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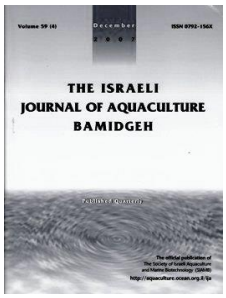

Effect of Dietary Supplementation of *Bacillus* spp. on Growth Performance, and Resistance of Pacific White Shrimp (*Litopenaeus vannamei*) to Acute Hepatopancreatic Necrosis Disease

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Keywords: *Litopenaeus vannamei*; probiotic; *Bacillus* spp.; disease resistance; acute hepatopancreatic necrosis disease; histopathology

Abstract

This study was conducted to investigate effect of dietary supplementation of three *Bacillus* spp. on disease resistance of *L. vannamei* against *Vibrio parahaemolyticus*, a main causative pathogen for acute hepatopancreatic necrosis disease (AHPND), and growth performance and feed efficiency. Four diets were prepared by supplementing powder forms of *Bacillus* spp. (1×10^{10} CFU/g) with a combination at 0.2% (*BS, B. subtilis* only), 0.4% (*BS/BP,* a mixture of *B. subtilis* and *B. pumilus*), and 0.6% (*BS/BP/BL,* a mixture of *B. subtilis*, *B. pumilus* and *B. licheniformis*) into a control diet (no supplement). Quadruplicate groups of shrimp (average body weight, 0.51 g) per each diet were hand-fed the diets for 33 days. Shrimp fed the *BS* diet had significantly higher growth performance than those fed the control diet or other diets (*BS/BP* and *BS/BP/BL*) ($P<0.05$). After the feeding trial, the shrimp were challenged with *V. parahaemolyticus* through an immersion method at a concentration of 2×10^5 CFU/ml water for 193 h. The shrimp groups fed the *Bacillus* supplemented diets showed significantly increased ($P<0.05$) cumulative survival rates compared to the control diet group. At the last sampling (193h), AHPND toxin was not detected in shrimp fed *BS* diet while no hepatopancreas was sampled due to 100% mortality in the control group. The findings indicate that growth performance and disease resistance of *L. vannamei* against AHPND could be improved when shrimp are fed the tested *Bacillus* spp.

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Introduction

Acute hepatopancreatic necrosis disease, AHPND (also known as early mortality syndrome, EMS) has caused losses in production in Asia and Central America (Mexico) (Lightner et al. 2012). Generally, AHPND has affected shrimp production at post-larval stage resulting in a mass mortality (up to 100%) within a month. The disease was also reported to affect *Penaeus monodon* at the last stage (Leobert et al. 2015). It is now well known that *V. parahaemolyticus* is the main pathogen for this disease targeting the hepatopancreas (Tran et al. 2013). Histopathological symptoms of AHPND are evident in the hepatopancreas, with R- and B-cell dysfunction, hematopoietic infiltration, and nuclear hypertrophy (FAO, 2013).

Intensive shrimp farming causes rapid propagation of bacterial diseases, which leads to an increase of antimicrobial use (Chumpol et al. 2017). Antibiotics have commonly been used in almost every production cycle of shrimp culture for prophylactic or therapeutic purposes. However, the use of antibiotics has continuously increased antibiotic-resistant bacterial strains including *V. parahaemolyticus* (Han et al. 2015a). Plasmid-mediated resistance of the *V. parahaemolyticus* strains against oxytetracycline or tetracycline was found thereby making general AHPND therapies very difficult (Han et al. 2015a).

