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Original Article

Promotion of *Lactobacillus plantarum* on growth and resistance against acute hepatopancreatic necrosis disease pathogens in white-leg shrimp (*Litopenaeus vannamei*)

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Abstract

Acute hepatopancreatic necrosis disease (AHPND), or early mortality syndrome (EMS), causes high mortality in cultivated penaeid shrimps, which leads to heavy losses for shrimp farming industry. *V. parahaemolyticus* with a toxin PirAB-encoding extrachromosomal plasmid are considered as common pathogenic agents. The present study aimed to assess the effects of potential probiotics on growth and resistance of shrimp to AHPND infection. Results confirmed typical clinical signs and histopathological features in AHPND-infected shrimps. Among 11 *Vibrio* isolates from AHPND-infected shrimps, strain XN9 was the most virulent and was identified as *V. paraheamolyticus* using AP3-based PCR amplification and API 20E kits. *In vitro* screening of potential probiotics was performed based on 26 marine and fermented food-derived bacteriocin-producing bacterial strains with broad inhibitory spectra from our previous research. Among these candidates, only two strains, *Lactobacillus plantarum* T8 and T13, exerted antimicrobial activities against all tested *Vibrio* isolates. Further *in vitro* trials showed that T8 enhanced significant growth and survival of shrimps after bath challenge with XN9. Although it had no significant effect on shrimp growth, T13 expressed better protection of shrimp from the AHPND pathogen immersed compared to the T8 treatment, or the control. This study presents the first evidence on the positive effects of probiotic candidates on growth and resistance of white-leg shrimp against *V. paraheamolyticus* causing AHPND.

Keywords: AHPND, aquaculture, *Lactobacillus plantarum*, *Litopenaeus vannamei*, probiotics, *Vibrio parahaemolyticus*

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Introduction

Acute hepatopancreatic necrosis disease (AHPND), or early mortality syndrome (EMS), is a serious bacterial disease in both penaeid shrimps *Penaeus monodon* and *Litopenaeus vannamei* (De Schryver et al., 2014), which leads to high mortality up to 100% in cultivated shrimp within 20-30 days (Lightner et al., 2012) or 46-96 days (Pena, 2015) after pond stocking. Hence, the disease has caused heavy losses for the shrimp aquaculture industry, especially in Asia (Flegel, 2012). AHPND was first described in China in 2009 (Yang et al., 2014), and then reported to spread to Vietnam, Malaysia, Thailand (Lai et al., 2015), Mexico (Nunan et al., 2014; Soto-Rodriguez et al., 2015) and the Philippines (Pena, 2015) in 2010-2012. For example, shrimp aquaculture in Thailand has been yet to recover from the first infection of EMS in 2012 and the production of 180 000 to 200 000 tonnes in 2014 is reduced by 25% compared with 2013 (FAO, 2015).

Major clinical signs and histopathological features of AHPND shrimps have been observed, including an atrophied pale hepatopancreas and a sloughing or necrosis of the hepatopancreas tubule epithelial cells in the early or later stage, respectively (Nunan et al., 2014; Soto-Rodriguez et al., 2015; Hong et al., 2016). The causative agent of AHPND was first identified as *Vibrio parahaemolyticus* (Tran et al., 2013), and then as *Vibrio harveyi* (Kondo et al., 2015), *Vibrio owensii* (Liu et al., 2015), or possibly a variety of species within the *Vibrio harveyi* clade (Xiao et al., 2017). Genome sequences of AHPND-causing *Vibrio* strains sampled from diverse areas have been published (Yang et al., 2014; Kondo et al., 2015; Liu et al., 2015). AHPND-causing *Vibrio* strains have been found to typically carry a large extrachromosomal plasmid that shares high identity among known strains and contains two toxin genes, PirA and PirB, which are absent in non-AHPND strains (Yang et al., 2014; Han et al., 2015). After shrimps were challenged with AHPND-causing bacteria, the bacteria initially colonized the stomach, secreted the PirB toxin alone in the hepatopancreas which could be sufficient to cause cellular damage at the early stage within 6 h after infection; whereas both the PirA and PirB toxins along with the bacteria were all detected in the hepatopancreas at the later stage (Lai et al., 2015).

Molecular diagnostic systems for AHPND were developed based on the amplification of DNA plasmid sequences present specifically in AHPND-causing *V. parahaemolyticus* strains. Two interim PCR detection methods were announced on 24 December 2013, updated in 2014 (Flegel and Lo, 2014; Lee et al., 2015) based on the sequences of AP1 and AP2; the AP2 method gave superior results to AP1 with 97% positive predictive value. Hence, a new method using a more sensitive and specific pair of primers AP3 based on the PirA-like ToxA gene was developed to improve this predictive value with no false positive or negative results found in comparison with AP1 and AP2-based PCR (Sirikharin et al., 2015). Another method for detection of AHPND-causing *V. parahaemolyticus* strains based on loop-mediated isothermal amplification (LAMP) combined with DNA-functionalized, ssDNA-labelled nanogold probe (AuNP) has recently been suggested to be used in shrimp hatchery and farm settings due to a decrease in diagnostic time, difficulty and cost compared to PCR methods (Arunrut et al., 2016). A rapid and sensitive AHPND-RPA assay has recently been developed for specific detection of the AHPND-causing *Vibrio owensii* (Liu et al., 2017).

