Effect of Formic Acid, β-Carotene and Vitamin E on Growth, Survival and Prevention to *Vibrio parahaemolyticus* in Rearing of Pacific White Shrimp (*Litopenaeus vannamei*)

Narupanat Yowaphui, Tirawat Rairat and Niti Chuchird*

ABSTRACT

A 60-day feeding trial was conducted to evaluate the effects of formic acid (Amasil® NA), β-carotene (Lucarotin® 10% Feed) and vitamin E (Lutavit® E 50 S) on growth, survival, prevention of *Vibrio* infection and immune parameters of Pacific white shrimp. In Experiment 1, postlarvae-12 were randomly distributed into six groups and then fed four times daily with the following diets as treatments: 0.3% formic acid, 50 ppm β-carotene, 50 ppm β-carotene + 0.3% formic acid, 400 ppm vitamin E and no feed additives (positive and negative control groups). After 30 days of the feeding trials, the body weight of shrimp fed with 50 ppm β-carotene was significantly higher than those of the control groups, whereas the survival rate was not different among all treatment groups. In Experiment 2, *Vibrio parahaemolyticus* was added to all tanks except the positive control to obtain a final concentration of 10^4 colony-forming units/ml. Each treatment group received the aforementioned diets for another 30 days. At the end of the trial, shrimp fed with 50 ppm β-carotene and 400 ppm vitamin E had significantly higher body weight compared to the negative control. Apart from the positive control, the highest survival rate was observed in shrimp fed a diet containing 0.3% formic acid, while immune parameters of shrimp fed a diet containing β-carotene were significantly improved. In summary, dietary formic acid and β-carotene exhibited positive effects on shrimp health.

Keywords: formic acid, β-carotene, vitamin E, Pacific white shrimp, *Vibrio parahaemolyticus*

INTRODUCTION

Pacific white shrimp (*Litopenaeus vannamei*) is the primary shrimp species cultured in Thailand (Limsuwan and Chanratchakool, 2004). Thai shrimp farmers have suffered major economic losses owing to early mortality syndrome (EMS) since 2012. 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 4 days after stocking (Munkongwongsiri *et al*., 2013), and *Vibrio parahaemolyticus* is the suspected agent that causes mass mortality (Tran *et al*., 2013). Because the usage of antibiotics in shrimp aquaculture is discouraged, it is necessary to find alternative agents to prevent

Aquaculture Business Research Center, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand
*Corresponding author, e-mail: ffisntc@ku.ac.th

bacterial infection. Organic acids, including formic acid, are among the most promising substances as they have been reported to possess anti-*Vibrio* spp. activity (Mine and Boopathy, 2011; Adams and Boopathy, 2013; Da Silva *et al*., 2013), and increase survival rate of shrimps (Walla *et al*., 2012; Su *et al*., 2014). In addition, the positive effect of other substances on health of shrimps has also been reported, including β-carotene and vitamin E, which may be classified as immunostimulants (Sakai, 1999; Lee and Shiau, 2004; Supamattaya *et al*., 2005; Maggini, 2010). Therefore, organic acids, β-carotene and vitamin E have the potential to be used in shrimp farming as feed additives. The objective of this study was to evaluate the effect of dietary supplementation of formic acid (Amasil® NA), β-carotene (Lucarotin® 10% Feed) and vitamin E (Lutavit® E 50 S) on growth, survival and prevention to *V. parahaemolyticus* infection in Pacific white shrimp under laboratory conditions. The effect of these substances on some immune parameters of shrimp was also examined.

MATERIALS AND METHODS

Experiment 1: The effect of formic acid (Amasil® NA), β-carotene (Lucarotin® 10% Feed) and vitamin E (Lutavit® E 50 S) on growth and survival of Pacific white shrimp postlarvae

