Replacement of fish oil and fish meal with *Shizochytrium* sp. oil and soybean meal in Pacific white shrimp (*Litopenaeus vannamei*) diets

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ABSTRACT

A 70-day growth trial was conducted with Pacific white shrimp (*Litopenaeus vannamei*) post-larvae (PL12) to study the use of soybean meal and oil from a single cell microorganism (thraustochytrid) as fish meal and fish oil substitutes, respectively, in practical diets for *L. vannamei*. The growth, survival and immune characteristics of shrimp were evaluated. Four experimental diets were designed with soybean meal as the primary protein source with each formulation containing 33% crude protein and 8% lipid. Fish oil was completely substituted with 3% soybean oil and different concentrations of oil from a single cell heterotrophy *Shizochytrium* sp. which is rich in docosahexaenoic acid (DHA). Commercial shrimp feed was used as the control. The final weights and survival rates of shrimp were not significantly different among all treatments. However, shrimp raised on diets supplemented with DHA showed improvements in immune parameters such as total hemocyte count, phenoloxidase activity, superoxide dismutase activity, bactericidal activity. These findings demonstrate that soybean meal and oil from thraustochytrid are potential alternative renewable sources of fish-based ingredients in shrimp diets.

Keywords: docosahexaenoic acid (DHA), *Shizochytrium* sp., Pacific white shrimp (*Litopenaeus vannamei*)

INTRODUCTION

Feed production for aquatic animal farming requires a significant amount of marine captured fisheries products to serve the continued growth of aquaculture and increasing demand for sea food worldwide. In 2006, the aquaculture sector consumed 3,724,000 tons (Mt) of fish meal (68.2% total global fish meal production in 2006) and 835,000 tons of fish oil (88.5% total reported fish oil production in 2006), or an equivalent of 16.6 million Mt of small pelagic forage fish (using a wet fish to fish meal processing yield of 22.5% and wet fish to fish oil processing yield of 5%) (Tacon and Metian, 2008). The worldwide top consumers of fish meal were marine shrimp, followed

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by marine fish, salmon, Chinese carps, trout, eel, catfish, tilapia, freshwater crustaceans and other freshwater fishes (Jackson, 2007). Despite increases in the total global consumption of fish meal and fish oil by the aquaculture sector, the average dietary fish meal and fish oil inclusion levels within compound aqua-feeds have been steadily declining due to the increase in prices of fish meal and fish oil. In addition to the higher cost of aqua-feeds production, environmental legislations and market pressure have forced the aqua-feeds industry to seek for an alternative dietary substitution to improve the overall sustainability of fishery resources (Deutsch *et al*., 2007).

Over the past few decades, efforts have been made to evaluate the suitability of various feed ingredients as alternative protein sources for fish meal. A variety of animal and plant proteins have been used as substitutes for marine sources with varying levels of successes (Lim and Dominy, 1990; Piedad-Pascual *et al*, 1990; Tidwell *et al*, 1993; Forster *et al*, 2003; Samocha *et al*, 2004). However, the use of alternative protein sources often was accomplished in combination with marine oils to supply essential fatty acids. Thraustochytrids are heterotrophic protists under subclass Thraustochytridea (Chromista, Heterokonta) (Cavalier-Smith *et al*., 1994). These single celled organisms are commonly found in marine environments. They can serve as a potential source of oil for aquaculture (Barclay and Zeller, 1996; Nichols *et al*., 1996; Lewis *et al*., 1998, 1999; Carter *et al*., 2003) as they produce lipids with high levels of docosahexaenoic acid (DHA) (Cohen *et al*., 1995; Behrens and Kyle 1996; Apt and Behrens, 1999). Oil from *Schizochytrium* sp. had been demonstrated as a potential candidate for oil replacement in shrimp feed (Browdy *et al*., 2006; Patnaik *et al*., 2006).

The objective of this current study was to evaluate the potential of terrestrial protein sources and oils from a product rich in DHA originating from a single cell source to replace fish meal and fish oil in the practical diets of Pacific white shrimp (*Litopenaeus vannamei*).