Probiotics have been used as an alternative therapeutic way to reduce antibiotic use in shrimp culture (Balcazar et al. 2006). Many studies have been reported on use of probiotics in shrimp diets as alternative treatments for AHPND (Chumpol et al. 2017, Felix et al. 2017, Kumar et al. 2014). Several *Bacillus* spp. have great potential as supplements in probiotic in diets for shrimp. *L. vannamei* fed a diet containing *B. subtilis* E20 demonstrated better growth performance than those fed a non-supplemented control diet (Liu et al. 2009; Pattukumar et al., 2013). Subsequently, it was found that *B. subtilis* improved survival and innate immunity of *L. vannamei* larvae when it was added into the rearing water (Liu et al. (2010). Similar results showing that dietary supplementation of *B. subtilis* enhanced growth performance and immune response of *L. vannamei* have been reported (Shen et al. 2010). However, no information is available on the dietary supplementation of *Bacillus* spp. as a preventative treatment for AHPND in *L. vannamei*.

Therefore, this study aimed to investigate the supplemental effects of three different probiotics (*B. subtilis*, *B. pumilus,* and *B. licheniformis*) on growth performance and feed efficiency. In addition, a challenge test against *V. parahaemolyticus* was conducted to provide data for the development of probiotic agents that help reduce the high mortality of *L. vannamei* by *V. parahaemolyticus*.

Materials and methods

Experimental diets and design

Experimental diets were prepared by supplementing powder forms of *Bacillus* spp. (1 x 10^{10} CFU/g) with a combination at 0.2% (*BS, B. subtilis* only), 0.4% (*BS/BP,* a mixture of *B. subtilis* and *B. pumilus*), and 0.6% (*BS/BP/BL,* a mixture of *B. subtilis*, *B. pumilus,* and *B. licheniformis*) into a fish meal-based control diet with no supplement. The tested *Bacillus* spp. (1 x 10^{10} CFU/g) was provided from the Applied Technology Center, CJ CheilJedang Corp. (Suwon, South Korea). The bacteria were originally isolated from intestinal microflora of shrimp. The ingredients were mixed in a feed mixer (NVM-14, Gyeonggido, South Korea) and pelleted (SP-50, Gumgang Engineering, Daegu, South Korea) after addition of fish oil and 15% distilled water. The pelleted diets were dried at 23-26ºC for 18h and stored at -20ºC until use. The proximate composition of the diets was analyzed by AOAC (2005) (Table 1).

Table 1. Dietary formulation and proximate composition of the experimental diets in the feeding trial of *L. vannamei* (% dry matter).

Ingredients	*Experimental diets*[1]			
	Control	*BS*	*BS/BP*	*BS/BP/BL*
Fish meal[2]	40.00	40.00	40.00	40.00
Soybean meal[3]	12.81	12.81	12.81	12.81
Squid liver meal	10.00	10.00	10.00	10.00
Wheat flour	25.61	25.61	25.61	25.61
Amygluten 110	3.00	3.00	3.00	3.00
Fish oil A/C	2.00	2.00	2.00	2.00
Amino acid[4]	0.42	0.42	0.42	0.42
Vitamin/Mineral premix[5]	5.96	5.96	5.96	5.96
Rice bran	0.20	0.00	0.00	0.00
BS ($1x10^{10}$)	0.00	0.20	0.00	0.00
BS/*BP* ($1x10^{10}$)	0.00	0.00	0.40	0.00
BS/*BP*/*BL* ($1x10^{10}$)	0.00	0.00	0.00	0.60
Proximate composition (% dry matter)				
Moisture	5.68	5.60	5.53	5.42
Crude protein	47.3	47.4	47.1	47.6
Crude lipid	7.31	7.49	7.39	7.38
Crude ash	6.01	6.12	6.14	6.11