Experimental diets

Feed additives used in this study consisted of formic acid (Amasil® NA, BASF, Ludwigshafen, Germany), β-carotene (Lucarotin® 10% Feed, BASF) and vitamin E (Lutavit® E 50 S, BASF). Shrimps were divided into six treatment groups which consisted of positive control, negative control, 0.3% formic acid, 50 ppm β-carotene, 50 ppm β-carotene + 0.3% formic acid, and 400 ppm vitamin E. Shrimp in the positive and negative control groups were fed with commercial pelleted feed with no feed additives. Although the conditions of positive and negative control treatments in Experiment 1 were exactly the same, both were prepared to be used differently in Experiment 2 (see "Shrimp and experimental protocol" in Experiment 2). The experimental shrimp in the other treatment groups were fed with commercial pelleted feed (36% crude protein, 6% lipid, Charoen Pokphand Foods Public Company Limited (CPF), Thailand) supplemented with feed additives as described above. All of these substances were applied by spraying and mixing with the commercial pelleted feed.

Shrimp and experimental protocol

The experiments were carried out at the Aquaculture Business Research Center Laboratory, Faculty of Fisheries, Kasetsart University, Thailand. Postlarvae-9 (PL-9) of Pacific white shrimp were obtained from a hatchery in Chachoengsao Province, Thailand. After three days of acclimation, the experimental shrimp (now PL-12 stage) were randomly distributed into 24 × 500-L fiberglass tanks (four replicate tanks per treatment). Each tank was stocked with 60 shrimp PLs. Each treatment group was fed with one of the six diets four times daily until satiation in line with a standard feeding rate for 30 days. The rate was adjusted in line

with shrimp weight throughout the 30-day experimental period, following a published protocol (Limsuwan and Chanratchakool 2004). Salinity throughout the experiment was maintained at 25 ppt, dissolved oxygen above 4 ppm, and water temperature at 29 ± 1°C. Leftover feed and feces were siphoned out daily, and 10% of the water was replaced every 3 days.

Growth and survival studies

The average body weight and survival rate of shrimp were recorded after the 30-day experimental period.

Experiment 2: The effect of formic acid (Amasil® NA), β-carotene (Lucarotin® 10% Feed) and vitamin E (Lutavit® E 50 S) on growth, survival, and immune responses of Pacific white shrimp challenged with *Vibrio parahaemolyticus*

Shrimp and experimental protocol

The experimental shrimp from each tank in Experiment 1 were randomly distributed into new (24 × 500-L) fiberglass tanks (four replicate tanks per treatment). The stocking density was 50 shrimp per tank. At the beginning of this experiment (Day 0), *Vibrio parahaemolyticus* isolated from a diseased *L. vannamei* containing approximately 10^4 colony-forming units (CFU)/ml, which is the normal concentration of *Vibrio* in shrimp farm water as described by Sung *et al*. (2001) and Lavilla-Pitogo *et al*. (1998), was added into all groups except the positive control. Each treatment group received the same diet as in Experiment 1 four times daily for another 30 days. Salinity, dissolved oxygen, and water temperature were maintained as in the Experiment 1. Leftover feed and feces were siphoned out every 2 days without water replacement.

Growth and survival studies

The body weight and survival rate of shrimp from each treatment were recorded on the 30^{th} day after being challenged with *V. parahaemolyticus* at 10^4 CFU/ml.

Immune parameters study

Immune parameters were measured at the end of the feeding trial. Ten 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 3^{rd} walking leg using a syringe containing 750 µL of precooled (4°C) anticoagulant (0.114 M trisodium citrate, 450 mM NaCl, 10 mM KCl, 10 mM HEPES at pH 7.4). The hemolymph-anticoagulant mixture was used to measure 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)

Statistical analysis

Results are presented as means ± standard deviation. One way ANOVA and Duncan's New Multiple Range test were used to compare data among treatments. Differences were considered significant if $p<0.05$.

RESULTS

Experiment 1: The effect of formic acid (Amasil® NA), β-carotene (Lucarotin® 10 % Feed) and vitamin E (Lutavit® E 50 S) on growth and survival of Pacific white shrimp postlarvae

After 30 days of dietary administration, shrimp fed with 50 ppm β-carotene had the highest average body weight (1.41 ± 0.13g), which was significantly greater than that of the control groups, but not significantly different from those fed with diets added with 400 ppm vitamin E (1.39 ± 0.07 g) and 50 ppm β-carotene + 0.3% formic acid (1.36 ± 0.06 g). The two lowest average body weights were observed in positive and negative control groups (1.21 ± 0.07 and 1.26 ± 0.07 g, respectively). The average survival rate of shrimp in all groups ranged from 91 to 97%, and was not significantly different from each other (Table 1; Figures 1-2).