MATERIALS AND METHODS

Experimental diets

Three diets were formulated for the present study and the performance of these diets was compared to a commercial diet (control) (Table 1). All diets were prepared to contain 33% crude protein and 8% lipid by completely replacing fish meal with soybean meal, fish oil with soybean oil and different concentrations of spray-dried heterotrophic marine algal meals high in DHA (AquaGrow GOLD; Advanced BioNutrition Corp., Columbia, MD, USA). A commercial diet was used as a positive control group. A diet in which fish meal and fish oil were replaced only with soybean meal and oil (Diet A) was used as a negative control group. Feeds were prepared at a commercial feedmill following commercial procedures and using ingredients which are readily available and commonly used in the manufacture of shrimp feeds in Thailand.

Table 1. Composition of experimental diets (g/100 g dry weight) used in the present study

RAW MATERIAL	Control	A	B	C
SOYBEAN MEAL 48% CP	15	40	40	40
RICE BRAN FRESH	14	14	14	14
CORN GLUTEN MEAL	9	9	9	9
WHEAT FLOUR	26.6	18.8	18.3	18.55
SOYBEAN OIL	0	3	3	3
FISH OIL	1.2	0	0	0
DICALCIUMPHOSPHATE	2	2	2	2
FISH SOLUBLE	2	2	2	2
LECITHIN	1.5	1.5	1.5	1.5
WHEAT GLUTEN	4	7	7	7
MINERAL PREMIX	2	2	2	2
VITAMIN PREMIX	0.3	0.3	0.3	0.3
CP FISH MEAL 60% CP	22	0	0	0
COLINECHLORIDE 75%	0.4	0.4	0.4	0.4
AG-GOLD	0	0	0.5	0.25
TOTAL	**100**	**100**	**100**	**100**

Experimental shrimp

Specific Pathogen Free (SPF) postlarvae (PL5) of *L. vannamei* were obtained from a hatchery in Chachoengsao province, Thailand, and acclimated at the Aquaculture Business Research Center laboratory, Faculty of Fisheries, Kasetsart University, Bangkok for one week. During the acclimation period, shrimp were fed five times per day with a formulated shrimp larval feed (Oversea Marenatrade Ltd, Thailand) until they reached PL15 stage. When the larvae reached PL12, 1,200 shrimps were randomly stocked in 24 500-liter fiberglass tanks with six replicate tanks per treatment. Each tank was stocked with 50 shrimps to achieve a density of 100 shrimps/m^2. The animals were fed four times daily to satiation according to standard feeding rates. Feeding rates were adjusted according to shrimp weight throughout the 70 days experimental period following a published protocol (Limsuwan and Chanratchakool, 2004). Salinity, pH and temperature during acclimation and experimental period were maintained at 25 ppt, 7.8-8.0 and 28°C respectively. Water

quality parameters such as dissolved oxygen (DO), ammonia and nitrite were measured weekly throughout the experiment using a standard protocol (APHA *et al*., 1995). Leftover feed and feces were siphoned daily and 10% of the water was exchanged every 3 days. At the end of the 70-day feeding trial, 150 shrimp from all treatment groups were counted and weighed. Another 150 shrimps from each treatment were randomly sampled to evaluate immune parameters, including total hemocyte count (THC), phagocytic activity, phenoloxidase (PO) activity, superoxide dismutase (SOD) activity, and bactericidal activity.

Immune parameters analysis

Preparation of hemolymph samples

Hemolymph sample of 0.5 ml was withdrawn from the base of the third walking leg of each shrimp by a syringe containing 1.5 ml anticoagulant (K-199 + 5% L-cysteine).

Total hemocytes

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

Phagocytotic activity

Phagocytotic activity was determined according to Itami *et al*. (1994). Two hundred microliters of hemolymph were collected from the base of the third walking leg of shrimp and were mixed with 800 µl of sterile anticoagulant. The collected hemocytes were rinsed with shrimp saline and the viable cell number adjusted to 1×10^6 cells/ml. The cell suspension (200 µl) was placed onto a glass cover slip. After 20 minutes, the cell suspension was removed and rinsed with shrimp saline three times. Heat-killed yeast (2 ml) was added and the suspension was incubated for 2 hours. After incubation, heat-killed yeast was removed, and the suspension was rinsed five times with shrimp saline, fixed with 100% methanol and then the cover slip was stained with Giemsa stain and mounted with permount.

