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Research article

Antibacterial *Bacillus* strains from the Mekong Delta: Combating *Vibrio parahaemolyticus* in intensive white-leg shrimp aquaculture

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Abstract

Vibrio parahaemolyticus is a major bacterial pathogen causing significant losses in the global shrimp farming industry, including in Vietnam. The overuse of antibiotics for controlling *V. parahaemolyticus* has led to increased bacterial resistance. This study aimed to isolate and screen indigenous Bacillus strains with inhibitory activity against *V. parahaemolyticus*, which causes Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPNS) in intensively farmed white-leg shrimp (*Litopenaeus vannamei*) in Bac Lieu province, Mekong Delta, Vietnam. A total of forty-two *Bacillus* strains were isolated from the intestines of healthy shrimp, pond mud, and pond water. The well diffusion agar method revealed that twenty out of the forty-two strains exhibited inhibitory effects against *V. parahaemolyticus*. Among these, four strains, identified as BLN7, STNC, BLB7, and BLN8, demonstrated the strongest antagonistic activity, with inhibition zone diameters of 24 ± 0.00, 12.03 ± 0.06, 11.97 ± 0.06, and 11.93 ± 0.12 mm, respectively. Additionally, the study found that these four strains possess extracellular enzyme activities, including amylase, cellulase, and protease. Molecular identification through 16S rRNA gene sequencing, along with morphological, physiological, and biochemical analysis, confirmed that strains BLN7 and STNC belong to *Bacillus* spp. These findings provide a foundation for developing biological products to prevent EMS disease in white-leg shrimp.

Keywords: AHPNS disease, Antibacterial activity, *Bacillus,* Probiotics, White-leg shrimp.

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INTRODUCTION

Vietnam, ranked fourth globally in aquaculture production in 2022, boasts significant strengths in cultivating and producing a wide variety of aquatic species (FAO, 2022). The Mekong Delta provinces, including Bac Lieu, Tra Vinh, Ca Mau, Soc Trang, Ben Tre, and Kien Giang, are recognized as critical aquaculture hubs, contributing to 80% of the nation's shrimp export output. Notably, the farming of white-leg shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*) is a cornerstone of the seafood export industry in Bac Lieu province (Dung et al., 2017). According to the Vietnam Association of Seafood Exporters and Producers (VASEP), the country's seafood production has grown at an average annual rate of 8%, surging from 1.3 million tons in 1995 to 8.4 million tons in 2020, a more than sixfold increase. Of this, aquaculture accounts for 54% of the output, with wild capture contributing 46%. Reports from relevant agencies estimate that by January 2024, Vietnam's seafood exports will reach USD 730 million, marking a 60.8% increase compared to the same period in 2023. This includes a notable 71% rise in shrimp exports (Department of Fisheries, 2024).

Nevertheless, intensive high-density farming has led to the outbreak of various shrimp diseases in recent years. These include Yellow Head Virus, White Spot Syndrome Virus, *Macrobrachium rosenbergii* nodavirus (Oanh and Phuong, 2012), Early Mortality Syndrome (EMS), also known as Acute Hepatopancreatic Necrosis Syndrome (AHPNS) (Flegel et al., 2012), and more recently, white feces disease caused by *Vibrio* spp. (Ut et al., 2021). Extensive research has identified that AHPNS is caused by *V. parahaemolyticus* (Vp AHPND, Loc et al., 2013). This disease was first reported in China in 2009 and subsequently in other shrimp-farming regions such as Vietnam, Malaysia, Thailand, South America, the United States, and Mexico (Flegel, 2012; Soto-Rodriguez et al., 2015; Restrepo et al., 2016; Dhar et al., 2019). AHPNS is characterized by its rapid spread and high mortality rates in shrimp farms (Zorriehzahra and Banaederakhshan, 2015). In affected regions, shrimp production has plummeted by approximately 60%, with the global shrimp farming industry incurring losses of $43 billion due to this disease (Kumar et al., 2021).

