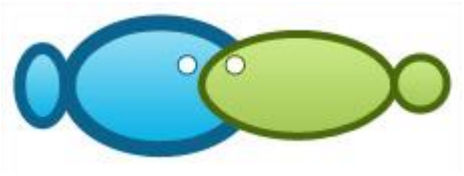


Isolation of potential probiotic *Bacillus subtilis* CM3.1 and its effects on the water quality and growth performance of the whiteleg shrimp *Litopenaeus vannamei* in the Mekong Delta, Vietnam

Quoc P. Truong, Thi C. T. Phan, Hung H. Vu, Thi T. N. Pham, Truong G. Huynh, Ngoc U. Vu

College of Aquaculture and Fisheries, Can Tho University, Can Tho City, Viet Nam.
Corresponding author: N. U. Vu, vnut@ctu.edu.vn

Abstract. The study aimed to screen the probiotic potential of the *Bacillus subtilis* isolated from intensive shrimp ponds and to assess its effect on the whiteleg shrimp (*Liptopenaeus vannamei*). Based on an initial screening of 83 isolates identified from sediment samples, 13 strains showed antibacterial activity and 3 of them (CM3.1, CM2.2 and TV1.2) were further screened for their ability to control the pathogen *Vibrio parahaemolyticus*. All three strains exhibited notable amylase, protease and cellulase activities, but the strain CM3.1, identified as *B. subtilis* CM3.1, showed the highest extracellular activities and thus was used for further studying. The water supplemented with 10^2 (T1), 10^3 (T2), and 10^4 (T3) CFU mL^{-1} of this strain was significantly reduced in concentrations of the total ammonia nitrogen (TAN) and nitrite-nitrogen (NO_2^--N). No significant differences were found between the control and treatment groups in final weight, weight gain and daily weight gain of the shrimp ($p>0.05$). However, a significant difference in the specific growth rate and survival rate was found between the control and the T3 treatment ($p<0.05$). Water supplemented with *B. subtilis* CM3.1 was found to be improved in quality, with lower TAN and NO_2^--N concentrations, and provided an enhancement of the survival rate and of the specific growth rate of shrimps.
Key Words: aquaculture, antibacterial, extracellular enzymes, sediment, survival.

Introduction. Whiteleg shrimp, *Litopenaeus vannamei*, is an economically important and widely cultured aquaculture species worldwide, and nowadays it is the most common cultured shrimp species in the Mekong Delta, Vietnam. According to VASEP (2020), whiteleg shrimp production increased rapidly by approximately 41% after 5 years, with an average increment of 9% per year. However, in the recent years, its production has been significantly reduced by disease problems, the most severe being the acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio parahaemolyticus*. Massive loss of shrimp was recorded in the Mekong Delta in 2012, due to an outbreak of AHPND (Oanh et al 2012) that has continued to date. The widespread use of antibiotics, chemicals and drugs to combat shrimp diseases has caused serious environmental and disease management problems (Srinivasan & Ramasamy 2009). Therefore, alternative methods must be developed to control harmful pathogens and presently probiotics are widely used in shrimp aquaculture to control infectious diseases, enhance growth and improve water quality without any side effects (Balcazar et al 2008).

Of the many possible probiotics, *Bacillus* and *Lactobacillus* species are the most commonly selected candidates for aquaculture species (Yi et al 2018). *Bacillus* species are commonly found in sediment and water and can also adhere to the digestive tract of shrimp (Kesarcodi et al 2008). In addition, the genus *Bacillus* can produce many extracellular compounds to improve feed digestion and absorption, and to combat pathogenic bacteria (Yilmaz et al 2006; Wang 2007; Liu et al 2009). Several previous

studies have demonstrated that *Bacillus* species have the ability to enhance growth performance, immune system function, disease management and water quality in shrimp aquaculture (Liu et al 2010; Arisa et al 2015; Kewcharoen & Srisapoome 2019).

The present study aimed to isolate and identify potential probiotic *Bacillus* spp. from sediments in shrimp ponds and determine their antimicrobial activity, their efficacy against *V. parahaemolyticus* and their extracellular enzyme production under *in vitro* experimental conditions. The isolate *Bacillus subtilis* CM3.1 obtained in this study shows strong potential as a probiotic candidate. Therefore, this strain was selected to perform in vivo experiments to evaluate the effects of water supplementation of *B. subtilis* CM3.1 on the water quality and growth performance of the *L. vannamei*. The results of this study could be used as a reference for application in the shrimp culture industry.

Material and Method

Isolation and identification of bacteria. Sediment samples with an average weight of 100 g were collected from extensive and semi-extensive shrimp ponds located in Tra Vinh, Soc Trang, Bac Lieu and Ca Mau provinces. All samples were stored at 4ºC in a cool box and transported to the laboratory for further processing. *Bacillus* spp. were isolated using the procedure of Boottanun et al (2017) with slight modifications. Briefly, 1 g of each sediment sample was homogenized in 9 mL of sterile saline solution (0.9% NaCl) and was serially diluted 10-fold. The tubes were placed in a water bath at 80°C for 20 min. Then, the tubes were cooled to room temperature and 100 µL of aliquots of each dilution were aseptically spread onto Nutrient Agar (NA) plates supplemented with 1.5% NaCl. The plates were incubated at 28°C for 24 h. After incubation, individual colonies were picked up and purified by the streaking method on a fresh plate. All the isolates were identified and stored at -80°C in nutrient broth (NB) supplemented with 20% glycerol for further experiments.

