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THE EFFECT OF TRACE MINERAL SUPPLEMENTATION IN LOW FISHMEAL DIETS ON THE GROWTH PERFORMANCE AND IMMUNE RESPONSES OF THE PACIFIC WHITE SHRIMP (*Litopenaeus vannamei*)

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Abstract: The issue of low fishmeal diets has been a hot-button topic in the animal industry, including the aquafeed industry, for the past thirty years due to insufficient fishmeal production and unsustainable fishery. Reducing fishmeal in diets that may lack some essential minerals, especially trace minerals such as zinc, copper, manganese, and selenium, will affect animal performance. Hence, several trace mineral concentrations in the diets of *Litopenaeus vannamei* were studied. The trial was assigned through a completely randomized design with 3 treatments and 10 replicates. The diets with 37% crude protein were formulated and topped up with different trace mineral levels of 1x, 2x, and 3x. All diets were fed to the shrimps for 4 weeks and the results showed no significant differences (P>0.05) on all growth performance parameters. The numerical values of growth performance, however, showed an improving trend when mineral concentrations were increased. Immune responses, such as phenoloxidase activity and glutathione, showed no significant difference between treatments (P>0.05), but the phenoloxidase activity showed an increase in numerical value when the mineral concentrations were higher. Therefore, the results showed that an increase of trace mineral concentrations in the diets of the Pacific white shrimp showed a promotion of growth performance and improvements in the immune responses.

Keywords: Trace minerals supplementation, low fishmeal diets, *Litopenaeus vannamei*, growth performance, immune response.

Introduction

The Pacific white shrimp (*Litopenaeus vannamei*) is a very important animal for the world's aquatic industry, especially in Southeast Asia, because it is among the most exported animal in the world. The Pacific white shrimp is an omnivorous species, receiving sustenance from both animal and plant sources, and it has the ability to digest and absorb nutrients well, including any raw material from any recipe. Fishmeal is a protein source that is a staple of the aquaculture industry, including the shrimp feed industry, due to the high-quality protein (Tacon & Metian, 2008), as well as rich minerals, vitamins, and other nutrients (Riche, 2015). However, the fishing industry is currently facing various problems (Naylor *et al*., 2009), resulting in the reduction of fishmeal production around the world, with a trend of continuous decline. Many feed factories around the world needed to adapt and adjust feed formulas in response to such changes. One issue that cropped up following the reduction of content in the fishmeal formula is of the elimination of many types of minerals, especially trace minerals, such as zinc, copper, manganese, and selenium. Animals are not able to produce these minerals by themselves and only acquire from the feed they consume. Therefore, the supplementation of trace minerals is an important thing to be concerned about and apply due to the decreased or removed fishmeal from the feed formula. Moreover, high usage of soybean meal in shrimp feed could reduce the bioavailability of protein and trace minerals, such as copper (Cu), zinc (Zn), manganese (Mn), and selenium (Se), as the phytic acid in soybean meal binds trace minerals, so animals cannot absorb the minerals and excrete them to the environment. Therefore, this research focuses on the supplementation of trace minerals (Cu, Zn, Mn, and Se) in the Pacific white shrimp (*L. vannamei*) diet formula, which uses low fishmeal and high soybean meal to enhance the growth and immune responses of the Pacific white shrimp (*L. vannamei*).

Objective

To study the effect of various levels of trace mineral supplementation in the Pacific white shrimp diet with a low fishmeal concentration (7.5%) on the opportunities and the ability to increase the growth performance and stimulation of the immune responses of the Pacific white shrimp.

