Effect of Giant Bladder Kelp (*Macrocystis pyrifera*) Feed Additive on Growth, Survival and Immune Responses of Pacific White Shrimp (*Litopenaeus vannamei*) Injected with *Vibrio parahaemolyticus*

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ABSTRACT

A 60-day feeding trial was conducted with Pacific white shrimp (*Litopenaeus vannamei*) postlarvae 12 (PL12) to ascertain the effectiveness of using a giant bladder kelp as a feed additive. Shrimp growth, feed efficiency, survival, and immune response were evaluated. Postlarvae (PL) 12 were stocked in 16 × 500-L fiberglass tanks at a density of 50 PLs • $tank^{-1}$. Water temperature and salinity were maintained at 29 ± 1 °C and 20-25 ppt, respectively. The shrimp were distributed into four groups (four replicates/treatment) and fed four times • day^{-1} with standard normal pelleted shrimp feed (control group) or feed mixed with 1, 2 and 3% by weight of the giant bladder kelp feed additive. The final weights and survival rates of shrimp were not significantly different among all treatments. However, shrimp raised on diets supplemented with giant bladder kelp showed significant improvement in their immune responses, including phenoloxidase and phagocytosis activities. The immune response of shrimp was significantly influenced by the percentage of the giant bladder kelp feed additive present in the shrimp feed. Shrimp fed with a diet containing 3% giant bladder kelp had significantly higher total hemocyte counts and percentages of phagocytosis, phenoloxidase, superoxide dismutase and bactericidal activities compared with the control group. Moroever, the survival rate of shrimp after challenging with *Vibrio parahaemolyticus* when fed diets containing 2 and 3% of giant bladder kelp were significantly increased compared with that of the control group. These findings demonstrated that the giant bladder kelp feed additive used in this study had a positive effect on the shrimp postlarval nonspecific immune responses and survival after being challenged with *Vibrio parahaemolyticus*.

Keywords: *Macrocystis pyrifera*, *Litopenaeus vannamei*, *Vibrio parahaemolyticus*, feed additive

INTRODUCTION

Currently, Pacific white shrimp (*Litopenaeus vannamei*), native to the Pacific coasts of Central and South America, is the major shrimp species cultured in China, Taiwan and Thailand (Limsuwan and Chanratchakool, 2004). Since 2012, shrimp farmers in Thailand have had a problem of early mortality syndrome (EMS) outbreaks. Affected shrimp show signs of a pale coloration due to pigment loss as well as an atrophied hepatopancreas. These signs may become apparent as early as four days after stocking (Munkongwongsiri *et al*., 2013), and *Vibrio parahaemolyticus* is the agent suspected of causing mass mortality (Tran *et al*., 2013). At present,

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many alternative methods to control pathogenic bacteria, including the use of probiotic bacteria or organic acid to reduce the pathogenic bacteria in the guts of the shrimp are being used for prevention (Tipsemongkol *et al*., 2009; Nayak *et al*., 2012; Walla *et al*., 2012; Jueliang *et al*., 2013; Yowaphui *et al*., 2016). The use of phytogenic compounds is one of the most promising solutions as it has several pharmacological properties, including antimicrobial and anti-inflammatory properties (Rairat *et al*., 2013; Niyamosatha *et al*., 2015).

The giant bladder kelp (*Macrocystis pyrifera*) is a macroalgae that grows in temperate or cold rocky shores from California (USA) to Mexico with an estimated biomass crop that reaches 99,626 tonnes during the summer months (Reyes *et al*., 2004). These macroalgae contain several active compounds, including fucoidan, alginates and carotenoids, which can improve animal resistance against bacterial and viral diseases (Takahashi *et al*., 1998; Hou and Chen, 2005; Balasubramanian *et al*., 2006; Yeh *et al*., 2006). The objective of this study was to evaluate, under laboratory conditions, the effect of dietary supplementation of giant bladder kelp on the growth, feed efficiency, and immune responses and tolerance of the Pacific white shrimp to *Vibrio parahaemolyticus*, which causes EMS infections.

