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ISOLATION AND EVALUATION THE EFFECT OF *BACILLUS SUBTILLIS* BLD01 STRAIN ON THE SURVIVAL RATES AND GUT MICROBIOTA OF *PENAEUS VANNAMEI* AFTER CHALLENGE WITH *VIBRIO PARAHAEMOLYTICUS*

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SUMMARY

Acute hepatopancreatic necrosis disease (AHPND) reasoned by *Vibrio parahaemolyticus* has caused severe damage to the shrimp farming industry of Vietnam. Probiotics are chosen as a prophylactic method to mitigate the outbreak of diseases. We reported in this article the isolation and evaluation the effect of a potential probiotic, *Bacillus subtilis* BLD01, which enhanced the survival rates and changed gut microbiota of whiteleg shrimp (*Penaeus vannamei*) after challenge with AHPND *V. parahaemolyticus*. After seven days of the challenge, the treatment where shrimps were fed with *B. subtilis* BLD01 strain (10^6 CFU/g) and challenge with AHPND *V. parahaemolyticus* (10^6 CFU/mL) showed high survival rates of 71% as compared to 33% in the treatment where shrimp were given standard feed without probiotics supplementation and challenged with AHPND *V. parahaemolyticus*. 16S rRNA amplicon data of the shrimp gut microbiome in four treatments were carried out using Illumina sequencing. A total of 231 436 reads were obtained, and a total of 14 phyla, 28 classes, 142 genera were revealed. The most abundant phyla in all subjects were Proteobacteria and Bacteroidetes. The sequence number of the *Vibrio* genus was the highest with 28% in treatment without *B. subtillis* BLD01 in-feed addition and shrimps challenged with AHPND *V. parahaemolyticus*. The sequence number of *Bacillus* genus was the highest with 3% in the treatment with *B. subtillis* BLD01 addition and without AHPND *V. parahaemolyticus* challenge. These results contribute to confirm the mechanism of action of *B. subtilis* against *V. parahaemolyticus* in the experimental model, creating a scientific basis for the development and use of probiotic products applied in shrimp farming in Vietnam.

Keywords: 16S rRNA, bacterial communities, Illumina sequencing, metagenome, probiotics

INTRODUCTION

Vietnam is one of the largest shrimp producers globally, with the total shrimp export value reached 3.85 billion USD (MARD, 2021). However, shrimp production in Vietnam is facing an increased risk of several diseases associated with aquaculture, such as white spot syndrome virus, white feces syndrome, and acute hepatopancreatic necrosis disease (AHPND). In recent years, AHPND caused by a strain of *V. parahaemolyticus* carrying

two *Photorhabdus* insect-related (Pir A and B) toxin genes had superseded other diseases, causing massive early mortality in all types of cultured shrimps (Lightner *et al*., 2012; Tran *et al*., 2013). The World Organisation for Animal Health (OIE) has been listed AHPND as a notifiable disease due to its highly virulent and rapidly spreading (FAO, 2020).

Antibiotics have been applied as an effective remedy to control or prevent shrimp diseases outbreak (Wang *et al*., 2020; Doan *et al*., 2020). However, this has led to antibiotic resistance in bacteria due to inappropriate or excessive use of antibiotics (Thornber *et al*., 2020). Furthermore, the application of antibiotics could adversely affect shrimp and consumers' health (Cheng *et al*., 2014). Probiotics are the best alternatives to antibiotics and have been increasingly used to protect shrimps against pathogens (Nguyen *et al*., 2018; Karthik *et al*., 2018). Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host'" (Hill *et al*., 2014). Some *Bacillus* species, such as *B. endophyticus*, *B. amyloliquefaciens*, *B. aryabhattai* and *B. subtilis*, have been used to modulate intestinal microbiota and shrimp resistance to *V. parahaemolyticus* (AHPND strains) (Luis-Villaseñor *et al*., 2013; Tepaamorndech *et al*., 2019; Knipe *et al*., 2020; Imaizumi *et al*., 2021). *B. subtilis* are Gram-positive spore-forming, widely used as probiotics for shrimp culture. *B. subtilis* can be isolated from the water, sediment, and digestive tract of shrimp (Le *et al*., 2019). However, little is known about the effects of probiotics on the gut microbiota of the shrimp in Vietnam.

Most microorganisms cannot be cultured and traditional techniques are limited to analysis of microbial diversity (Huang *et al*., 2014). In recent years, next-generation sequencing techniques have been widely applied to investigate bacterial composition in a range of complex environments. Among next-generation sequencing techniques, 16S rRNA amplicon sequencing can rapidly determine the microbial community of shrimp and aquatic environments (Angthong *et al*., 2020).