Materials and Methods

Experimental Feed, Animal and Pond Preparation

The feed composition is presented in Table 1. Three experimental feed was formulated to be isonitrogenous (37% protein) and isolipidic (5% lipid) based on NRC (2011) requirements. The basal diet was formulated to contain 7.5%, representing low fish meal feed and graded levels of trace minerals 1x, 2x, and 3x. The coarse raw materials were finely ground and passed through a 500µM mesh. All the dry ingredients were thoroughly mixed by a mixer machine and mash was produced after adding fish oil, lecithin, and deionized water. The dough was pelleted through a 2 mm die, using a mincer machine. The pellets were steamed at 100 degrees Celsius for 6 minutes and dried at 90 degrees Celsius in a hot air oven for 6 hours to a moisture content of 9% - 10%, then cooled down and after that sealed in plastic bags and stored at room temperature until used. The chemical analysis results of the experimental feed analyzed by the AOAC (2000) method are provided in Table 2. The experiment proceeded at a private farm in Samut Songkhram province, Thailand. The Pacific white shrimp, *L. vannamei*, was obtained from commercial hatcheries in Samut Songkhram province, Thailand. The 1,800 healthy shrimp (*L. vannamei*) with an average weight of 0.6997 ± 0.0039 grams were selected and randomly transferred into 30 cages (1x1x1.2 m cages), 10 replicates per treatment at a density of 60 shrimp per cage, which were paved with polyethylene whole ponds. All shrimp were fed by an auto-feeder five times per day at 8:00, 10:00, 14:00, 16:00, and 20:30. During the trial period, water quality parameters were monitored and maintained at a salinity of 15 ppt, dissolved oxygen was higher than 5 mg/L, pH 7.5-8.5, water temperature ranged from 28 to 30 degree Celsius, and nitrite was lower than 0.5 mg/L throughout the culture period for 4 weeks.

Sample Collection and Data Analysis

Growth Performance and Feed Utilization

At the end of the culture, the growth and feed utilization parameters of the Pacific white shrimp were recorded and evaluated, such as the final weight, total production, percent weight gain (WG), average daily growth (ADG), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER). The parameters were calculated as follows:

A. Percent weight gain (WG, %) = 100 × (Wt −Wi) / Wi

B. Average daily gain (ADG, g day-1) = Wt −Wi / t

C. Specific growth rate (SGR, % day-1) = 100 × (LnWt − LnWi) / t,

D. Feed conversion ratio (FCR) = feed consumed (g) / weight gain (g)

E. Protein efficiency ratio (PER) = weight gain (g, wet weight) / protein intake (g, dry weight)

Where Wt is the total final body weight (g), Wi is the total initial body weight (g), and t is the experiment duration in days.

Immune Response Analysis

At the end of the culture, the hemolymph of sixty shrimp per experimental treatment (six shrimp per replicate) was randomly collected to analyze the total hemocyte count, the

Table 1: The analysis results of the experimental shrimp feed composition (%)

Ingredients composition	1x	2x	3x
Fish meal 60% CP	7.50	7.50	7.50
Animal protein 58% CP	9.00	9.00	9.00
Soybean meal 49% CP	40.00	40.00	40.00
Soy protein concentrate 62% CP	4.00	4.00	4.00
Wheat flour	33.327	33.267	33.207
Fish oil	2.00	2.00	2.00
Lecithin	0.50	0.50	0.50
Cholesterol	0.003	0.003	0.003
Methionine	0.10	0.10	0.10
Multivitamin	1.30	1.30	1.30
Sodium phosphate	0.25	0.25	0.25
Calcium carbonate	1.20	1.20	1.20
Magnesium sulfate	0.30	0.30	0.30
Potassium chloride	0.30	0.30	0.30
Iron amino acid complex	0.10	0.10	0.10
Organo Mix-P	0.12	0.18	0.24
Total	100.00	100.00	100.00

Remark: Organo Mix-P is the commercial mixture of trace minerals 1x=0.06% including zinc, manganese, copper, and selenium.

Table 2: The chemical analysis results of the experimental shrimp feed

Analysis parameters	1x	2x	3x
Proximate analysis			
Moisture (%)	10.8	9.75	11.5
Crude protein (%)	36.7	37.1	36.4
Crude lipid (%)	5.03	5.50	4.59
Ash (%)	7.19	7.30	7.09
Minerals analysis			
Zinc (mg/kg)	126	175	219
Manganese (mg/kg)	87.0	139	178
Copper (mg/kg)	17.3	24.2	29.6
Selenium (mg/kg)	1.51	1.88	2.07

s no significant difference (*P*>0.05).

phenoloxidase enzyme activity according to the method by Encarnacion *et al*. (2012), the superoxide dismutase enzyme activity, and the total glutathione using the Assay Kit of SIGMA-ALDRICH (19160-1KT-F and CS0260-1KT, respectively).