MATERIALS AND METHODS

The experimental seaweed diets of kelp meals used in this study were produced from sustainably managed and harvested wild, natural stocks of giant bladder kelp (*Macrocystis pyrifera*) from the Pacific coast of Baja California, Mexico. The fresh algal biomass was harvested from the source and processed into a marine brown algal chemical hydrolysate product using a mixture of organic acids within 24 hours (Nutrikelp; Algasy Bioderivados Marinos, S.A. de C.V.; Albiomar, Ensenada, Mexico).

Experimental animals

Pacific white shrimp post-larvae 10 (PL 10) were used in this study. The larvae were transported from the hatchery and acclimated in fiberglass tanksat the Aquaculture Business Research Center laboratory, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. After two days of acclimatization, the shrimp (PL12) were used for the experiment. The initial body weight of shrimp at PL 12 was 0.003 g. A total of 16 500-L tanks were used in this experiment, and the shrimp were stocked in each tank at the density of $100 \cdot m^{-2}$ or $50PL \cdot tank^{-1}$. Water temperature and salinity were maintained at 29 ± 1°C and 20-25 ppt, respectively. The four experimental groups consisted of a control group in which the shrimp were fed only commercial pelleted feed, and three treatment groups in which the shrimp were fed pelleted feed mixed with 1, 2 and 3% of giant bladder kelp, respectively. Each treatment group had four replications.

Growth and Survival

The shrimp were fed four times daily until satiation. The feeding rate was adjusted according to shrimp weight throughout the 60-day experimental period. Survival rates and body weights were recorded every 10 days, while the feed conversion ratio (FCR) were recorded at the end of the experiment.

Water analysis

Water quality parameters, such as pH, dissolved oxygen, ammonia and nitrite were analyzed weekly throughout the experiment as described according to APHA (2012).

Immune parameters

The immune parameters were measured at the end of the feeding trial. Forty shrimp per treatment were used for the immunological tests. A hemolymph sample of 250 µL from each shrimp was withdrawn from the base of the 3rd walking leg with a syringe containing 750 µL of precooled (4°C) anticoagulant (0.114 M trisodium citrate, 450 mM NaCl, 10 mM KCl and 10 mM HEPES at pH 7.4). The hemolymph-anticoagulant mixture was used to measure the total hemocyte count

(THC), phagocytosis activity, phenoloxidase (PO) activity, superoxide dismutase (SOD) activity and bactericidal activity using the methods described by Nonwachai *et al*. (2010).

Challenge test with *Vibrio parahaemolyticus*

Ten healthy shrimp from each experimental tank were sampled and acclimated in new 16 200-L fiberglass tanks at the laboratory for seven days. The salinity and temperature were maintained at the same level as in the previous growth and survival trials. A virulent strain of *V. parahaemolyticus* causing EMS cultured in Tryptic Soy Agars with 1.5% NaCl (w/v) was injected into the muscle of the shrimp at the concentration of 2×10^4 CFU $\cdot ml^{-1}$ (LD50). The number of dead shrimp was recorded and collected daily for 10 days.

Statistical analysis

Data from all the trials were analyzed using SPSS statistical software version 20. Differences among the treatment levels were tested for statistical significance using Duncan's New Multiple Range test, with P set at 0.05.

RESULTS

After the shrimp were raised on experimental diets for 60 days, no significant differences were observed in the average final body weights and survival rates among the treatments (Table 1). The average body weights of shrimp ranged from 8.29 to 9.14 g for the various experimental groups. The survival rate of shrimp ranged from 89.5 to 91.5% among the experimental groups. The growth rate of the shrimp was typical of shrimp reared under research conditions. There was no indication of the feed being rejected or having reduced palatability when the shrimp were fed the various test diets relative to the control. No differences were found in the average FCRs among the treatments. The average FCR ranged from 1.35 to 1.49 (Figure 1).