This study evaluated the survival and gut bacterial communities of the whiteleg shrimp *Penaeus vannamei* in experimental challenge with *V. parahaemolyticus* based on the probiotic *B. subtilis* strain BLD01. This study provides valuable and practical strategies that can be applied in the shrimp culture industry, which is now declining from harmful shrimp diseases, especially from AHPND.

MATERIALS AND METHODS

Isolation of *Bacillus* spp. strains

Several sediment samples from shrimp ponds in Dong Hai district, Bac Lieu province, Vietnam, were collected to isolate probiotic bacteria as described in detail by Le *et al*., (2019). Briefly, 1 g of sample was homogenized in 10 mL of nutrient broth (NB) and the mixture was heated at 80°C for 10 min to eliminate vegetative cells. Ten-fold serial dilution of the supernatant was spread-plated onto nutrient agar (NA). The NA plates were incubated at 37°C for 24 h, and individual colonies were streaked across the plate to obtain pure isolated colonies. After that, bacterial isolates were subjected to gram stain, and positive isolates were stored in 50% glycerol at -80°C. Isolated colonies were selected and checked for antagonist effects to *V. parahaemolyticus* using agar well diffusion plate assay method described by Liu *et al*., (2015). *V. parahaemolyticus* was incubated in LB broth at 37ºC overnight, and 100 µL of this culture (10^7-10^8 CFU /mL) was spread over the LB agar plates. Wells of 5 mm depth and 6 mm in diameter were punched in the agar with a sterile tip. *Bacillus* spp. strains were grown at 37°C overnight in LB broth and 100 µL of *Bacillus* spp. strains (10^7-10^8 CFU /mL) were directly filled into the wells of agar plates and incubated for a period of 24 h at 37°C while 100 µL of LB broth without *Bacillus* spp. strains served as the negative control. Antibacterial activity was defined as the diameter (mm) of the clear

inhibitory zone formed around the well. All isolated strains were spot inoculated in triplicate on nutrient agar.

Bacterial DNA extraction and 16S rRNA sequencing

Total DNA from bacterial isolates was extracted by the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. The universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lee *et al*., 2017). The total volume of 25 µL of the PCR reaction consisted of 12.5 µL of 2X GoTaq® G2 Hot Start Green Master Mix (Promega, USA), 0.5 µL of 10 pmol of each primer, 1 µL of DNA (50 ng/µL) and 10.5 µL of distilled water. The PCR reaction was performed with 30 cycles of 94ºC for 1 min, 55ºC for 1 min, 72ºC for 2 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany) and then sequenced at the Institute of Biotechnology, Vietnam Academy of Science and Technology. The nucleotide sequences were analyzed by BioEdit v.7.0.5.3 (Hall, 1999) and were then BLAST searched against GenBank databases. The 16S phylogenetic tree was constructed by the neighbour – joining method using NEIGBOR program Unweighted Pair Group Mean Average (UPGMA) methods with MEGA X version 10.2.5 (Kumar *et al*., 2018). Bootstrap values were equal to or greater than 50% derived from1000 iterations.

Experimental setup

Whiteleg shrimps (PL40) were obtained from a commercial hatchery at a size about 2 g. The shrimp had not been exposed to shrimp diseases and were deemed AHPND negative by standard molecular techniques. The shrimp were acclimated for 7 days in glass tanks of laboratory conditions before the start of the experiments.

The pathogenic bacterium, *V. parahaemolyticus* CT01, was previously isolated from AHPND infected shrimps obtained from the College of Aquaculture and Fisheries, Can Tho University, Can Tho, Vietnam, and was recovered and grown for 24 h at 28ºC in nutrient broth (NB) supplemented with 1.5% NaCl. The density of bacteria was determined by optical density (OD) at 610 nm.

Probiotic *B. subtilis* strain BLD01 were grown in Luria Bertani (LB, Difco, USA) medium using a shaking incubator at 30°C for 48 h. The cultures were later centrifuged at 3000 g for 10 min at 4°C, the supernatant was discarded, the bacterial pellet was re-suspended and washed in sterile Normal Saline Solution (NSS) supplemented with 0.9% NaCl. The *B. subtilis* strain BLD01 density was identified using spectrophotometer at 600 nm and correlated to the colony-forming unit (CFU) using the spread-plate technique. After that, the suspension was kept at 4°C until used.

Commercial pellets (GroBest, Vietnam), containing 40% protein, 5% carbohydrate, and 3% lipid, were used as the basal diet. *B. subtilis* strain BLD01 suspension was mixed with shrimp feed to give a final concentration of approximately 10^6 CFU/g. The density of *B. subtilis* strain BLD01 in the feed was determined using the spread-plate technique as follows: take 1 g of the feed and serially 10-fold diluted in phosphate-buffered saline solution (PBS, pH 7.2) and 100 µL of each dilution was then spread on LB agar (LB agar, Difco, USA) to estimate the probiotic concentration (CFU/g).