Table 1. Average body weight and average survival rate of Pacific white shrimp after 30 days of feeding with formic acid, β-carotene and vitamin E.

Treatment groups	Average body weight (g)	Average survival rate (%)
Positive control	1.21 ± 0.07^{c}	91.25 ± 3.44^{a}
Negative control	1.26 ± 0.07^{bc}	92.50 ± 1.67^{a}
0.3% Formic acid	1.29 ± 0.07^{abc}	93.75 ± 2.5^{a}
50 ppm β-carotene	1.41 ± 0.13^{a}	94.58 ± 3.7^{a}
50 ppm β-carotene + 0.3% Formic acid	1.36 ± 0.06^{ab}	96.67 ± 2.72^{a}
400 ppm Vitamin E	1.39 ± 0.07^{ab}	96.25 ± 1.6^{a}

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

Experiment 2: The effect of formic acid (Amasil® NA), β-carotene (Lucarotin® 10% Feed) and vitamin E (Lutavit® E 50 S) on growth, survival, and immune responses of Pacific white shrimp challenged with *Vibrio parahaemolyticus*

At the end of the feeding trial, the average body weights of shrimp fed with an additive of 50 ppm β-carotene and 400 ppm vitamin E were the highest, at 3.42 ± 0.09 and 3.40 ± 0.08 g, respectively. These average body weights were significantly higher than that of the negative control group (2.99 ± 0.13 g), but not significantly different from the positive control and all the other groups. However, apart from the positive control group which had the highest survival rate (81.00 ± 4.76%), the survival rates of shrimp fed with 0.3% formic acid (58.00 ± 2.83%) and 50 ppm β-carotene + 0.3% formic acid (54.50 ± 4.43%) were significantly higher than all of the remaining groups (Table 2; Figures 3-4).

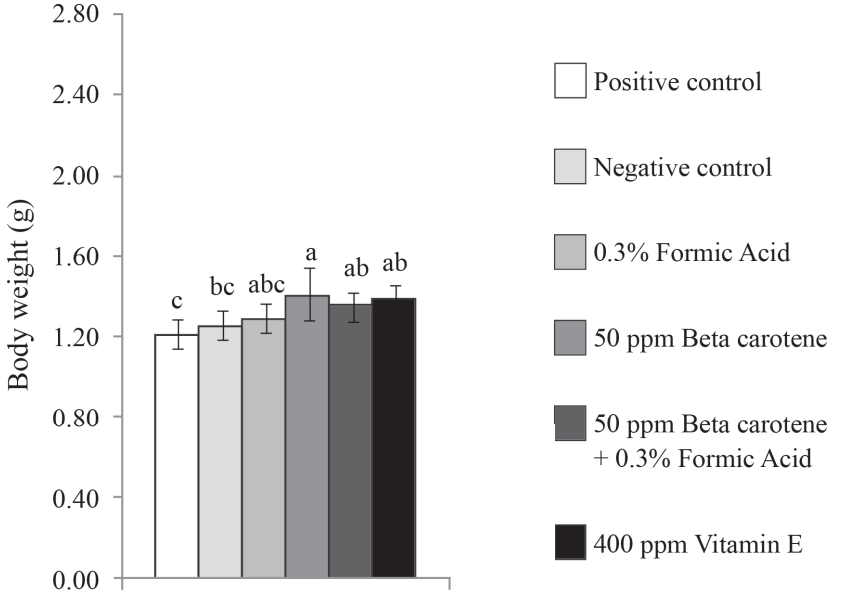

Figure 1. Average body weight of Pacific white shrimp after 30 days of feeding with formic acid, β-carotene and vitamin E.

