The Effects of Probiotic, β-1,3-glucan and Organic Acid on Pacific white shrimp's (*Litopenaeus vannamei*) Immune System and Survival upon Challenge with *Vibrio harveyi*

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ABSTRACT

A study of the effects of β-1,3-glucan, probiotic and organic acid on the growth, non-specific immune characteristics and survival of Pacific white shrimp (*Litopenaeus vannamei*) was conducted under laboratory conditions. Pacific white shrimp (average 6-7 g) was subjected to four treatments (six replicates/treatment). Each replicate consisted of 30 shrimp in 500-liter tanks. Shrimp were fed four times daily at 3% of body weight for 60 days with pelleted feed mixed with β-1,3-glucan probiotic at the rate of 2g:1kg, and organic acid at 1.2g:1kg of feed. Commercial pelleted feed without any supplement was used as the control diet. After 60 days of dietary administration, shrimp fed with β-1,3-glucan had the highest average body weight (20.93±4.40 g) which was not significantly different ($p>0.05$) from the other groups. Survival rates of shrimp in the 3 treatments. The glucan group had significantly higher ($p<0.05$) THC, percentage phagocytosis, bacteriacidal activity, phenoloxidase activity, superoxide dismutase activity than the other groups (probiotic, organic acid and control). However, shrimp fed with β-1,3-glucan and probiotic showed significantly higher survival rate after challenge with *Vibrio harveyi* than the organic acid and control groups. These results suggest that β-1,3-glucan and probiotic could be recommended to be used as a diet supplement to strengthen immunity of shrimp and control Vibriosis in shrimp culture.

Keywords: Probiotic, β-1,3-glucan, Organic Acid, Nonspecific Immune Characteristics, *Vibrio harveyi*, Pacific white shrimp

INTRODUCTION

At present, many countries are producing Pacific white shrimp of white leg shrimp at an industrial scale. However, the shrimp aquaculture industry is beset with disease, mostly due to bacteria (especially luminous *Vibrio harveyi*) and viruses. The high density of shrimp in hatchery tanks and ponds is conducive to the spread of pathogens. The aquatic environment, with regular applications of protein-rich feed, provide a suitable culture media for bacteria. In 2011, Thailand faced the White Feces

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Syndrome causing losses to shrimp farmers all over the country (Limsuwan, 2011). Most of the shrimp that had white feces showed high quantities of *Vibrio* bacteria in their hemolymph (Somboon *et al*., 2012) leading to some farms using antibiotics to control the disease. On the other hand, the use of or it resulted in an increase in virulence of pathogenic bacteria, further posing concerns in promoting the transfer of antibiotic resistance to human pathogens.

Nowadays there are many alternative methods to control pathogenic bacteria including the use of probiotics or organic acid or β-1,3-glucan (immunostimulant). Probiotics contain live microorganisms, confer a health benefit for the host (Fuller, 1989, Moriarty, 1996, 1998). The role of beneficial bacteria or probiotics is to replace the pathogens by competitive processes which are being used in the animal industry as a better remedy than administering antibiotics. Probiotic is now gaining acceptance for the control of pathogens in aquaculture, such as the *Bacillus* spp. which could possibly stimulate the immune response of aquatic animals to enteric pathogens, as reported in shrimp (Gullian *et al*., 2004). Several studies have proved that inclusion of organic acids in diets suppressed the pathogenic bacterial growth in gastrointestinal tracts of poultry and swine (Dibner and Buttin, 2002; Denil *et al*., 2003; Ali *et al*., 2011). In the intestinal tract of aquatic animals, organic acids inhibit the growth of gram-negative bacteria by penetrating through the bacterial cell wall (Defoirdt *et al*., 2009). Furthermore Anuta *et al*. (2011) reported that the addition of 0.4–2% commercial organic acid, based on calcium sulfate, did not change the performance parameters of *L. vannamei*; however, it resulted in an elevated immune response and a change in intestinal microbiota.

The application of immunostimulants in shrimp aquaculture is increasingly gaining interest as an environmentally safe alternative to antibiotics and chemotherapeutics (Song *et al*., 1997). The most active immunostimulant is β-1,3- glucan (Leung *et al*., 2006), a natural polymer isolated from the cell wall of yeast and mold. β-glucans has been used to enhance the non-specific defense mechanisms in crustaceans (Song *et al*., 1997; Chang *et al*., 1999, 2002, 2003; Sajeevan, 2009; Shivananda Murthy *et al*., 2009). Shrimp possess an innate immune system, consisting of cellular and humoral elements. Hemocytes play a central role in the non-specific immune response of shrimp, which rely mainly on phagocytosis, melanization, encapsulation, cytotoxicity, and clotting (Sritunyalucksana *et al*., 1999). Humoral defense factors, such as clotting proteins, agglutinins, hydrolytic enzymes, and antimicrobial peptides are released upon lysis of hemocytes, which is induced by lipopolysaccharides (LPS), peptidoglycans, and β-1,3-glucans (Chisholm and Smith, 1995; Destoumieux *et al*., 2000; Johansson and Söderhäll, 1989; Muta and Iwanaga 1996; Söderhäll *et al*., 1994).