Two hundred hemocytes were counted. Phagocytotic activity, defined as percentage Phagocytosis, was expressed as:

$$\text{percentage phagocytosis} = \frac{\text{phagocytotic hemocytes}}{\text{total hemocytes}} \times 100$$

Phenoloxidase activity assay

The 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). Hemocyte lysate (HLS) was prepared from hemocytes in a cacodylate buffer pH 7.4 by using the sonicator at 30 amplitude for 5 seconds. The suspension was then centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant was collected as HLS. Then 200 µl of 0.25% trypsin in cacodylate buffer was mixed to the 200 ml HLS followed by 200 µl of L-dihydroxyphenylalanine (L-DOPA) at 4 mg/ml as the substrate. Enzyme activity was measured as the absorbance of dopachrome at 490 nm wavelength. The amount of protein in HLS was determined using the method of Lowry *et al*. (1951). The phenoloxidase activity was calculated as the increase in optimum density (OD) per minute per mg of protein, as expressed in this equation:

$$\text{1 unit of phenoloxidase} = \Delta \text{ OD490 /min/mg protein}$$

Superoxide dismutase activity assay

Superoxide dismutase activity was carried out with the RANSOD kit (Randox, USA). This method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5 -phenyltetrazolium chloride (INT) and superoxide radical, which is assayed 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. Then 0.5 ml of each serum dilution and 0.5 ml of NaCl as the control were used in the study. *Vibrio harveyi* suspension of 0.1 ml (8.2 x 10^6 CFU/ml) was added into each serum dilution and the control. The treatments were incubated at room temperature for 3 h before enumerating the number of bacteriaby a spread plate technique. The dilution which could decrease *V. harveyi* by 50% was compared with the control.

Statistical analysis

The data were subjected to one-way analysis of variance followed by Duncan s multiple range test. Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Effect of diets on growth and survival of Pacific white shrimp under laboratory conditions

After 70 days of raising shrimp on experimental diets, no significant differences were observed in average body weight and survival rate among treatments (Figures 1 and 2). The average body weight of shrimp ranged from 7.51 to 7.58 g for various treatments, while shrimps in the control group had an average body weight of 7.67 g. The survival rate of shrimp ranged from 83 to 86% depending on the treatments. Their growth was observed to be typical for shrimp reared under laboratory

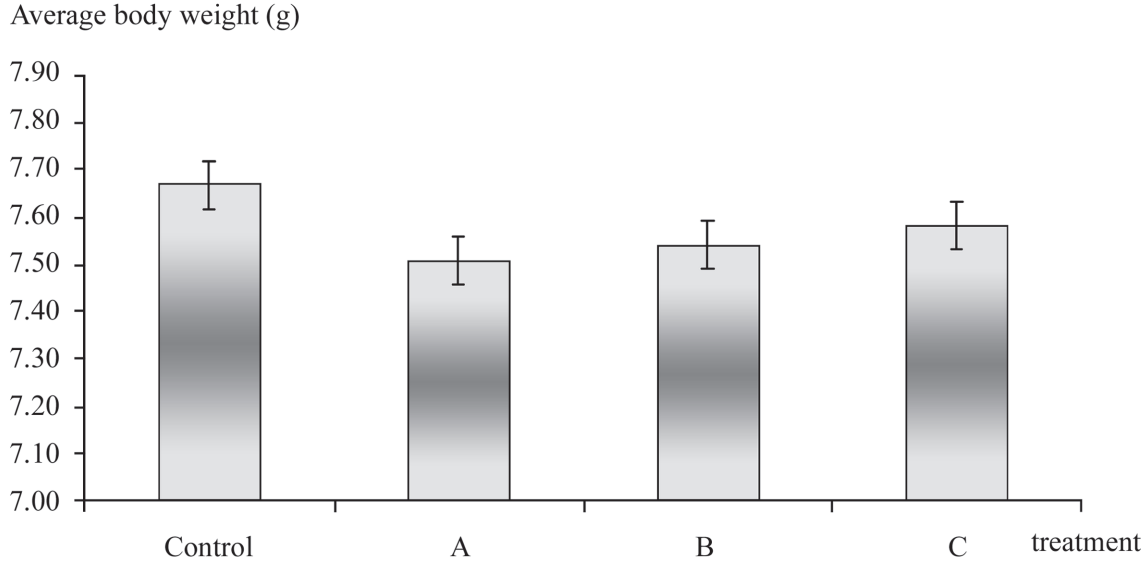

Figure 1. Average body weight of *L. vannamei* after 70 days of feeding with experimental diets