A total of 100 shrimps of each treatment were placed in a plastic tank containing 100 L of 20 ppt seawater. Shrimps were fed with *B. subtilis* strain BLD01, for 30 days before challenging with AHPND *V. parahaemolyticus* strain. Challenge experiments were carried out following the protocol described by Tran *et al*., (2013). Shrimps were challenged with *V. parahaemolyticus* at a density of 10^6 CFU/mL. In the negative control treatment, shrimps were immersed in sterilized TSB medium supplemented with 1.5% NaCl and later placed in tanks without bacteria supplementation. Shrimps were fed four times daily, and no water exchange was performed during the challenge. Dead shrimps were regularly

removed from tanks. Four experimental groups with three replicates/treatment (total of 12 plastic tanks) were used in the experiment as follows:

In treatment one (NC), shrimps were given standard feed with neither probiotic supplementation nor AHPND *V. parahaemolyticus* challenge.

In treatment two (VP), shrimps were given standard feed without probiotic supplementation and were challenged with AHPND *V. parahaemolyticus*.

In treatment three (BS), shrimps were given standard feed supplemented with *B. subtilis* BLD01 only.

In treatment four (VPBS), shrimps were given standard feed supplemented *B. subtilis* BLD01 and were challenged with AHPND *V. parahaemolyticus*.

Sample collection and DNA extraction

Genomic DNA was extracted in triplicate (the guts from each treatment's shrimp) by a QIAamp DNA mini kit (Qiagen, Germany). The genomic DNA was monitored by agarose gel electrophoresis and a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) to check its concentration and quality.

Library preparation and sequencing

DNA mixtures containing equivalent amounts of DNA from the pooled samples were used for PCR. The specific primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') were used in the amplification of the V3-V4 regions of 16S rRNA genes (Yu *et al*., 2005). PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, UK). Afterward, sequencing libraries were constructed using the TruSeq® DNA PCR-Free Library Preparation Kit (Illumina, USA) according to the manufacturer's recommendations. The library was subjected to quality assessment on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, USA) and the Agilent Bioanalyser 2100 system (Agilent, USA). Then, the library was sequenced, and 250 bp paired-end reads were constructed on an Illumina HiSeq 2500 platform (Illumina, USA).

Sequence data analysis

Paired-end reads were assigned to samples based on unique barcodes and truncated by trimming the primer sequences and barcodes. The raw tags were produced by merging the trimmed paired-end reads through the utilization of FLASH version 1.2.7 (Magoč, Salzberg, 2011). The raw tags were subjected to default filtering conditions for quality controls to obtain high-quality clean tags with QIIME (Version 1.7.0) (Bokulich *et al*., 2013; Caporaso *et al*., 2010). Then, the clean tags were compared to Gold Database using the UCHIME algorithm to detect and remove chimeric sequences. OTUs cluster and sequence annotation were performed using UPARSE software v7.0.1001 (Edgar *et al*., 2011). The sequences that showed more than 97% of similarity were grouped to the same OTUs. The RDP (Version 2.2) classifier algorithm (Wang *et al*., 2007) was used for species annotation of the representative sequence for each OTU based on the GreenGenes Database (DeSantis *et al*., 2006). Subsequent analyses of alpha diversity and beta diversity were all performed based on the rarefied OTU table. OTUs from each sample were used to estimate and compare bacterial diversity in samples through 5 indices, namely: observed species (OTUs), Good's coverage, Chao1, Shannon, and Simpson. The diversity indices were determined using the QIIME (Version 1.7.0) and displayed using R software (Version 2.15.3). A heatmap was produced to show species composition and abundance among samples by R software (Version 2.15.3).

All sequencing libraries generated in this article were deposited in the NCBI GenBank database under the accession numbers SAMN19065122 to SAMN19065125.

RESULTS

Isolation and characterization of *Bacillus* spp. strains

A total of 20 *Bacillus* spp. strains were isolated from 6 sediment samples from Dong Hai district, Bac Lieu province. All isolates were Gram-positive bacilli, spore-forming, with different characteristics in colony morphology. Isolated colonies were selected and tested for antibacterial activity against *V. parahaemolyticus* using agar well diffusion plate assay (Table 1).

Table 1. Morphology and antagonistic activity against *V. parahaemolyticus* of 20 *Bacillus* spp. in this study.