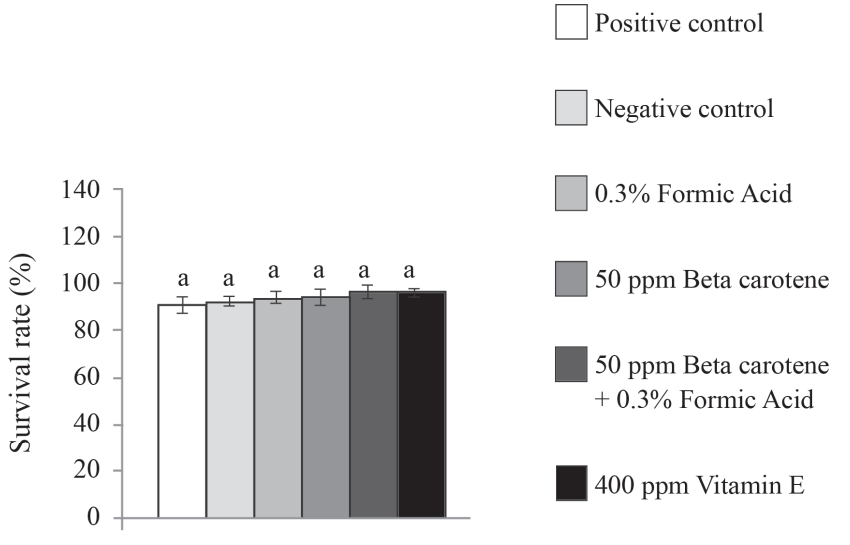

Figure 2. Average survival rate of Pacific white shrimp after 30 days of feeding with formic acid, β-carotene and vitamin E.

Table 2. Average body weight and average survival rate of Pacific white shrimp after challenge with *V. parahaemolyticus* (10^4 CFU/ml) 30 days.

Treatment groups	Average body weight (g)	Average survival rate (%)
Positive control	3.13 ± 0.19^{ab}	81.00 ± 4.76^{a}
Negative control	2.99 ± 0.13^{b}	36.50 ± 3.42^{c}
0.3% Formic acid	3.26 ± 0.41^{ab}	58.00 ± 2.83^{b}
50 ppm β-carotene	3.42 ± 0.09^{a}	41.00 ± 5.77^{c}
50 ppm β-carotene + 0.3% Formic acid	3.33 ± 0.12^{a}	54.50 ± 4.43^{b}
400 ppm Vitamin E	3.40 ± 0.08^{a}	41.50 ± 4.43^{c}

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

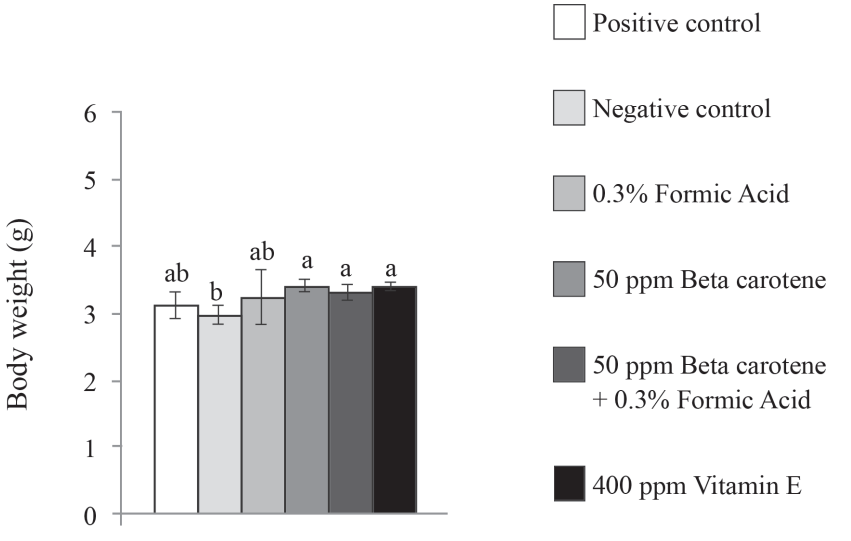

Figure 3. Average body weight of Pacific white shrimp after challenging with *V. parahaemolyticus* (10^4 CFU/ml) 30 days.