V. parahaemolyticus is a gram-negative, curved rod bacterium belonging to the Vibrionaceae family, and is commonly found in the environment (Drake et al., 2007). It is hypothesized that Vp AHPND shares characteristics with other *V. parahaemolyticus* strains present in seafood. Shrimp infected with Early Mortality Syndrome (EMS) exhibit clinical signs such as empty stomach and midgut, pale to white hepatopancreas (HP), and HP atrophy (Hong To et al., 2020). In affected shrimp ponds, mortality rates can approach 100% within a few days of the disease onset (Tran et al., 2013). Various control methods for *V. parahaemolyticus* have been employed, including biofloc, immunostimulants derived from medicinal plants, bacteriophages, and nanotechnology (Hoa et al., 2023; Elias et al., 2023). However, treatment remains challenging and often ineffective due to the lack of a specific immune system in shrimp. Moreover, infected shrimp become primary vectors for disease transmission to healthy shrimp through water, feces, and carcasses, facilitating rapid disease spread.

To date, many shrimp farmers resort to antibiotics to manage *V. parahaemolyticus* when signs of hepatopancreatic necrosis disease appear (Thinh et al., 2020; Tuan et al., 2021). However, the use of antibiotics has led to the emergence of antibiotic-resistant bacterial strains in aquaculture (Oanh et al., 2022; Zhang et al., 2024). Additionally, antibiotic use not only poses health risks to consumers but also eliminates beneficial bacteria in shrimp ponds (Bojarski et al., 2020). Consequently, the application of probiotics containing *Bacillus* strains is on the rise (Elshaghabee et al., 2017). Numerous studies have demonstrated that most *Bacillus* species are non-toxic to animals and humans, making them ideal candidates for probiotic production (Olmos et al., 2019). Bacillus bacteria are crucial in aquaculture due to their ability to produce secondary metabolites, such

as antibiotics, that inhibit or kill pathogenic microorganisms in shrimp, fish, and other aquatic animals (Pereira et al., 2022; Proespraiwong et al., 2023). Furthermore, these bacteria enhance the pond environment and stimulate the digestive systems of aquatic animals by producing extracellular enzymes like amylase, cellulase, and protease (Truong et al., 2021).

Despite the potential benefits, research and application of *Bacillus* in shrimp farming in Bac Lieu remain limited. This study aimed to isolate and select indigenous *Bacillus* strains with inhibitory activity against *V. parahaemolyticus*, the causative agent of AHPNS, in intensively farmed white-leg shrimp. The novelty of this research lies in its approach to leveraging local microbial biodiversity to develop effective and environmentally adapted probiotic solutions, addressing the urgent need for sustainable disease management in shrimp aquaculture. Given the increasing prevalence of antibiotic-resistant bacteria and the harmful effects of antibiotics, this study's focus is both timely and necessary. By identifying *Bacillus* strains that inhibit *V. parahaemolyticus*, the research provides a foundation for probiotic treatments that can reduce high mortality rates and economic losses in shrimp farming. The potential impacts include promoting sustainable farming practices, enhancing shrimp health, improving economic resilience in the aquaculture sector, and contributing to food security and environmental sustainability.

MATERIALS AND METHODS

Sample source used to isolate *Bacillus* spp.

A total of 36 samples were randomly selected from the ponds at each sampling site, including healthy shrimps (*Litopenaeus vannamei*, n = 16), sludge (n = 10), and pond water (n = 10), collected from intensive shrimp ponds across various districts (Vinh Loi, Bac Lieu City, Hoa Binh, Gia Rai, and Dong Hai) in Bac Lieu province (Figure 1). In each pond, 5-10 healthy shrimp, each weighing approximately 15-20 grams, were randomly selected from ponds with a high density of over 60 shrimp/m^2. The water temperature, salinity, and pH of the ponds were recorded, ranging from 26-30°C, 10-20%, and 6.5-7.2, respectively, depending on the farming season. Samples of pond sludge and water were collected from three distinct locations within each pond, combined, placed into sterilized bottles, and transported to the laboratory for *Bacillus* isolation.

Isolation of *Bacillus* spp.