This study was aimed to compare the efficacy of probiotic, β-1,3-glucan and organic acid on growth, survival, non-specific immune characteristics and survival rate upon the challenge with *Vibrio harveyi* of Pacific white shrimp (*Litopenaeus vannamei*) under laboratory conditions.

MATERIALS AND METHODS

Experimental diets

Shrimp were fed four times daily at 3% body weight for 60 days with 2 g of probiotic mixed with 1 kg of pelleted feed, 2 g of β-1,3-glucan mixed with 1 kg of pelleted feed, 1.2 g of organic acid mixed with 1 kg of pelleted feed and normal commercial pelleted feed without any supplement for the control group.

Probiotic used in this study consisted of *Bacillus velezensis*, *B. amyloliquifaciens*, *B. subtilis*, *B. megaterium* and *Brevibacillus* parabrevis as described Jueliang (2011). The component of organic acid used in this study included formic acid, benzoic acid and hydroxyl methylthio 2-butanoic acid similar to Walla (2012). β-1,3-glucan was a polysaccharides extract from yeast cell wall (Tipsemonkol, 2009).

Experimental shrimp

A total of 1,000 farm-reared shrimp were transported from Chanthaburi Province to the Aquaculture Business Research Center Laboratory at Kasetsart University, Bangkok, and acclimated for one week. During the acclimation period, shrimp were fed four times daily with commercial pelleted feed. After acclimation, 720 shrimp were randomly stocked in 24 500-L fiberglass tanks, i.e. for the four treatments with six replicates per treatment. Shrimp were stocked at a density of 30 animals per tank to achieve a density of 63 shrimp/m. They were fed four times daily according to the standard feeding rate (Limsuwan and Chanratchakool, 2004). Salinity, pH and temperature during the experiment were maintained at 25 ppt, 7.8-8.0 and 29±1ºC, respectively. Water quality parameters such as dissolved oxygen, ammonia and nitrite were measured weekly throughout the experiment using standard protocol (APHA *el al*., 1995), 30% water exchange every 10 days. At the end of the 60-day feeding trial, 240 shrimp from all treatment groups were counted and weighed. Another 120 shrimp from each treatment group were randomly sampled to evaluate immune parameters, including total hemocyte count (THC), phagocytosis activity, phenoloxidase (PO) activity, superoxide dismutase (SOD) activity, and bacteriacidal activity.

Immune parameters analysis

Hemolymph (0.5 ml) was withdrawn from the base of the 3rd walking leg of each shrimp with a 3 ml sterile syringe containing 1.5 ml of precooled (4ºC) anticoagulant solution (M-199 + 5% L-cysteine).

Total hemocytes

After collecting the hemolymph, hemocytes were counted using a hemocytometer and calculated as the number of hemocyte cells (total hemocytes/mm^3).

Phagocytosis activity

Phagocytosis activity was determined according to Itami *et al*. (1994). Hemolymph (200µl) was collected from the base of the 3rd walking leg and with 800 µl of sterile anticoagulant. The collected shrimp hemocytes were rinsed with shrimp saline (solution composed of NaCl 28.4g, MgCl 1g, $MgSO_4$ 2g, CaCl 2.25g, KCl 0.7g, glucose 1g, hepes 2.38g and dissolved in 1000 ml of distilled water) and the viable cell number adjusted to 1×10^6 cells.ml^{-1}. The cell suspension (200 µl) was inoculated onto the cover slip. After 20 min, the cell suspension was removed and rinsed with shrimp saline three times.

Heat-killed yeast preparation (2 ml) was added and incubated for 2 hours. Next, the heat- killed yeast preparation was removed and the cell suspension rinsed with shrimp saline five times to reach the concentration of 5×10^8 cells.ml^{-1} and fixed with 100% methanol. The cover slip was then stained with Dip Quick and mounted with Permount slide mounting fluid. Two hundred hemocytes were counted for each sample. Phagocytosis activity, defined as percentage phagocytosis, was expressed as:

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

Phenoloxidase activity assay

Phenoloxidase activity was measured spectrophotometrically by recording the formation following a modification of a published protocol (Supamattaya *et al*., 2000). The hemolymph-anticoagulant mixture was washed three times with shrimp saline and centrifuged at 1000 rpm at 4ºC for 10 min. Hemocytelysate was prepared from hemocytesin a cacodylate buffer (pH 7.4) by using a sonicator at 30 amplitude for 5 seconds. The suspension was centrifuged at 10,000 rpm at 4ºC for 20 min, after which the supernatant collected. The 200 µl of 0.25% trypsin in cacodylate buffer was mixed into the 200 µl of the hemocytelysate followed by 200 µl of L-dihydroxyphenylalanine (L-DOPA) at 4 mg.ml^{-1}as substrate. Enzyme activity was measured by the absorbance of dopachrome at 490 nm wave length. The protein content in hemocytelysate was measured following a published protocol (Lowry *et al*., 1951). The phenoloxidase activity was calculated as the increase in optimum density (OD) per minute per mg of protein.