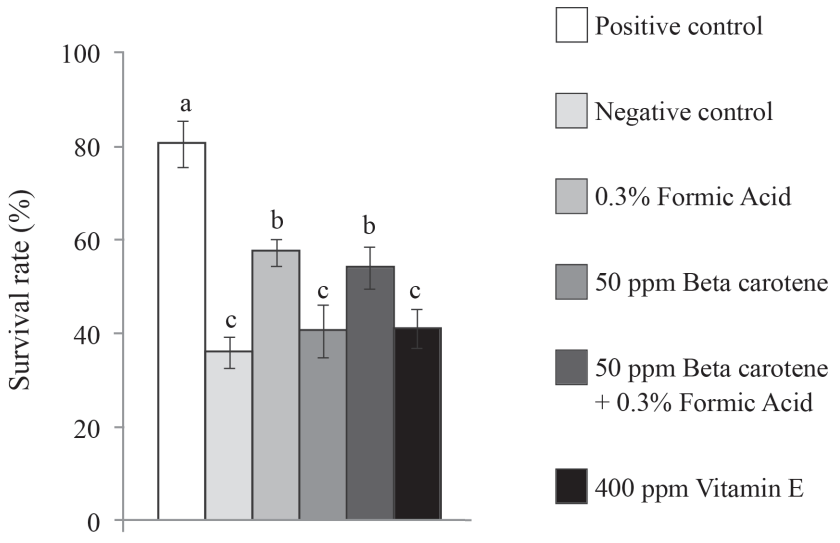

Figure 4. Average survival rate of Pacific white shrimp after challenging with *V. parahaemolyticus* (10^4 CFU/ml) 30 days.

The levels of immune parameters in the β-carotene-fed groups (namely, 50 ppm β-carotene and 50 ppm β-carotene + 0.3% formic acid) were significantly higher than all the other groups in every parameter investigated, i.e. total hemocyte count (THC), phagocytosis activity, phenoloxidase (PO) activity, superoxide dismutase (SOD) activity, bactericidal activity. On the contrary, the immune parameters in positive control, negative control, 0.3% formic acid, and 400 ppm vitamin E groups were lower and not significantly different from each other (Table 3; Figures 5-8).

Table 3. Immune parameters of Pacific white shrimp after being challenged with *V. parahaemolyticus* at (10^4 CFU/ml) feeding with eight experimental diets for 30 days.

Treatment groups	THC (10^5cells/ml)	Phagocytosis (%)	PO (units/min/ mg protein)	SOD (SOD units/ml)	Bactericidal activity
Positive control	3.68 ± 0.15^b	9.00 ± 1.15^b	63.98 ± 1.46^b	5.76 ± 1.50^b	1:4
Negative control	4.05 ± 0.39^b	9.50 ± 1.00^b	63.17 ± 1.73^b	5.81 ± 0.80^b	1:4
0.3% Formic acid	3.60 ± 0.60^b	9.00 ± 1.15^b	65.68 ± 1.82^b	5.89 ± 1.37^b	1:4
50 ppm β-carotene	6.53 ± 0.45^a	15.50 ± 1.00^a	88.38 ± 1.79^a	8.92 ± 0.35^a	1:8
50 ppm β-carotene + 0.3% Formic acid	6.08 ± 1.13^a	15.50 ± 1.00^a	86.09 ± 1.11^a	8.85 ± 0.87^a	1:8
400 ppm Vitamin E	4.05 ± 0.93^b	10.50 ± 1.00^b	64.33 ± 1.09^b	5.73 ± 1.05^b	1:4

