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Decreased Severity of Acute Hepatopancreatic Necrosis Disease in White Shrimp (*Litopenaeus vannamei*) by Mixed Culture of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus megaterium*

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Highlights

- A diet supplemented with the mixed culture of *Bacillus subtilis*, *B. licheniformis*, and *B. megaterium* could decrease AHPND severity in white shrimp (*L. vannamei*).

- Infected shrimp fed with a diet supplemented with the mixed culture of *Bacillus* strain revealed higher percent survival, lower percent of *Vibrio* AHPND strain detected by PCR, and a small amount of *Vibrio* sp. viability count in hepatopancreas than those other groups of infected shrimps.

- The mixed culture of these *Bacillus* strains is able to control the dissemination of *Vibrio* AHPND strain to hepatopancreas as a target tissue of AHPND.

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Decreased Severity of Acute Hepatopancreatic Necrosis Disease in White Shrimp (*Litopenaeus vannamei*) by Mixed Culture of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus megaterium*

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Running head: Decreased Severity of AHPND in White Shrimp

Abstract. The objective of this study was to investigate the mixed culture of *Bacillus subtilis, B. licheniformis,* and *B. megaterium* to control acute hepatopancreatic necrosis disease (AHPND) or EMS (Early Mortality Syndrome) in white shrimp *Litopenaeus vannamei* as a model. The infected shrimps with *Vibrio parahaemolyticus* AHPND strain were divided into tanks and different feeding with either *B. subtilis, B. licheniformis*, or *B. megaterium* and feeding with the mixed culture of all *Bacillus* strains. The infected shrimps were fed with the mixed culture of *Bacillus* that showed the significant highest survival rate and revealed lower percent detection of *V. parahaemolyticus* AHPND strain by Polymerase Chain Reaction (PCR) (57.14%) with a small amount of viability count in their hepatopancreas. In contrast, the infected shrimp feeding with each of *B. subtilis, B. licheniformis*, or *Bacillus megaterium*, revealed the spread of *V. parahaemolyticus* AHPND strain in all tissue by PCR detection (86.67-100%) with a large amount of viability count (3.53-4.24x10^3 CFU/g). This study indicated that the mixed culture of *Bacillus subtilis, B. licheniformis,* and *B. megaterium* be able to control the dissemination of *V. parahaemolyticus* in shrimps, especially in hepatopancreatic that is the target tissue of AHPND in white shrimp *(L. vannamei*). The result of this study revealed the efficiency and mechanism of the mixed culture

of *Bacillus subtilis, B. licheniformis,* and *B. megaterium* to control the virulence of AHPND and support the application of this mixed culture in aquaculture of shrimp farms to avoid chemical and antibiotic treatment by using it as a biological control.

Keywords: acute hepatopancreatic necrosis disease, *Litopenaeus vannamei***,** *Bacillus subtilis, Bacillus megaterium*, *Bacillus licheniformis*

INTRODUCTION

White shrimp (*Litopenaeus vannamei)* is one of the significant economic seafood products. However, the disease outbreak in the shrimp farming industry caused by microbial pathogens results in losses of production. Acute Hepatopancreatic Necrosis Disease (AHPND) also known as Early Mortality Syndrome (EMS) commonly affects shrimp post-larvae or juveniles and leads to 100% shrimp death within 30-35 days after stocking (Velázquez-Lizárraga *et al.* 2019; De Schryver *et al.* 2014). The causative agent of AHPND is *Vibrio parahaemolyticus* AHPND strain, the gram-negative bacteria with the halophilic property and toxin production. This pathogen has a plasmid harboring virulent genes that encode homologous of the Photorhabdus insect-related (Pir) toxins, PirA and PirB (or $PirAB^{VP}$) (Tinwongger *et al.* 2014). The route of infection is the exposure of mouth, gills, feed, and tissue damage to *V. parahaemolyticus* AHPND strain (Prachumwat *et al.* 2019; Lai *et al.* 2015). Subsequently, the disease develops by defects in the immune system and a large amount of this pathogen in their environment. The severity of AHPND has led to the study of how to prevent and treat this disease. The use of microbial culture as a probiotic in the treatment of organic waste in water and soil to reduce the risk factor of *V. parahaemolyticus* AHPND strain proliferation has been increasing interested (Kumar *et al.* 2016; Hlordzi *et al.* 2020). The genus of *Bacillus* has previously been reported as a potential probiotic supplemented in a diet for shrimp. A diet containing *B. subtilis* was fed to *L. vannamei* and demonstrated better growth than a non-supplemented control group (Liu *et al.* 2009). Additionally, *B. subtilis, B. licheniformis,* and *B. megaterium* could inhibit the growth and toxin production of *Vibrio harveyi* leading to a higher survival rate of shrimp with a diet containing these probiotics than those a control group with non-supplemented (Nakayama *et al.* 2009). Moreover, aquaculture of shrimp farms with probiotics demonstrated better quality of water in the pond (Kumar *et al.* 2016; Hlordzi *et al.* 2020). However, little is known about the mechanism of these *Bacillus* species and *V. parahaemolyticus* AHPND strain in host interaction. The purpose of this study was to investigate the effect of *Bacillus subtilis, Bacillus*

licheniformis, Bacillus megaterium, and their mixed culture on the disease severity of AHPND in the infected shrimp model.