[1]Experimental diets were prepared by supplementing powder forms of *Bacillus* spp. (1×10^{10} CFU/g) with a combination at 0.2% (*BS, B. subtilis* only), 0.4% (*BS/BP,* a mixture of *B. subtilis* and *B. pumilus*) and 0.6% (*BS/BP/BL,* a mixture of *B. subtilis*, *B. pumilus* and *B. licheniformis*), and a fish meal-based diet was prepared as a control diet (no supplement).
[2]CJ Cheilhedang Co. Ltd., South Korea (crude protein: 67%)
[3]South America (crude protein: 44%)
[4]Amino acid mixture composition (g/100.4 g dry weight mixture; all L-form amino acids unless otherwise indicated): arginine, 8.88; histidine, 3.00; isoleucine, 4.32; leucine, 7.80; lysine hydrochloride, 9.64; methionine, 2.76; phenylalanine, 4.32; threonine, 4.56; tryptophan, 1.50; valine, 6.12; aspartic acid, 10.6; glutamic acid, 19.4; glycine, 10.8; alanine, 6.72.
[5]Vitamin/Mineral premix (g/kg of mixture): retinol, 3.0; cholecalciferol, 1.0; ascorbic acid, 20.0; tocopherol, 20.0; menadione, 2.0; thiamine, 4.0; riboflavin, 6.0; pyridoxine, 5.0; cobalamin, 6.0; inositol, 54.0; panththenic acid, 12.0; biotin, 0.2; niacin amide, 40.0; folic acid, 2.0; ferrous sulfate, 10.0; copper sulfate, 1.0; zinc sulfate, 30; manganous sulfate, 2.0; cobalt chloride, 10.; potassium iodide, 1.0; potassium, 6.0; sodium selenite, 0.01.

Shrimp and feeding trial

L. vannamei at the post larval stage were purchased at a local shrimp farm (Tamla shrimp, Jeju, South Korea) and transported to the Institute of Marine Sciences of Jeju National University (Jeju, South Korea). The shrimp were fed a commercial shrimp diet (SAJO DongA One, Seoul, South Korea) for a month. The shrimp (initial mean body weight, 0.51±0.01 g) were then randomly selected and distributed into 110 L capacity 16 acrylic tanks at a density of 30 shrimp per tank. Four replicate groups of shrimp were fed one of the four diets at a ratio of 4-12% body weight (four times a day, 08:30, 12:00, 15:30, and 19:00h) for 33 days. At the end of the feeding trial, all the shrimp in each tank were individually weighed for calculation of final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and survival.

Salinity was maintained at 30 ppt during the feeding trial. Water quality was maintained within a standard range for *L. vannamei* as follows; temperature (30-32℃), pH (7 - 8), dissolved oxygen (6.5 - 7.0 mg/L) and ammonia (0.05 - 0.10 mg/L).

Bacterial immersion challenge test

The causative strain associated with AHPND was selected by conventional PCR targeting pirA- and pirB-like genes (Han et al. 2015b). A preliminary test was conducted to verify the right time of exposure and proper concentration of the strain (*Vp*AHPND) in tanks prior to the challenge test. After the feeding trial, eleven shrimp (average weight, 3.6 g) were randomly selected from the respective dietary tanks and distributed into 16 acrylic 110 L capacity tanks keeping with four replicates per dietary treatment conducted in a quarantine room. For the infection, *V. parahaemolyticus* was cultured in TSB^{+} overnight (30h) and centrifuged (150 rpm) to reach 2×10^{5} CFU/ml water. The bacterial

culture solution of 50 ml (OD_{600}, 2.1) was then added to each tank for the immersion challenge. Shrimp were monitored for mortality every 1 hour. After immersion for 10h, 50% of the rearing water of each tank was exchanged. Four replicate groups of shrimp were fed their own respective diet at a ratio of 10% body weight (three times a day, 08:30, 13:30, and 18:30h) and monitored for 193h.

DNA extraction and AHPND PCR assays

We used AHPND-affected *L. vannamei* in this study to determine the quantities of virulent plasmid. The hepatopancreas was sampled before the challenge (0h), during the challenge (10h and 24h), and at the end of challenge (193h). qPCR analysis was used to detect the pirA gene from the laboratory bioassay (Table 2). DNA of tissue was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). We used the TaqMan Faster Universal PCR Master Mix (Life Technologies) on 1 cycle at 95°C for 20 sec, 95°C for 3 sec, and 40 cycles at 60°C for 30 sec. All samples were analyzed by Qiagen Rotor-Gene Q real-time PCR Detection System (Qiagen, Hilden, Germany).