Statistical Analysis

The results were presented as means ± standard deviation. A completely randomized design (CRD) was assigned and all data were analyzed by one-way ANOVA (analysis of variance). Duncan's procedure was used for multiple comparisons on the differences between the treatment means. Differences were regarded as significant when $P<0.05$. The alphabetical notation was used to mark the differences at a significant level of alpha 0.05.

Results and Discussion

Growth Performance and Feed Utilization

No significant ($P>0.05$) differences were found for the final body weight, total production, weight gain (WG), average daily gain (ADG), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) between shrimp fed trace minerals 1x, 2x, and 3x diets for 4 weeks (Table 3). The final body weight ranged between 6.06 ± 0.66 g to 6.10 ± 0.91 g, total production ranged between 321.5 ± 39.4 g to 324.2 ± 35.8 g, WG ranged between 665.9 ± 93.8 g to 673.9 ± 85.5 g, ADG ranged between 0.18 ± 0.01 g/shrimp/day to 0.18 ± 0.03 g/shrimp/day, SGR ranged between 7.18 ± 0.37 to 7.20 ± 0.33, FCR ranged between 0.93 ± 0.12 to 0.96 ± 0.20, and PER ranged from 2.92 ± 0.57 to 2.97 ± 0.38.

In this 4-week study, the growth performance of shrimp fed with low fishmeal (7.5%) diets with increasing trace minerals from 1x to 2x and 3x exhibited the same performance, which seems to show that that the trace minerals had no effect on shrimp growth due to many reasons. Firstly, this trial used a 7.5% fishmeal to represent the low fishmeal content feed, and other animal protein was used as the protein source for protein and amino acids in the *L. vannamei* shrimp feed. Secondly, soybean meal and soy protein concentrate were added to the formula to act as an alternative protein source for protein and amino acids at levels approximately 40 percent and 4 percent, respectively, which is enough for shrimp requirements. Thirdly, trace minerals were supplemented to each diet in various concentrations from 1x, 2x, and 3x, in a short period of 4 weeks. The increase of trace minerals by 0.06% in the diet results showed that all parameters of the shrimp performance were not significantly different ($P>0.05$).

Lim and Dominy (1990) found that weight gain, FCR, and survival rate of *L. vannamei* showed no difference between the shrimp fed 11 percent fishmeal and 28 percent fishmeal diets ($P>0.05$). Similarly, Ghorbani *et al*. (2017) reported that the *L. vannamei* shrimp fed with f 11 percent fishmeal with 42 percent soybean meal diets exhibited no significant difference from shrimp fed with commercial feed ($P>0.05$)

Table 3: The growth performance and feed utilization of the Pacific white shrimp (*L. vannamei*) fed with low fishmeal diets and supplemented with varying levels of trace mineral concentrations for 4 weeks under normal conditions

Parameters	1x	2x	3x	*P*-value
Final weight (g/shrimp)	6.06 ± 0.66	6.10 ± 0.91	6.10 ± 0.59	0.991
Total production (g)	321.5 ± 39.4	323.7 ± 54.5	324.2 ± 35.8	0.991
Weight gain (%)	665.9 ± 93.8	672.3 ± 130.1	673.9 ± 85.5	0.985
Average day growth (g/day)	0.18 ± 0.02	0.18 ± 0.03	0.18 ± 0.01	0.992
Specific growth rate (%/day)	7.18 ± 0.37	7.18 ± 0.51	7.20 ± 0.33	0.988
Feed conversion ratio	0.95 ± 0.14	0.96 ± 0.20	0.93 ± 0.12	0.952
Protein efficiency ratio	2.93 ± 0.41	2.92 ± 0.57	2.97 ± 0.38	0.978

Remark: Data without superscript letters in the same row indicate and there were no adverse effect to the final weight, final production, feed conversion ratio, protein efficiency ratio, and survival rate. Bulbul *et al*. (2016) found that there were no differences in final body weight, SGR, FCR and feed intake between kuruma shrimp fed high fishmeal diets (40%) and low fishmeal diets (6%) (P>0.05).