MATERIALS AND METHODS

Experimental Shrimp

Healthy shrimp (*Litopenaeus vannamei*, PL21) were provided by Kung Krabaen Bay Royal Development Study Center, Chantaburi, Thailand. They were maintained in the tank (500L) at 25–27°C, the salinity of 23 ppt. with aeration, and were fed commercial feeds 2 times a day. Some shrimp were collected to confirm for uninfected of *V. parahaemolyticus* by PCR detection (Tinwongger *et al.* 2014).

Pathogen Inoculum Preparation

The *V. parahaemolyticus* AHPND strain was kindly provided by Kung Krabaen Bay Royal Development Study Center, Chantaburi, Thailand. This bacterial strain was originally isolated from the shrimp farm in Chantaburi. Confirmation of the virulence *V. parahaemolyticus* AHPND strain was performed using the challenge test and histopathological examination as previously described (Tinwongger *et al.* 2014). The bacteria were single colony isolated on Thiosulfate Citrate Bile-salt Sucrose (TCBS) agar and grow in Tryptic Soy Broth (TSB) supplemented with 2 % NaCl at 37 °C for 18 hr on an incubator shaker. The media was removed by centrifugation at 10,000 rpm for 1 min. The bacterial cells were resuspended in normal saline for absorbance measurement. An absorbance value of 1 at 540 nm, corresponding to a cell density of approximately 10^9 CFU/ml. The bacterial concentration at 10^8 CFU/ml was used as the infective dose as previously described (Khimmakthong & Sukkarun 2017).

Bacillus Inoculum and Feed Preparation

Bacillus subtilis, *Bacillus megaterium*, and *Bacillus licheniformis* were purchased from the Thailand Institute of Scientific and Technological Research (TISTR). These bacterial strains were cultured on nutrient agar (NA) and incubated at 35 °C for 24 hr. Subsequently, starter cultures were prepared by inoculated one loopful of each strain in 40 ml of nutrient broth (NB) with a magnetic stirrer and incubated at room temperature for a day. The mixed culture of *Bacillus* strains was performed by adding 40 ml of each strain in 4 L of minimal medium (MM) and cultured for 36

- 72 hr at room temperature. A pure culture of each strain was prepared by using 120 ml of each strain in 4 L of MM. The total plate counts were performed to determine the number of viable bacterial cells in microbial culture. Either pure culture or mixed culture of these *Bacillus* strains were prepared in 10^7 CFU/ml to supplement in feeding. Feed preparation by adding 5 ml of each bacterial suspension (10^7 CFU/ml) to 10 g of commercial feeds, mixed, and air-dried. The control feeding was only commercial feeds that did not mix with any *Bacillus* strain.

Vibrio parahaemolyticus **Infection Model with** ***Bacillus*** **sp. Treatments**

To investigate the effect of *Bacillus* culture for control severity of AHPND in infected shrimps. All shrimp were infected with *V. parahaemolyticus* AHPND strain as previously described (Khimmakthong & Sukkarun 2017). Briefly, approximately 300 shrimps were immersed in seawater containing *V. parahaemolyticus* AHPND strain at 10^8 CFU/ml for 1 h. After exposure, the shrimp were washed with sterile seawater and placed in new sterile seawater without seawater exchange. The uninfected group, 60 shrimps were no *V. parahaemolyticus* AHPND strain treatment. The infected shrimps were divided into 60 shrimps per tank for 5 tanks and each tank containing 75 L of seawater. The infected shrimps were cultured with normal feed (control+*V.parahaemolyticus*) (tank1), feed supplement with mixed culture of *Bacillus subtilis*, *B. megaterium*, and *B. licheniformis* (BS+BL+BM) (tank2), feed supplement with *B. subtilis* (BS) (tank3), feed supplement with *B. licheniformis* (BL) (tank4), and feed supplement with *B. megaterium* (BM) (tank5). Additionally, the 60 uninfected shrimps were treated with normal feed in tank6 (control-*V. parahaemolyticus*). All tanks were fed 10 g of feeding diet per tank, 3 times a day. After infection, tanks were visually monitored every day. Dead shrimp were collected for DNA extraction to confirm AHPND strain infection by PCR and recorded indicating the time at which mortality occurred. For survival analysis, the shrimps were observed for 22 days after *V. parahaemolyticus* administration, and percent survival was calculated by dividing the number of survival shrimp by the initial number of shrimp x100. At the endpoint of the experiment, all survival shrimps were sacrificed at 22 days after *V. parahaemolyticus* administration. At the time of euthanasia by lacking oxygen, the various tissues (hepatopancreas, muscle, and intestine) were collected for DNA extraction and were fixed with 70% ethanol.