Table 2. Sequences of primers used for quantitative real time PCR.

Primers/probe	*Target*	*Sequence*	*Size*	*Reference*
VpPirA-F		TTG GAC TGT CGA ACC AAA CG		
VpPirA-R	*Pir*A	GCA CCC CAT TGG TAT TGA ATG	135	Han et al. (2015c)
Probe		(FAM)- AGA CAG CAA ACA TAC ACC TAT CAT CCC GGA -	-	

Histological analysis

The sampled (0, 10, 24, and 193h) hepatopancreas were used for histological analysis by hematoxylin and eosin (H & E) staining method. To minimize tissue damage, Davidson's alcohol-formalin-acetic acid (AFA) was injected into the hepatopancreas of the sampled shrimp using a 1 ml syringe right before the tissue sampling. The dissected hepatopancreas was fixed in 1.5 ml Eppendorf tube containing Davidson's AFA for 24h and stored in ethyl alcohol (70%). The fixed tissue was cut into suitable sized shape for tissue specimen preparation (about 2-3 mm), placed in a cassette for 13h, and then the standard method was followed. After completion of the staining, the slides were photographed 200X using a microscope program (TCapture, Tucen Photonics) and a phase contrast microscope (BX50, Olympus, Japan).

Statistical analysis

The feeding trial was designed to be completely randomized. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the mean difference was compared with Duncan's multiple range tests. Statistical significance was determined at $P < 0.05$. Data are presented as mean ± SD. Percentage data were analyzed after transformation into arcsine.

Results

Growth, feed efficiency, and survival of shrimp fed the diets are shown in Table 3. Growth (FBW, WG and SGR) improved significantly in shrimp fed *BS* diet compared to the shrimp fed other diets. FCR was numerically lower in shrimp fed *BS* diet than shrimp fed other diets although it was not significant. No significant difference was observed in survival among all the groups.

Table 3. Growth performance and feed efficiency of *L.vannamei* (average body weight: 0.51g) fed four experimental diets for 33 days.

	FBW[1]	*WG*[2]	*SGR*[3]	*FCR*[4]	*Survival (%)*
Control	3.49±0.12^{b}	592±18.9^{b}	6.04±0.09^{b}	1.30±0.26	80.0±3.33
BS	3.86±0.18^{a}	677±31.9^{a}	6.37±0.13^{a}	1.19±0.12	71.1±6.94
BS/BP	3.48±0.17^{b}	589±38.3^{b}	6.03±0.17^{b}	1.34±0.20	75.6±5.09
BS/BP/BL	3.57±0.12^{b}	607±24.2^{b}	6.11±0.11^{b}	1.39±0.06	80.0±3.33

Values are mean of quadruplicates and presented as mean ± SD. Values with different superscripts in the same column are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Final body weight (g)

[2]Weight gain (%) = [(final mean body weight-initial mean body weight)/initial mean body weight] × 100

[3]Specific growth rate (%) = 100× [(ln(final body weight)-ln(initial body weight)]/days

[4]Feed conversion ratio = dry feed fed/wet weight gain.

During the AHPND challenge studies, the survival was significantly affected by the dietary supplementation of *Bacillus* spp. (Fig. 1). The shrimp became lethargic and showed erratic swimming, and reduced diet consumption immediately after the infection of *V. parahaemolyticus*. *Bacillus* supplemented diets showed significantly increased cumulative survival compared to the control diet. All shrimp fed the control diet were dead 37h after the infection.