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

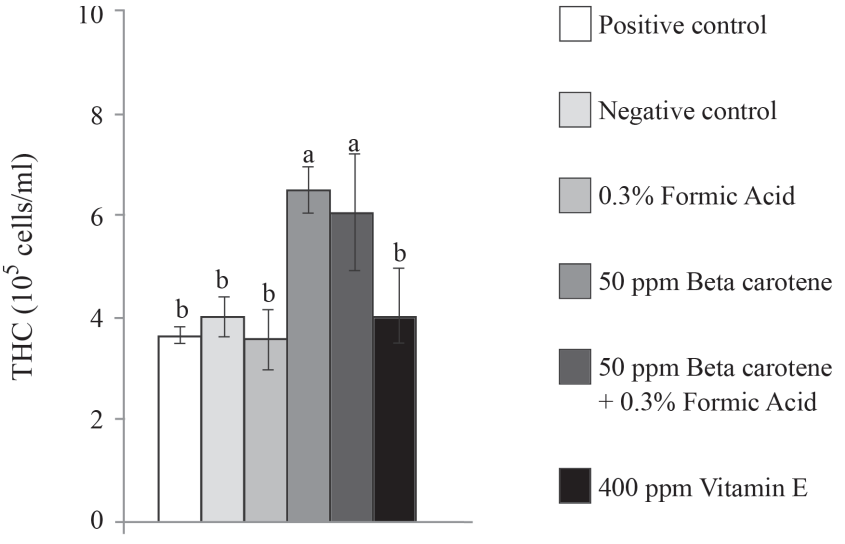

Figure 5. The total hemocyte count (10^5 cells/ml) of Pacific white shrimp (n=10) after being challenged with *Vibrio parahaemolyticus* at 10^4 CFU/ml fed with six experimental diets for 30 days.

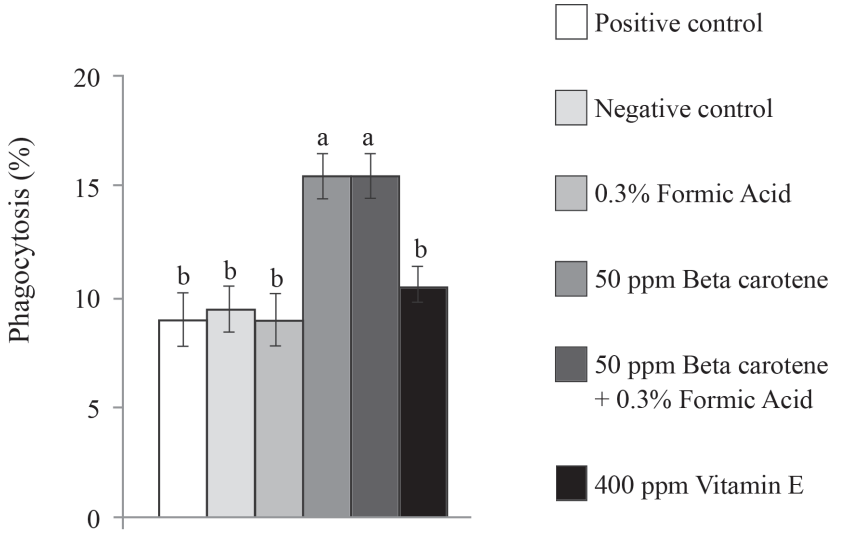

Figure 6. The phagocytosis activity (%) of Pacific white shrimp (n=10) after being challenged with *Vibrio parahaemolyticus* at 10^4 CFU/ml fed with six experimental diets for 30 days.

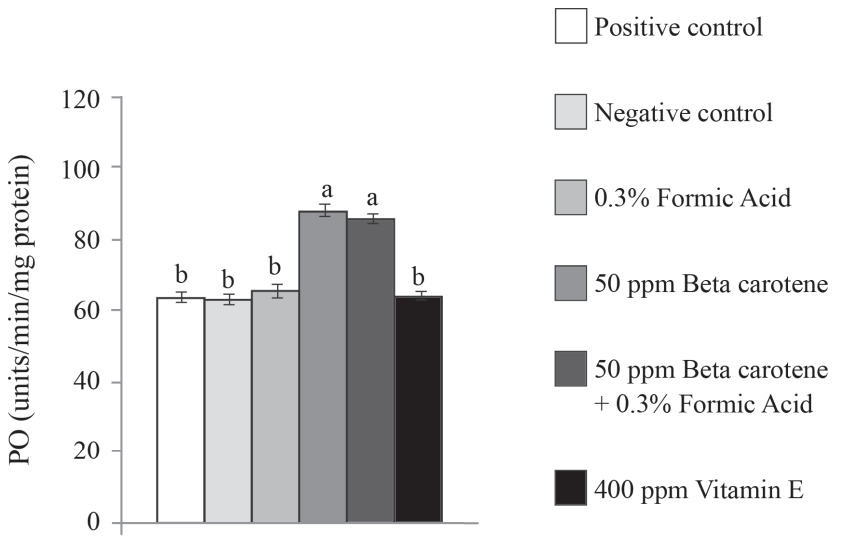

Figure 7. Phenoloxidase activity (units/min/mg protein) of Pacific white shrimp (n = 10) after being challenged with *Vibrio parahaemolyticus* at 10^4 CFU/ml and fed with six experimental diets for 30 days.