Detection of *V. parahaemolyticus* AHPND strain by PCR

DNA extraction was performed using the boiling method. Briefly, the shrimp tissue was homogenized by micropestle in a 1.5 ml microcentrifuge tube with vortex regularly and 900 µl of TSB supplement with 2 % NaCl were added and incubated at 37 °C for 24-48 hr. Subsequently, aliquoted 700 µl of culture media in a new tube and then centrifuged at 12,000 rpm for 5 min. The cell pellet was collected for the extraction of DNA. The 200µl of distilled water was added to the cell pellet for resuspending. The cell suspension was boiled for 5 min and centrifuged at 12,000 rpm for 5 min. The supernatant was used as a DNA template and stored at -20 °C until used. The DNA extraction of bacterial pure culture was performed by resuspending a single colony in 200 µl of distilled water in a microcentrifuge tube. The bacterial suspension was boiled for DNA extraction as described above. The set of primers used in this study was shown in Table 1. Two primers sets were used to detect the plasmid that was specific for AHPND strain (TUMSAT-Vp1F, TUMSAT-Vp1R, and TUMSAT-Vp3F, TUMSAT-Vp3R), and species-specific primer set, flaE gene or flagella gene in *V. parahaemolyticus* chromosome (Vp-flaE-79F, Vp-flaE-34R) were used as previously described (Tinwongger *et al.* 2014). A tube of PCR reaction (20 µl) included 2 µl of DNA template, 2.4 µl of 10 µM Forward/Reverse Mix primer (Vp-flaE, Vp1, Vp3), 10 µl of 2x PCR Master Mix (i-TaqTM plus, Korea) and 5.6 µl PCR grade-water (Invitrogen, USA). All PCR was conducted under the following condition: initial denature 94 °C for 3 min, then amplified for 29 cycles at 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec. The cycling was terminated at 72 °C for 2 min (Tinwongger *et al*. 2014). PCR products were determined by gel electrophoresis on 1.5% agarose gel with RedSafe (iNtRON, Korea) at 100 V for 30 min. The percent of *V. parahaemolyticus* detection in tissue was calculated as the number of detected tissues divided by the number of all tissue x 100.

Table 1 Primer used in this study.

Primer name	Sequence (5'-3')	Product size (bp)
Vp-flaE-79F	5'-GCAGCTGATCAAAACGTTGAGT-3'	897
Vp-flaE-34R	5'-ATTATCGATCGTGCCACTCAC -3'	
TUMSAT-Vp1F	5'-CGCAGATTTGCTTTTGTGAA -3'	500
TUMSAT-Vp1R	5'-AGAAGCTGGCCGAAGTGATA -3'	
TUMSAT-Vp3F	5'-GTGTTGCATAATTTTGTGCA -3'	360
TUMSAT-Vp3R	5'-TTGTACAGAAACCACGACTA -3'	

The Viability Count of *Vibrio parahaemolyticus* in Tissue

The amount of viable *V. parahaemolyticus* in the tissue of shrimp was performed on TCBS agar. Briefly, the tissues were weighed and grounded by using a micropestle. Ten-fold serial dilution was performed and spread on TCBS agar plates. All plates were incubated at 35 °C for 24-48 hr Only green colonies were counted in colony-forming units per gram of tissue (CFU/g).

Determination of Water Quality Parameter

The water in all experimental tanks was sampled for determination of water quality by pH, alkalinity (mg/L), salinity (ppt), ammonia (mg/L), nitrite (mg/L), and dissolved oxygen (DO) (mg/L) as previously described (Kyeong-Jun *et al.* 2019).

Statistical Analysis

The mean ± SD was used for data presentation. Survival analyses were evaluated with the log-rank test. The statistically significant difference between 2 groups and more than 2 groups were examined by the t-test and one-way analysis of variance (ANOVA) with Tukey's multiple comparisons, respectively. The *p*- values < 0.05 were considered statistically significant. SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS

Decreased Severity of Hepatopancreatic Necrosis Disease in White Shrimp *(Litopenaeus vannamei*) by Mixed Culture of *Bacillus* strain

The infected shrimp were fed with a mixed culture of *Bacillus subtilis, B. licheniformis,* and *B. megaterium* (BS+BL+BM) revealed a high survival rate but infected shrimp without any bacteria contained in the feeding (Control+*V.parahaemolyticus*) show lower survival rate and significant difference in survival rate between these groups were observed (*p*-value <0.0001) (Figure 1A) with indicated that the mixed culture of *Bacillus subtilis* (BS), *Bacillus megaterium* (BM) and *Bacillus licheniformis* (BL) be able to control disease severity of hepatopancreatic necrosis disease in white shrimp by decrease mortality of infected shrimp. However, the survival rate of infected shrimp fed with each strain of *Bacillus* sp**.** shown not different survival rate with

Control+*V.parahaemolyticus* group except *B. Megaterium* (*p*-value 0.0036) and suggested that only one strain of *Bacillus* could not control disease severity and *B. megaterium* maybe play as a key role to control *V. parahaemolyticus* in this infected shrimp model**.** Additionally, all survival shrimps were weighed at the endpoint of the experiment, the result found that the infected shrimps with and without *Bacillus* strain contained in the feeding were not significantly different weights (Figure 1B). However, the weight of uninfected shrimp was higher than all groups of infected shrimps with significant differences (*p*-value <0.0001).