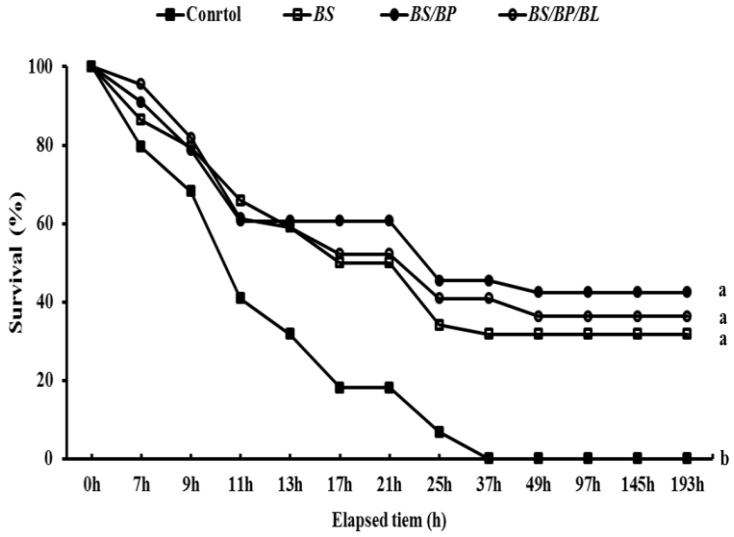

Fig. 1. Survival of *L. vannamei* after challenge with *V. parahaemolyticus*. The shrimp were immerged with *V. parahaemolyticus* suspension containing 2 × 10^5 CFU/mL. Quadruplicate groups of shrimp were hand-fed with one of the test diets three times a day during the challenge period. Experimental diets were prepared by supplementing powder forms of *Bacillus* spp. (1 × 10^{10} CFU/g) with a combination at 0.2% (*BS, B. subtilis* only), 0.4% (*BS/BP,* a mixture of *B. subtilis* and *B. pumilus*) and 0.6% (*BS/BP/BL,* a mixture of *B. subtilis*, *B. pumilus* and *B. licheniformis*), and a fish meal-based diet was prepared as a control diet (no supplement). Different letters indicate significant differences in survival at the end of the trial (P<0.05).

The results of the qPCR analysis of AHPND toxin in the hepatopancreas are shown in Table 4. The cycle threshold (Ct) was compared in all treatments. The lower the Ct value means the greater the amount of toxin. AHPND toxin was not detected in all the shrimp before the challenge test (0h). At 10h after the infection, DNA extracted from pooled samples proved to be AHPND-positive in all diet groups and their Ct values were 25.7, 31.3, 24.4, and 28.3 for the control in the *BS*, *BS*/*BP* and *BS*/*BP*/*BL* diets, respectively. At the last sampling (193h), no hepatopancreas samples were collected due to 100% mortality in the control group, while AHPND was not detected in shrimp fed *BS* diet. The Ct value was over 30.0 in shrimp fed the *BS*/*BP* and *BS*/*BP*/*BL* diets (30.3±1.49 and 30.3±0.93, respectively) at 193h after the infection.

Table 4. The cycle threshold (Ct) values of the hepatopancreas of shrimp sampled at 0, 10, 24, and 193 (final) h after the *V. parahaemolyticus* infection.

Treatments	*Ct values*			
	0h	*10h*	*24h*	*193h*
Control	nd[1]	25.7±0.50[c]	24.6±0.87[c]	-[2]
BS	nd[1]	31.3±0.12[a]	nd[1]	nd[1]
BS/*BP*	nd[1]	24.4±0.20[d]	33.6±0.27[a]	30.3±1.49
BS/*BP*/*BL*	nd[1]	28.3±0.10[b]	27.9±1.01[b]	30.3±0.93

Values are mean of quadruplicates and presented as mean ± SD. Values with different superscripts in the same column are significantly different (*P*<0.05).
[1]nd, not detected of toxin
[2]At the last sampling (193h), no hepatopancreas sample was obtained due to 100% mortality in the control group.