The genomic DNA of *Bacillus* isolate was extracted using a genomic DNA purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction and stored at -20^{o}C until use for sequence analysis. The 16S rRNA gene was amplified using the primer 16S–F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-R (5'-TAC GGTTACCTTGTTACGACTT-3') (Weisburg et al 1991). PCR products were detected using 1.5% agarose gel electrophoresis. The purified products were sequenced by the Nam Khoa Biotek company, Ho Chi Minh City, Vietnam. The sequences of the 16S rRNA gene from the isolates were compared with the available sequence of *Bacillus* species in the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov).

Antimicrobial activity. The antimicrobial activity of the *Bacillus* isolates was studied against a targeted shrimp pathogen (*V. parahaemolyticus,* VP_{AHPND}) by a cross-streak technique according to Chythanya et al (2002). In brief, *Bacillus* isolates and VP_{AHPND} were grown in Tryptone Soy Broth (TSB) supplemented with 1.5% NaCl. Following overnight incubation at 30°C, the bacteria cell density was approximately adjusted to 10^6 CFU mL^{-1} with sterile saline solution. Mueller Hinton Agar (MHA, HiMedia Laboratories, India) plates were prepared and inoculated with various species of *Bacillus* (approximately 10^6 CFU mL^{-1}) by a single streak of inoculum in the center of the plate and then incubated at 30°C for 24 h. Then the plates were seeded with indicator bacteria by a single streak at a 90° angle to the *Bacillus* isolates and were incubated further at 30°C for 24 h. The microbial interactions were analyzed by determining the size of the inhibition zone (Lorian 1995).

Co-culture assay. Three isolates (CM3.1, CM2.2 and TV1.2) that exhibited high antimicrobial activity were selected for this experiment. The co-culture of these isolates and pathogenic bacterium (VP_{AHPND}) was evaluated on TSB medium using the protocols previously described by Kewcharoen & Srisapoome (2019). Briefly, 100 mL of TSB were distributed in an Erlenmeyer flask and autoclaved at 121°C for 20 min. The broth was inoculated with 1 mL of each isolate and 1 mL of an indicator bacteria (VP_{AHPND}) with an

inoculation rate of 10^5 CFU mL^{-1}, followed by incubation under agitation at 30°C. Positive controls of each *Bacillus* isolate and pathogenic bacterium were conducted separately with similar conditions. The number of bacteria was evaluated using NA agar for *Bacillus* spp. and TCBS agar for *Vibrio* spp. For enumeration of *Bacillus* spp., the samples were heated at 80°C for 20 min before being spread on TSA plates (Di et al 2019). The results were recorded at 0, 24, 48, 72, 96, and 120 h. The experiment was conducted in triplicate.

Extracellular enzyme assay. The selected strains (CM3.1, CM2.2 and TV1.2) were cultured in TSB medium supplemented with 1% NaCl. After 24 h of incubation, cultures were centrifuged at 3,000 rpm for 10 min at 4°C, and the cell-free supernatant (CFS) was collected to determine the extracellular alpha-amylase, cellulase and protease production. The *Bacillus* isolates were tested by the alpha-amylase assay using the method described by Bernfeld (1955), with slight modifications. Briefly, 1 mL of CFS of *Bacillus* isolates was added to 1 mL of 1% soluble starch in a test tube and incubated at 37°C for 20 min. After that, 2 mL of 3,5-Dinitrosalicylic acid (DNS) reagent was added to each tube to stop the reaction and then placed in a boiling water bath for 5 min. Then, the tubes were cooled and the volume was made up to 10 mL using distilled water. The absorbance was read at 540 nm in a spectrophotometer. Blanks were obtained by adding DNS reagent prior to incubation. The amylase activity was expressed as 1 mg of maltose produced per min at 37°C. To determine the extracellular protease production, 100 μL of CFS of *Bacillus* isolates was added to 100 μL of a 1% casein solution (prepared in Tris-HCl buffer, pH 7.0). The reaction of the mixture was incubated for 10 min at 37°C. 500 μL of 5% (v/v) trichloroacetic acid were then added to stop the reaction (Huynh et al 2018). After 20 min, it was centrifuged at 3,000 rpm and 4°C for 20 min, and the optical density of the supernatant was measured by a modified Lowry's method (Lowry et al 1951). One unit of protease was equivalent to the amount of enzyme required to release 1 μg mL^{-1} min^{-1} of tyrosine, under standard assay conditions. Cellulase activity was conducted according to the method described by Ghose (1987). In brief, the reaction mixture contained 0.5 mL of 1% sodium carboxymethyl cellulose solution (prepared in 0.05 M citrate buffer; pH 5.0) and 0.5 mL of the crude enzyme solution, and was incubated at 50°C for 30 min. After incubation, 1.5 mL of DNS solution was added to stop the reaction and the test tube was kept in a boiling water bath for 10 min. The absorbance was read at 540 nm in a spectrophotometer. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmoL of glucose per minute under the standard assay conditions.

Safety evaluation of probiotic isolates. Based on the screening results of the antimicrobial activity and on the extracellular enzyme assays, the *Bacillus* strain CM3.1 was chosen to evaluate its safety for use with shrimp. Two experimental groups (a control group, without probiotic supplement, and a probiotic-added group exposed to 5×10^8 CFU CM3.1 mL^{-1}) were tested in triplicate. Juvenile shrimp were obtained from a local commercial hatchery located in Can Tho City, Vietnam. Shrimp were cultured to an average weight of 5 g before being used for this experiment. A total of 60 healthy *L. vannamei* were randomly allocated into six 10 L glass tanks. Each tank contained 10 shrimp in 5 L brackish water (15‰, 29±1℃). Shrimp were fed with commercial pellets (40% protein) at 3% body weight per day for seven days. One hundred percent of the water was replaced with fresh brackish water on a daily basis. Then, 5 mL of bacterial suspension ($5x10^8$ CFU mL^{-1}) were added into a probiotic-added group, while 5 mL of sterile saline was added to the control group (Yun et al 2019). After 7 days of culture, the survival rate of the shrimp was recorded.