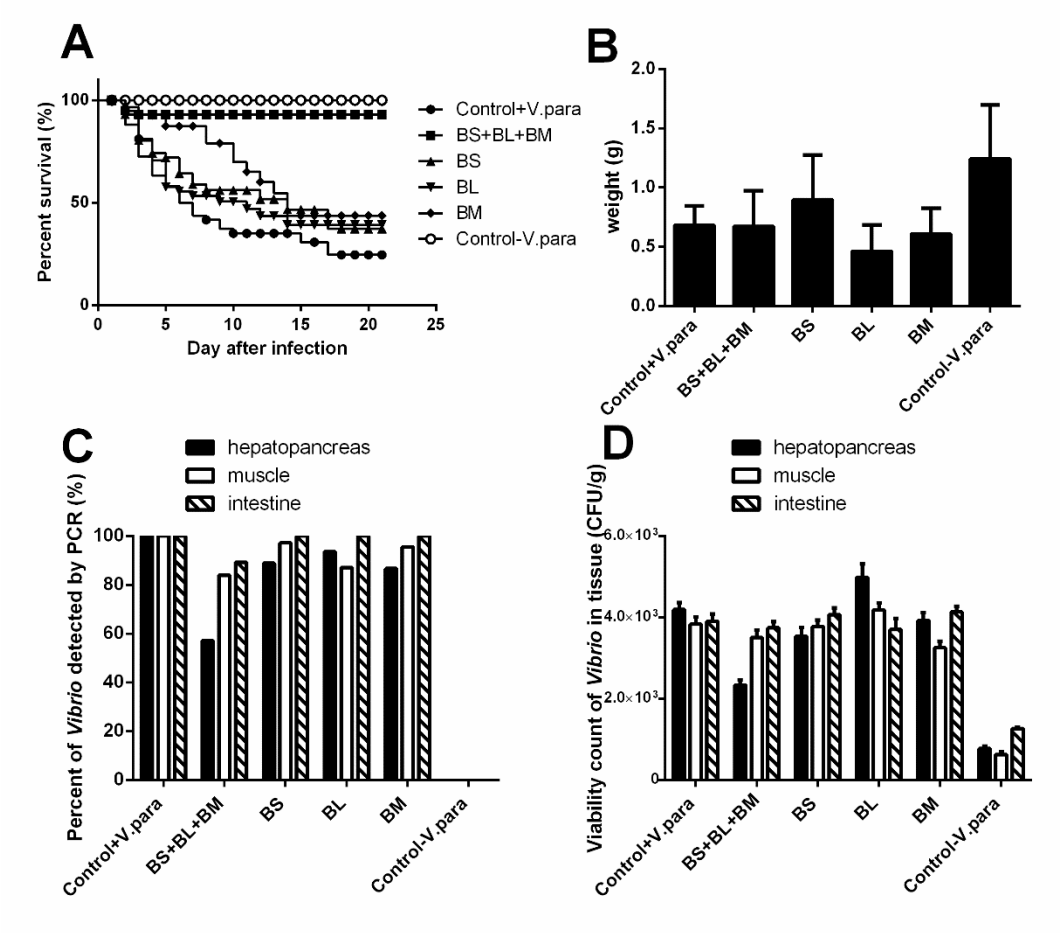

Figure 1. A: percent survival of *Vibrio*-infected shrimps that feeding with various *Bacillus* strain. B: weight of *Vibrio*-infected shrimps that feeding with various *Bacillus* strain after endpoint of the experiment. C: percent of *Vibrio*-detected in various tissue by PCR. D: viability count of *Vibrio* in various tissue.

The Mixed Culture of *Bacillus* strain able to Control Disseminated of *V. Parahaemolyticus* to Hepatopancreas as a Target Tissue of AHPND

To determine the effect of *Bacillus* strain in controlling disseminated of *V. Parahaemolyticus* in shrimp model**.** The hepatopancreas, muscle, and intestine were collected from each infected shrimp and *V. parahaemolyticus* AHPND strain was detected by PCR in tissue**.** All dead shrimp were caused by *V. parahaemolyticus* AHPND strain infection that revealed 3 specific bands consisting of 897, 500, and 360 base pairs (bp) on agarose gel electrophoresis in hepatopancreas (Figure 2). At the endpoint of the experiment, shrimp were sacrificed to determine *V. parahaemolyticus* AHPND strain infection by PCR and the viable count of *Vibrio* sp. in various tissue**.** The infected shrimp without any bacteria contained in the feeding (Control+*V. parahaemolyticus*) reveal all tissues were detected *V. Parahaemolyticus* AHPND strain (Figure 3A and Figure 1C, 1D). The infected shrimp were fed with a mixed culture of *Bacillus* sp**.** (BS+BL+BM) revealed a lower percent of *Vibrio* detection by PCR (57.14%) and a lower amount of *Vibrio* sp. in hepatopancreas (Fig. 3B and Fig. 1C, 1D), while other groups of infected shrimps revealed higher percent of *V. Parahaemolyticus* AHPND detection and amount of viability of *Vibrio* sp. in all tissue (Figure 1C and 1D). These results suggested that aquaculture of infected shrimp with the mixed culture of *Bacillus* spp**.** in feeding (BS+BL+BM) could decrease the severity of AHPND by decreased dissemination of *V. parahaemolyticus* AHPND strain to hepatopancreas which is the target tissue of this disease**.**