Bacterial count in the shrimp hepatopancreas (Table 5) indicated that higher number of beneficial microbes (*Bacillus* spp.) were found in shrimp fed *Bacillus* supplemented diets than in shrimp fed the non-supplemented control diet. Shrimp fed BS/BP diet showed almost 7-fold greater numbers of the microbes than shrimp fed the control diet.

Table 5. The bacterial counts in the hepatopancreas of *L. vannamei* fed four experimental diets. The hepatopancreas of *L. vannamei* was sampled at the end of the feeding trial.

	Bacillus (TSA$^+$)
Control	3.0 x 10^3
BS	1.6 x 10^4
BS/*BP*	2.0 x 10^4
BS/*BP*/*BL*	7.0 x 10^3

A G-grading system was adapted to classify the severity of the sampled hepatopancreas (Lightner 1996). The G-grading system uses from G0 as a negative to G4 as the highest severity of AHPND. Normal tissue morphology (G0) was observed in all the treatment groups sampled before the challenge test (Fig. 2a and 2b). The sampled hepatopancreas at 10h showed the most severe damage (G4) in shrimp fed the control diet (Fig. 2c), whereas sloughing and lack of B and R cells in hepatopancreas tubule epithelial cells were found in shrimp fed all the *Bacillus* supplemented diets (G2-3, G1-2 and G1 for *BS*, *BS*/*BP* and *BS*/*BP*/*BL*, respectively) (Fig. 2d). At 24h, sloughing and lack of B and R cells in hepatopancreas tubule epithelial cells were observed in shrimp fed *BS*/*BP* and *BS*/*BP*/*BL* diets (G2-3) (Fig. 2e and 2f). At the end of the challenged point (193h), only a few inflammatory cells were observed instead of the tissue necrosis by AHPND toxin in shrimp fed the *Bacillus* supplemented diets indicating almost recovered hepatopancreas (Fig. 2g and 2h).