Application of a selected probiotic bacteria to improve water quality and the growth of L. vannamei. The effects of the probiotic *B. subtilis* CM3.1 on *L. vannamei* juvenile were evaluated in this experiment by adding the probiotic to the rearing water. *L. vannamei* specimens were obtained from a private shrimp hatchery and were acclimatized for a week. After acclimatization, 100 shrimps (0.5±0.05 g) were randomly

stocked into each tank, with a capacity of 500 L. Four concentrations of probiotic were tested: 0 (control), 10^2 (T1), 10^3 (T2), and 10^4 CFU mL^{-1} (T3). For treatments T1, T2 and T3, the probiotic was added once a week from the start to the end of the experiment. Each treatment was run in triplicate. During the experiment, the shrimps were fed three times a day, with a commercial diet, at a rate of 3-5% of the body weight and the water was not exchanged. However, tanks were siphoned daily to remove uneaten food and faeces, and dead shrimps were removed to prevent cannibalism. Water quality parameters, such as temperature, pH and dissolved oxygen (DO) were determined daily using HI9828 (Hanna, Romania). Alkalinity, total suspended solids (TSS), nitrite (NO_2^--N) and total ammonium nitrogen (TAN) were analyzed weekly using the standard methods of APHA (2017). Total *Bacillus* and *Vibrio* counts in the culture water were analyzed every 7 days using the same protocols as described above. The experiment lasted for 56 days. At the end of the experiment, the survival rate, final weight, weight gain (WG), daily weight gain (DWG) and specific growth rate (SGR) for all groups were calculated using the following equations:

$$\text{Survival rate (\%)} = (\text{Final numbers} / \text{Initial numbers}) \times 100$$

$$\text{Weight gain (g shrimp}^{-1}) = \text{Final weight (g)} - \text{Initial weight (g)}$$

$$\text{Daily weight gain (g day}^{-1}) = (\text{Final weight} - \text{Initial weight}) / \text{number of days}$$

$$\text{Specific growth rate (\% day}^{-1}) = ([\ln \text{final wt} - \ln \text{initial wt}] / \text{number of days}) \times 100$$

Statistical analysis. Data were presented as mean ± standard error (SE). All data were analyzed using one-way analysis of variance (ANOVA), and the Tukey's test with multiple comparisons was then performed to identify the significant differences between treatment groups. A significant difference was found among treatments when the p-value was less than 0.05. For the antibacterial activity index, however, a significance level of 0.01 was applied. Statistical analyses were conducted using the SPSS software (version 20.0).

Results

Isolation and identification. A total of 83 *Bacillus* isolates were recorded from the whiteleg shrimp ponds and a preliminary screening was applied using NA agar. Most of the colonies were round, 0.3-3.1 mm in diameter, with domed or of flat elevation, with irregular or entire margin, and of a creamy white color (Figure 1). Of the 83 strains isolated, 44 were Gram-positive bacilli, rod-shaped (short rod accounted for 50.6% and long rod made up 20.5%), motile, spore-forming, catalase-positive and oxidase-positive. During the secondary screening, only 13 isolates (BT1.2, CM2.2, CM3.1, CN1.3, DH2.1, NH5.1, NH5.2, TN2.2, TN5.1, TN5.3, TV1.2, TV3.1, and TV3.3) exhibited effective colonization and strong effects against the target shrimp pathogens (Figure 2). The strain CM3.1 had the highest antagonistic ability and enzyme activity, and was selected for further study. The result from 16S rRNA gene sequences analysis revealed 99.9% identity with *B. subtilis* for CM3.1.

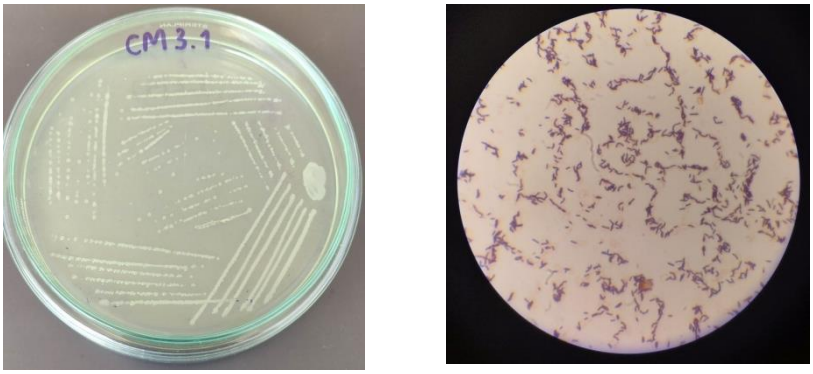


Figure 1. Colony morphology and gram-staining property of *Bacillus* colonies on NA medium.

Antimicrobial activity. Antimicrobial activities of 13 *Bacillus* isolates against *V. parahaemolyticus* (VP_{AHPND}) are shown in Figure 2. The three isolates, CM3.1, CM2.2 and TV1.2 showed inhibition zones of 13.05±0.35, 12.5±0.3 and 9.90±0.2 mm in diameter against VP_{AHPND}, respectively. CM3.1 and CM2.2 isolates displayed significantly higher antagonistic effects against VP_{AHPND} compared to the others ($p<0.01$). However, there were no significant differences in the antimicrobial activity between CM3.1 and CM2.2 ($p>0.01$). The lowest antagonistic effect against VP_{AHPND} was recorded for CN1.3 and TN 5.1.