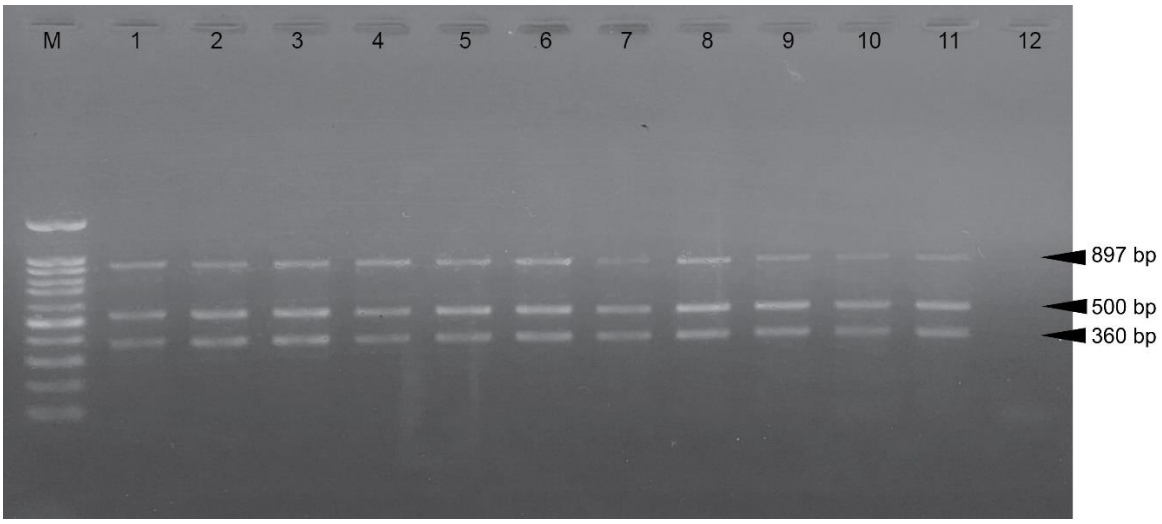

Figure 2. Agarose gel electrophoresis of dead shrimp caused by *V. Parahaemolyticus* AHPND strain infection that revealed 3 specific bands consist of 897, 500, 360 bp from hepatopancreas specimen. Lane M: 100 pb DNA ladder; Lane 1: positive control (DNA from pure culture of *V. parahaemolyticus* AHPND strain); Lane 2: sample 1 from tank1 with normal feed (Control+V.para); Lane 3: sample 2 from tank1 with normal feed (Control+V.para); Lane 4: sample 1 from tank2 was feed supplement with the mixed culture of *Bacillus* strain (BS+BL+BM); Lane 5: sample 2 from tank2 was feed supplement with the mixed culture of *Bacillus* strain (BS+BL+BM); Lane 6: sample 1 from tank3 was feed supplement with *Bacillus subtilis* (BS); Lane 7: sample 2 from tank3 was feed supplement with *Bacillus subtilis* (BS); Lane 8: sample 1 from tank4 was feed supplement with *B. licheniformis* (BL); Lane 9: sample 2 from tank4 was feed supplement with *B. licheniformis* (BL); Lane 10: sample 1 from tank5 was feed supplement with *B. megaterium* (BM); Lane 11: sample 2 from tank5 was feed supplement with *B. megaterium* (BM); Lane 12: negative control (Distilled water).