No known treatment for AHPND/EMS is available to date. The FAO recommended water quality management practices and other aquaculture practices which include water disinfection before stocking, regular bottom disinfection with 1,3-Dibromino-5,5-dimethylhydantoin (DBDMH), use of feed additives to reduce pH in the gut, and use of low organic fertilizer (FAO, 2013). The FAO also suggested responsible use of antimicrobial treatment and non-antimicrobial therapies such as probiotics, prebiotics, bacterial biofloc, bioremediators, immunostimulants, vitamins, phage therapy and microbial population control studies (FAO, 2013). However, all such therapies have not yet been studied fully in the case of AHPND/EMS. A recent study has shown that both AHPND virulent and non-virulent *V. parahaemolyticus* strains were resistant to several antibiotics (Lai et al., 2015), which suggests that the use of antibiotics in shrimp culture should be more strictly regulated, and hence, non-antimicrobial therapies could be a long-term and effective approach for sustainable shrimp aquaculture. In addition, new solutions such as delayed first day of feeding, polyculture and water ageing have been suggested to promote shrimp protection against AHPND outbreak (Boonyawiwat et al., 2016).

The present study describes probiotic candidates with positive effects on growth and survival of white-leg shrimp *L. vannamei* exposed to AHPND-causing *V. parahaemolyticus* XN9 isolated from a AHPND outbreak in Ninh Thuan province, Vietnam in 2015.

Materials and Methods

Sampling: A total of 30 *L. vannamei* samples (20 shrimps per sample) were collected on farms that had experienced massive death within 45 days after stocking in Ninh Thuan province, Vietnam. Diseased shrimps showed clinical signs including empty gut, anorexia, lethargy, discoloration, and pale and shrunk hepatopancreas (HP). Live/moribund shrimps were collected from infected ponds and transported in plastic bags filled with pond water and proper aeration. The samples were immediately transported to the laboratory for further bacteriology and histopathology analyses.

The shrimps were analyzed individually and disinfected with 70% ethanol. The abnormalities were recorded. The shell was removed and the HP was dissected aseptically. The HP of shrimp was individually removed by using sterile scissors and subsequently excised into two pieces. One piece was fixed in 90% ethanol for PCR analysis while the second piece was used for bacterial isolation on agar plates.

Histological analysis: Ten shrimps were used for histopathology analyses to identify hepatopancreas

damages related to AHPND. Hepatopancreatic tissues were preserved in the Davidson's fixative solution (33% ethanol, 22% formalin and 11% acetic acid in distilled water) for 48 h before processing by routine histology methods described by Lightner (1996). The tissue sections were then stained with hematoxylin and eosin (H&E).

Bacterial isolation: The excised HP was streaked on Thiosulfate Citrate Bile Salts Agar (TCBS) (Difco, Detroit, MI, USA) agar plates using sterile loop. The streaked plates were incubated at 30°C for 24 h to allow vibrios to grow. Then, CFU was counted, and yellow colonies and green colonies were recorded. The green colonies from the TCBS agar plates were further purified in CHROMagar Vibrio (CHROMagar, Paris, France). The plates were incubated at 30°C for 18-24 h. Mauve colonies were selected and then cryopreserved at -80°C in Nutrient Broth (NB) (Difco) with 30% (v/v) glycerol. After pure cultures were obtained, bacterial identification was conducted using AP3-based PCR methods as described below and confirmed using a miniaturized API 20E kit (Biomérieux, Lyon, France).

Genomic DNA extraction and AP3-based PCR amplification: Bacterial DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA extract was used as template in AP3-based PCR detection method according to a recently published report (Sirikharin et al., 2014) to screen for AHPND pathogen. The AP3-PCR method has been used for the detection of the AHPND *toxA* toxin gene from AHPND-causing *V. parahaemolyticus* strains, but not from *V. parahaemolyticus* strains or other bacteria that do not cause AHPND. The AP3-PCR primers and the PCR cycling conditions were designed as described by Sirikharin et al. (2014).

Potential probiotic bacterial strains and culture conditions: Twenty-six bacteriocinogenic bacteria strains isolated from marine animals and traditional Vietnamese fermented cabbage with inhibitory spectra against diverse Gram positive and Gram negative bacteria including *Vibrio* spp. strains in our previous research (Nguyen et al., 2014[a,b]; Pham et al., 2014) were hypothesized as potential probiotics against AHPND/EMS pathogens in this study. They included *Lactobacillus plantarum* T8 and T13, *Bacillus pumilus* B3.10.2B, *Bacillus cereus* D9, *Proteus* spp. B3.10B, D15, D10, T14, CT1.1, G1, B3.7A, B3.10.2, N1.4, T9, B3.7.1 and B3.10A, *Providencia stuartii* B3.7.4, *Klebsiella* spp. M2, L5 and V1.1, *Alcaligenes faecalis* D16 and D18, *Enterobacter cloacae* H77 and *Cronobacter sakazakii* H9, H51 and H61. The strains were cultured in de Man, Rogosa and Sharpe (MRS) (Difco) for T8 and T13, and in Marine Agar (MA) (Difco) for the other strains, at 30°C and stored in the same media containing sterile glycerol (30% v/v) at -80°C.