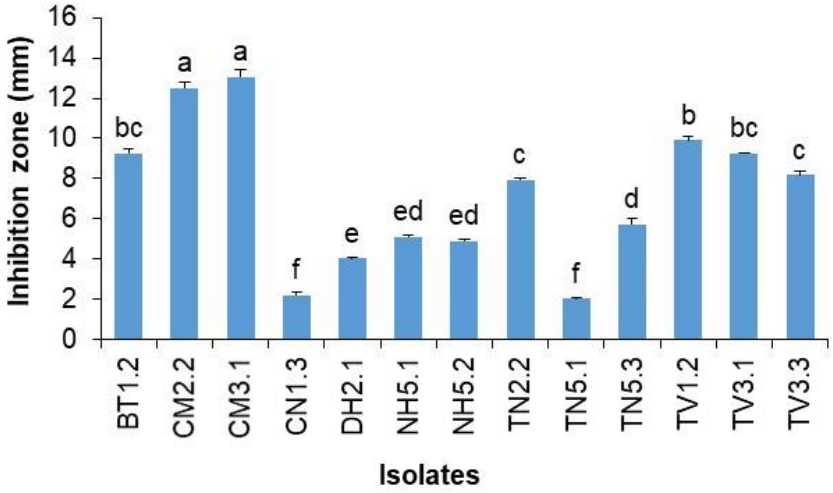
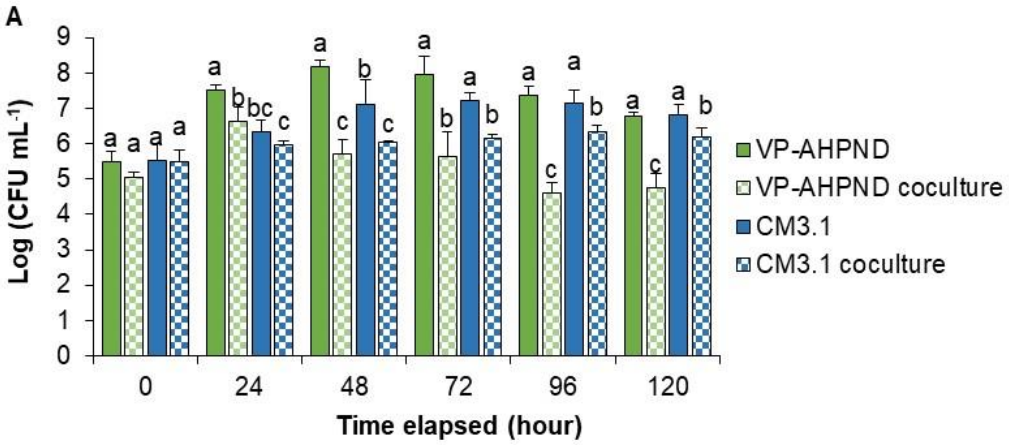


Figure 2. Inhibition zone of *Bacillus* isolates towards *Vibrio parahaemolyticus*. The different letters on each bar indicate significant differences among treatments ($p<0.01$).

Co-culture assay. The *Bacillus* isolates (CM3.1, CM2.2 and TV1.2) were evaluated under co-cultured conditions. The results showed that all these strains could effectively inhibit the target pathogen (VP_{AHPND}). The number of CM3.1 and CM2.2 colonies was significantly greater than that of VP_{AHPND} colonies after 96 to 120 h, at the end of the trial ($p<0.05$) (Figure 3A and 3B). Although the number of TV1.2 colonies was higher than that of VP_{AHPND} colonies after 96 h and 120 h in co-culture, no significant difference was found between them ($p>0.05$) (Figure 3C).