Strain	Gram	Morphology	Colony features	Antagonistic activity (diameter of the inhibition zone, mm)
BLD01	+	Rod	White, round-shaped, shiny	11.9 ± 0.76
BLD02	+	Rod	Cream, irregular, shiny	ND
BLD03	+	Rod	Cream, round-shaped, transparent	8.9 ± 0.66
BLD04	+	Rod	Cream, round-shaped, transparent	9.2 ± 0.43
BLD05	+	Rod	White, Irregular, transparent	ND
BLD06	+	Rod	White, round-shaped, transparent	ND
BLD07	+	Rod	White, round-shaped, transparent	ND
BLD08	+	Rod	Cream, irregular, transparent	ND
BLD09	+	Rod	Cream, irregular, transparent	ND
BLD10	+	Rod	White, round-shaped, shiny	ND
BLD11	+	Rod	White, round-shaped, shiny	ND
BLD12	+	Rod	Cream, round-shaped, shiny	ND
BLD13	+	Rod	Cream, irregular, transparent	ND
BLD14	+	Rod	Cream, irregular, transparent	ND
BLD15	+	Rod	White, round-shaped, shiny	7.6 ± 0.54
BTN16	+	Rod	White, round-shaped, transparent	ND
BTN17	+	Rod	White, round-shaped, transparent	ND
BTN18	+	Rod	Cream, irregular, transparent	ND
BTN19	+	Rod	Cream, irregular, transparent	ND
BTN20	+	Rod	Cream, round-shaped, transparent	7.8 ± 0.38

ND: not determined.

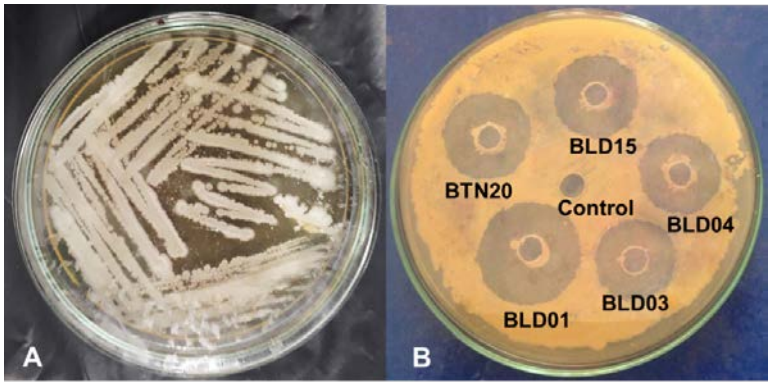

Figure 1. *Bacillus* spp. were grown on LB agar (A); *B. subtilis* BLD01 showed the highest antibacterial activity against *V. parahaemolyticus.* LB broth served as the negative control (B).