Assay for antimicrobial activity: Antimicrobial activity was determined by agar-well diffusion method as described by Nguyen et al. 2014[b] with an exception that the strains were grown on MRS or MA medium and incubated at 30°C for 8-24 h.

Animal experiment 1: Pathogen challenge trial: Ethical approval was not required because white-leg shrimps are popularly cultured animals and not endangered or protected species. All efforts were made to minimize suffering.

(i) Pathogenic bacterial inoculation: Bacterial inoculation was obtained from AP3-PCR-positive/negative AHPND isolates. The bacteria were recovered from cryo-vials, inoculated in 10 ml of NB supplemented with 2.0% NaCl, and incubated overnight at 30°C. Each 100 µL was inoculated into flasks containing 30 ml of sterile NB plus 2.0% NaCl, and the flasks were then placed in a rotary shaker and incubated at 30°C for 18 h. After 18 h of incubation, Tryptic Soy Broth (TSB) (Difco) suspensions were checked using a spectrophotometer at an optical density OD_{600} from 0.6 to 0.8 to determine bacterial density (~10^8 CFU/ml on TCBS agar).

(ii) Immersion challenge with the isolates: Challenges were conducted with selected AHPND+ and AHPND- isolates as described by Tran et al. (2013) with some modifications. Briefly, 150 ml of fresh bacterial culture containing approximately 10^8 CFU/ml was used for immersion of 30 individual shrimps (1-2 g body weight) for 15 min before transferring to 30 L of filtered seawater with aeration to obtain a final bacterial density of approximately 10^6 CFU/ml with three replicates. Shrimps in the control group were immersed in 150 ml sterile TSB supplemented with 1.5% NaCl in 30 L of seawater. The seawater parameters were maintained with salinity of 30-32 ppt, at temperature of 28°C and pH 8. Cumulative mortality (dead and moribund shrimps) was recorded and mean time to death was calculated for isolates that gave 100% mortality within 7 days.

(iii) Observation and sampling for re-isolation of bacteria: The shrimps were fed twice daily with commercial feed TT661 (Thang Long company, Vietnam) containing 35% protein during the whole experimental period. They were checked every hour until all of the shrimp died. Moribund shrimp were disinfected with 70% ethanol, and the HP was dissected aseptically for bacteriological and histological analyses as well as molecular identification as previously described.

Animal experiment 2: Probiotic supplementation and pathogen challenge trial:

(i) Probiotic preparation: Two probiotic preparations, *L. plantarum* T8 and T13, were made for animal experiment 2 as follows. The strains were fermented in a BioFlo 110 fermentor (New Brunswick, NJ, USA) with MRS broth at pH 6.5 and 37°C for 8 h. Late exponential phase cells at the concentration of 1.0×10^8 CFU/ml were harvested by centrifugation (3200 g for 10 min at 4°C), washed in 0.9% NaCl solution and then obtained at the same centrifugation. Finally, probiotic biomass was mixed with shrimp feeds for daily diets, then naturally dried in a sterile incubator for 30 to 60

min and finally covered by squid oil (10-20 ml/kg feed).

(ii) Culture system and experimental animals: Stocks of specific pathogen-free (SPF) *P. vannamei* shrimp (Postlarvae 15) were purchased from local hatcheries in Khanh Hoa province, Vietnam and maintained in aerated tanks of 5 m^3 for 2 weeks before divided into biological experiments. The shrimps were fed commercial feed TT661 as described above.

Seawater was firstly filtered, treated with chlorine of 25-30 ppm, aerated for 3 days, then neutralized the redundancy chlorine with natriumthiosulfat (1:1), filtered again with algae scrubber and finally used for the animal experiment. Favorite conditions for shrimps were maintained during the whole experiment as follows: pH at 8.0-8.2, temperature at 23-25.5°C (morning) and 24-26.5°C (afternoon), salinity at 30-32% , alkalinity at 122-157 mg Ca/l, concentration of NH_4^+ at 0.25-0.4 mg/l and NO_2^- at 0.1-0.3 mg/l.

(iii) Probiotic supplementation: Shrimps from different acclimation tanks were merged and then randomly redistributed into 6 composite tanks of 500 L at a density of 300 animals per tank. The tanks were randomly assigned to one of the three probiotic treatments: treatment A with no probiotic supplementation as the control, treatment B with *L. plantarum* T8 administration and treatment C with *L. plantarum* T13 administration. Each treatment was performed in duplicate.

The shrimps were fed twice daily a ratio of 6-8% of weight of daily feeds. Water in each tank was continuously aerated and independently filtered through a biological seawater filter system at a rate of not less than 0.5 L/min. All uneaten food and feces were removed daily by siphon cleaning. About 10-20% water was refreshed daily. The amount of water lost during siphoning was refilled into each tank to retain the water level. The shrimps were fed probiotics for 3 weeks and then without probiotics for 7 weeks. A total of 15 shrimps from each treatment were weekly harvested for growth measurement based on body length (mm) and weight (g).