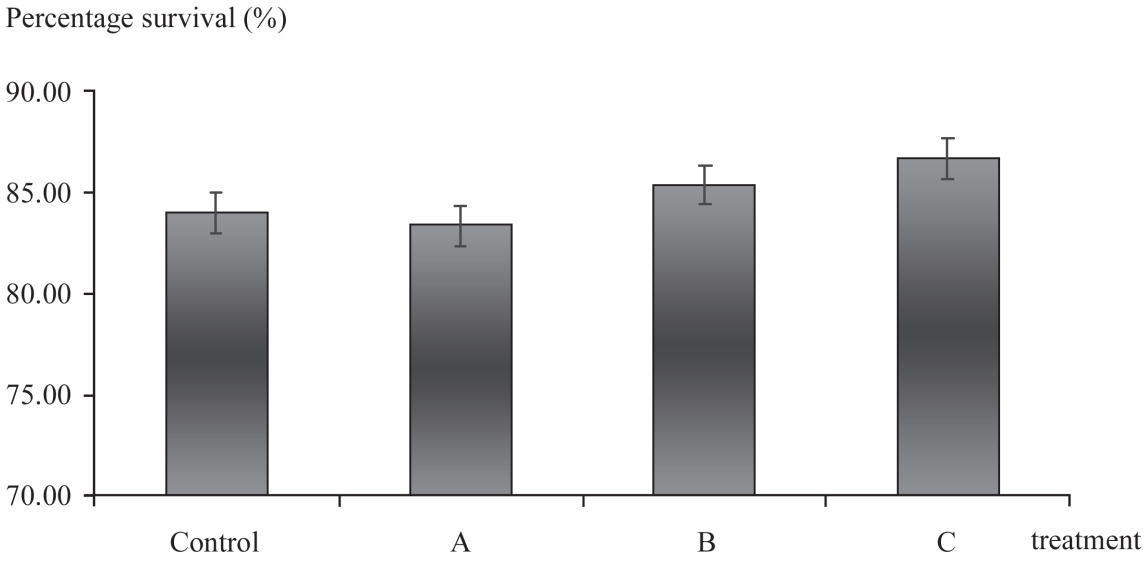

Figure 2. Percentage survival of *L. vannamei* after 70 days of feeding with experimental diets

conditions. There was no indication of feed being rejected or reduced palatability to shrimp fed various test diets. The replacement of fish meal and fish oil with soybean meal and algal meals rich in DHA did not adversely affect the final weight and survival of the shrimp, suggesting that the nutrient quality of the diets was suitable and sufficient for *L. vannamei.*

The present study was to determine the effect of complete replacement of fish meal and fish oil from the practical diets of Pacific white shrimp (*L. vannamei*) and the effect on growth, survival and non-specific immunity. The results showed that shrimp raised on diets containing even the lowest level of algal meal was adequate to support good growth and survival, as reported by Patnaik *et al*. (2006). This also means that the complete replacement of fish meal with co-extruded soybean poultry by-product meal, and fish oil with heterotrophic algal sources rich in DHA (*Schizochytrium* sp.) did not negatively affect the growth and survival of *L. vannamei* under experimental conditions. However, the replacement of marine protein sources in practical diets for *L. vannamei* has been variable. Researchers have demonstrated that 40% of a marine protein mix could be replaced by solvent -extracted cottonseed meal (Lim, 1996) and solvent-extracted soybean meal (Lim and Dominy, 1990), but higher levels of replacements resulted in reduced growth. In contrast, co-extruded soybean poultry by-product meal with egg supplement could be used successfully as a replacement for fish meal in the diet without any apparent effect on survival or growth of shrimp both in indoor research systems and outdoor green-water systems (Davis and Arnold, 2000; Samocha *et al*., 2004). Although these replacement strategies removed fish meal, the diets still utilized marine oils to deliver essential fatty acids.

Effect of diets on the immune characteristics of Pacific white shrimp under laboratory conditions

Immune parameters of shrimp were influenced by the replacement of fish oil with AG-GOLD. Shrimp fed only with soybean meal and oil (group A) had the lowest levels of all immunological parameters tested in this study. In contrast, shrimp fed diets containing soybean meal and oil supplement with AG-GOLD (groups B and C) showed the highest immune responses. The THC and phagocytic activity of shrimp are shown in Tables 2 and 3. Shrimp fed with soybean meal supplement with two concentrations of AG-GOLD (groups B and C) had significantly ($P<0.05$) higher THC and percentage phagocytosis than the groups fed only with soybean meal and oil (group A) and control diet.