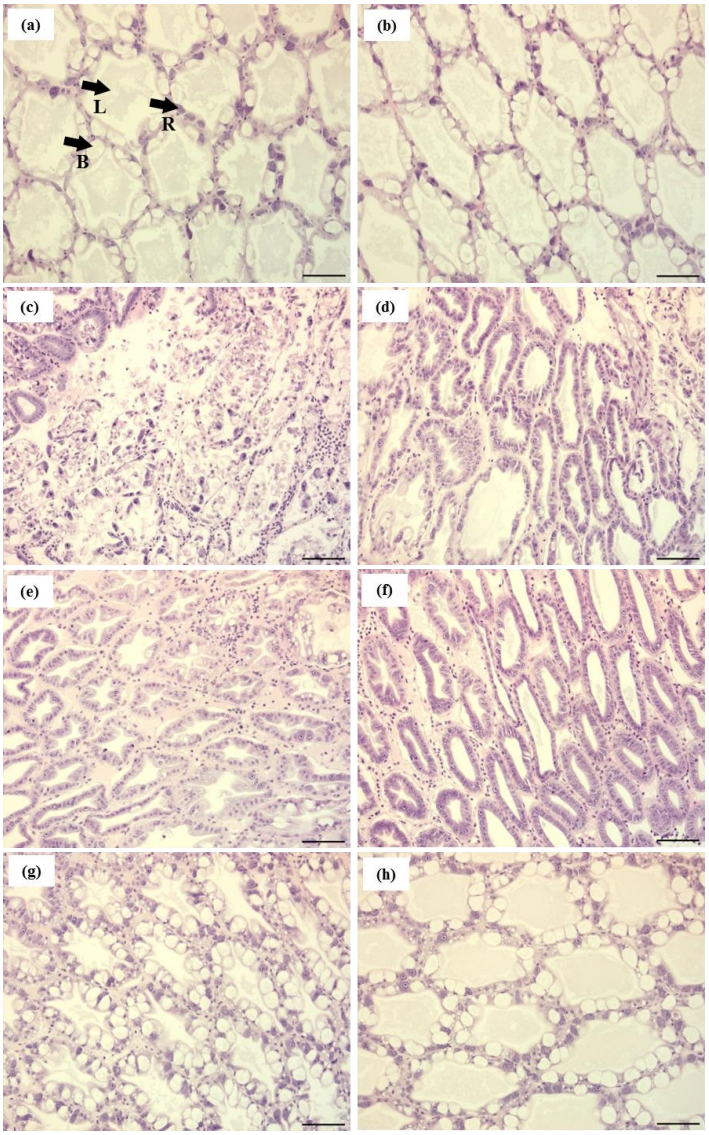

Fig. 2. Detection of *V. parahaemolyticus* in hepatopancreas tissues in the shrimp at (a) 0h control, (b) 0h *BS*/*BP*/*BL*, (c) 10h control, (d) 10h *BS*, (e) 24h *BS*/*BP*, (f) 24h *BS*/*BP*/*BL* and (g) 193h *BS*, (h) 193h *BS*/*BP*. Two representative figures are listed by sampling time. Hepatopancreas was stained with H & E method. Scale bars, 200 μm. At the last sampling (193h), no hepatopancreas sample was obtained due to 100% mortality in shrimp fed the control diet.

Discussion

The shrimp in this study grew very well on the experimental diets, and even showed better growth rate than those reported for similar sized (initial mean body weight, 0.5-0.6g) *L. vannamei* (Zhu et al. 2018). Growth performance of *L. vannamei* (0.67-4.0g) was significantly improved by the supplementation of two different *B. subtilis* strains (Zokaeifar et al. 2012) and a mix of *Bacillus* spp. (*B. licheiformis* and two different strains of *B. subtilis* at a ratio of 1:1:1) in diets compared to those fed a non-supplemented control diet (Sanchez-Ortiz et al. 2016). However, in the present study, the supplementation of *B. subtilis* alone, positively affected the growth of shrimp rather than when mixed with other *Bacillus* spp. A number of studies have demonstrated that *Baciilus* spp. as a probiotic in the form of dietary supplements improve the growth performance of several shrimp species (NavinChandran et al. 2014). Probiotics are known to assist the digestion processes of shrimp by either producing extracellular enzymes or providing some growth factors (Arellano-Carbajal & Olmos-Soto 2002). Probiotics can also help establish a balanced microbial flora in shrimp intestines and thereby improve the digestive process and function. These beneficial effects of probiotics enhance the growth, feed utilization efficiency, and eventually the innate immunity of the host animals including shrimp (Shen et al. 2010). Improved growth and feed efficiency of *L. vannamei* might be due to a prevention of intestinal disorders or lowering levels of some anti-nutritional factors in formulated feeds (Balcazar et al. 2006). In this regard, the improved growth performance of the shrimp fed *B. subtilis* in the present study could be explained. However, differences in performance of aquatic animals could occur due to different preparation process or strain of *B. subtilis,* and by the different application methods in the forms of either dietary additives or direct treatments to the culture water.

Bacillus spp. showed great potential as a dietary therapeutic probiotic for *L. vannamei* in the present study. It is well known that shrimp rely completely on their innate immunity to protect them from pathogens as they lack adaptive immunity (Tassanakajon et al. 2013). In recent years, immune-stimulants and probiotics have been used as dietary supplements for shrimp aquaculture to improve their innate immunity. The decrease in the immunity is the decisive cause of diseases and improvement in immunity is directly related to enhancement of disease resistance. In the present study, significantly higher disease resistance against *V. parahaemolyticus* was observed in shrimp fed the *Bacillus* spp. in either sole or mixed forms. Improved disease resistance of shrimp to *Vibrio* species has been reported with the addition of *Bacillus* spp. into diets. Resistance against *V. parahaemolyticus* was reportedly increased when *L. vannamei* were fed four different probiotics - *V. alginolyticus, B. subtilis, P. aestumarina* and *Roseobacter gallaeciensis* (Balcazar et al. 2007). In other studies, the disease resistance of *L. vannamei* to *V. parahaemolyticus* increased when a mixed probiotic of *R. sphaeroides* and *Afifella marina* (Chumpol et al. 2017) or *Dunaliella* sp. (Felix et al. 2017) was added to their diets. *B. subtilis* has also been reported to improve disease resistance of *L. vannamei* aginst *V. alginolyticus* and *V. harveyi* (Tseng et al. 2009; Liu et al. 2014).