(iv) Pathogen challenge: At the 12th week (2 weeks of acclimation, 3 weeks of probiotic supplementation and 7 weeks without probiotics), the shrimps were challenged with *V. parahaemolyticus* XN9 by immersion method. The shrimps were redistributed into six plastic tanks of 120 L at a density of 50 animals per tank (two replicates for each treatment). The shrimps were acclimated for 3 days before immersing with *V. parahaemolyticus* XN9 at 1×10^5 and 1×10^6 CFU/ml. Number of dead shrimps was counted every 2 h after challenge until 100% died in all treatments. Cumulative mortality rate is a measure of the number of shrimps that die over a specified period per the initial number. After the experiment finished, bacterial re-isolation from gut samples of the tested shrimps on TCBS and MRS was performed.

Statistical analysis: Differences between the treatments in survival rate, cumulative mortality rate, body length and weight of shrimps were determined using the product-limit (Kaplan-Meier) estimator and confirmed with an ANOVA. A post-hoc Duncan's test was used to detect homogeneity subsets among the groups of means. All statistical analyses were performed using the statistical software Excel 2010 (Microsoft) and SPSS 22.0 (IBM) standardized at a significance level of $\alpha = 0.05$.

Results

Clinical signs and histopathological features of AHPND: AHPND occured at 35-45 days post stocking in white-leg shrimps farmed in Ninh thuan, Vietnam. Infected shrimps showed clinical signs as follows: lethargy, anorexia, erratic swimming, and empty guts. Diseased shrimps typically presented HP discoloration, shrinking and stiffness (*data not shown*). Histopathologically, the infected shrimps also showed a severe necrosis of the HP tubules with a massive sloughing of epithelial cells that caused disorganization and a total loss of tissue structure (Fig. 1). The tubular epithelium was necrotic with severe desquamation of the cells, which accumulated in the lumen as dead cells (Figs. 1B and 1C). The severely affected tubules showed hemocytic infiltration as a response to the necrotic epithelium (Fig. 1C). The inter-tubular tissue from the HP tubules showed a severe inflammatory response. The tubular epithelium became entirely necrotic, which caused proliferation of bacterial masses inside the tubule lumens (Figs. 1B and 1C). The interstitial spaces from these tubules showed increased hemocytic infiltration that developed hemocytic capsules around the tissues. Some tubules presented melanization from necrotic material (Fig. 1A). No B- or R-cells were apparent.

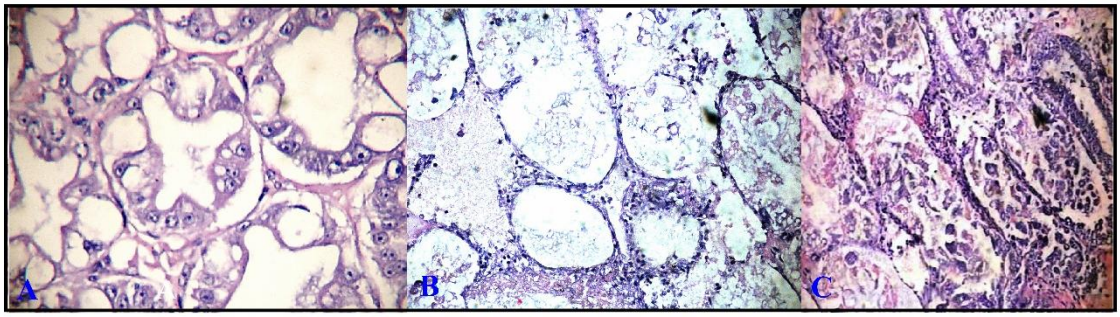

Figure 1 Histopathological features of AHPND white shrimps (*Penaeus vannamei*). (A) Normal shrimp showing normal HP structure. (B-C) AHPND-infected shrimp showing no B-, F-, and R-cells, and sloughing of hepatopancreas (HP) tubule epithelial cells surrounded by hemolytic infiltration. 200 X.

Table 1 Cultural characteristics and preliminary identification of bacterial isolates from AHPND white-leg shrimps in Ninh Thuan province, Vietnam

No.	Bacterial isolate	Growth on TCBS	Growth on CHROMagar *Vibrio*	Culture-based preliminary identification	Detection by AP3-based PCR	Confirmed identification using API 20E kit
1	NH0906C3	Green colony	+	*Vibrio* sp.	-	
2	HH4/1	Green colony	+	*Vibrio* sp.	-	
3	PT250964P	Green colony	+	*Vibrio* sp.	-	
4	HH07044/6	Green colony	+	*Vibrio* sp.	-	
5	XN9	Green colony	+	*Vibrio* sp.	+	*V. parahaemolyticus*
6	H2509E1B	Green colony	+	*Vibrio* sp.	-	
7	PT2509E1W	Green colony	+	*Vibrio* sp.	-	
8	C2	Green colony	+	*Vibrio* sp.	-	
9	XN8	Green colony	+	*Vibrio* sp.	+	*V. parahaemolyticus*
10	PT2509E1V	Green colony	+	*Vibrio* sp.	-	
11	NH0906C4	Green colony	+	*Vibrio* sp.	-	

Isolation and identification of AHPND pathogens in white-leg shrimps in Vietnam: A total of 11 bacterial isolates were preliminary identified as *Vibrio* spp. based on the growth on selective media TCBS and CHROMagar Vibrio (Table 1). Then, these bacterial isolates were initially screened using the AP3-based PCR method. An expected 336 bp amplicon was detected in two AHPND-causing isolates only (XN8 and XN9). Finally, the results of API 20E kits indicated that these two isolates shared the most identical biochemical characteristics with *Vibrio parahaemolyticus* (Table 1), which confirmed the results from the PCR test. Only two characteristics including positive tryptophane deaminase activity and negative indole production were reverse to typical *V. parahaemelyticus* strains.