Bacillus isolation from shrimp intestines was conducted following the protocol described by Jlidi et al. (2022). Briefly, shrimp were surface-disinfected with 70% alcohol. The intestines were then homogenized and enriched overnight in Nutrient Broth (NB) medium (Himedia, India) supplemented with 1.5% NaCl. The enriched culture was heat-treated at 80°C for 20 minutes, followed by serial dilution with physiological saline (0.9% NaCl) to a dilution factor of 10^{-6}. Subsequently, 100 µL of each dilution was spread onto Nutrient Agar (NA) plates (Himedia, India) supplemented with 1.5% NaCl.

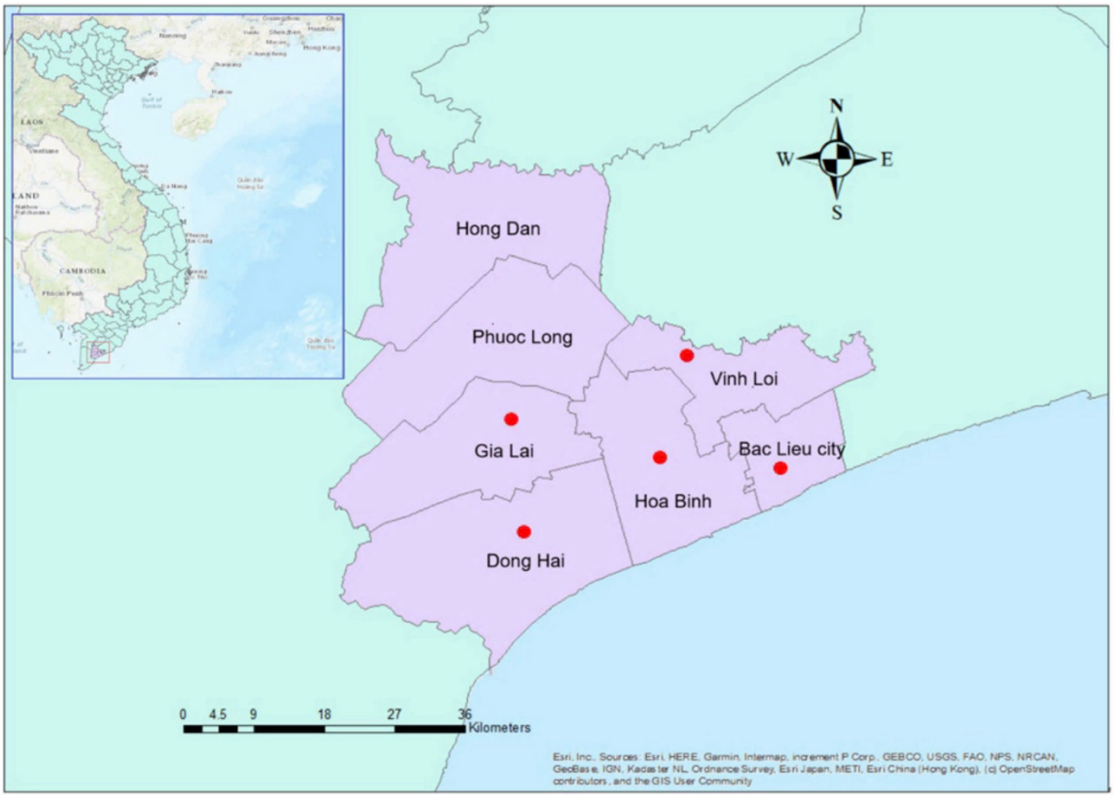

Figure 1 (A) Sample collection places in Bac Lieu province, Vietnam (shown by a red spot) for *Bacillus* isolation.

For *Bacillus* isolation from water samples and pond sludge, the method by Chen et al. (2016) was employed. Pond water samples were heat-treated at 80°C for 20 minutes, then diluted with physiological saline (0.9% NaCl) to a dilution factor of 10^6, and 100 µL of each dilution was spread onto NA plates supplemented with 1.5% NaCl. Similarly, 10 grams of pond sludge were homogenized in 90 mL of distilled water and shaken overnight at room temperature on a shaker at 110 rpm. The sample was then heat-treated at 80°C for 20 minutes, and the isolation procedure was conducted as with the water samples.