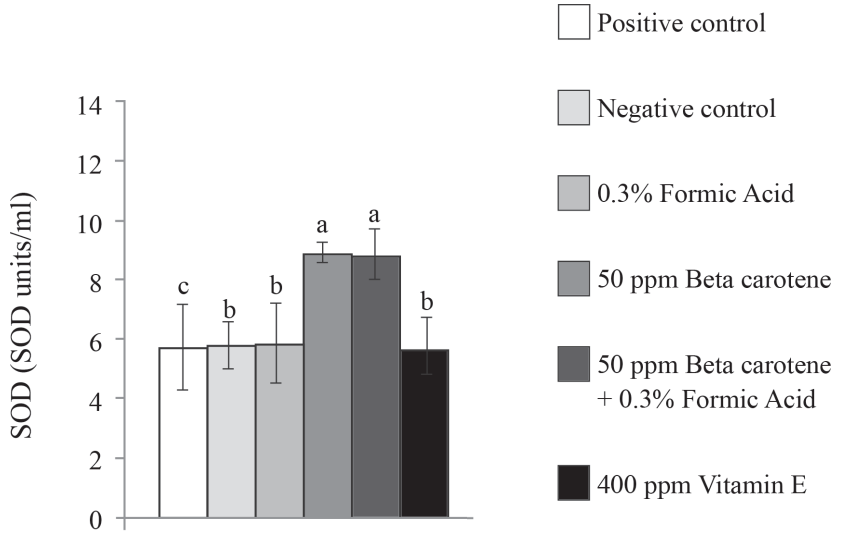

Figure 8. Superoxide dismutase activity (SOD units/ml) of Pacific white shrimp (n=10) after being challenged with *Vibrio parahaemolyticus* at 10^4 CFU/ml and fed with six experimental diets for 30 days.

DISCUSSION

Organic acids are widely used as animal feed additives and preservatives. As a group these compounds primarily include the saturated straight-chain monocarboxylic acids and their derivatives (Ricke, 2003). Organic acids possess antimicrobial activity against several pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp., and *Vibrio* spp. (Ricke, 2003; Papatsiros and Billinis, 2012; Da Silva *et al*., 2013). Undissociated forms of organic acids can easily penetrate bacterial cell membranes, and dissociate into anions and H^+ within the cytoplasm. Once inside the bacterial cells, they reduce intracellular pH and disrupt the cytoplasmic membrane, protein synthesis system, genetic materials, and metabolic enzymes. In addition, because the bacterial cell uses ATP to pump the excess H^+ out of cells, organic acids also deplete ATP levels and affect the cell's ability to maintain pH homeostasis (Ricke, 2003; Beales, 2004; Lückstädt and Mellor, 2011). However, not all organic acids have effects on bacteria. In fact, organic acids associated with specific antimicrobial activity are short-chain acids (C1-C7) such as formic, acetic, propionic, butyric acid, lactic, malic, tartaric, and citric acids (Dibner and Buttin, 2002; Papatsiros and Billinis, 2012).

Although organic acids are mainly used as feed additives for improving growth performance of pigs and poultry (Dibner and Buttin, 2002; Franco *et al*., 2005; Lückstädt and Mellor, 2011; Papatsiros and Billinis, 2012), there are also reports on the benefit of organic acids in aquatic animals, including hybrid red tilapia (Ng *et al*., 2009) and Pacific white shrimp (Walla *et al*., 2012; Da Silva *et al*., 2013; Su *et al*., 2014; Rorkwiree *et al*., 2014). Despite no clear improvements in growth and survival of uninfected shrimps in our study, the use of formic acid significantly increased the survival rate of *V. parahaemolyticus*-infected shrimp compared with the negative control group, possibly by antibacterial activity as described above. The antimicrobial effect of formic acid against *Vibrio* spp. was reported *in vitro* as well (Mine and Boopathy, 2011; Adams and Boopathy, 2013; Da Silva *et al*., 2013).