Bacterial challenge pre-test: The primary investigation into shrimps immersed in the cell culture broth of the AHPND (+) *V. paraheamolyticus* strains XN8 and XN9 at 10^6 CFU/ml showed that XN9 was the most virulent (*data not shown*). In particular, XN9 caused the first mortality of immersed shrimps within 6 h. The experiment was terminated after 42 h because cumulative mortalities reached 100%. Gross signs presented by the challenged shrimp included lethargy, empty stomach and gastrointestinal tract, whitish and atrophied HP, and soft shells. Histopathologically, the HPs of shrimp treated with the AHPND (+) pathogen showed HP tubule cell sloughing, no B-, F- and R-cells, and hemolytic infiltrates.

In vitro screening of probiotic candidates with antimicrobial activity against AHPND-causing V. paraheamolyticus XN9: Among the 26 tested strains, only two strains, *L. plantarum* T8 and T13, exerted strong antimicrobial activities with all 11 isolates from AHPND white-leg shrimps in Ninh Thuan province, Vietnam (Fig. 2). Interestingly, both T8 and T13 were found to inhibit the growth of AHPND-causing *V. paraheamolyticus* XN9 with the inhibitory zone diameter of 8.2 and 10.2 mm, respectively (Fig. 2B).

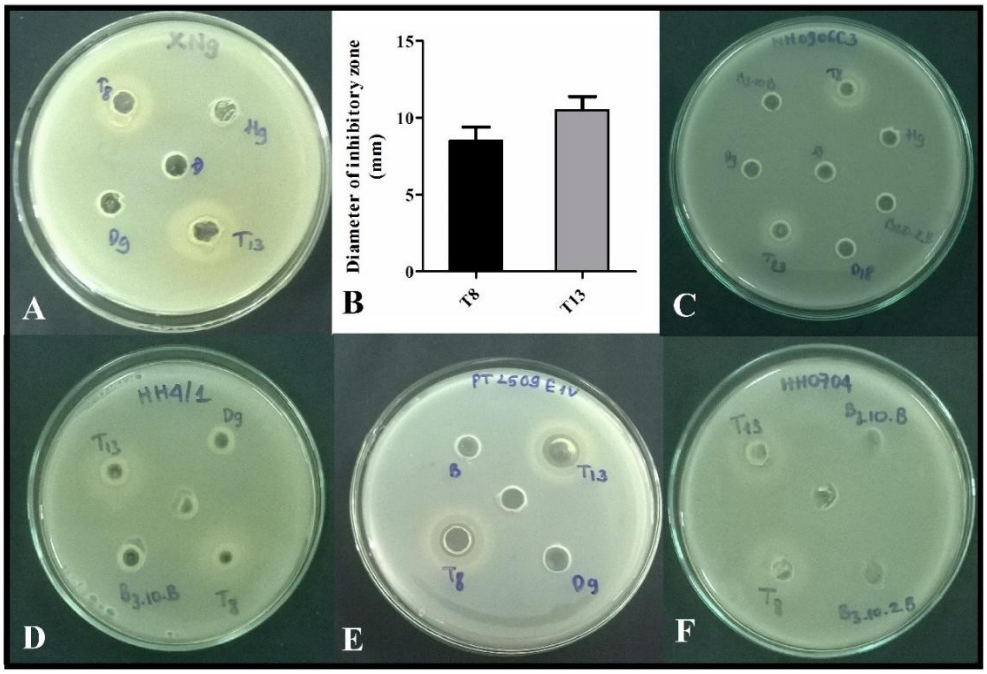

Figure 2 **Inhibition zones (A, C-F) and their diameters (B) caused by antimicrobial activity of *Lactobacillus plantarum* T8 and T13 against AHPND *Vibrio* strains.** Pathogens: *V. parahaemolyticus* XN9 (A-B), *Vibrio* sp. HH4/1 (C), *Vibrio* sp. NH0906C3 (D), *Vibrio* sp. HH0704 (E), *Vibrio* sp. PT2509E1V (F).

Effect of probiotic candidates on growth of white-leg shrimps: At the initial week, the average body length and weight of all shrimps were 5.4 ± 2.01 (mm) and 0.01 ± 0.00 (g), respectively. The shrimps were randomly distributed to 3 treatments including the T8 treatment, the T13 treatment, as described above, and the control, without probiotic administration. Their growth in body length and weight was measured until the 12th week. No significant differences (P > 0.05) were found among the 3 treatments after 5 weeks (*data not shown*) and even between the control and the T13 treatment after 12 weeks (Fig. 3). However, after 12 weeks, the shrimps from the T8 treatment expressed a significant difference (P < 0.05) in both body length and weight compared to the control. In particular, the average body length and weight of the shrimps treated with T8 were 72.73 ± 7.06 (mm) and 3.21 ± 0.84 (g) compared to those of the control of 66.03 ± 3.97 (mm) and 2.36 ± 0.43 (g), respectively.