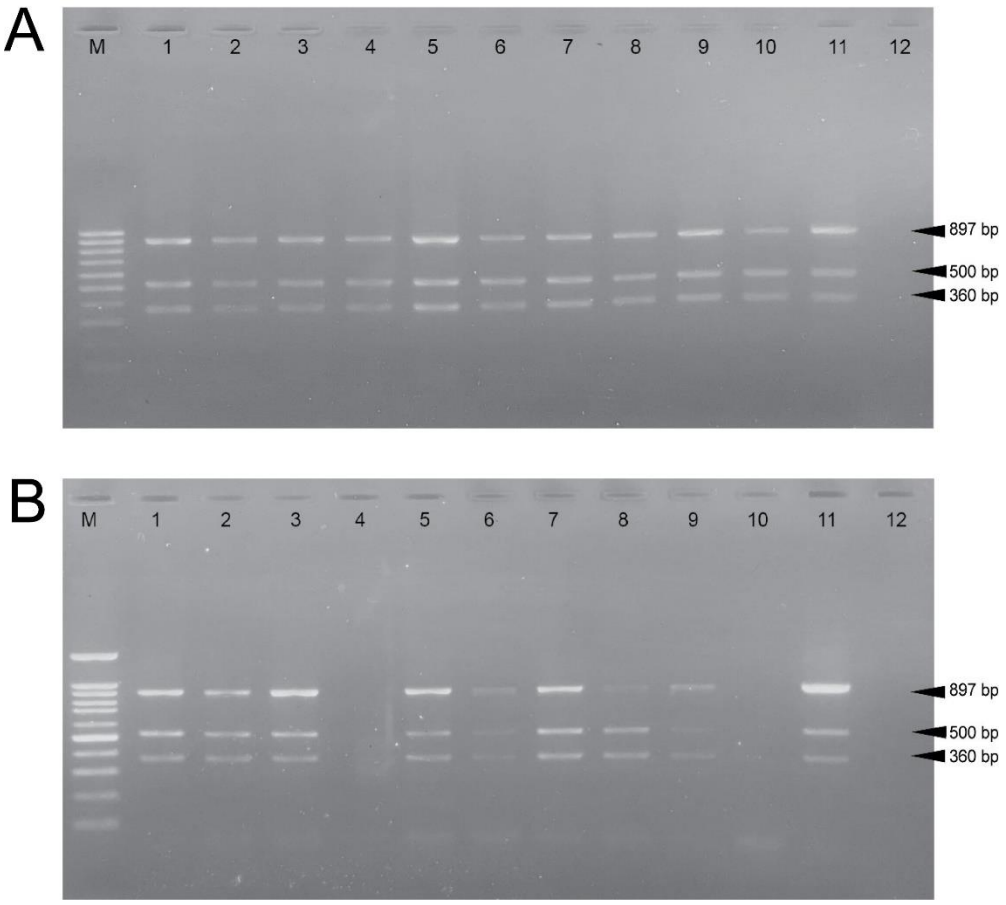

Figure 3. Agarose gel electrophoresis of *V. parahaemolyticus* AHPND strain detection that revealed 3 specific bands consist of 897, 500, 360 bp. 3A: The infected shrimp without any bacteria contained in the feeding (Control+V.para). 3B: The infected shrimp were feed with a mixed culture of *Bacillus* sp. (BS+BL+BM). Lane M: 100 pb DNA ladder; Lane 1: positive control (DNA from pure culture of *V. parahaemolyticus* AHPND strain); Lane 2: hepatopancreas - sample 1; Lane 3: muscle-sample 1; Lane 4: hepatopancreas - sample 2; Lane 5: muscle - sample 2; Lane 6: hepatopancreas - sample 3; Lane 7: muscle - sample 3; Lane 8: hepatopancreas - sample 4; Lane 9: muscle - sample 4; Lane 10: hepatopancreas - sample 5; Lane 11: muscle - sample 5; Lane 12: hepatopancreas – uninfected sample (Control-V.para).

The Water Quality Did Not Affect the Survival of Infected Shrimp During the Experiment

To determine whether the water quality may affect the survival of *Vibrio*-infected shrimp. The pH, alkalinity, salinity, ammonia, nitrite, and dissolved oxygen (DO) were investigated (Figure 4). The results found that the range of pH, alkalinity, salinity, ammonia, nitrite, and DO was 8.0-8.1 (Figure 4A), 112-155 mg/L (Figure 4B), 27-33 ppt (Figure 4C), 0.000-0.053 mg/L (Figure 4D), 0.000-0.280 mg/L (Figure 4E), and was 5.0-6.0 mg/L (Figure 4F), respectively. These results were within acceptable values.