Superoxide dismutase activity assay

Superoxide dismutase activity was measured by its ability to inhibit superoxide radical-dependent reactions using a Ransod Kit (Randox, Crumlin, UK). This method is based on the formation of red formazan during reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl- tetrazolium chloride (INT) and superoxide radical, which is assayed in a spectrophotometer at 505 nm. In the presence of xanthine oxidase, superoxide and uric acid are produced from the xanthine. The superoxide radicals then reacted with INT to produce a red formazan dye. The hemolymph-anticoagulant mixture was centrifuged at 3000 rpm and 4ºC for 10 min. Plasma was removed, and the pellet was resuspended with 3ml of 0.9% NaCl and centrifuged again. The supernatant was discarded, and the pellet was resuspended with 2 ml of triple distilled water at 4 ºC. A 50 µl aliquot of resuspended hemocytes was placed in each semi-micro cuvette (10×4 mm) that contained 200 µl of reaction mixture. Fifty microliters of xanthine oxidase solution was added to each semi-micro cuvette, then the absorbance was measured at 505nm and 37 ºC. The rate of reaction was estimated from the absorbance readings of 0.5 and 3 min after adding xanthine oxidase. A reference standard of SOD was supplied with the Ransod Kit. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. The specific activity was expressed as SOD units/ml.

Bacteriacidal activity

Bacteriacidal activity was measured as described by Supamattaya *et al*. (2000). Serum was separated from the hemocytes of each shrimp sample before diluting in 2.6 % NaCl at the following ratios: 1:2, 1:4, 1:8, 1:16 and 1:32. Then 0.5 ml of each serum dilution was used for the assay. For the negative control, 0.1 ml of NaCl was used in the assay. One tenth of a milliliter of *Vibrio harveyi* suspension (10^6 CFU.ml^{-1}) was added to each serum dilution and the control. The treatments were incubated at room temperature for 3h before enumerating the bacteria. The results were recorded from a dilution that could decrease 50% of *V. harveyi* compared to the control.

Effect of probiotic, β-1,3-glucan and organic acid on survival of Pacific white shrimp upon challenge with V. harveyi

On day-60 of the growth trial, 30 shrimp from each treatment were sampled and stocked into 3 100-L tanks (10 shrimp/tank). Shrimp were challenged with a virulent strain of *V. harveyi* which was cultured in tryptic soy agar supplemented with 1.5% NaCl (w/v) for 24h at 35ºC. After 24h of growth, bacterial colonies were transferred to10 ml tryptic soy broth supplemented with 1.5 % NaCl and incubated for 24h at 35ºC. Then, the bacterial culture was centrifuged at 1000 rpm for 15 min at room temperature. The supernatant was removed, and the bacterial pellet was resuspended in saline solution at a concentration of 10^6 CFU.ml^{-1}. All shrimp were injected with *V. harveyi* suspension at 10^6 CFU.ml^{-1} for two consecutive days. Shrimp injected with 2% saline served as the control. Mortalities were recorded up to 96 h post injection.

RESULTS AND DISCUSSION

Effects of probiotic, β-1,3-glucan and organic acid on growth and survival of Pacific white shrimp under laboratory conditions

After 60 days of dietary administration, the weight of shrimp fed with β-1,3-glucan was not significantly different ($p>0.05$) from the other groups. Survival rates of shrimp in the 3 treatments ranged from 80.67-86.00%, which were significantly higher ($p<0.05$) than that of the control group (76.00%) (Table 1). Results showed that shrimp fed with probiotic had the highest survival rate. As reported earlier, probiotic and organic acid cloud supported the survival of shrimp (Yankomut, 2010; Jueliang, 2011; Walla *et al*., 2012), and β-1,3-glucan supported the elevated immune response of shrimp but there was no significant effect on shrimp growth (Scholz *et al*., 1999; Supamattaya *et al*., 2000b; Burgents *et al*., 2004; Tipsemomkol *et al*., 2009).

Table 1. Average body weight and survival rates of Pacific white shrimp after 60 days of feeding with probiotic, β-1,3-glucan and organic acid

Treatments	Average body weight (g)	Survival rate (%)
Probiotic	19.27 ± 3.07^{a}	86.00 ± 2.79^{a}
β-1,3-glucan	20.93 ± 4.40^{a}	80.67 ± 2.79^{a}
Organic acid	19.55 ± 3.63^{a}	81.33 ± 2.98^{a}
Control	19.56 ± 3.63^{a}	76.00 ± 2.79^{b}

Mean values within the same column sharing the same superscript are not significantly different at p<0.05

Effects of probiotic, β-1,3-glucan and organic acid on non-specific immune characteristics of Pacific white shrimp under laboratory conditions