After spreading the samples, the plates were incubated at 37°C for 24 hours. The suspected *Bacillus* colonies were purified by subculture on nutrient agar and confirmed by colony morphology, gram and spore staining, motility, and oxidase and catalase activities. (Barrow and Feltham, 1993; Buller, 2014). The growth temperature range in LB medium was tested at 10, 20, 30, 40, 50, and 60°C, while pH tolerance was assessed at pH 2, 4, 6, 8, 10, and 12. Additionally, NaCl tolerance was evaluated in LB medium at concentrations of 0.5, 1, 2, 4, 6, 8, and 10% w/v (Barrow and Feltham, 1993; Buller, 2014).

Inhibitory activity isolated *Bacillus* against *V. parahaemolyticus*

The inhibitory activity of the isolated *Bacillus* strains against *V. parahaemolyticus* was evaluated using the well diffusion agar method (Amarantini et al., 2019). The indicator strain *V. parahaemolyticus* (VPBT23), isolated from AHPNS-infected white shrimp (Thi et al., 2025), was prepared in physiological saline (0.9% NaCl) to a density of approximately 9×10^8 CFU/mL, comparable to the McFarland 0.5 standard. This bacterial suspension was then evenly spread onto TSA medium plates (Trypticase Soy Agar, Himedia, India) supplemented with 1.5%

NaCl. These agar plates were punctured by a sterile technique for agar wells with a diameter of 6 mm.

Simultaneously, the isolated *Bacillus* strains were cultured in TY medium (5 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone, pH 7.0) and incubated on a shaker at 120 rpm at room temperature for 24-48 hours. The cultures were then centrifuged at 10,000 rpm for 5 minutes, and 80 µL of the supernatant was introduced into the wells of the TSA plates. The plates were incubated at 30°C for 24 hours. The antibacterial activity of the *Bacillus* isolates was assessed by measuring the diameter of the inhibition zone (D - d) around each well, where "D" represents the total diameter of the clear zone (mm) and "d" is the well diameter (6 mm).

Extracellular enzyme activity

The ability of isolated *Bacillus* strains to produce extracellular enzymes was assessed following the method described by Harley et al. (2001). The *Bacillus* isolates were cultured in liquid LB medium (Luria-Bertani, Himedia, India) at 37°C for 24 hours. After the incubation period, the bacterial growth solution was inoculated onto LB agar plates supplemented with specific substrates to detect the presence of cellulase, amylase, protease, and lipase. The substrates used were 0.5% carboxymethyl cellulose (CMC) for cellulase, 1% starch for amylase, 1% gelatin for protease, 1% casein for protease, and 1% v/v olive oil for lipase. The plates were then incubated overnight at 37°C, and the formation of clear halos around the colonies indicated enzyme activity.

Bacterial identification

Bacterial identification was carried out using the API 20E kit (BioMerieux, France), following the manufacturer's instructions. Additionally, representative bacterial isolates were subjected to PCR and sequencing of the 16S rRNA gene segment using the primer pairs 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Heuer et al., 1997). DNA from the isolated bacteria was extracted using the iVAaDNA Extraction Kit P (Thermo Scientific, USA), adhering to the manufacturer's protocol. The PCR reaction mixture consisted of 12.5 µL iStandard iVAPCR Master Mix (Thermo Scientific, USA), 9.5 µL double distilled water, 0.5 µL primer 27F (20 pmol), 0.5 µL primer 1492R (20 pmol), and 2 µL of sample DNA. The PCR cycling conditions were as follows: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, extension at 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. Post-amplification, the PCR products were subjected to 1.5% agarose gel electrophoresis, and the results were visualized using the Analytik Jena gel imaging system. Lastly, 1,500 bp of PCR results were delivered to DNA Sequencing Company (Vietnam) for sequencing.

Data analysis

Descriptive statistical methods were used to determine the mean values and standard deviations. At a significance level of 5%, an ANOVA was performed using the MiniTab 20 software to investigate the variation in inhibitory activity amongst bacterial strains. The sequence similarity of the bacterial strains was determined using the BLASTn tool to compare their sequences with those in the National Center for Biotechnology Information (NCBI) database. This comparison helps to identify the closest matches and possible identities of the isolated strains. For multiple sequence alignment, the CLUSTAL W program was employed (Thompson et al., 1997). This step aligns the bacterial sequences with one another to identify conserved regions and variations among the strains.