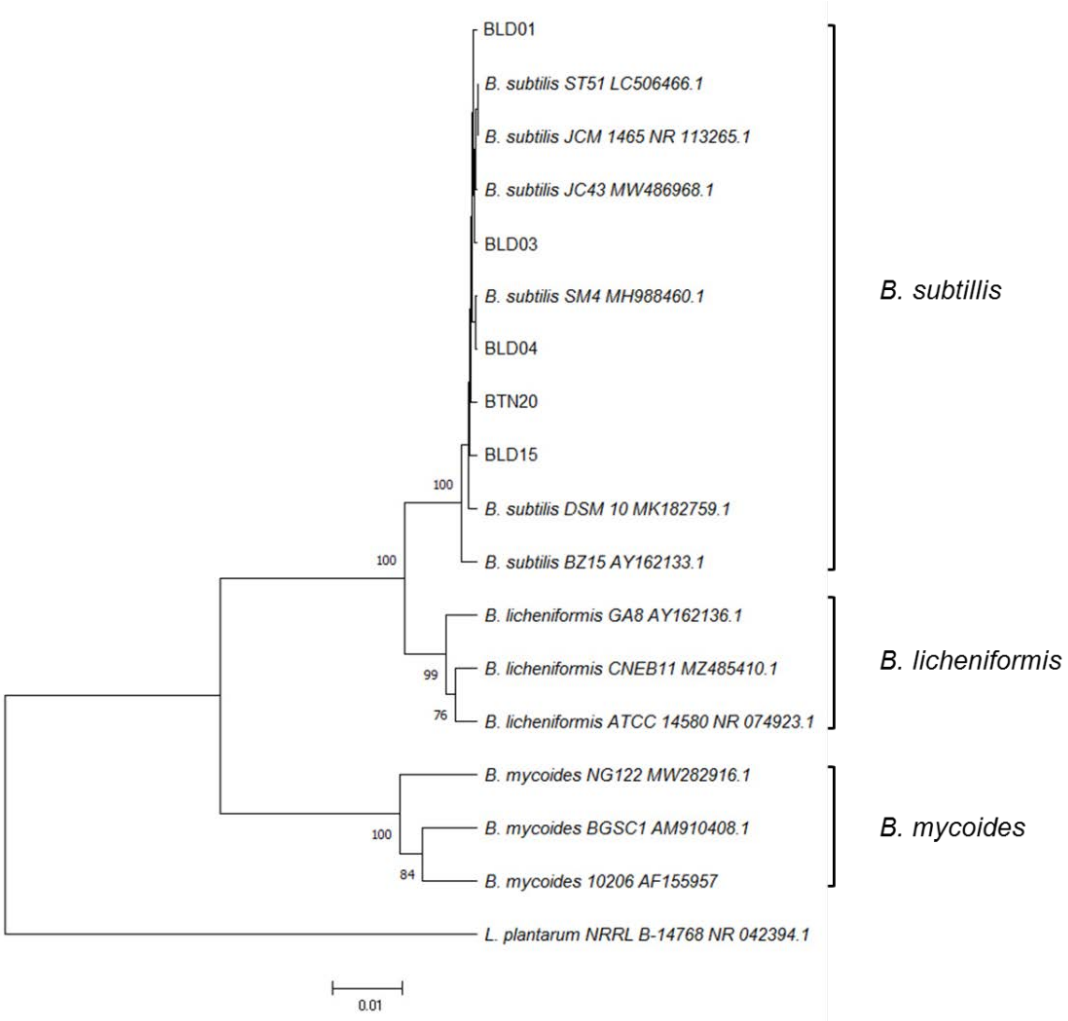

Figure 2. Phylogram obtained from MEGA X software analyses of the 16S rRNA gene. Strains BLD01, BLD03, BLD04, BLD15, and BTN20 group among *Bacillus* species. *Lactobacillus plantarum* is the outgroup.

Among the 20 isolates, five strains showed antagonistic effects against *V. parahaemolyticus*, namely BLD01, BLD3, BLD4, BLD15, and BTN20 (Figure 1). These strains were identified as *B. subtilis* based on the physiological identification and 16S rRNA sequence analysis (Figure 2). Among them, strain BLD01 showed significant antimicrobial performance ($p < 0.05$) and was chosen for further investigations.

Effect of *B. subtilis* BLD01 on survival rate of whiteleg shrimps after challenge with *V. parahaemolyticus*

To determine the effect of *B. subtilis* BLD01 in-feed addition on the survival rate of whiteleg shrimps challenged with AHPND *V. parahaemolyticus* strains, four treatments were designed. The highest survival rate is obtained in treatment BS, which is 93.3%, followed by treatment NC (89.3%), treatment VPBS (71%), and treatment VP (33%) (Figure 3).

Effect of *B. subtilis* BLD01 on the gut microbiota of whiteleg shrimps after challenge with *V. parahaemolyticus*

16S rRNA gene amplicon sequencing was performed to determine the changes in microbial communities in the guts of whiteleg shrimps. A

total of 231,436 reads were obtained from the gut of shrimps in 4 treatments by sequencing the V3-V4 region of the 16S rRNA gene (Table 2).

Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity. The number of OTUs in four treatments NC, BS, VP, and VPBS was 174, 185, 239, and 172, respectively. Bacterial richness and diversity were estimated by Good's coverage, Chao1, Shannon, and Simpson indexes. Good's coverage index in all four samples was 0.999 suggesting the obtained OTUs from each library represented most bacteria in the gut of shrimps in each treatment. Chao1 was the highest in the treatment VP (245.577), followed by VPBS (201.062), BS (194.37), and NC (191.647). Bacterial diversity in NC, BS, VP, and VPBS with Shannon indexes of 3.874, 3.816, 4.439, and 4.13, respectively. Simpson index in the four treatments was 0.865 in NC, 0.827 in BS, 0.89 in VP, and 0.899 in VPBS, respectively (Table 2).