After 60 days, shrimp fed with probiotic, β-1,3-glucan and organic acid were analyzed for their immune responses by THC, percentage phagocytosis, PO activity, SOD activity and bacteriacidal activity. The immunological profile of the shrimp in all treatment groups showed an increasing trend after feeding with probiotic, β-1,3-glucan and organic acid . The highest THC, phagocytosis activity and PO values were observed on shrimp of β-1,3-glucan group. The shrimps fed with β-1,3-glucan showed significantly higher immune response compared to the control and other treatment groups ($p<0.05$) (Figures 1, 2 and 3). β-1-3-glucan stimulated immune response of shrimp (Supamattaya, 2000a; Siripaisan, 2006; Purivirojkul, 2006; Sung *et al*., 1996; Burgents *et al*., 2004). Tipsemonkol (2009) reported shrimp fed with 0.25% of β-1-3-glucan had highest THC when compared with other group. β-1-3-glucan is a long polysaccharide of glucose (Dijkgraaf *et al*., 2002) to stimulate immune response in plasma, pathogen associated molecular pattern (PAMPs) and also stimulate β-glucan binding protein, (Vargas-Albores and Yepiz-Plascencia, 2000) which remain in digestive tract and humoral function of shrimp to produce β-1,3-glucan-binding protein complex (Sritunyalucksana *et al*., 2002). These protein activate the specific membrane receptor of hemocyte to destroy the pathogenic agents (Vargas-Albores *et al*., 1998). Increase of hemocyte was enhanced by elevated immune response especially phagocytosis activity and humoral immune response to produce enzyme for example phenoloxidase, superoxide dismutase and bacteriacidal activity (Rukpratanporn, 1999; Srichana, 2004; Jantarapan, 2008; Vargas-Albores *et al*., 1998).

At the beginning of the experiment, shrimp showed no significant difference in SOD in all the treatments. After 60 days of culture with the supplemented diets, SOD of shrimp in the β-1,3-glucan group was highest at 46.23±7.81 SOD units.ml^{-1} and significantly different ($p<0.05$) from probiotic, organic acid and control group which had 37.57±8.79, 34.55±13.45 and 37.90±8.98 SOD units.ml^{-1}, respectively (Figure.4).

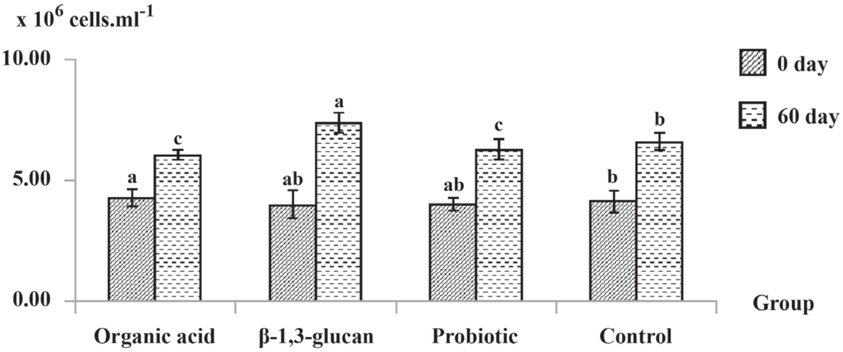

Figure 1. Total hemocyte count (THC) of Pacific white shrimp before and after 60 days of feeding with probiotic, β-1,3-glucan and organic

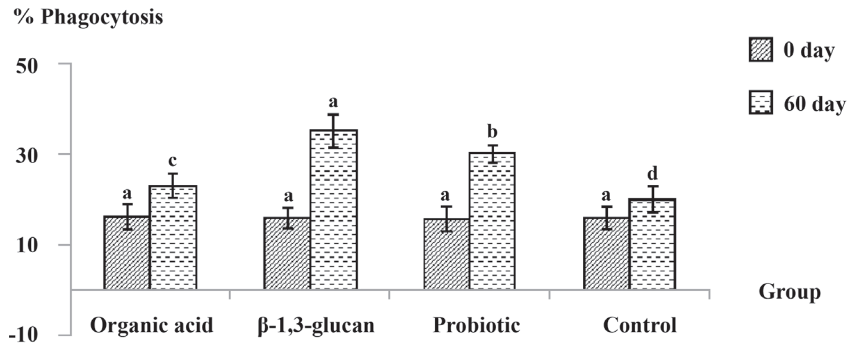

Figure 2. Percentage of phagocytosis of Pacific white shrimp before and after 60 days of feeding with probiotic, β-1,3-glucan and organic acid