To illustrate the genetic relationships between the bacterial strains, a phylogenetic tree was constructed using the MEGA X software. The phylogenetic analysis was based on the neighbor-joining method (Saitou and Nei, 1987), which clusters sequences based on genetic distance. The robustness of the phylogenetic

tree was tested with bootstrap values calculated from 1,000 replications to ensure the reliability of the inferred evolutionary relationships (Tamura, 2013). The resulting tree provides a visual representation of the genetic links and evolutionary history among the isolated *Bacillus* strains.

RESULTS

Bacterial isolation

The data in Table 1 reveal that the majority of *Bacillus* isolates were obtained from the intestines of healthy shrimp, accounting for nearly half (45.23%) of the total isolates. This suggests that the intestinal microbiota of healthy shrimp is a rich source of *Bacillus* strains. Pond sludge also yielded a significant proportion of isolates (30.95%), indicating that the sediment in shrimp ponds harbors diverse *Bacillus* species. Pond water contributed the least number of isolates (23.80%), which may reflect a lower density or diversity of *Bacillus* in the water column compared to the shrimp intestines and pond sludge.

The distribution of the samples and isolates across different sources highlights the ecological niches where *Bacillus* strains thrive in shrimp farming environments. The relatively higher number of isolates from shrimp intestines suggests a potential role of these *Bacillus* strains in the gut health and disease resistance of shrimp. This information is critical for developing probiotic applications aimed at enhancing shrimp health and productivity in aquaculture systems.

Table 1 Origin of isolated *Bacillus* strains

Source of isolation	Number of ponds	Number of samples	Number of isolates
Healthy shrimp	6	16	19
Sludge	5	10	13
Pond water	5	10	10
Total	16	36	42

Morpho-physiological, and biochemical characteristics of isolated bacteria

The morphological and biochemical analysis of the isolated *Bacillus* strains highlights several key characteristics, as illustrated in Figure 2. In each pond, the healthy shrimp (Figure 2a& 2b) are randomly collected. The colonies exhibited a round shape, serrated edges, smooth or slightly wrinkled surfaces, and an opaque white color (Figure 2c). These features are typical of *Bacillus* species, indicating successful isolation. Gram staining revealed that the bacteria were long rod-shaped and Gram-positive (Figure 2d), confirming their classification within the *Bacillus* genus. Spore staining demonstrated the presence of endospores (Figure 2e), indicating that these bacteria can form resistant spores, allowing them to survive in harsh environmental conditions.

The observed motility and positive results for catalase and oxidase tests further support the identification of these strains as *Bacillus*, as these traits are common within this genus. The ability of the isolates to grow over a wide temperature range (20°C to 60°C) and pH range (4.0 to 10.0) demonstrates their adaptability, making them suitable candidates for various applications in aquaculture. However, their inability to grow at NaCl concentrations above 8% suggests a limitation in high-salinity environments, which is an important consideration for their use in shrimp farming. Overall, these findings provide a comprehensive profile of the isolated *Bacillus* strains, supporting their potential use as probiotics in aquaculture to enhance shrimp health and disease resistance.