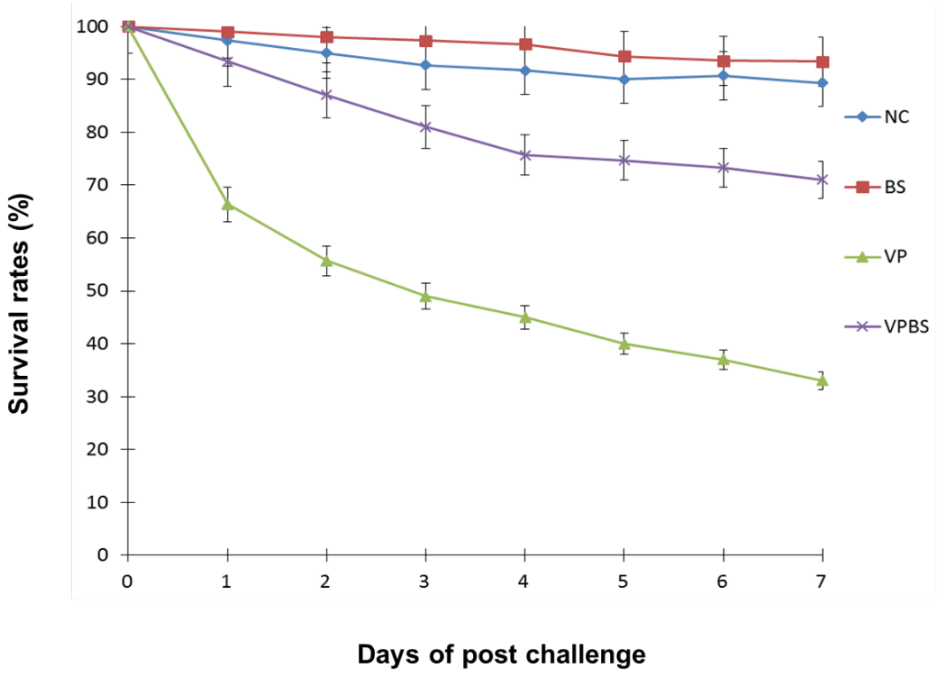

Figure 3. Cumulative survival rates of the whiteleg shrimp fed dietary supplemented *B. subtilis* BLD01 after challenge with *V. parahaemolyticus.* NC: Negative control; VP: *V. parahaemolyticus*; BS: *B. subtilis*; VPBS: *V. parahaemolyticus* and *B. subtilis.*

Table 2. Illumina high-throughput data, bacterial diversity richness (OTUs), sample coverage (Good's coverage), diversity (Shannon, Simpson), and estimated OTU richness (Chao1) within samples.

	NC	BS	VP	VPBS
Number of sequences	55 559	62 333	59 192	54 352
OTUs (97%)	174	185	239	172
Good's coverage	0.999	0.999	0.999	0.999
Chao1	191.647	194.37	245.577	201.062
Shannon	3.874	3.816	4.439	4.13
Simpson	0.865	0.827	0.89	0.899

NC: Negative control; VP: *V. parahaemolyticus*; BS: *B. subtilis*; VPBS: *V. parahaemolyticus* and *B. subtilis.*

Bacterial reads in the four treatments were classified into 13 major phyla, 25 classes, and 94 genera. Proteobacteria was the most abundant bacterial phylum in all four treatments NC, BS, VP, and VPBS, with the relative abundance of 52.4%, 59.3%, 76.9 %, and 52.7%, respectively. The second dominant phylum was Bacteroidetes with 38.3% in NC, 28.1% in BS, 12.7% in VP, and 32.6% in VPBS (Figure 4).

Among Proteobacteria phylum, the *Alphaproteobacteria* formed the most dominant class (with 38.2% in NC, 47.7% in BS, 36.9% in VP, and 36.3% in VPBS), followed by *Gammaproteobacteria* (with 14.1 % in NC, 11.4% in BS, 39.6% in VP, and 15.9% in VPBS). Among *Alphaproteobacteria* class, *Rhodobacteraceae* was the major family (with 37.0% in NC, 46.6% in BS, 36.1% in VP, and 31.4% in VPBS). Among *Gammaproteobacteria* class, the family *Vibrionaceae* was the major representative (with 7.5% in NC, 3.3% in BS, 28.7% in VP, and 8.2% in VPBS).

Within the Bacteroidetes phylum, bacteria from class *Flavobacteriia* were most major (26.2 % in NC, 21% in BS, 11.4% in VP, and 11.0% in VPBS). The class *Flavobacteriia* was dominated by the family *Flavobacteriaceae* (with 25.7% in NC, 20.8% in BS, 11.3% in VP, and 11.0% in VPBS).