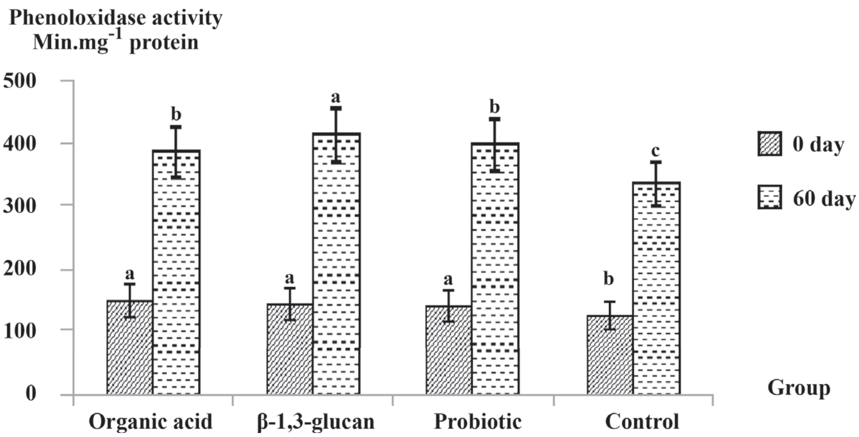

Figure 3. Phenoloxidase activity of Pacific white shrimp before and after 60 days of feeding with probiotic, β-1,3-glucan and organic

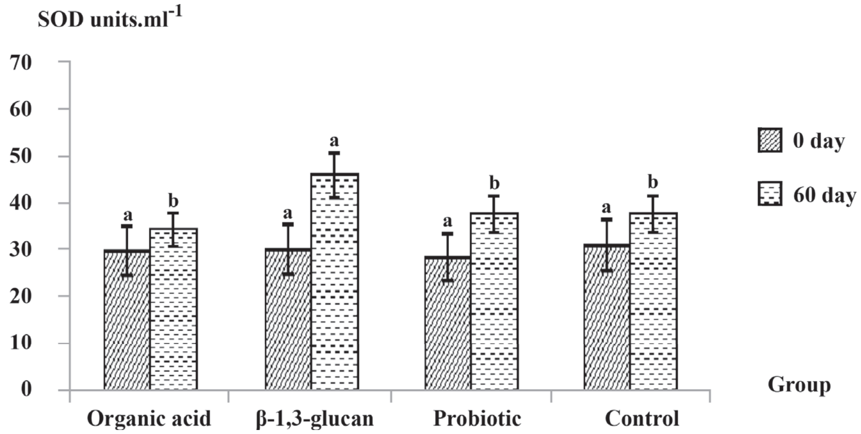

Figure 4. Superoxide dismutase activity (SOD) of Pacific white shrimp before and after 60 days of feeding with probiotic, β-1,3-glucan and organic acid

Shrimp fed with β-1,3-glucan had the highest level of bacteriacidal activity at the serum dilution of 1:16, followed by probiotic which had bacteriacidal activity at serum dilution of 1:8, then organic acid and control group had bacteriacidal activity at serum dilution of 1:4 (Table 2).

Table 2. Bacteriacidal activity of Pacific white shrimp before and after 60 days of feeding with probiotic, β-1,3-glucan and organic acid

Treatments/culture period	0 day	60 days
Probiotic	1:4	1:8
β-1,3-glucan	1:4	1:16
Organic acid	1:4	1:4
Control	1:4	1:4

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) (Muñoz *et al*., 2000; Rodríguez and Le Moullac, 2000). Destroying the phagocytized materials involves the intracellular production of free radicals. 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). The present study showed that the phagocytosis activity, PO, SOD and bacteriacidal activity of shrimp in β-1,3-glucan group increased when compared with the control. The immunological profile of shrimp in probiotic group increased as well, but SOD level of probiotic group was not different from the organic acid and control groups.

Effect probiotic, β-1,3-glucan and organic acid on survival of Pacific white shrimp upon challenge with *V. harveyi*

After challenge with *V. harveyi* at 10^6 CFU.ml^{-1}, shrimp fed with β-1,3-glucan and probiotic had significantly higher ($p<0.05$) survival rates than those fed with organic acid and control diet (Table 3). This is similar to the report by Chang *et al*. (2002, 2003) and Cheng, *et al*. (2005) which demonstrated that dietary supplementation of β-1,3-glucan cloud improved immune response and disease resistance of black tiger shrimp and Pacific white shrimp under laboratory conditions. Similar results were reported by feeding peptidoglycan, a type of β-glucan to Kuruma shrimp (Itami *et al*., 1998) as well as Pacific white shrimp (Wang *et al*., 2004). Probiotic cloud increased survival of shrimp after challenge with *V. harveyi* (Vieira *et al*., 2010). In addition, a similar result with *Penaeus monodon* was observed, wherein survival to *V. harveyi* infection was higher when *Bacillus* S11 was added to culture water (Rengpipat *et al*., 1998). High survival of shrimp fed with probiotic might be related to an immune reactive effect of probiotics on the host. Lactic acid bacteria are known to produce extracellular compounds that can stimulate the immune response in vertebrates (Marteau *et al*., 2002; Gill, 2003).