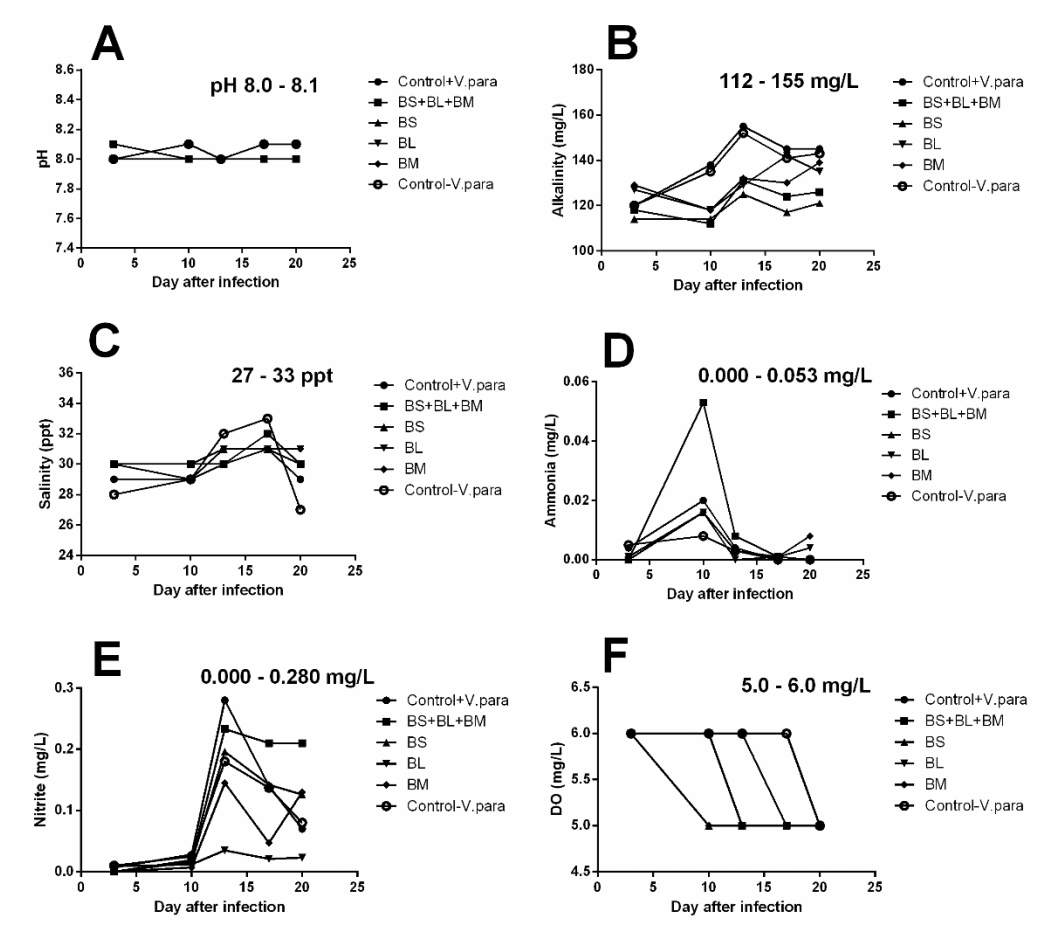

Figure 4. Water quality during the experiment. A: pH, B: Alkalinity, C: Salinity, D: Ammonia. E: Nitrite. F: Dissolved oxygen (DO)

DISCUSSION

The effect of *Bacillus subtilis, B. licheniformis*, and *B. Megaterium* to control disease severity of AHPND caused by *Vibrio parahaemolyticus* infection were investigated in white shrimp as a model**.** White shrimp *(Litopenaeus vannamei)* were infected with *V. Parahaemolyticus* AHPND strain, subsequently, were feed with a diet supplemented with different *Bacillus* strain and their mixed culture**.** The infected shrimp that was feed with a mixed culture of *Bacillus* strain (*B. subtilis* + *B. licheniformis* + *B. megaterium*), revealed a higher survival rate than those that were feed with a diet containing each of *Bacillus* strain and non-supplemented control group. This result was supported by the previous study that *B. subtilis, B. licheniformis,* and *B. Megaterium* were found to inhibit growth and toxin production of *Vibrio harveyi* resulting in a higher survival rate of shrimp with a diet containing these probiotics than those a control group with non-supplemented (Nakayama *et al.* 2009). The mechanism of this result may be associated with AHPND pathogenesis of the model in this study.

A previous study by Khimmakthong and Sukkarun (2017) investigated the *Vibrio parahaemolyticus* dissemination in the tissue of *Litopenaeus vannamei*, they found *V. parahaemolyticus* in the gills, hepatopancreas, intestine, muscles, and hemolymph. Later, after 6 h of infection found only small amounts of this pathogen in the hepatopancreas and intestine with abnormal histopathology. This study suggested that *V. Parahaemolyticus* could spread quickly by using the hepatopancreas as the target tissue (Khimmakthong & Sukkarun 2017). Similarly, the present study investigated the dissemination of *V. parahaemolyticus* AHPND strain in the presence of a diet supplemented with different *Bacillus* strain and their mixed culture in a shrimp model**.** At the endpoint of the experiment (22 days after infection), the infected shrimp without any bacteria contained in a diet (Control+*V. parahaemolyticus*) demonstrated the hepatopancreas, intestine, and muscle could be detected *V. Parahaemolyticus* AHPND strain with 100 % detection by PCR (Figure 1C) and revealed the large amount of *Vibrio* spp. viability in all tissues (Figure 1D). Interestingly, the infected shrimp were fed with a diet supplemented with the mixed culture of *Bacillus* sp. (*B. subtilis* + *B. licheniformis* + *B. megaterium*) shown a lower percent of *Vibrio* detection by PCR (57.14%) (Figure 1C) with a small amount of *Vibrio* in hepatopancreas while other groups of infected shrimps reveal higher percent of *Vibrio* detection with a large amount of *Vibrio* viability in all tissues (Figure 1D). These results suggested that aquaculture of infected shrimp with the mixed culture of *Bacillus* spp**.** (*B. subtilis* + *B. licheniformis* + *B. megaterium*) in a diet could decrease the severity of AHPND

by decreased dissemination of *V. parahaemolyticus* AHPND strain to hepatopancreas which is the target tissue of this disease. Additionally, the infected shrimps with a diet supplemented *Bacillus* strain and the non-supplement groups were not significantly different in weight. In contrast, the weight of uninfected shrimp was higher than all groups of infected shrimps with significant differences (*p*-value <0.0001). This result suggested the disease progression in the infected shrimps affected the function of their digestive tract and result in weight loss.

Kyeong-Jun *et al.* (2019) demonstrated the effect of dietary supplementation of three *Bacillus* spp. consist of *B. subtilis, B. pumilus,* and *B. licheniformis* on growth performance and disease resistance of *L. vannamei*. They found that shrimp fed with a diet supplemented with only *B. subtilis* had significantly higher growth performance than those feed non-supplement or supplemented with the mixed culture of *B. subtilis* and *B. pumilus* or the mixed culture of three strains (Kyeong-Jun *et al.* 2019). However, different *Bacillus* strains in the mixed culture maybe affect the shrimp growth and survival. Recently, Nguyen *et al.* (2021) proposed that *Bacillus subtilis* DSM33018 strain was shown to degrade AHPND toxins *in vitro,* as detected by Western blots and $PirB^{VP}$ toxin is more susceptible to degradation by this *Bacillus* strains than $PirA^{VP}$ (Nguyen *et al.* 2021). Similarly, the previous study explained the properties of *B. subtilis* which is an exoenzyme producing bacteria such as protease and amylase that could digest the mucus coated the gram-negative bacterial pathogen. Additionally, *B. subtilis* could produce some antimicrobial molecules that destroy the cell structure of the pathogen leading to inhibition of growth (Vaseeharan & Ramasamy 2003). Moreover, a diet supplemented with *B. subtilis* demonstrated the enhancing of growth and immune response in *L. vannamei* (Shen *et al.* 2010). In contrast, in this present study, the infected shrimp with a diet supplemented only *Bacillus subtilis* (BS) shown lower percent survival with higher percent *Vibrio*-detected by PCR and higher number of viability count of *Vibrio* spp. than those supplemented with the mixed culture.