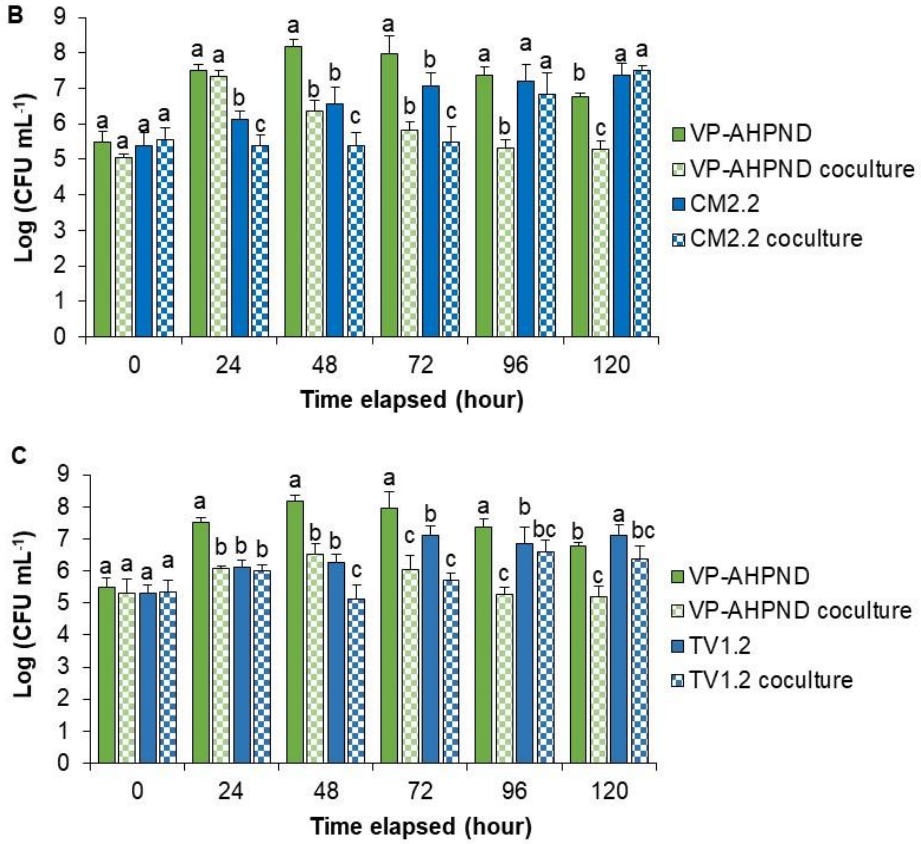


Figure 3. The inhibitory ability of *Bacillus* strains CM3.1 (A), CM2.2 (B) and TV1.2 (C) against *Vibrio parahaemolyticus* was indicated by co-culture conditions in TSB broth medium. Values are presented as mean ± SE. The lowercase letters on each bar indicate significant differences among treatments ($p<0.05$).

Extracellular enzyme production. Extracellular enzyme production of the bacterial strains including CM3.1, CM2.2 and TV1.2 are shown in Table 1. The amylase activity of CM3.1 was significantly higher than in CM2.2 and TV1.2 ($p<0.05$), while no significant difference in the amylase activity was found between CM2.2 and TV1.2 ($p>0.05$). Likewise, CM3.1 had a significantly higher cellulase activity than the other two isolates ($p<0.05$). There was no significant difference in the protease activities among the 3 isolates.

Table 1

Extracellular enzyme activities of the three *Bacillus* isolates

No.	*Bacillus isolates*	*Enzyme (U mL^{-1})*		
		α-amylase	*Protease*	*Cellulase*
1	CM3.1	153.7±8.2^{a}	141±7.1^{a}	658.7±30.2^{a}
2	CM2.2	32.5±8.9^{b}	140.4±8.9^{a}	25.8±7.7^{c}
3	TV1.2	18.3±2.8^{b}	129.8±3.5^{a}	180.1±35.3^{b}

Values shown are mean ± SE. Mean values within a column followed by the same letters show that there is no significant difference among the groups ($p>0.05$).

Safety evaluation of probiotic isolates. After 7 days of culture, the survival rate of the shrimp in the control and probiotic supplement treatments was of 83.3% and 100%, respectively. In addition, no symptoms of disease or unusual swimming activity were observed in the probiotic supplement group. This suggested that the *B. subtilis* CM3.1 strain would not harm *L. vannamei*.

Water quality and bacteriological analysis. No significant difference was observed in temperature, pH, DO, alkalinity and TSS among treatments from the beginning until the

end of the trial (p>0.05). The values of these parameters were 27.8-29.4 (temperature), 7.5-8.2 (pH), 4.5-5.5 mg L^{-1} (DO), 96-142 mg$CaCO_3$ L^{-1} (alkalinity) and 50-240 mg L^{-1} (TSS). However, TAN concentration in the control was significantly higher than that of T1, T2 and T3 at all-time intervals (p<0.05), except day 1 and day 35 (Figure 4). Similarly, the concentration of NO_2^--N in the control was also significantly higher than in the other groups on day 35 and 42 (p<0.05) (Figure 5).

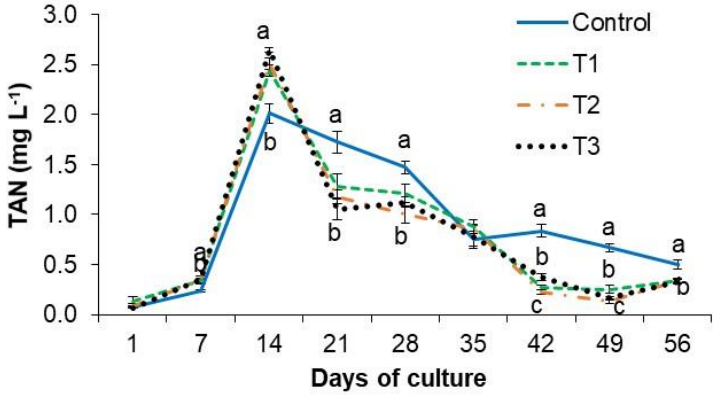


Figure 4. Changes in the level of TAN in different experimental groups.

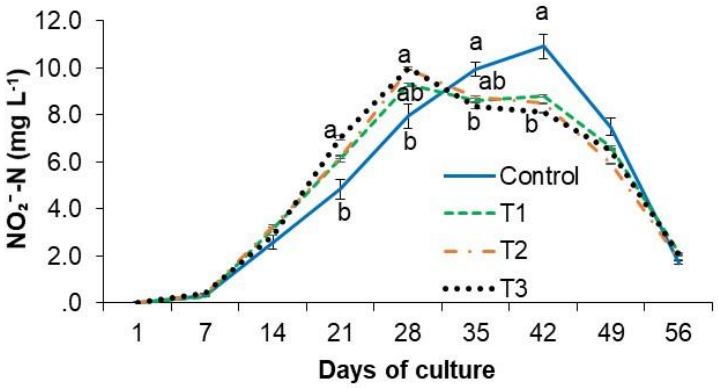


Figure 5. Changes in the level of NO_2^--N in different treatments.

Total *Bacillus* counts in the control were significantly lower than that in other groups during the experiment period (p<0.05). On the last day of the trial, the total *Bacillus* counts in the control, T1, T2, and T3 were 3.105, 3.512, 3.640 and 3.841 Log CFU mL^{-1}, respectively (Figure 6). As for total *Vibrio* counts, T3 group had significantly fewer *Vibrio* than the control group in most sampling periods (Figure 7).