Table 3. Percentage survival of Pacific white shrimp after challenged with *V. harveyi* 10^6 CFU.ml^{-1} at 96 hours

Treatments	Survival rate (%)
Probiotic	76.67 ± 0.58^a
β-1,3-glucan	80.00 ± 0.00^a
Organic acid	63.33 ± 0.58^b
Control	53.33 ± 0.58^c

Mean values within the same column with the same superscripts are not significantly different at $p<0.05$

CONCLUSION

The study of effects of probiotic, β-1, 3-glucan and organic acid on immune system and survival upon challenge with *V. harveyi* on Pacific white shrimp showed highest THC, phagocytosis activity, phenoloxidase, SOD and bacteriacidal activity in shrimp fed with β-1,3-glucan fed group. In addition, the immunological profile of shrimp in probiotic and organic acid treatment groups showed an increasing trend, however this was less than those fed with β-1,3-glucan. Shrimp fed with β-1,3-glucan and probiotic showed a significantly higher survival rate after challenge with *V. harveyi* at 96 hours.

The results indicated that probiotics support growth and survival of shrimp. The study indicated that there is potential in the use of probiotic for supporting the growth of shrimp while the diet supplement with β-1,3-glucan could be used to stimulate immune response and increase survival rate of shrimp after exposure to *V. harveyi*. Supplementing the diet with organic acid could improve the survival rate of shrimp against Vibriosis.

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LITERATURE CITED

Ali S. A., H. N. Reza, R. Enayat and S. Jalal. 2012. Herbal additives and organic acids as antibiotic alternatives in broiler chickens diet for organic production. **Afr. J. Biotechnol.** Vol.11 (8): 2139-2145.

Anuta, D.J., A. Buentello, S. Patnaik, A.L. Lawrence, A. Mustafa, M. Hume, D.M. Gatlin III, and M.C. Kemp. 2011. Effect of dietary supplementation of acidic calcium sulfate (Vitoxal) on growth, survival, immune response and gut microbiota of the Pacific White Shrimp, *Litopenaeus vannamei*. **J. World Aquacult Soc.** 42, 834–844.

APHA, AWWA and AWCA. 1995. **Standard Methods for the Examination Water and Wastewater.** 20^{th} ed. United Book Press, Maryland.

Burgent, J.E., K.G. Burnett and L.E. Burett. 2004. Disease resistance of Pacific white shrimp, *Litopenaeus vannamei*, following the dietary administration of a yeast culture food supplement. **Aquaculture** 231: 1-8.

Chang, C.F., M.S. Su, H.Y. Chen, C.F. Lo, G.H. Kou, and I.C. Liao. 1999. Effect of dietary beta-1,3-glucan on resistance to white spot syndrome virus (WSSV) in post larval and juvenile *Penaeus monodon*. **Dis. Aquat. Org.** 36: 163–168.

Chang, C.F., H.Y. Chen, M.S. Su, and I.C. Liao. 2002. Immunomodulation by dietary β-1,3-glucan in the brooders of the black tiger shrimp, *Penaeus monodon*. **Fish Shellfish Immunol.** 10:505–514.

Chang, C.F., M.S. Su, H.Y. Chen, and I.C. Liao. 2003. Dietary β-1,3-glucan effectively improve immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. **Fish Shellfish Immunol.** 15: 297–310.

Chen, W., C. Liu, C. Kuo and J. Chen. 2005. Dietary administration of sodium alginate enhances the immune ability of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio* alginolyticus. **Fish Shellfish Immunol.** 18:1-12.

Chisholm, J.R.S. and V.J. Smith. 1995. Comparison of antibacterial activity in the hemocytesof different crustacean species. **Comp. Biochem. Physiol.** 110A, 39–45.

Denil, M., F. Okan and K. Celik. 2003. Effect of dietary probiotic, organic acid and antibiotic supplementation to diets on broiler performance and carcass yield. **Pak. J. Nutr.** 2: 89-91.

Defoirdt, T., N. Boon, P. Sorgeloos, W. Verstraete, and P. Bossier. 2009. Short-chain fatty acids and poly-β-hydroxyalkanoates: (new) biocontrol agents for a sustainable animal production. **Biotechnology Advances** 27, 680–685.

Destoumieux, D., M. Munñoz, C. Cosseau, J. Rodríguez, P. Bulet, M. Comps and E. Bachère. 2000. Penaedins, antimicrobial peptides with chitin binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. **J. Cell Sci.** 113, 461–469.

Dibner J.J. and P. Buttin. 2002. Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. **J. Appl. Poult. Res.** 11: 453-463.

Dijkgraaf, G.J.P., H. Li and H. Bussey. 2002. Cell wall β–glucans of *Saccharomyces cerevisiae*, pp. 179-213. *In* S. De Baets, E.J. Vandamme and A. SteinbÜchel, eds. **Biopolymers Polysaccharides II. Polysaccharides from Eukaryotes.** Wiley-VCH, Weinheim.

Fuller, R. 1989. Probiotics in man and animals. **J. Appl. Bact.** 66, 365-378.