Even though growth performance parameters displayed no significant differences among the treatment, but the higher dose of trace mineral concentrations showed increasing trends of numerical values, such as the final body weight, total production, and weight gain, which is related to the research by Katya *et al*. (2016), in which *L. vannamei* shrimp fed with 0.5% trace mineral (Cu, Mn, and Zn) premix feed had significantly higher final body weight and weight gain compared with shrimp fed with basal feed. David *et al.* (1993a) reported that weight gain in the Pacific white shrimp increased in response to copper supplementation of up to 32 mg of Cu/kg of diet.

Lee and Shiau (2002) found that shrimp fed with diets supplemented with 10 and 20 mg of Cu/kg of diet had significantly (P<0.01) greater weight gain and protein efficiency ratio (PER) than those fed the unsupplemented control diet. These indicated that an optimum dietary copper requirement ranges between 10-30 mg/kg of diet, or no more than 34 mg of total Cu/kg of diet (Lee & Shiau, 2002; David *et al.,* 1993a). Yuan *et al*. (2019) showed that weight gain and specific growth rate (SGR) in shrimp fed with copper sulfate and copper amino acid complex diets was significantly higher than shrimp fed only copper sulfate diets. These minerals are an essential element for all organisms, and they function as a cofactor in several enzyme systems, including glucose oxidation, amino acid metabolism, and fatty acid metabolism (Lall, 2002), as well as a cofactor and a component of metalloenzymes, such as alkaline phosphatase and DNA polymerases (NRC, 2011).

Zinc had an effect on the growth and survival of *L. vannamei* shrimp, especially organic zinc (ZnMet), which showed the highest weight gain parameters when compared with no added zinc and other forms (Lin *et al*., 2013). David *et al.* (1993b) found that zinc supplemented at levels of 33 mg/kg showed maximized zinc storage in the hepatopancreas of shrimp, whereas 200 mg/kg overcame the depressed bioavailability of zinc. Huang *et al*. (2017), who supplemented and adjusted the mineral balance of recipes using f 12% fishmeal close to the formula used in 30% fishmeal, found that growth rates, survival rates, levels of copper, manganese, and zinc accumulation in the shrimp body of both formulas were not significantly different (P>0.05.) However, FAO stated that the basic recommended mineral levels for shrimp survival and growth in normal condition is 80-120 mg/kg for zinc, 8-12 mg/kg for copper, 40-60 mg/kg for manganese, and 0.17-0.25 mg/kg for selenium (Tacon, 1987).

Immune Response Analysis

The immune response of the Pacific white shrimp fed with low fishmeal diet with different trace mineral concentrations is shown in Table

Table 4: The immune response of the Pacific white shrimp (*L. vannamei*) fed with low fishmeal diet and supplemented with varying trace mineral concentrations for 4 weeks under normal conditions

Parameters	1x	2x	3x	*P*-value
Total hemocyte count ($x10^4$ cell/ml)	13.5 ± 5.07	12.2 ± 2.47	12.0 ± 6.54	0.923
Phenoloxidase (unit/min)	81.0 ± 36.31	100.6 ± 33.81	111.6 ± 49.19	0.662
Superoxide dismutase (inhibition rate %)	90.77 ± 0.09^c	90.46 ± 0.01^b	90.16 ± 0.14^a	0.001
Glutathione (nM)	0.55 ± 0.07	0.43 ± 0.09	0.53 ± 0.10	0.261

Note: Data without superscript letters in the same row indicates no significant difference (P>0.05).

4. Most of the parameters showed no significant differences between treatments (*P*>0.05). The total hemocyte count ranged from 12.0x10^4 ± 6.54 to 13.5x10^4 ± 5.07 cell/ml. The phenoloxidase enzyme activity ranged from 81.0 ± 36.31 to 111.6 ± 49.19 unit/min. The glutathione ranged from 0.43 ± 0.09 to 0.55 ± 0.07 nM, while the superoxide dismutase enzyme activity showed a significant difference (*P*<0.05). The treatment of 1x showed the highest value of 90.77 ± 0.09 %, followed by 90.46 ± 0.01 % for the 2x treatment, and 90.16 ± 0.14 % for the 3x treatment.