Shrimp fed with 3% giant bladder kelp had significantly higher THCs ($p<0.05$) compared with the control group. However, no difference was observed among the THCs of shrimp fed with 1, 2 and 3% giant bladder kelp (Figure 2). There was no difference among the percentage phagocytosis of shrimp fed with 1, 2 and 3% giant bladder kelp. However, the percentage of phagocytosis of the experimental shrimp from these groups was significantly higher ($p<0.05$) than that of the control (Figure 3). Shrimp fed with 3% giant bladder kelp had significantly higher PO activities ($p<0.05$) compared with those of other groups. There was no difference between the PO activities of shrimp fed with 1 and 2% giant bladder kelp. The PO activities of shrimp from these three groups were significantly different ($p<0.05$) from those of the control group (Figure 4). Although the shrimp fed with 3% giant bladder kelp had the highest SOD activities, it was not significantly different from that those of shrimp fed with 1 and 2% giant bladder kelp. Moreover, shrimp in the control group had significantly lower ($p<0.05$) SOD activities than the group fed with 3% giant bladder kelp (Figure 5). Shrimp fed with diets containing 3 and 2% giant bladder kelp had bactericidal activities at the serum dilution of 1:8, which was higher than that of shrimp fed with 1% giant bladder kelp and the control group, which had a serum dilution of 1:4 (Table 2).

For the challenge test, the infected shrimp showed pale hepatopancreas and empty gut. The survival rates of the shrimp were significantly influenced by the dietary level of giant bladder kelp i.e. shrimp fed diets containing 3% giant bladder kelp displayed the highest average survival rate followed by the group fed with 2% giant bladder kelp. The average survival rates of shrimp in these groups were significantly higher than those of the group fed with 1% giant bladder kelp and the control ($p<0.05$). Those in the control group had the lowest average survival rate (Table 3).

Water quality parameters such as pH, dissolved oxygen, ammonia and nitrite were maintained at optimal levels for rearing shrimp as described by Limsuwan and Chanratchakool (2004) (Table 4).

Table 1. The average body weight, survival rate and feed conversion ratio (FCR) of Pacific white shrimp after 60 days of the shrimp being fed with different diets.

Experiment group	Body weight (g)	Survival rate (%)	FCR
control	8.30 ± 0.75[a]	89.5 ± 4.43[a]	1.49 ± 0.22[a]
1% giant bladder kelp	8.29 ± 0.67[a]	90.5 ± 3.00[a]	1.49 ± 0.13[a]
2% giant bladder kelp	9.14 ± 0.15[a]	91 ± 4.76[a]	1.35 ± 0.05[a]
3% giant bladder kelp	8.60 ± 1.19[a]	91.5 ± 2.52[a]	1.47 ± 0.24[a]

The data are presented as mean ± standard deviation. The means in the same column with different superscripts are significantly different from each other ($p<0.05$).

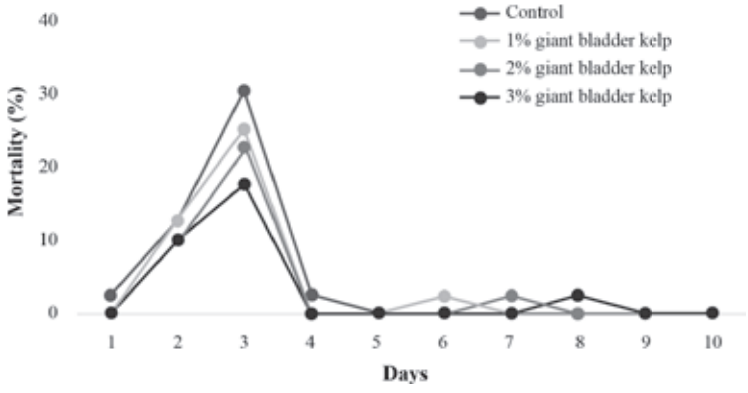

Figure 1. Percent mortality of Pacific white shrimp injected with *Vibrio parahaemolyticus*

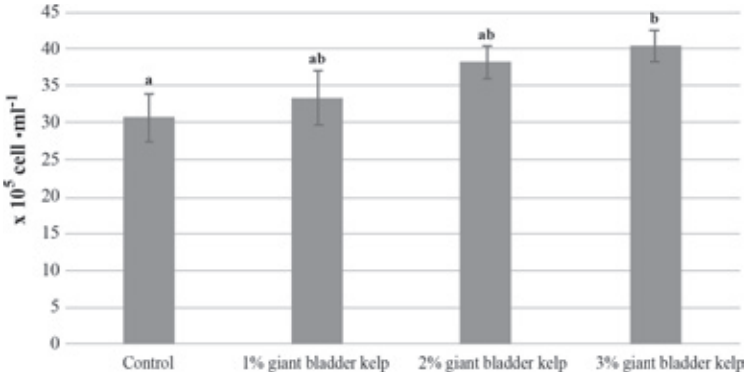

Figure 2. Total hemocyte count (THC) of Pacific white shrimp after feeding with diets having different levels of giant bladder kelp additive