Gill, H.S. 2003. Probiotic to enhance anti-infective defences in the gastrointestinal tract. **Best. Pract. Res. Clin. Gastroenterol.,** 17:755-773

Gullian, M., F. Thompson and J. Rodriguez. 2004. Selection of probiotic bacteria and study on their immunostimulatory effect in *Penaeus vannamei*. **Aquaculture** 233: 1-14.

Itami T., Y. Takahashi, E. Tsuchihira, H. Igasu and M. Kondo. 1994. Enhancement of disease resistance of Kuruma prawn *Penaeus japonicas* and increase in phagocytic activity of prawn hemocytes after oral administration of b 1,3-glucan (Schizophyllan). *In*: **Proceeding of the third Asian fisheries forum, Singapore, 26-30 October 1992.** The Asian Fisheries Society Manila; Philippines.

Itami T., M. Asano, K. Tokushige, K. Kubono, A. Nagkawan and N. Takeno. 1998. Enhancement of disease resistance of Kuruma shrimp *Penaeus japonicas*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. **Aquaculture**, 164: 227-288.

Jantarapan P., 2008. Growth, Survival and Immune Characteristics of Pacific white shrimp (*Litopenaeus vannamei*) Fed with Aquanin plus (Beta-Cyclodextrin Cysteamine Hydrochloride). **Thesis Master Degree, (in Thai).** Kasetsart University, Thailand. 95 pp.

Johansson, M.W. and K. Söderhäll. 1989. A cell adhesion factor from crayfish haemocyte has degranulating activity towards crayfish granular cells. **Insect Biochem.** 19,183–190.

Jueliang, P. 2011. Application of Spore-forming bacteria for Controlling Pathogenic bacteria *Vibrio harveyi* in *Litopenaeus vannamei* culture. **Thesis Master Degree (in Thai).** Kasetsart University, Thailand. 89 pp.

Limsuwan, C. 2011. White feces syndrome and early mortality syndrome in Pacific white shrimp. **The Practical Asian Aquaculture** Vol.2 (7): 4-8.

Limsuwan, C. and P. Chanratchakool. 2004. **Shrimp farming industry in Thailand.** National Research Council of Thailand, Bangkok, Thailand 206 pp.

Lowry, O.H., N.J. Rosebrough, A.L. Farrand and R.J. Randall. 1915. Protein measurement with folin phenol reagent. **J. Biol. Chem.** 193:265-275.

Marteau, P., P. Seksin and R. Jian. 2002. Probiotics and intestinal health effects: a clinical perspective. **Br. J. Nutr.,** 88:S51-S57.

Moriarty, D. J. W. 1996. Microbial Biotechnology: a key ingredient for sustainable aquaculture. **INFOFISH International** 4: 29-33.

Moriarty, D. J. W. 1998. Control of luminous *Vibrio* species in penaid aquaculture ponds. **Aquaculture** 164: 351-358.

Muta, T. and S. Iwanaga. 1996. The role of hemolymph coagulation in innate immunity. Curr. Opin. **Immunol.** 8, 41–47.

Muñoz, M., R. Cedeño, J. Rodríguez, W. P. W. van der Knaap, E. Mialhe and E. Bachère. 2000. Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*. **Aquaculture,** 191:89-107.

Purivirojkul, W. 2006. Immunoenhancement of black tiger shrimp *Penaeus monodon* Fabricius. **Thesis Doctor of Pyhilosophy (Zoology) (in Thai).** Kasetsart University, Thailand. 134 pp.

Rengpipat, S., W. Phianphak and S. Piyatiratitivorakul. 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. **Aquaculture,** 167:301 -313.

Roch, P. 1999. Defense mechanisms and disease prevention in farmed marine invertebrates. **Aquaculture,** 172:125 -145.

Rodríguez, J. and G. Le Moullac. 2000. State of the art of immunological tools and healthcontrol of penaeid shrimp. **Aquaculture,** 191:109-119.

Rukpratanporn, S. 1999. Immunoenhancement in black tiger shrimp, *Penaeus monodon* by Bacillus Strain S11. **Thesis Master. Science (Industrial Microbiology) (In Thai).** Chulalongkorn University. Bangkok. (Thailand).

Sajeevan, T.P., R. Philip, and I.S. B. Singh, 2009. Dose/frequency: A critical factor in the administration of glucan as immunostimulant to Indian white shrimp *Fenneropenaeus indicus*. **Aquaculture**, 287: 248–252.

Scholz, U., G. Garcia Diaz, D. Ricque, L.E. Cruz Suarez, F.Vargas Albores and J. Latchford. 1999. Enhancement of vibriosis resistance in juvenile *Litopenaeus vannamei* by supplementation of diets with different yeast products. **Aquaculture** 176: 271-283.

Shivananda Murthy H., P. Li, A. L. Lawrence and D. M. Gatlin III. 2009. Dietary β-glucan and nucleotide effects on growth, and immune responses of Pacific white shrimp, *Litopenaeus vannamei*. **J APPL. Aquaculture,** 21(3): 160-168.