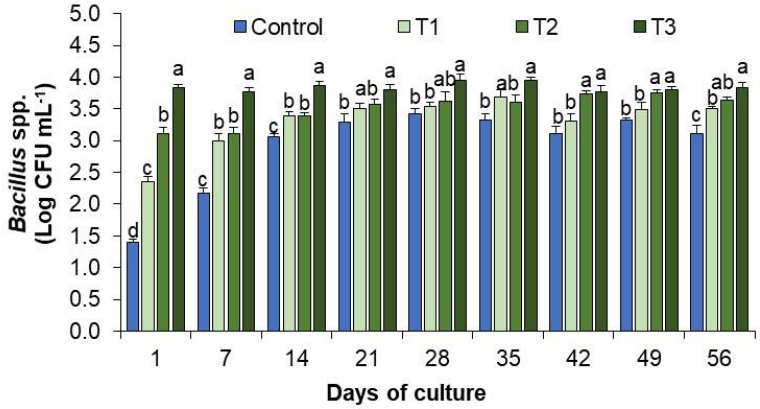


Figure 6. Total number of *Bacillus* count in different experimental groups. The lowercase letters on each bar indicate significant differences among treatments (p<0.05).

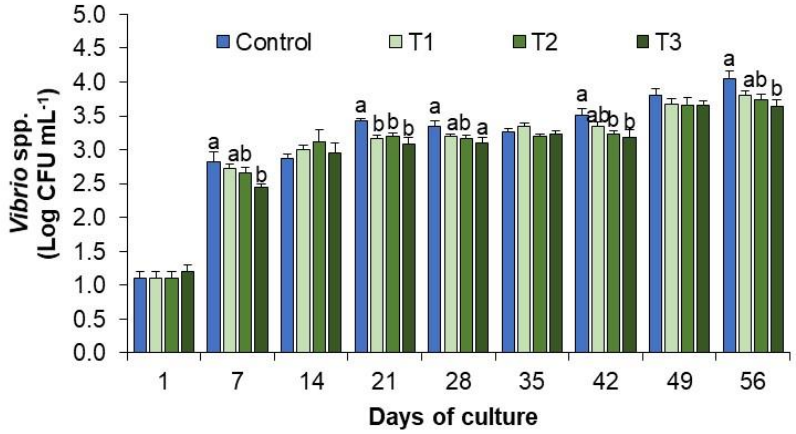


Figure 7. Total number of *Vibrio* count in different experimental groups. The lowercase letters on each bar indicate significant differences among treatments ($p<0.05$).

Growth performance. The growth performance and survival rate of shrimp in all treatments are presented in Table 2. There were no significant differences in the final weight, WG and DWG between the control and T1, T2 and T3 groups ($p>0.05$). The specific growth rate increased marginally with increasing levels of CM3.1 added to the culture tanks, but only the specific growth rate in treatment T3 was significantly higher than that of the control treatment (Table 2). However, the statistical analysis showed that shrimp in T3 grew significantly faster than those in the control ($p<0.05$). Similarly, the survival rate of shrimp in T3 (68.7±3.2%) was significantly higher than that of the control (57.7±2.8%) ($p<0.05$). There was no significant difference in the survival rate between the control and probiotic supplement groups (T1 and T2) ($p>0.05$).

Table 2

Growth performance and survival of *Litopenaeus vannamei* cultured with the probiotic *Bacillus subtilis* CM3.1

Parameters	*Treatments*			
	Control	*T1*	*T2*	*T3*
Initial weight (g)	$0.59±0^{a}$	$0.59±0.01^{a}$	$0.58±0.01^{a}$	$0.57±0.01^{a}$
Final weight (g)	$6.22±0.07^{a}$	$6.58±0.1^{a}$	$6.56±0.18^{a}$	$6.59±0.08^{a}$
WG (g)	$5.64±0.07^{a}$	$6.00±0.10^{a}$	$5.99±0.18^{a}$	$6.02±0.08^{a}$
DWG (g day^{-1})	$0.094±0.001^{a}$	$0.100±0.002^{a}$	$0.100±0.003^{a}$	$0.100±0.001^{a}$
SGR (% day^{-1})	$3.94±0.02^{b}$	$4.03±0.05^{ab}$	$4.05±0.04^{ab}$	$4.08±0.03^{a}$
Survival rate (%)	$57.7±2.8^{b}$	$67±3.2^{ab}$	$69.3±3.1^{a}$	$68.7±3.2^{a}$

Values are mean ± SE. Mean values within a row followed by the same letters show that there is no significant difference among the groups ($p>0.05$).

Discussion. There are a very large number of *Bacillus* strains, but not all are equally effective as probiotics. For example, 83 strains from shrimp ponds in the Mekong Delta were isolated, but only three (CM3.1, CM2.2 and TV1.2) demonstrated a strong in-vitro inhibitory effect against our target pathogen, *V. parahaemolyticus*. Of these, strain CM3.1, which is 99.9% identical to *B. subtilis* in terms of rRNA, produced significantly more extra-cellular alpha-amylase, protease and cellulase. These enzymes play an important role in inhibiting the activity of and reducing colonization by pathogens (Desriac et al 2010; Hammami et al 2012; Joseph et al 2013), and high protease production by the strain *B. subtilis* E20 has been shown to stimulate immune responses and disease resistance of *L. vannamei* to the pathogenic bacterium, *Vibrio alginolyticus* (Tseng et al 2009). Although the ability of *B. subtilis* CM3.1 to produce quorum-quenching enzymes was not assessed, other species of *Bacillus* have the capacity to produce these quorum-sensing blockers to support the control of infectious diseases (Zhao et al 2015), and it is likely that *B. subtilis* CM3.1 also has this capacity. In addition, *B. subtilis* has been reported to secrete digestive enzymes that help the large units of

food breakdown during the digestion process and improve the absorption surface area, which in turn contributes to an increased nutrients absorption (Kewcharoen & Srisapoome 2019).