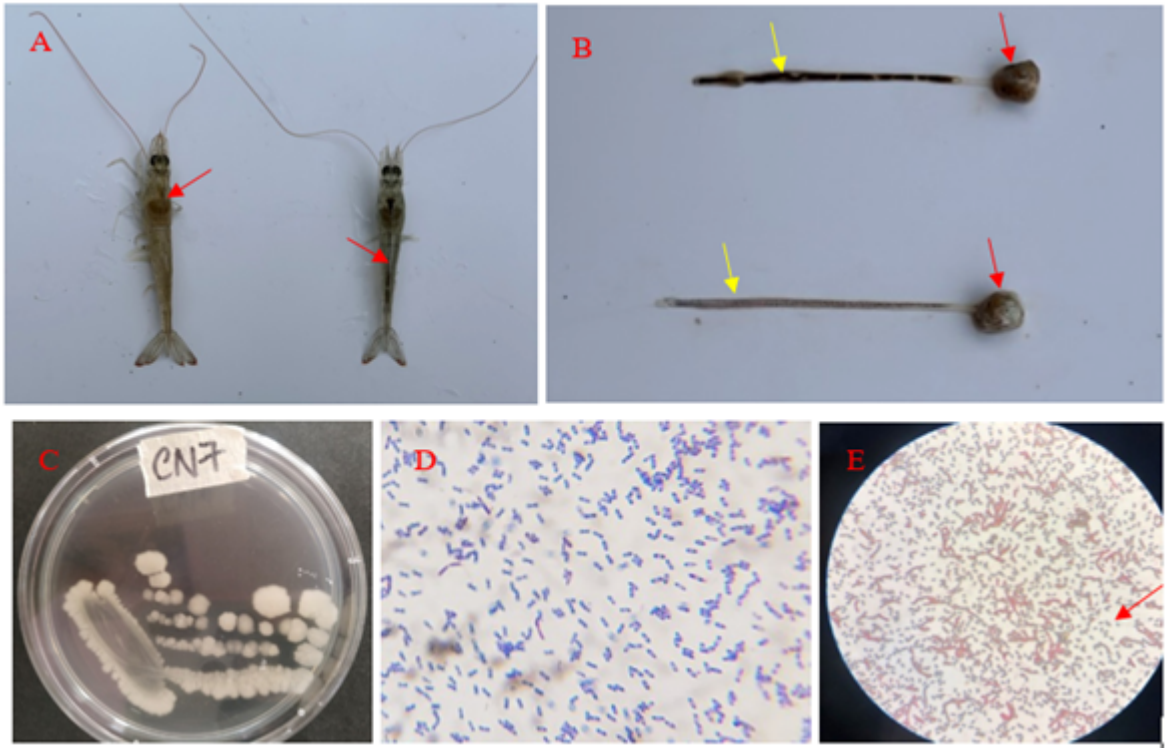

Figure 2 Morphological and biochemical characteristics of an isolated *Bacillus*.
A. Healthy shrimp samples with normal hepatopancreas and intestine (red arrow); B. The hepatopancreas (red arrow) and shrimp intestine of healthy shrimp (yellow arrow); C. Bacillus bacterial colonies on NA medium; D. Gram stain (100X); E. Spore staining (blue malachite green spores, red arrow, 100X)

Inhibitory activity of isolated *Bacillus* against *V. parahaemolyticus*

The analysis of the antibacterial activity data indicates a diverse range of inhibitory effects among the isolated *Bacillus* strains. Figure 3 visually demonstrates the presence of clear zones around the wells, indicating the antagonistic activity of certain *Bacillus* strains against *V. parahaemolyticus*. The variation in halo diameters highlights the differences in the potency of each strain.

Figure 4 provides a detailed comparison of the inhibition zones produced by each strain. Strain BLN7 stands out with the largest inhibition zone (24 mm), indicating its strong antibacterial potential. Strains BLB7, BLN8, and STNC also show significant inhibitory activity, with a consistent halo diameter of 12 mm. The moderate activity group, with inhibition zones between 5 mm and 10 mm, includes a significant portion of the isolates, suggesting that many strains have a potential for moderate antibacterial applications.

The weaker activity group, with halo diameters below 5 mm, still contributes valuable information about the variability of antibacterial properties within the *Bacillus* isolates. The presence of even weak activity may be beneficial in certain contexts or in combination with other treatments.

Overall, the data underscore the potential of specific *Bacillus* strains as effective biocontrol agents against *V. parahaemolyticus*, with the strongest strains (BLN7, BLB7, BLN8, and STNC) showing the most promise for further development and application in aquaculture settings to combat bacterial infections.

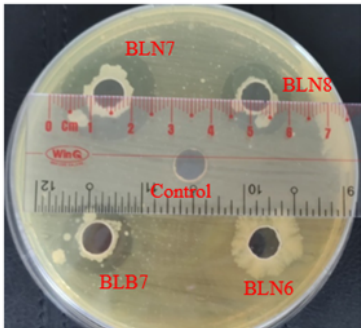

Figure 3 Antibacterial activity of *Bacillus* strains using the well diffusion agar method.