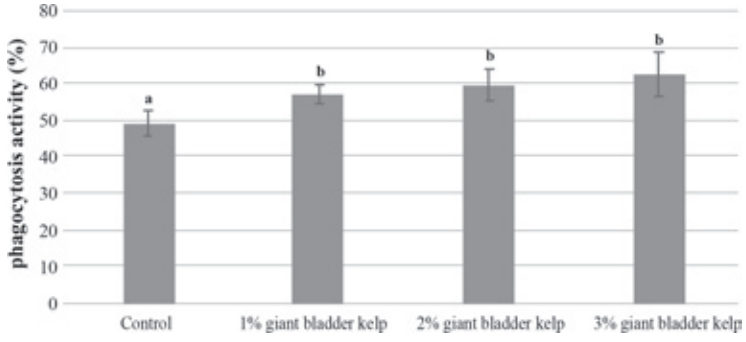

Figure 3. The percentage of phagocytosis activity of Pacific white shrimp after feeding with diets having different levels of giant bladder kelp additive

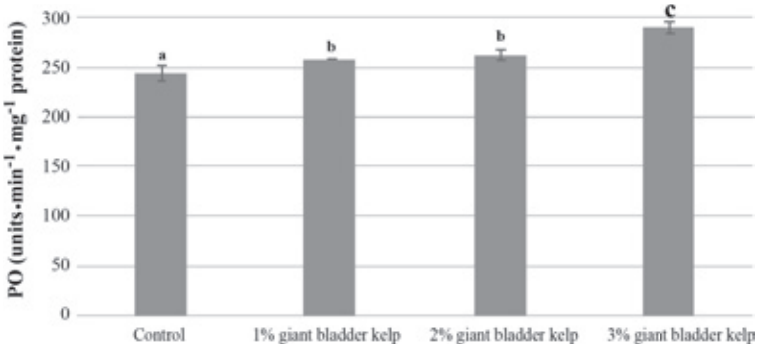

Figure 4. Phenoloxidase (PO) activity (units • min^{-1} • mg^{-1} protein) of Pacific white shrimp after feeding with diets having different levels of giant bladder kelp additive

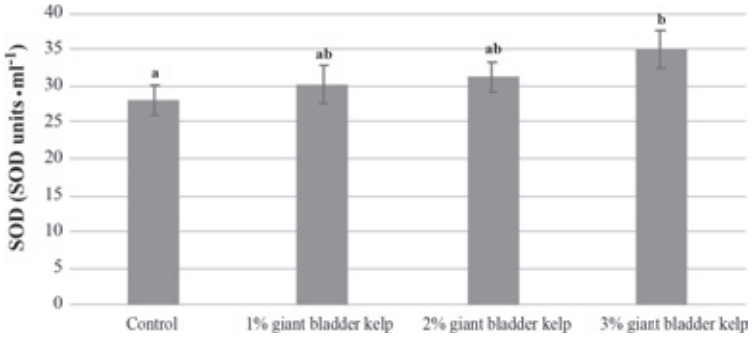

Figure 5. Superoxide dismutase activity (SOD; units • ml^{-1}) of Pacific white shrimp after feeding with diets having different levels of giant bladder kelp additive

Table 2. Bactericidal activity of Pacific white shrimp serum after feeding with diets having different levels of giant bladder kelp additive

Experiment group	Bactericidal activity
control	1:4
1% giant bladder kelp	1:4
2% giant bladder kelp	1:8
3% giant bladder kelp	1:8

The data are presented as mean ± standard deviation. The means in the same column with different superscripts are significantly different from each other ($p<0.05$).

