23^{b}	33.23 ± 2.14^{ab}	35.36 ± 1.62^{a}	29.41 ± 1.32^{c}
Bactericidal activity	1:8	1:8	1:8	1:4

Data are presented as mean ± standard deviation. Means in the same row with different superscript letters are significantly different from each other ($P < 0.05$)

Previous studies on FM replacement have focused on growth performance and the apparent digestibility coefficient, but immune parameter studies have been lacking. Our study showed that the replacement of FM with different levels of FSBM affected the shrimp immune response. A significant decrease was seen in the immune response of the shrimp that were fed 100% FSBM. This decrease may be associated with poor growth performance due to a lower feed intake, a lower feed efficiency, or the oxidative stress related to 100% of the dietary protein deriving from plants. Krogdahl *et al*. (2010) indicated that nutritional imbalances and anti-nutritional factors caused by FM replacement are potential stressors that have adverse effects on aquatic animals' immune responses. Ding *et al*. (2015) reported that completely replacing FM with FSBM may cause oxidative stress. In that study, a high proportion of FSBM in the *Macrobrachium nipponense* diet induced oxidative stress and decreased immunity.

Nordberg and Arner (2001) observed that organisms develop antioxidant defense mechanisms to reduce oxidative stress and protect their biological systems from free radical toxicity. In our study, the negative effect on the immune parameters such as total hemocyte count, phagocytic activity, phenol oxidase activity, superoxide dismutase activity, and bactericidal activity of the shrimp that were fed 100% FSBM may be related to the shrimp's defense mechanisms against the oxidative stress caused by a 100% plant-derived protein diet. A decrease in dietary FM levels may affect shrimp production of nitric oxide (NO), which would negatively affect all the immune parameters. This theory is supported by Xie *et al*. (2016) who reported that NO concentrations decreased with decreasing FM levels, down to 10%, indicating that the immune responses of shrimp that were fed a low-FM diet were damaged by reduced NO synthesis (NO from the amino acid L-arginine plays an important role in innate immunity). Type-2 NO synthase (iNOS or NOS2) is expressed in activated macrophages and controls the replication or killing of intracellular microbial pathogens (Bogdan *et al*., 2000). This is in accordance with Yang *et al*. (2004), who indicated that high plant protein content reduced both the immune and antioxidative defense systems in *M. nipponense.*

CONCLUSION

The optimum FM replacement with FSBM in the diets of *L. vannamei* was 50%. This dietary group exhibited significant improvements in growth performance, weight gain, feed efficiency, and immunological parameters compared to both the control group and the group that was fed 100% FSBM. Therefore, it is believed that FSBM has a great potential for use as a FM replacement in shrimp diets.

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