β-carotene are pigments that belong to carotenes class of carotenoids. Their function in crustaceans is to provide pigmentation, as a source of provitamin A, and as antioxidant (Liñán-Cabello *et al*., 2002). Antioxidant activity of carotenoids may be involved in the immunomodulatory effect, by quenching singlet oxygen and free radicals, carotenoids can protect white blood cells from oxidative damage (Bendich 1989). Furthermore, the effects of carotenoids on enhancing cell-mediated and humoral immune responses of vertebrates are also documented (Bendich, 1989; Chew and Park, 2004).

Several studies have reported that dietary carotenoids could increase immune parameters, enhance survival rate, or improve resistance to pathogens of many aquatic animals such as common carp (Sowmya and Sachindra, 2013; Anbazahan *et al*., 2014), rainbow trout (Amar *et al*., 2001), Pacific white shrimp (Flores *et al*., 2007; Niu *et al*., 2009; Rorkwiree *et al*., 2014), black tiger shrimp (Supamattaya *et al*., 2005), and kuruma prawn (Chien and Shiau, 2005). Rather consistent with these previous studies, our result also showed the significantly

increased immunity in shrimp fed with 50 ppm β-carotene in all immunological parameters investigated, yet it could not enhance the survival rate of *V. parahaemolyticus*-infected shrimp. This result clearly indicated that the increased immune responses of β-carotene-fed shrimp may not be sufficient to cope with *V. parahaemolyticus* infection. Furthermore, the present study revealed that a combination of 0.3% formic acid and 50 ppm β-carotene was no better than using formic acid alone. Therefore, formic acid seems to be more suitable for the prevention of *Vibrio* infection in Pacific white shrimp.

Among fat-soluble vitamins, vitamins A, D, and E (but not vitamin K) have been proven to be essential for penaeid shrimps. Shrimp fed on a vitamin E-deficient diet had lower survival rates, poor growth, inappetence, and exhibited histopathological change in the hepatopancreas (He *et al*., 1992; Reddy *et al*., 1999).

Like carotenoids, vitamin E also possesses a potent antioxidant activity which may be responsible for its immunostimulatory effect (Sakai, 1999; Maggini, 2010; Verlhac-Trichet, 2010). It plays an important role in preventing oxidation of polyunsaturated fatty acids in membrane phospholipids and plasma lipoproteins (Traber, 2009; Dasgupta and Klein, 2014). However, the immunomodulation property of vitamin E was not detected in our study nor improved shrimp survival rate. In fact, there were some reports about the positive effect of vitamin E on health and immunity of shrimp (Gimenez *et al*., 2004; Lee and Shiau, 2004; Liu *et al*., 2007). The absence of immunostimulation in the present study might be due to inappropriate dosage regimen. For example, the dose of vitamin E used in our study (400 ppm) was higher than 85-89 ppm recommended by Lee and Shiau (2004). Nevertheless, it did increase the average body weight of *V. parahaemolyticus*-infected shrimp to some degree. Given the fact that vitamin E enhanced neither survival rate nor immunity of shrimp in the laboratory condition, the use of vitamin E as feed additive in shrimp farming is apparently less beneficial than using formic acid and β-carotene.

CONCLUSION

The present study revealed the positive effects of formic acid, β-carotene and vitamin E on health of *Vibrio parahaemolyticus*-infected shrimp but in different aspects, i.e. 0.3% formic acid increased survival rate, 50 ppm β-carotene stimulated shrimp's immunity, 50 ppm β-carotene and 400 ppm vitamin E enhanced growth slightly. However, in the absence of *V. parahaemolyticus* infection, the survival rate among all treatment groups were similar, while the highest body weight was seen in 50 ppm β-carotene fed shrimp. Feed supplemented with formic acid was far more superior than those with β-carotene and vitamin E in the prevention of *V. parahaemolyticus* infection; therefore, it can be useful in shrimp aquaculture.

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