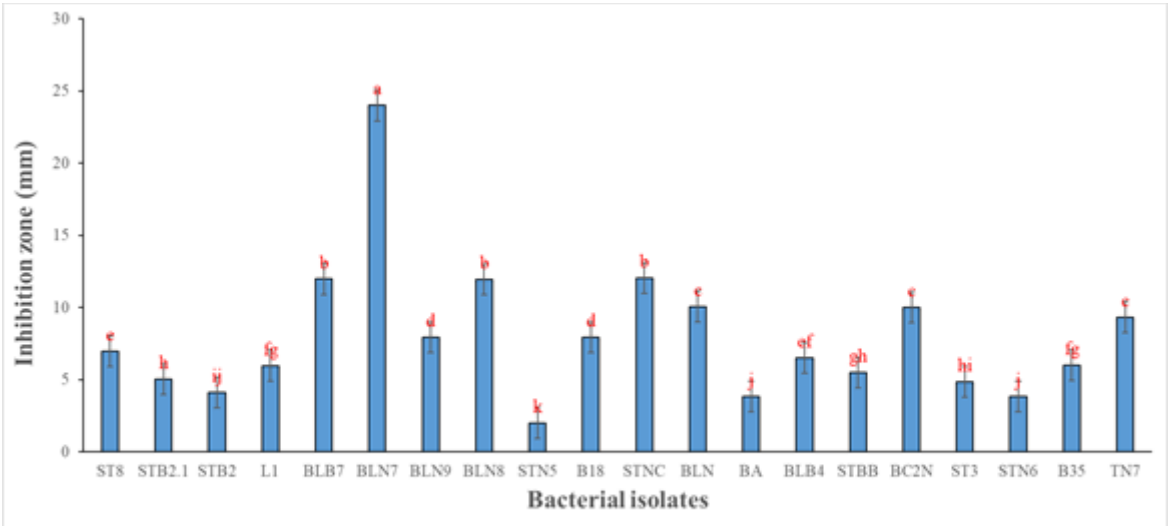

Figure 4 Differences in the inhibitory activity of *Bacillus* strains against *V. parahaemolyticus* (significant variations between treatments are shown by different letters on each bar ($p<0.05$).

Extracellular enzyme activity

The findings showed that the four strains BLN7, STNC, BLB7, and BLN8 exhibited extracellular enzyme activities, such as protease, amylase, and cellulase (Figure 5), except lipase enzyme activity was not present in the four bacterial strains (Table 2).

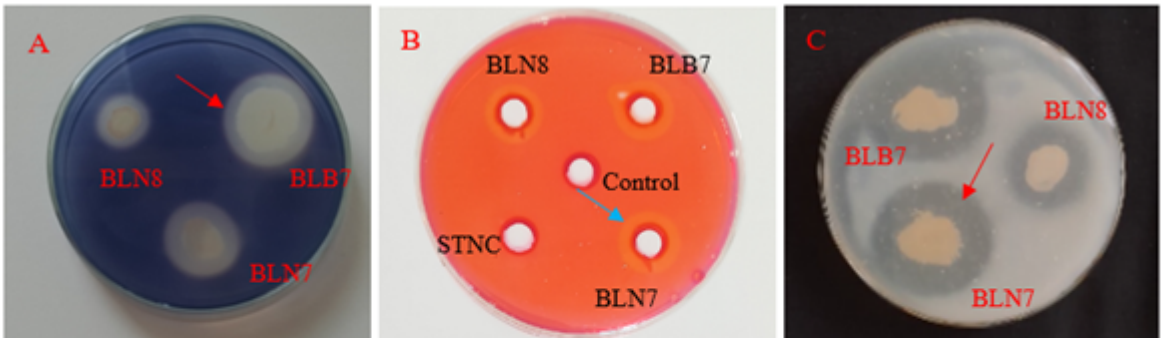

Figure 5 Extracellular enzyme activity of isolated *Bacillus* strains.
A. Amylase activity (arrow); B. Cellulase activity (arrow); C. Protease activity (arrow)