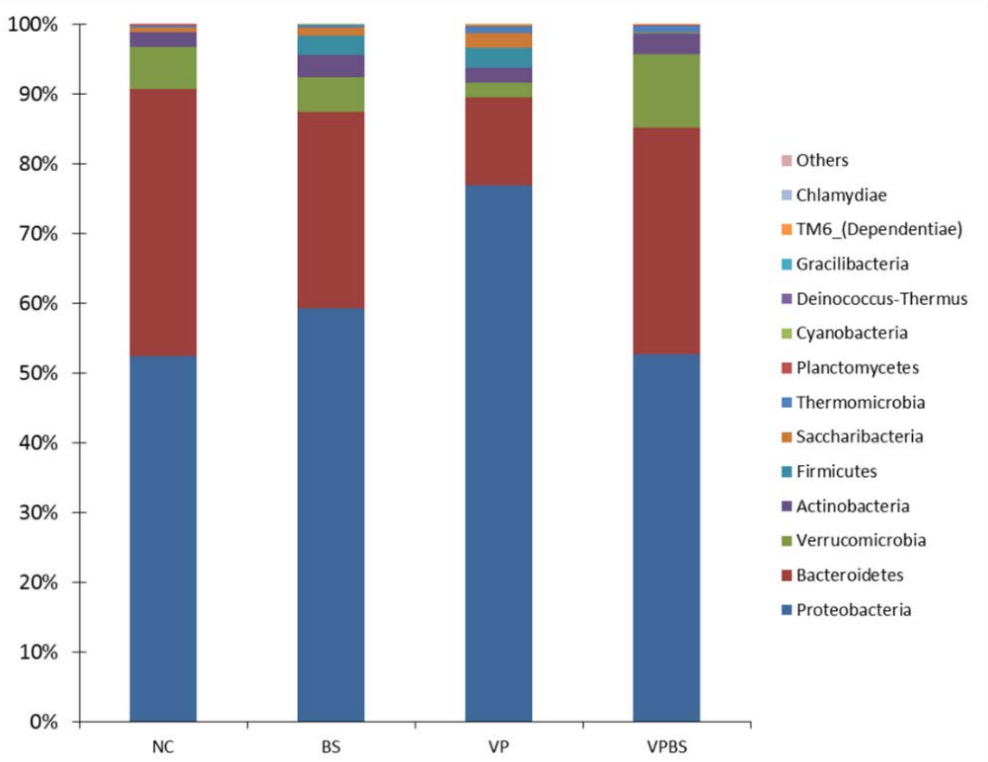

Figure 4. Relative abundances of bacterial phyla. The top 13 phyla are shown, and the other phyla are included as "Others". NC: Negative control; VP: *V. parahaemolyticus*; BS: *B. subtilis*; VPBS: *V. parahaemolyticus* and *B. subtilis.*

Figure 5 represents the frequency of 35 dominant genera among all treatments. At the genus level, the *Ruegeria* genus was predominant across treatments NC, BS, VP, and VPBS with 29.64%, 37.97%, 10.05%, and 21.84%, respectively. *Vibrio* followed this genus with 7.34% in NC, 3.25% in BS, 28.10% in VP, and 8.24% in VPBS. There was a difference in *Flavobacterium* abundance among all treatment VP and VPBS, with 0.44 and 0%, respectively. The colour in the heatmap indicates

that the percentage of *Bacillus* genus was the highest with 2.6% in treatment BS compared to 0% in treatment NC, 0.47% in treatment VP, and 0.02% in treatment VPBS.

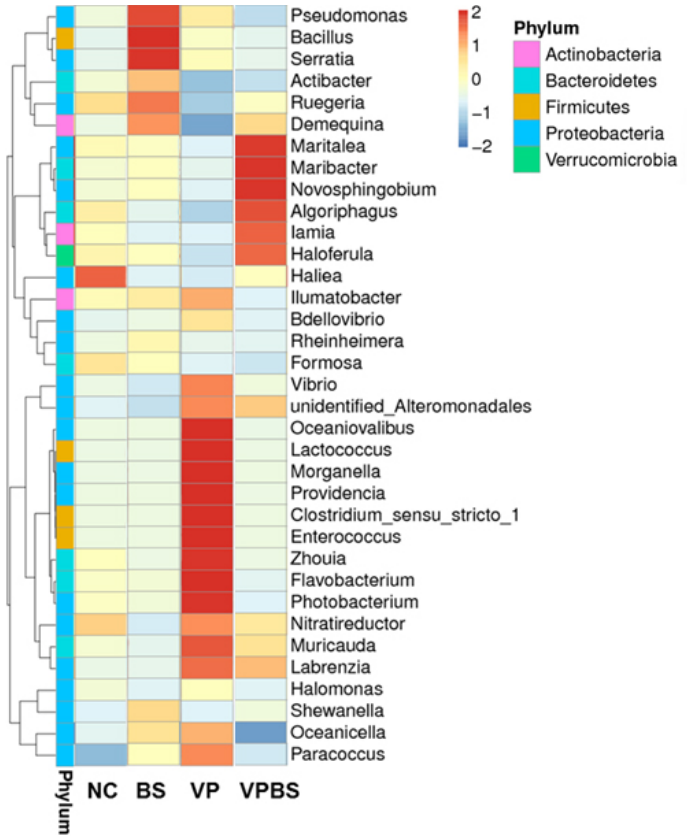

Figure 5. Species abundance heatmap shows the distribution of the dominant 35 genera among all samples. The colors represent the relative percentage of the microbial genus assignments within each sample. Square colors shifted towards red indicates higher abundance. NC: Negative control; VP: *V. parahaemolyticus*; BS: *B. subtilis*; VPBS: *V. parahaemolyticus* and *B. subtilis*.