The increased resistance to AHPND found in the present study can be explained with the AHPND toxin level and histopathologic changes in hepatopancreas. Quantitative PCR has become a method that can quantitatively compare disease infections (Han et al. 2015c). In the present study, qPCR analysis was used to confirm the anti-AHPND ability of probiotic in the challenge test. The highest mortality was observed at 10h after infection at which time the toxin was observed in all shrimp groups. Shrimp fed *BS* diet seemed to recover from AHPND at 24h and 193h after the infection as the AHPND toxin was not detected in the hepatopancreas of the shrimp.

Histological examination can easily confirm AHPND bacteria in the hepatopancreas by showing damage in the infected tissues. In the present study, normal hepatopancreas tissues were identified in shrimp before the challenge test against *V. parahaemolyticus*. At 10h after infection, damage in the hepatopancreas was clear and the most severe damage was found in shrimp fed the control diet while tissue necrosis progressed in shrimp fed all the *Bacillus* supplemented diets. The hepatopancreas is responsible for immune response and is a target organ for bacteria and virus in shrimp. *V. parahaemolyticus* was reported to be detected in the gills and hepatopancreas when the

shrimp were challenged (Aguirre-Guzmán et al. 2010). In addition, *V. parahaemolyticus* was concentrated in hepatopancreas and intestine rather than other organs (Khimmakthong & Sukkarum 2017). The innate immunity which eventually improves resistance to AHPND can be activated by pattern recognition receptors (PRRs) when pathogens were detected in hepatopancreas (Takeuchi & Akira 2010).

Probiotics can modulate the microflora in the gastrointestinal tracts of host animals leading to beneficial microbes being dominant (Burr et al. 2005). Gram-positive bacteria including *Bacillus* spp. are known to have some specific molecules present in their cell walls. The molecules possess microbe-associated molecular patterns (MAMPs) which can interact with PRR for immune responses (Bron et al. 2012). The PRR signaling can trigger the innate immune responses in the host animals (Baarlen et al. 2013). Recent genomic approaches and analyses have already proven the fact that probiotic microorganisms are closely involved in a modulation of the immune system. Lectin, a type of PRRs, facilitates recognition and phagocytosis of a pathogen by an opsonization in crustaceans. Many studies have reported that lectins in AHPND-survived prawns and in the stomach of shrimp post *V. parahaemolyticus* challenged are upregulated (Thepnarong et al. 2015) and have indicated that lectins involved in immune response activity in shrimp stomach and hepatopancreas (Ge et al. 2017). Therefore, another reason for the increased resistance to AHPND in the present study is likely to be due to the presence of some specific molecules such as peptidoglycans and capsular polysaccharides in the cell walls of the *Bacillus* spp. and increased beneficial microbes (Table 5) in the host microflora. The *Bacillus* spp. used in this study was derived from marine waters and these probiotics could have settled in the shrimp intestine more easily. Beneficial microbes such as *Bacillus* spp. compete with pathogenic microbes and eventually inhibit their proliferation in the host intestines (Nayak 2010).

In conclusion, the addition of *Bacillus* spp. such as *B. subtilis, B. pumilus,* and *B. licheniformis* as a mixture in shrimp diets can increase the disease resistance of the shrimp to AHPND. Moreover, the sole dietary supplementation of *B. subtilis* at the level of approximately 0.2% could be a promising practice to improve growth and feed efficiency of *L. vannamei*.

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