From the results of this experiment, the total hemocyte count (THC) showed no significant difference among the group (*P*>0.05), and it ranged between 12.0 ± 6.54 to 13.5 ± 5.07 x10^4 cell/ml. Bulbul *et al*. (2016) found that the use of low fishmeal (6%) in kuruma shrimp feed did not affect the total haemocyte count (*P*>0.05). Shiau and Jiang (2006) found that zinc concentration of 35.0 and 48.0 mg/kg diets exhibited the highest values of THC and the higher concentration (more than 48 mg/kg) showed a decreasing trend of tTHC, which is related to this experiment, which shows that the higher treatments of 2x and 3x showed no improvement of THC values, and in fact, there was a little decrease. The phenoloxidase enzyme activity (PO), although no significant difference was found when comparing between the treatments of 1x, 2x, and 3x (*P*<0.05), exhibited increasing values when the dose of trace minerals was increase. The phenoloxidase enzyme system is the major defense system in shrimp. This enzyme produces the strong oxidizing agent of phenol for the oxidization or killing of pathogens infecting the shrimp, which ultimately leads to the melanization of pathogens and damaged tissues.

The process of melanization depends on the activation of the phenoloxidase (PO) enzyme, which is controlled by the prophenoloxidase (proPO) activation system. The numerical value of the phenoloxidase enzyme activity of the 3x treatment showed the highest values of 111.6 ± 49.19 units/min, followed by 100.6 ± 33.81 units/min in the 2x treatment, and 81.0 ± 36.31 units/min in the 1x treatment. Lin (2013) reported that organic-zinc 30 ppm could enhance PO activity to 0.9 units/mL in *L. vannamei* shrimp when compared with the control treatment, which resulted in a PO activity 0.3 units/mL (*P*<0.05). Activities of the two major antioxidant enzymes SOD and glutathione responsible for combating the effects of ROS vary widely due to many factors. Superoxide dismutase is an indicator of the immune responses in the Pacific white shrimp.

Haemocytes have high SOD activity and muscle increases by 1.4-1.5 fold after immune stimulants, like beta-glucan and sulfated polysaccharide, are applied for 6 hours, or due to pathogen infection. Hence, in normal conditions, SOD does not increase. The values of superoxide dismutase (SOD) showed significant differences (*P*<0.05), even though the values range from 90.16 ± 0.14 to 90.77 ± 0.09 %, which showed that there were not many differences between the groups and the only differences were at the decimal level. Selenium is capable of generating glutathione because it is an important component of glutathione peroxidase, which is characterized as a tetrameric protein with four atoms of Se per molecule (Rotruck *et al.*, 1973). Wang (2006) showed that Se intakes of 1 mg/kg did not affect the generation of oxidative stress scavenging enzyme, such as SOD and glutathione, in *L. vannamei* and there were no differences when compared with the control group that had no added Se in the diets, which is the same as the results of this experiment, as the glutathione activity showed no significant difference (*P*>0.05). Moreover, the research by Musharraf and Khan (2019) showed that zinc levels of 51.42 ppm can enhance superoxide dismutase and glutathione peroxidase activities when compared with the control group (*P*<0.05), but doses higher than 51.42 ppm, such as 71.91 ppm, 83.14 ppm, and 112.32 ppm, showed no difference when compared with zinc levels of 51.42 ppm (*P*>0.05), which is the same as the results of this experiment, which showed that the zinc levels of the diets ranged from 126 to 219 ppm and the glutathione activity exhibited no difference between the groups (*P*>0.05).

Conclusion

This research strongly suggests that the trace mineral concentration of 1x is suitable and enough for the raising of shrimp in 15 ppt salinity seawater, whereas the increasing of trace mineral concentrations in the Pacific white shrimp (*L. vannamei*) feed showed an increasing numerical trend in terms of growth performance, as shrimp fed with 2x and 3x trace minerals showed a little increase in numerical trends, such as the final body weight, average daily growth, and total production when compared with the 1x group, even though there were no significant differences (*P*>0.05). It could also increase the activity of some immune parameters, such as phenoloxidase enzyme (*P*>0.05), in the period of short-term cultivation (4 weeks). However, the properties of trace minerals should also be considered and balanced to reach the basic recommended levels when low fishmeal is used in the feed.

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