Recent studies reported that two *Bacillus* spp., *B. amyloliquefaciens* and *B. subtilis* AQAHBS001, were safe for use as probiotics in aquaculture (Nandi et al 2017a,b; Kewcharoen & Srisapoome 2019). Likewise, we found no adverse effect of *B. subtillis* CM3.1 on the survival rate, other visual indicators of shrimp health or growth rate and we conclude that this probiotic is safe to use with *L. vannamei*. However, while *B. subtilis* CM3.1 strongly inhibited *V. parahaemolyticus* in-vitro, this translated into a relatively small increase in the growth rate, with only a statistically significant (but nonetheless small) difference in specific growth rate between the T3 treatment and control. On the other hand, the survival rate was significantly higher (in our case, by about 10%) after the addition of *B. subtilis* CM3.1 to the culture water; consequently, despite the disappointing improvement in the growth rate, this probiotic nevertheless provided a significant benefit in terms of potential yield of *L. vannamei*.

As found in previous studies of the use of *Bacillus* as a probiotic (Lalloo et al 2007; Reddy et al 2018), the total ammonium nitrogen (TAN) and nitrite (NO_2^--N) in the culture water were reduced significantly by the *B. subtilis* CM3.1 used in our study. This is a significant benefit of the treatment because high levels of TAN and NO_2^--N reduce the survival rate of *L. vannamei* (Gross et al 2004; Schuler & Boardman 2010).

Conclusions. Given the synergies between the strong in-vitro inhibition of *V. parahaemolyticus* by *B. subtilis* CM3.1, its high level of extra-cellular enzyme production, its improvement of water quality and its enhancement of survival rates, we conclude that *B. subtilis* CM3.1 is a strong potential probiotic candidate for use in the shrimp culture in the Mekong Delta, Vietnam.

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Conflict of interest. The authors declare no conflict of interest.

References

Arisa I. I., Widanarni W., Yuhana M., Muchlisin J. A., Muhammadar A. A., 2015 The application of probiotics, prebiotics and synbiotics to enhance the immune responses of vannamei shrimp (*Litopenaeus vannamei*) to *Vibrio harveyi* infection. AACL Bioflux 8(5):772-776.

Balcazar J. L., Vendrell D., Blas I. D., Zarzuela I. R., Girones O., Muzquiz J. L., 2008 Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. Aquaculture 278:188-191.

Bernfeld P., 1955 Amylase, α and β. In: Methods in enzymology. Colowick S. P., Kaplan N. O. (eds), pp. 149-158, New York, USA.

Boottanun P., Potisap C., Hurdle J. G., Sermswan R. W., 2017 Secondary metabolites from *Bacillus amyloliquefaciens* isolated from soil can kill *Burkholderia pseudomallei*. AMB Express 7(1):16.

Chythanya R., Karunasagar I., Karunasagar I., 2002 Inhibition of shrimp pathogenic Vibrios by a marine Pseudomonas I-2 strain. Aquaculture 208:1-10.

Desriac F., Defer D., Bourgougnon N., 2010 Bacteriocin as weapons in the marine animal-associated bacteria warfare: inventory and potential applications as an aquaculture probiotic. Marine Drugs 8:1153-1177.

Di J., Chu Z., Zhang S., Huang J., Du H., Wei Q., 2019 Evaluation of the potential probiotic *Bacillus subtilis* isolated from two ancient sturgeons on growth performance, serum immunity and disease resistance of *Acipenser dabryanus*. Fish & Shellfish Immunology 93:711-719.

Ghose T. K., 1987 Measurement of cellulose activities. Pure and Applied Chemistry 59(2):257-268.