Table 3. Average survival rates of Pacific white shrimp after challenging with *V. parahaemolyticus* causing EMS

Experiment group	Average survival rate (%)
control	52.5 ± 5.00^{a}
1% giant bladder kelp	57.5 ± 5.00^{ab}
2% giant bladder kelp	65.0 ± 5.77^{bc}
3% giant bladder kelp	70.0 ± 8.16^{c}

The data are presented as mean ± standard deviation. The means in the same column with different superscripts are significantly different from each other ($p<0.05$).

Table 4. Water quality parameters during the 60 days feeding trial

Parameter	Dissolved oxygen (mg • L^{-1})	pH	Total ammonia (mg • L^{-1})	Nitrite (mg • L^{-1})
control	6.22-6.50	7.25-7.37	0.34-0.57	0.03-0.08
1% giant bladder kelp	6.24-6.88	7.16-7.31	0.23-0.80	0.01-0.09
2% giant bladder kelp	6.42-6.70	7.15-7.29	0.21-0.84	0.01-0.07
3% giant bladder kelp	6.31-6.74	7.28-7.34	0.21-0.67	0.01-0.07

DISCUSSION

In aquatic animals, seaweed has been commonly used as a dietary feed supplement for both fish and shrimp (Valente *et al*., 2006; Hashin & Mat-Saat, 1992; Moss, 1994; Cruz-Suarez *et al*., 2000; Cruz-Suarez *et al*., 2009). It has been reported that supplementing shrimp diets with kelp meals, such as *Macrocystis pyrifera*, *Ascophyllum nodosum* and *Sargassum* sp., resulting in higher feed intakes and better growth performance (Cruz-Suarez *et al*., 2000; Cruz-Suarez *et al*., 2009). These types of seaweed contain some active compounds, such as alginates, carotenoids, fatty acids, fucoidan and specific amino acids (Cruz *et al*., 2000). Moreover, seaweeds have been successfully used as dietary supplements for direct human consumption and in animal feeds (Mansilla *et al*., 2009). The proximate analysis of the giant bladder kelp (*M. pyrifera*) feed additive used in this study is shown in Table 5.

The aim of the present study was to determine the effect of the giant bladder kelp feed additive on the growth, feed efficiency, and nonspecific immunity and survival of Pacific white

Table 5. The proximal analysis of the giant bladder kelp (*M. pyrifera*) used in this study.

Moisture	90.2 %
Crude protein (N×6.25)	0.85 %
Total lipid (acid hydrolysis)	1.61 %
Ash	5.22 %
Crude fiber	0.3 %
Carbohydrate (by difference)	1.82 %
Carbohydrates	% or mg · kg^{-1} (dry matter basis)
Alginate	13.9 %
D-Mannitol	4.8 %
Fucoidan	1.3 %
Sterols and Tocopherols	mg · kg^{-1} or % of total lipid
Alpha Tocopherol (Vitamin E)	385 mg · kg^{-1}
Total Tocopherols	394 mg · kg^{-1}
Carotenoids	mg · kg^{-1} (dry matter basis)
Zeaxanthin	5 mg · kg^{-1}
Trans-beta Carotene	4 mg · kg^{-1}
Total Carotenes	9 mg · kg^{-1}
Fatty Acid (FA)	Mg FA · g^{-1} oil
Total FA	407.1
Total Saturates	52.6
Total Monounsaturates	60.8
Total Polysaturates	124.6
Total Omega 3	74.7
Total Omega 6	49.9
Total Omega 9	47.4
Amino acid profile	% amino acid dry matter basis
Total Amino acids	7.14

shrimp (*L. vannamei*) after being challenged with the bacterial pathogen *V. parahaemolyticus*. As for the growth performance, some studies have demonstrated that macroalgal inclusion in shrimp feed may vary according to consumer species and geographical distribution of the algae. Briggs and Funge-Smith (1996) observed a significant reduction in the growth of *Penaeus monodon* fed with diets containing 300 g · kg^{-1} of red seaweed (*Gracilaria* spp). Cruz-Suarez *et al*. (2000) found a significant increase in the growth of *L. vannamei* fed with diets containing Mexican kelp meal (*M. pyrifera*), although, when Chilean kelp meal (*M. pyrifera*) was tested on *L. vannamei*, a slight increment in weight gain was observed. In the present study, there was no difference between the average weight of shrimp fed with kelp meal and the control diet.