DISCUSSION

In recent years, the emergence of AHPND, mainly caused by *V. parahaemolyticus*, has resulted in a decline in shrimp production (Kumar *et al*., 2020). The use of probiotics in shrimp farming is a practical alternative to promote shrimp health and limit the detrimental effects of AHNPD (Pinoargote *et al*., 2018; Restrepo *et al*., 2021). *Bacillus* species have been reported to have the antagonistic activities against pathogenic bacteria, such as *V. parahaemolyticus* (Liu *et al*., 2015). In Vietnam, there is a lack of knowledge on using probiotic bacteria that have antagonistic activity towards the AHPND *V. parahaemolyticus* in shrimp aquaculture, with few studies carried out (Truc *et al*., 2019; Le *et al*., 2019). This study explored the effect of *B. subtillis* BLD01 strain on the survival and bacterial communities in the gut of whiteleg shrimps exposed to probiotic treatments after challenge with *V. parahaemolyticus*. The result showed that the growth of AHPND *V. parahaemolyticus* was inhibited by the *B. subtillis* BLD01 strain. In addition, the treatment VPBS showed a high survival rate (71%) than the treatment VP (33%) ($p < 0,05$). The survival rate of shrimp in treatment BS fed with *B. subtillis* BLD01 strain was the highest with 93.3% and slightly higher than NC treatment

(89.3%). Therefore, the *B. subtillis BLD01* strain presented as a potential probiotic against AHPND *V. parahaemolyticus* in shrimp aquaculture.

Next-generation sequencing results revealed how probiotics, in general, may alter the diversity of microbial communities. Proteobacteria and Bacteroidetes were dominant phyla across all samples in this study. *Alphaproteobacteria* was the dominant class in the gut of whiteleg shrimp in all treatments, followed by *Gammaproteobacteria* and *Flavobacteriia*. The phylum and family distribution trends observed in this study were similar to those reported in previous research (Pinoargote *et al*., 2018). A high proportion of bacteria was identified as the family *Rhodobacteraceae* in this study. It has been reported that the members of the *Rhodobacteraceae* family, such as *Ruegeria* spp. can reduce the pathogenic *Vibrio* their antagonistic activity (Pilotto *et al*., 2018). *Ruegeria* was the relatively higher abundant genus found in treatments BS (37.97%) and VPBS (21.84%) compared to treatments (NC 29.64%) and VP (10.05%). The beneficial bacteria genus, such as *Bacillus*, was abundant in the treatment BS (2.6%) compared to 0% in treatment NC. These results suggested that members of the *Ruegeria* genus might have positively interacted with *Bacillus* genus in the gut of shrimps, resulting in the high survival rate of shrimp in treatment BS and VPBS compared to the low survival rate of shrimp in treatment VP. Previous studies have reported that members of the *Flavobacterium* can cause bacterial disease in shrimp (Flegel, 2012). In this study, the relative abundance *Flavobacterium* genus was highly abundant in the treatment VP (0.44%) and was absent in the treatment VPBS (0%). The relative abundance of *Vibrio* was enriched in the treatment VP (28.10%) and weakened in the treatment VPBS (8.24%). Therefore, the results of this study suggested *B. subtillis* might colonize and reduce *Flavobacterium* and *Vibrio* in the gut of shrimps. However, the mechanisms of *B. subtillis* in the gut of whiteleg shrimp to control the *V. parahaemolyticus* are needed for further investigations.

CONCLUSION

In conclusion, the findings in this study demonstrated that *B. subtillis* BLD01 strain could mitigate the detrimental effects associated with AHPND infection in whiteleg shrimps. The shrimps were given standard feed without probiotic supplementation and were challenged with AHPND *V. parahaemolyticus* showed a low survival rate of 33%, while shrimps were given feed supplemented *B. subtilis* BLD01 and were challenged with AHPND *V. parahaemolyticus* showed a high survival rate of 71%. Additionally, shrimp fed *B. subtilis* BLD01 strain showed diverse microbial composition with a high portion of *Bacillus* genus in the gut. Therefore, the suitable forms and application of *B. subtillis* BLD01 should be further evaluated to confirm its effectiveness and achieve its potential application.

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