Siripaisan, C. 2006. Application of Betaglucan as Immunostimulant in Pacific white shrimp (*Litopenaeus vannamei* Boone). **Thesis Master of Science (In Thai).** Kasetsart University, Thailand. 55 pp.

Somboon, M., W. Puriwirojkul, C. Limsuwan and N. Chuchird. 2012. Effect of *Vibrio* spp. in white feces infected shrimp in Chantaburi, Thailand. **KU. Fish. Res. Bull.** 36(1):7-15.

Song, Y.L., J.J. Liu, L.C. Chan and H.H. Sung. 1997. Glucan induced disease resistance in tiger shrimp (*Penaeus monodon*). **Dev. Biol. Stand.** 90, 413–421.

Söderhäll, K., L. Cerenius, and M. W. Johnson. 1994. The prophenoloxidase activity system and its role in invertebrate defense: foundations for the invertebrate immune system. **Ann. Ny. Acad. Sci.** 712, 155–166.

Srichana, T. 2004. Some Components of Humoral Immune System in Giant tiger prawn (*Penaeus monodon*, Fabricius) at Various Stages. **Thesis Master of Science (in Thai).** Kasetsart University, Thailand. 90pp.

Sritunyalucksana, K., L. Cerenius, and K. Söderhäll. 1999. Molecular cloning and characterisation of prophenoloxidase in the black tiger shrimp, *Penaeus monodon*. **Dev. Comp. Immunol.** 23, 179–186.

Sritunyalucksana, K., S.Y. Lee and K. Söderhäll. 2002. A ß-1,3-glucan binding protein from the black tiger shrimp, *Penaeus monodon*. **Dev. Comp. Immunol.** 26: 237-245.

Supamattaya, K., J. Pongmaneerat and T. Klowklieng. 2000a. The effect of β-glucan (MacroGard®) on growth performance, immune response and disease resistance in black tigers hrimp, *Penaeus monodon* Fabricius. **Songklanakarin J. Sci. Technol.** 22:677-688.

Supamattaya, K., W. Phroomhunthong, C. Tantikitti and R.W. Hoffmann. 2000b. The immune system in black tiger shrimp, *Penaeus monodon* Fabricius: II. Cell and tissues involved the remove of foreign particles in blacker shrimp (*Penaeus monodon* Fabricius). **Songklanakarin J. sci. Technol.** 22(Suppl.): 581-588.

Sung, H.H., Y.L. Yung and Y.L. Song. 1996. Enhancement of microcidal activity in the black tiger shrimp prawn *Penaeus monodon* via immunostimulation. **J. Crust. Biol.** 16(2): 278 – 284.

Tipsemonkol, C.,W. Purivirojkul, N. Chuchrid and C. Limsuwan. 2009. Effects of activate DVAQUA® on the growth, survival and immune characteristics of Pacific white shrimp (*Litopenaeus vannamei*). **KU. Fish. Res. Bull.** 33(1):15-27.

Vargas-Albores, F. and G. Yepiz-Plascencia. 2000. Beta glucan binding protein and its role in shrimp immune response. **Aquaculture** 191: 13-21.

Vargas-Albores, F., P. Hinojosa-Baltazar, G. Portillo-Clark and F. Magallon-Barajas. 1998. Influence of temperature and salinity on the yellow leg shrimp. *Penaeus californisis* Holmes, phenoloxidase system. **Aquaculture** 29(8): 549-553.

Vieira, F.N., C.C. Buglione, J.P.L. Mouriño, A. Jatobá, M.L. Martins, D.D. Schleder, E.R. Andreatta, M.A. Barraco and L.A. Vinatea. 2010. Effect of probiotic supplemented diet on marine shrimp survival after challenge with *Vibrio harveyi*. **Arq. Bras. Med.Vet.Zootec.,** 6(3):631-638.

Walla, W. 2012. Effects of Activate DA on Growth, Survival and Population of *Vibrio harveyi* in Rearing of Pacific white shrimp (*Litopenaeus vannamei*). **Thesis Master of Science (In Thai).** Kasetsart University, Thailand. 104 pp.

Walla, W., W. Purivirojkul, N. Chuchrid and C. Limsuwan. 2012. Effects of activate DA on growth, survival and the total number of bacteria and *Vibrio* spp. in rearing of Pacific white shrimp (*Litopenaeus vannamei*). **KU. Fish. Res. Bull.** 36(2):14-22.

Wang, X., X. Song and J. Huang. 2004. Effect of peptidoglycan (PG) preparation on humoral immune factors of *Litopenaeus vannamei*. **J. Fish. Sci. China,** 11:26-30.

Yankomut, A. 2010. Effect of MERA™Cid on Growth, Survival and Preventing *Vibrio harveyi* in Rearing of Pacific white shrimp (*Litopenaeus vannamei*). **Thesis Master Degree (in Thai).** Kasetsart University, Thailand. 138 pp.