Shrimp resistance to *Vibrio* spp. infection is influenced by its immune status. The first step in the immune process is the recognition of pathogens. This process is carried out by hemocytes through molecules that have the ability of recognizing structures in the cell walls of pathogens, such as attachment proteins, and by the recognition of β-1, 3-glucans, lipopolysaccharides, and peptidoglycans (Lin *et al*., 2006; Vargas-Albores and Yepiz-Plascencia, 2000). Once pathogens are detected, hemocytes get activated then a whole series of mechanisms is triggered to control or remove the pathogens. The phenoloxidase system has been recognized as an efficient defense mechanism against the non-self. This system is stored and produced by semi-granular and granular hemocytes, and it can be activated by a presence of microbes. Activation of the prophenoloxidase system results

in the production of melanin, a dark-brown pigment responsible for inactivating pathogens, and preventing their spread throughout the host body, as well as for healing cuticle damages (Sritunyalucksana and Söderhäll, 2000). Destroying the phagocytized materials involves the intracellular production of free radicals. During contact with and recognition of the pathogen, host enzymes like NADPH-oxidase are activated, which in turn increase oxygen consumption, resulting in the production of free radicals such as superoxide anions (O^{2-}) and hydrogen peroxide (H_2O_2), among others (Muñoz *et al*., 2000; Rodríguez and Le Moullac, 2000). These free radicals can directly kill the invading organism or work in combination with nitrogen compounds (nitric oxide), or exert a synergistic effect with lysozymes (Roch, 1999). Phagocytosis is the most common reaction of defense cell mechanisms. By this process, cells (hemocytes) ingest and destroy invading pathogens (Secombes, 1996). Encapsulation and nodule formation are processes by which several hemocytes cooperate with each other aiming to stop the action of invading pathogens (Söderhäll and Cerenius, 1992).

Our data showed that shrimp raised on diets that contained kelp feed additives demonstrated good immune responses and survivability after being challenged with *V. parahaemolyticus* causing EMS. In a recent study, Cheng *et al*. (2005) showed that sodium alginate extracted from brown algae at 0.5 $g \cdot kg^{-1}$ diet has been reported to enhance the immune responses (phagocytic activity, PO activity and SOD activity) of *L. vannamei* and increase its resistance to *V. alginolyticus* infection. Similarly, Kitikiew *et al*. (2013) reported that fucoidan at the dose of 1 $g \cdot kg^{-1}$ diet could effectively provoke the shrimp's innate immunity by increasing the circulation of hemocytes, PO activity, phagocytic activity and resistance against *V. alginolyticus*. Moreover, Nonwachai *et al*. (2010) showed that the use of heterotrophic algae as a source of essential fatty acids in the feed significantly affected the immune response of *L. vannamei* and improved the resistance of shrimp against *V. harveyi*. In the present study, the immune parameters of the shrimp were influenced by the percentage of giant bladder kelp in the shrimp feed. The shrimp fed diets containing 3% of giant bladder kelp showed the highest THC, percentage of phagocytosis activity, PO activity, SOD activity and bactericidal activity followed by shrimp in the group fed with 2% and 1% of giant bladder kelp, respectively. In contrast, the control group had the lowest immune responses.

Because the giant bladder kelp (*Macrocystis pyrifera*) used in this study contained many natural active compounds, such as 13.9% alginate, 1.3% fucoidan, 9% total carotenes and high amounts of important amino acids and fatty acids, the mechanism explaining why the kelp meal improved the survival and immune parameters of the shrimp in this study remains unclear. However, it is possible that a synergic effect from these compounds present in the kelp meal could play a crucial role in shrimp health by stimulating the immune parameters of the shrimp and enhancing the resistance of the shrimp to *Vibrio* infection. Further studies on determining the effect of each specific compound, such as alginate and fucoidan, to confirm the function of these two compounds on the immunity of shrimp are recommended.

CONCLUSION

In conclusion, the present study documents that feeding *L. vannamei* a diet containing 3% giant bladder kelp (*Macrocystis pyrifera*) significantly increased shrimp immune abilities by increasing their THC, PO activity, phagocytosis activity, SOD activity and bactericidal activity together with an increase in their resistance against *V. parahaemolyticus* causing EMS.

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