Although a diet supplemented with each strain of *Bacillus* sp**.** including *Bacillus subtilis, B. licheniformis* revealed no significantly different in percent survival with Control+*V.parahaemolyticus* group, except *B. megaterium* contained a diet (*p*-value 0.0036). In addition, it seems to slowly decrease the percent survival when compared to each *Bacillus* strain in this study (Figure 1A). This result suggested that only one strain of *Bacillus* in this study could not control disease severity and *B. Megaterium* maybe play a key role to control *V. Parahaemolyticus* in this infected shrimp model**.**

B. megaterium could be isolated from the digestive tract of *L. vannamei* and was reported the properties of the extracellular enzyme (protease, amylase, lipase) with high antimicrobial production. The *in vivo* study evidenced that a diet supplemented with *B. megaterium* BM1 strain could be beneficial for growth of *L. vannamei* by giving a significantly higher specific growth rate compared to other diets (Yuniarti *et al.* 2013). This evidence suggested that *B. megaterium* could be better adaptive in the digestive tract of *L. vannamei* and had beneficial properties as described above.

Additionally, *B. licheniformis* was reported the beneficial effects with *L. vannamei* by the number of *Vibrio* spp. was significantly decreased after administration of *B. licheniformis* in *L. vannamei* and revealed improved immune that indicated by haemocyte, phenolxidase, and superoxide dismutase were significantly higher than those the control (Li *et al.* 2007). Similarly, Fan *et al.* (2021) reported that pathogen susceptibility and immune suppression in shrimp are caused by nitrite stress which is one of the pollutants commonly found in aquaculture water. They indicated that after nitrite stress of *L. vannamei*, a diet supplemented with *B. licheniformis* revealed improved weight, growth rate, and survival rate (Fan *et al.* 2021)

Although, *Bacillus subtilis, B. licheniformis*, and *B. megaterium* reported beneficial properties to *L. vannamei*, however, the result of the present study shows a significant difference in percent survival of infected shrimps fed with a diet supplemented with *B. megaterium* only and their mixed culture. However, the mixed culture of these *Bacillus* strains resulted in higher percent survival of infected *L. vannamei*, the mechanism of the mixed culture of *Bacillus subtilis, B. licheniformis*, and *B. Megaterium* in controlling *V. Parahaemolyticus* AHPND strain in shrimp was required for further exploration of their interaction.

In addition, several studies evident that the genus of *Bacillus* is not only used as an effective probiotic but also used for the treatment of organic waste in aquaculture environments to reduce the risk factor of *V. parahaemolyticus* AHPND strain infection (Kumar *et al.* 2016; Hlordzi *et al.* 2020; Liu *et al.* 2009; Nakayama *et al.* 2009). *Bacillus subtilis, B. licheniformis*, and *B. megaterium* have reported a role in water quality including Biochemical oxygen demand (BOD), Dissolved oxygen (DO), ammonia, alkalinity, and pH (Elsabagh *et al.* 2018; Reddy *et al.* 2018; Cha *et al.* 2013) that demonstrated by the better quality of water in the pond (Kumar *et al.* 2016, Hlordzi *et al.* 2020). The present study used a diet supplemented *Bacillus* strain to feed infected shrimp, several factors were measured for monitoring water quality during the experiment. The results found that the range of pH, alkalinity, salinity, ammonia, nitrite, and dissolved oxygen (DO) were within acceptable values.

CONCLUSION

The present study concluded that a diet supplemented with the mixed culture of *Bacillus subtilis*, *B. licheniformis*, and *B. megaterium* could decrease AHPND severity in white shrimp (*L. vannamei*). This mixed culture was supported by higher percent survival, lower percent of *Vibrio* AHPND strain detected by PCR, and a small amount of *Vibrio* sp. viability count in hepatopancreas than those other groups of infected shrimps. These results suggested that the mixed culture of *Bacillus* spp. in a diet could decrease the severity of AHPND by reduced dissemination of *V. parahaemolyticus* AHPND strain to hepatopancreas which is the target tissue of this disease. However, further study about the mechanism of the mixed culture from these *Bacillus* strains interacts with *V. parahaemolyticus* AHPND strain in shrimp was required for understanding their interaction.

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AUTHOR CONTRIBUTIONS

S.S., K.S., W.P., C.K., and P.L. designed experiments. S.S., K.S., C.K., P.K. and A.K. performed experiments. S.S., K.S., C.K., and P.K. prepared the manuscript. All authors discussed the results and commented on the manuscript.

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