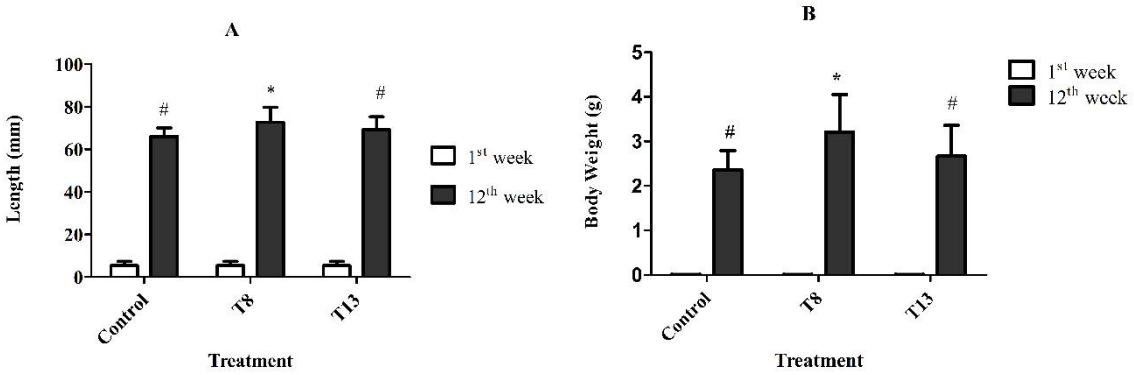

Figure 3 **Average body lengths (A) and weights (B) of shrimps after probiotic administration.** Body lengths and weights at initial time (white column) and 12th week (black column) after probiotic supplementation were measured and given. Treatments including control without any probiotic supplementation, supplementation with T8, and supplementation with T13 are presented. Body lengths and weights were expressed as Means ± SD. Symbols * and # indicate significant differences (P < 0.05) among the treatments.

Effect of probiotic candidates on survival rate of white-leg shrimps challenged with AHPND pathogen: With the challenged density of *V. parahaemolyticus* XN9 at 10^5 CFU/ml, the shrimps stopped feeding just some hours post infection and a few died at 6 h post infection. The average cumulative mortality rate of shrimps during the challenge trial is shown in Fig. 4. At 48 h after infection, the average cumulative mortality rate of the control approached 100%, whereas the death rates in the treatment with T8 and T13 were 78% and 76%, respectively, indicating that the survival rate was 22-24%. This shows a significant difference (P < 0.05) between each probiotic treatment and the control, but no significant difference (P > 0.05) between the two probiotic treatments T8 and T13.

With the higher challenged density of XN9 at 10^6 CFU/ml, the shrimps also began to die at 6 h after infection but then the mortality increased more rapidly. The average cumulative mortality rate of the control approached 100% in 24 h post the first death (Fig. 4). At the same time, 80% and 72% of the shrimps treated with T8 and T13 died, respectively. This means that 20-28% of the shrimps treated with probiotic candidates were alive 24 h after the challenge. Statistical analysis indicated significant differences (P < 0.05) among all 3 treatments and suggested a protective effect of T8 and T13 on shrimps exposed to AHPND pathogen and the better effect of T13 24 h after infection.

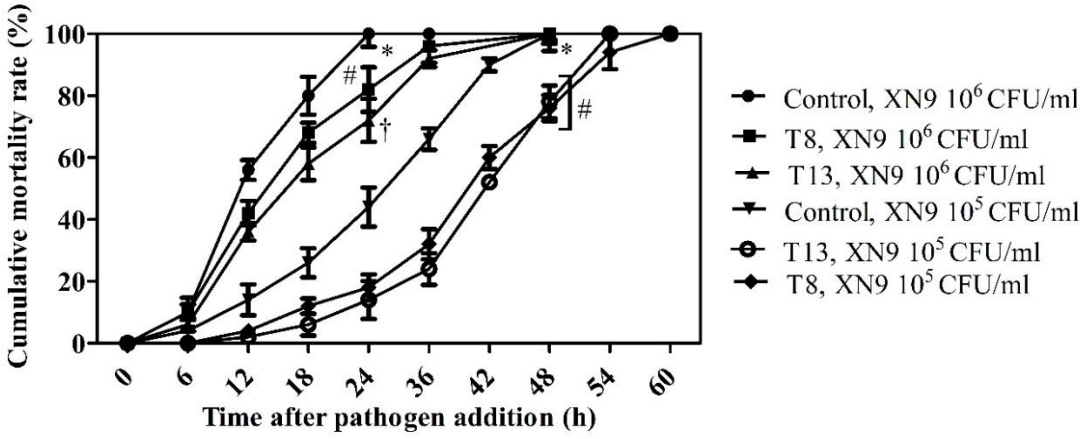

Figure 4 **Cumulative mortality rate of shrimps after pathogen challenge with *Vibrio parahaemolyticus* XN9 at density of 10^5 and 10^6 CFU/ml.** Cumulative mortality rate was expressed as Means ± SD. Symbols *, # and † indicate significant differences (*P* < 0.05) among the treatments.