Gross A., Abutbul S., Zilberg D., 2004 Acute and chronic effects of nitrite on white shrimp, *Litopenaeus vannamei*, cultured in low salinity brackish water. Journal of the World Aquaculture Society 35(3):315-321.
Hammami I., Jaouadi B., Ben Bacha A., Rebai A., Bejar S., Nesme X., 2012 *Bacillus subtilis* bacteriocin Bac 14B with a broad inhibitory spectrum: purification, amino acid sequence analysis, and physicochemical characterization. Biotechnology and Bioprocess Engineering 17:41-49.
Huynh T. G., Chi C. C., Nguyen T. P., Tran T. T. H., Cheng A. A., Liu C. H., 2018 Effects of synbiotic containing *Lactobacillus plantarum* 7-40 and galactooligosaccharide on the growth performance of white shrimp, *Litopenaeus vannamei*. Aquaculture Research 46:2416-2428.
Joseph B., Dhas B., Hena V., Raj J., 2013 Bacteriocin from *Bacillus subtilis* as a novel drug against diabetic foot ulcer bacterial pathogens. Asian Pacific Journal of Tropical Biomedicine 3:942-946.
Kesarcodi W. A., Kaspar H., Lategan M. J., Gibson L., 2008 Probiotic in aquaculture: the need, principles and mechanisms of action and screening processes. Aquaculture 274:1-14.
Kewcharoen W., Srisapoome P., 2019 Probiotic effects of *Bacillus* spp. from Pacific white shrimp (*Litopenaeus vannamei*) on water quality and shrimp growth, immune responses, and resistance to *Vibrio parahaemolyticus* (AHPND strains). Fish & Shellfish Immunology 94:175-189.
Lalloo R., Ramchuran S., Ramduth D., Gorgens J., Gardiner N., 2007 Isolation and selection of *Bacillus* spp. As potential biological agents for enhancement of water quality in culture of ornamental fish. Journal of Applied Microbiology 103:1471-1479.
Liu C. H., Chiu C. S., Lin P. L., Wang S. W., 2009 Improvement in the growth performance of white shrimp, *Litopenaeus vannamei*, by a protease producing probiotic, *Bacillus subtilis* E20 from natto. Journal of Applied Microbiology 107:1031-1041.
Liu K. F., Chiu C. H., Shiu Y. L, Cheng W., Liu C. H., 2010 Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. Fish & Shellfish Immunology 28:837-844.
Lorian V., 1995 Antibiotics in laboratory medicine. In: Disk susceptibility test. 4th edition. Acar J. F., Goldstein F. W. (eds), pp. 1-51, London, William and Walkins Awaverly.
Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., 1951 Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193:265-275.
Nandi A., Banerjee G., Dan S. K., Ghosh P., Ghosh K., Ray A. K., 2017a Screening of autochthonous intestinal microbiota as candidate probiotics isolated from four freshwater teleost's. Current Science 113(4):25.
Nandi A., Dan S. K., Banerjee G., Ghosh P., Ghosh K., Ringo E., Ray A. K., 2017b Probiotic potential of Autochthonous bacteria isolated from the gastrointestinal tract of four freshwater teleost's. Probiotics and Antimicrobial Protein 9:12-21.
Oanh D. T., Phuong N. T., 2012 [Dangerous diseases in shrimp culture in the Mekong Delta]. Science Jounal Can Tho University Vietnam 22:106-118. [In Vietnamese].
Reddy K. V., Reddy A. V. K., Babu B. S., Lakshmi T. V., 2018 Applications of *Bacillus* sp in aquaculture waste water treatment. International Journal of Science Research in Science and Technology 4:1806-1812.
Schuler D. J., Boardman G. D., 2010 Acute toxicity of ammonia and nitrite to Pacific white shrimp *Litopenaeus vannamei*, at low salinities. Journal of the World Aquaculture Society 41(3):438-446.
Srinivasan P., Ramasamy P., 2009 Occurrence, distribution and antibiotic resistance patterns of Vibrio species associated with viral diseased shrimp of south Indian aquaculture environment. International Journal of Agriculture and Biology 1(2):1-10.
Tseng D. Y., Ho P. L., Huang S. Y., Cheng S. C., Shiu Y. L., Chiu C. S., Liu C. H., 2009 Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus*

vannamei, by the probiotic, *Bacillus subtilis* E20. Fish & Shellfish Immunology 26:339-344.
Wang Y. B., 2007 Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquaculture 269:259-264.
Weisburg W. G., Barns S. M., Pelletier D. A., Lane D. J., 1991 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173:697-703.
Yi Y. L., Zhang Z. H., Zhao F., Liu H., Yu L. J., Zha J., Wang G., 2018 Probiotic potential of *Bacillus velezensis* JW: antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on *Carassius auratus*. Fish & Shellfish Immunology 78:322-330.
Yilmaz M., Soran H., Beyatli Y., 2006 Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil. Microbiology Research 161:127-131.
Yun L., Yu Z., Li, Y., Luo P., Jiang X., Tian Y., Ding X., 2019 Ammonia nitrogen and nitrite removal by a heterotrophic *Sphingomonas* sp. strain LPN080 and its potential application in aquaculture. Aquaculture 500:477-484.
Zhao J., Chen M., Quan C. S., Fan C. D., 2015 Mechanisms of quorum sensing and strategies for quorum sensing disruption in aquaculture pathogens. Journal of Fish Diseases 38:771-786.
*** APHA, American Public Health Association, 2017 Standard methods for the examination of water and wastewater. 23th edition. American Public Health Association 800 I Street, NW, Washington DC, 1546 p.
*** Basic Local Alignment Search Tool, BLAST, http://blast.ncbi.nlm.nih.gov.
*** VASEP, 2020 Review on Vietnam aquaculture. http://vasep.com.vn/1192/OneContent/tong-quan-nganh.htm. [In Vietnamese].

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Authors:
Quoc Phu Truong, Department of Aquatic Pathology, College of Aquaculture and Fisheries, Can Tho University, Campus II, 3/2 Street, Ninh Kieu District, Can Tho City, Viet Nam, e-mail: tqphu@ctu.edu.vn
Thi Cam Tu Phan, Department of Applied Hydrobiology, College of Aquaculture and Fisheries, Can Tho University, Campus II, 3/2 Street, Ninh Kieu District, Can Tho City, Viet Nam, e-mail: ptctu@ctu.edu.vn
Hung Hai Vu, Department of Applied Hydrobiology, College of Aquaculture and Fisheries, Can Tho University, Campus II, 3/2 Street, Ninh Kieu District, Can Tho City, Viet Nam, e-mail: vhhai@ctu.edu.vn
Thi Tuyet Ngan Pham, Department of Applied Hydrobiology, College of Aquaculture and Fisheries, Can Tho University, Campus II, 3/2 Street, Ninh Kieu District, Can Tho City, Viet Nam, e-mail: pttngan@ctu.edu.vn
Truong Giang Huynh, Department of Applied Hydrobiology, College of Aquaculture and Fisheries, Can Tho University, Campus II, 3/2 Street, Ninh Kieu District, Can Tho City, Viet Nam, e-mail: htgiang@ctu.edu.vn
Ngoc Ut Vu, Department of Applied Hydrobiology, College of Aquaculture and Fisheries, Can Tho University, Campus II, 3/2 Street, Ninh Kieu District, Can Tho City, Viet Nam, e-mail: vnut@ctu.edu.vn

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