Table 2. Total hemocyte count (THC) of *L. vannamei* after 70 days of feeding with experimental diets

Treatment	THC (x 105 cells/ml)
Control	17.63 ± 1.88^{b}
A	16.16 ± 2.26^{b}
B	22.44 ± 4.42^{a}
C	23.25 ± 3.88^{a}

Average values in the same column with different superscripts are significantly different ($P<0.05$).

Table 3. Percentage phagocytosis of *L. vannamei* after 70 days of feeding with experimental diets

Treatment	percentage phagocytosis
Control	15.00 ± 2.50^{b}
A	14.17 ± 2.57^{b}
B	23.08 ± 2.12^{a}
C	24.08 ± 2.08^{a}

Average values in the same column with different superscripts are significantly different ($P<0.05$).

After 70 days of feeding, there were no significant differences in PO and SOD activities of shrimp fed with soybean meal and oil (group A), diets containing two concentrations of AG-GOLD (groups B and C) and the control group (Tables 4 and 5). However, shrimp fed diets containing two concentrations of AG-GOLD showed slightly higher PO and SOD activities than those in group A and the control group.

Table 4. Phenoloxidase activity of *L. vannamei* after 70 days of feeding with experimental diets

Treatment	Phenoloxidase activity (min/mg protein)
Control	272.77 ± 5.37 [a]
A	271.29 ± 5.50 [a]
B	274.00 ± 6.01 [a]
C	274.49 ± 5.63 [a]

Average values in the same column with different superscripts are significantly different ($P<0.05$).

Table 5. Superoxide dismutase activity of *L. vannamei* after 70 days of feeding with experimental diets

Treatment	Superoxide dismutase (SOD units/ml)
Control	29.70 ± 4.59 [a]
A	28.54 ± 5.45 [a]
B	30.35 ± 3.87 [a]
C	30.54 ± 4.82 [a]

Average values in the same column with different superscripts are significantly different ($P<0.05$).

The effects of diets containing AG-GOLD (groups B and C) on bactericidal activity of shrimp are shown in Table 6. Shrimp fed with diets containing two concentrations of AG-GOLD had bactericidal activity at the serum dilution of 1:8, which was higher than shrimp fed with soybean meal and oil (group A) and the control group, which had a serum dilution of 1:4.

Table 6. Bactericidal activity of *L. vannamei* serum after 70 days of feeding with experimental diets

Treatment	Bactericidal activity
Control	1:4
A	1:4
B	1:8
C	1:8

The present study also showed that the use of heterotrophic algae as a source of eicosapentaenoic acid (EFA) in the feed significantly affected the immune responses of shrimp. In addition, shrimps fed with diets containing DHA showed higher immune responses compared to the control group. However, the impact of DHA on immune parameters of shrimp in this study remains unclear. It is possible, though, that the source of DHA used in this study came from thraustochytrids which are potential sources of carotenoids such as ß-carotene, and oxygenated carotenoids such as the xanthophylls, astaxanthin, and canthaxanthin (Aki *et al*. 2003). Astaxanthin is used in aquaculture to enhance flesh coloration of salmonids and other animals, and thraustochytrids probably produced carotenoids to prevent the storage of fats from oxidation (Burja *et al*., 2006).

Previous research has demonstrated that carotenoid pigments are involved in many physiological systems in aquatic animals especially the antioxidant activity (Esterman, 1994; Hunter, 2000). Supamattaya *et al*. (2005) reported that astaxanthin could elevate humural factors such as serum complement and lysozyme activity, as well as cellular factors such as phagocytosis and nonspecific cytotoxicity of shrimp and enhance the resistance of shrimp to white spot syndrome virus (WSSV). In the present study, thraustochytrids such as *Shizochytrium* could be a source of both polyunsaturated fatty acids (PUFAs) and xanthophylls.

CONCLUSION

A complete replacement of fish meal and fish oil using soybean meal and oil sources from a single cell microorganism did not significantly affect the growth and survival rate of shrimp. In addition, algal oil enhanced immune responses of shrimp.

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