Discussion

The genus *Vibrio* includes many important pathogens for marine culture that provoke a high mortality in sea animals (Nguyen et al., 2013). The present study confirms that *V. parahaemolyticus* strains XN8 and XN9 isolated from a disease outbreak in Ninh Thuan province, Vietnam are AHPND pathogens found in *L. vannamei*. Regarding 9 other *Vibrio* strains, further studies should be performed to understand their associations with AHPND outbreak. They could be potential AHPND pathogens because there have recently been increasing observations on non-*V. parahaemolyticus* causative agent of AHPND, including *Vibrio harveyi* (Kondo et al., 2015), *Vibrio owensii* (Liu et al., 2015), and other species within the *Vibrio* Harveyi clade (Xiao et al., 2017).

The application of probiotics in aquaculture is increasing (Farzanfar, 2006; Nguyen et al., 2013) and this study followed on *in vitro* and *in vivo* tests to explore the possibility of using potential strains available in our collections as probiotics in shrimp farming. In regard to *in vitro* tests, of the 26 potential probiotic strains tested, only two strains, *L. plantarum* T8 and T13, showed antimicrobial activity against AHPND-causing *V. parahaemolyticus* XN9. It should be noted that these 26 strains showed broad antimicrobial activity against diverse food spoilage and animal pathogenic bacteria in the previous studies (Nguyen et al., 2014[a,b]; Pham et al., 2014). Some of them were even found before to inhibit the growth of different *V. parahaemolyticus* strains, e.g. D10, D15, T14, B3.10.2B, B3.7.1, T8 and T13 against *V. parahaemolyticus* C1 (Nguyen et al., 2014[a,b]); D9, D10, and D15 against *V. parahaemolyticus* VP2865 (Nguyen et al., 2014[a]). This indicates that antimicrobial activity is strain-dependent, thus the screening of probiotics against AHPND pathogens becomes more challenging.

Resistance to acids and bile salts is also considered as an important selection criteria for probiotics since probiotics have to survive and go through the stomach with pH 2-4 for 2 h and the small intestine contains bile salts at the concentration of 0.1-0.3%, which is toxic for living cells (Liong and Shah, 2005). Lactic acid bacteria were found to be resistant to 0.3% bile salt (Erkkila and Petaja, 2000) or grow in MRS agar supplemented with 0.3% bile salt (Pennacchia et al., 2004) but this characteristic is also strain-dependent (Lee et al, 2011; Ramos et al., 2013). Interestingly, our unpublished data showed that T8 and T13 were resistant to bile salt at the concentration of 0.1%, but they could not survive at 0.2% or 0.3% bile concentration. Also, both of them were tolerant to pH 2 for 1 h and pH 3-4 for 2 h (M. Leelakriangsak et al., unpublished data). These results are in accordance with the results of previous studies (Tokatl et al., 2015).

In regard to *in vivo* trials, the results showed that while T8 could enhance growth and reduce mortalities in shrimps after immersion challenge with AHPND-causing *V. parahaemolyticus* XN9, T13 could not stimulate shrimp growth significantly but expressed a better protective effect against this pathogen attack in shrimp. The used potential probiotic strains *L. plantarum* T8 and T13 were isolated from traditional Vietnamese fermented cabbage, and their delivery to shrimps was achieved through amendment of commercial feeds. This suggests that the observed beneficial effects were mostly due to their activity while localized in the digestive system of shrimps. The results also agree well with our previous study that *L. plantarum* T13 along with two other strains, *Bacillus pumilus* B3.10.2B and *B. cereus* D9, reduced mortalities in lobster juveniles after immersion challenge with pathogenic *V. owensii* DY05 (Nguyen et al., 2014[a]).

Moreover, two concentrations of L. plantarum T13 prepared and mixed with commercial shrimp feed to give final concentrations of approximately 10^9 CFU/g and 10^5 CFU/g were studied on growth performance and digestive enzyme activity of pacific white shrimp. Shrimp fed diets containing L. plantarum T13 at the concentration of 10^9 CFU/g significantly improved final weight, weight gain, specific growth rate, feed conversion ratio and protease activity compared to control after 8 weeks of administration. Significant differences in weight gain and protease activity in the 10^9 CFU/g feed compared to the 10^5 CFU/g feed were also found (M. Leelakriangsak et al., unpublished data). The improvement in shrimp growth factors by *L. plantarum* T13 may be due to the induction of digestive enzyme of the host.

It is not surprising that *Lactobacillus* are increasingly employed as probiotics in shrimp species (Farzanfar, 2006) because they not only expressed antimicrobial, enzyme and bacteriocin activities but also were found to be associated with digestive tracts of cultivated and wild adult shrimp, including *L. vannamei*, *Metapenaeus brevicornis*, *P. merguiensis* and *P. monodon* (Kongnum and Hongpattarakere, 2012; Rungrassamee et al., 2014; Nguyen, 2016; Nguyen and Nguyen, 2017). The presence of *Lactobacillus* detected in *the i*ntestines of these shrimp species suggests that the bacterium can withstand *shrimp* gut and aquatic environments (Nguyen et al., 2013). Finally, the strains T8 and T13 become highly potential probiotics for shrimp aquaculture because they belong to the commercially important species *L. plantarum*, which has been used as probiotics for human and animals for centuries and is generally regarded as safe (GRAS) by the US Food and Drug Administration (FDA).

Back to the case of AHPND/EMS, even the results of the present study will become more significant for disease management when a current study has shown that both AHPND virulent and non-virulent *V. parahaemolyticus* strains are resistant to a series of antibiotics such as Chloramphenicol, Enrofloxacin, Ofloxacin, Ampicillin, Streptomycin, Sulfamethoxazole, Fosfomycin, and Bicozamycin (Lai et al., 2015). Therefore, the FAO's recommendation for the use of antibiotics to control this disease in shrimp aquaculture (FAO, 2013) must be followed more carefully. Non-antimicrobial therapies like probiotics to support beneficial health effects on cultivated shrimps, in turn, should be stimulated strongly for use in a sustainable shrimp farming industry.

Conclusion

AHPND occurring in white-leg shrimp within 35-45 days after pond stocking in Ninh Thuan province, Vietnam have expressed typical clinical signs and histopathological features, which is in accordance with the findings of previous studies. A total of 11 bacterial isolates from AHPND white-leg shrimps were preliminary identified as *Vibrio* spp. based on selective media, in which XN8 and XN9 were identified as *V. paraheamolyticus* using the AP3-based PCR amplification and API 20E kits. XN9 was found to be the most virulent among the tested isolates.

Among 26 bacteriocinogenic bacteria strains isolated from marine animals and traditional Vietnamese fermented cabbage with inhibitory spectra against diverse bacteria in our previous research, only two strains, *L. plantarum* T8 and T13, exerted antimicrobial activities with all 11 tested *Vibrio* isolates. Interestingly, both T8 and T13 were resistant to bile salt at the concentration of 0.1% but not at 0.2% or 0.3% and tolerant to pH 2 for 1 h and pH 3-4 for 2 h but not at pH 1. After *in vitro* screening, T8 and T13 were supplemented through feed for 3 weeks to further *in vivo* screen in white-leg shrimps. The results showed that after 12 weeks of probiotic administration, T8 enhanced significantly ($P < 0.05$) both body length and weight of shrimps relative to the control. No significant effects ($P > 0.05$) were found between the treatment with T13 and the control in the same condition. Meanwhile both T8 and T13 reduced significantly 20-28% in mortality rate of shrimps after challenged with XN9 at the density of 10^5 or 10^6 CFU/ml after 48 h or 24 h of infection, respectively.

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บทคัดย่อ

Lactobacillus plantarum ส่งเสริมการเติบโตและการรอดตายจากการติดเชื้อก่อโรค AHPND ในกุ้งขาว (*Litopenaeus vannamei*)

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กลุ่มอาการตับและตับอ่อนตายเฉียบพลัน (Acute hepatopancreatic necrosis disease, AHPND) หรือกลุ่มอาการตายด่วน (Early mortality syndrome, EMS) เป็นสาเหตุการตายของกุ้งในอัตราที่สูง ซึ่งส่งผลต่ออุตสาหกรรมการเพาะเลี้ยงกุ้งอย่างมาก แบคทีเรีย *V. parahaemolyticus* ที่มีพลาสมิดของยีนผลิตเป็นสารชีวพิษ PirAB เป็นตัวการสำคัญในการก่อโรค งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของโพรไบโอติกต่อการเติบโตและการอยู่รอดจากเชื้อก่อโรค AHPND ในกุ้ง ผลการศึกษาได้ยืนยันลักษณะการติดเชื้อโรค AHPND ของกุ้งจากสัญญาณทั่วไปทางคลีนิคและการตรวจทางพยาธิวิทยา จาก 11 ไอโซเลตในกลุ่ม *Vibrio* ที่คัดแยกจากกุ้งติดเชื้อ พบว่า XN9 เป็นสายพันธุ์ที่ร้ายแรงที่สุดและระบุว่าเป็น *V. paraheamolyticus* จากการตรวจสอบโดย AP3-based PCR และชุดทดสอบ API20E โพรไบโอติกที่มีศักยภาพได้ถูกคัดเลือกมาจากแบคทีเรียที่สามารถผลิตแบคเทอริโอซินได้จากการคัดแยกจากทะเลและอาหารหมัก ที่แสดงถึงความสามารถในการยับยั้งเชื้อก่อโรค จากงานวิจัยก่อนหน้านี้ มีเพียงสองสายพันธุ์ ได้แก่ *Lactobacillus plantarum* T8 และ T13 ที่แสดงผลการยับยั้งเชื้อ *Vibrio* ทั้งหมดที่ทดสอบ จากการทดสอบพบว่า T8 เพิ่มการเติบโตและการอยู่รอดของกุ้งอย่างมีนัยสำคัญหลังจากทดลองการติดเชื้อด้วย XN9 ถึงแม้ว่า T13 ไม่ส่งผลต่อการเติบโตของกุ้งอย่างมีนัยสำคัญ แต่แสดงผลในการป้องกันกุ้งเมื่อแช่ในเชื้อก่อโรคดีกว่าเมื่อเปรียบเทียบกับ T8 หรือกลุ่มควบคุม การศึกษานี้เป็นการศึกษาแรกที่แสดงถึงผลในเชิงบวกของโพรไบโอติกตัวอย่างต่อการเติบโตและการมีชีวิตรอดของกุ้งหลังจากทดสอบด้วยเชื้อก่อโรค AHPND

คำสำคัญ: AHPND การเพาะเลี้ยงสัตว์น้ำ แลคโตบาซิลลัส แพลนทารัม ลิโทพีเนียส แวนาไม โพรไบโอติก วิบริโอ พาราฮีโมไลติคัส

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