Table 2 Extracellular enzyme activities of isolated *Bacillus* isolates

Bacterial isolates	Isolate BLN7	Isolate STNC	Isolate BLB7	Isolate BLN8
Amylase	+	+	+	+
Cellulase	+	-	+	+
Protease	+	+	+	+
Lipase	-	-	-	-

+: positive; -: negative

Bacterial identification by the API 20E kit

Using the API 20E kit, the bacterial isolates showed negative reactions for the following criteria: ortho-nitrophenyl galactosidase, lysine, ornithine, H_2S, urease, indole, glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and arabinose. Conversely, the bacterial strains exhibited positive reactions for arginine, citrate, tryptophane, sodium pyruvate, and gelatin. The detailed results of *Bacillus* identification using the API 20E kit, alongside colony morphology and biochemical characteristics, are presented in Table 3.

The API 20E kit results revealed distinct biochemical profiles for the *Bacillus* isolates. The bacteria showed consistent negative reactions to multiple substrates, including ortho-nitrophenyl galactosidase, lysine, and ornithine, among others. However, they demonstrated positive reactions for arginine, citrate, tryptophane, sodium pyruvate, and gelatin, indicating specific metabolic capabilities.

The morphological and biochemical characteristics of the isolates, such as Gram-positive staining, long-rod cell shape, and motility, further confirm their classification as *Bacillus* species. These strains were capable of growing in a wide range of NaCl concentrations (up to 8%), various pH levels (4 to 10), and temperatures (20°C to 60°C), highlighting their adaptability to different environmental conditions.

This comprehensive characterization using the API 20E kit, coupled with morphological and biochemical analysis, underscores the potential of these *Bacillus* strains for application in aquaculture, particularly for their robustness and metabolic versatility. These traits are essential for developing effective probiotic formulations aimed at enhancing shrimp health and mitigating bacterial diseases in aquaculture systems.

Bacterial identification by PCR

The findings demonstrated that all selected bacterial strains successfully amplified the 16S rRNA gene segment, with a DNA band appearing at a size of 1,500 bp (Figure 6).

Sequencing results showed that strain BLN7 had high similarities (>98%) to *B. siamensis* strain FL14 (KY818932.1), *B. amyloliquefaciens* strain R1 (JX177675.1), *B. velezensis* strain SB2 (MH603331.1), *B. licheniformis* strain HT-22 (KJ526832.1), and *B. subtilis* strain MS07903 (ON870847.1). Similarly, strain STNC is over 98% similar to *B. amyloliquefaciens* strain JA3 (MZ148632.1), *B. velezensis* strain HSB1 (MT626060.1), *Bacillus* sp. DH-4 (KF646676.1), *B. methylotrophicus* strain L02 (JN700125.1), and *B. subtilis* strain S29 (OQ504777.1) in the NCBI database.

The PCR amplification results (Figure 6) clearly indicate the successful amplification of the 16S rRNA gene segment, with all bacterial strains displaying a distinct band at approximately 1,500 bp, which is consistent with the expected size for *Bacillus* species. This result confirms the presence of *Bacillus* DNA in the isolates. The sequencing data further support the identification of the *Bacillus* strains. Strain BLN7 shows high similarity to multiple *Bacillus* species, including *B. siamensis* and *B. amyloliquefaciens*, which are known for their beneficial roles in various environments. Strain STNC also aligns closely with several *Bacillus* species, such as *B. amyloliquefaciens* and *B. velezensis*, indicating its potential utility in aquaculture.

Table 3 Colony morphological, physiological, and biochemical characteristics of isolated *Bacillus*

Characteristics		Isolate BLN7	Isolate STNC	Isolate BLB7	Isolate BLN8
Gram staining		Gram-positive	Gram-positive	Gram-positive	Gram-positive
Cell shape		Long-rod	Long-rod	Long-rod	Long-rod
Motility		+	+	+	+
Oxidase		+	+	+	+
Catalase		+	+	+	+
Oxidation/Fermentation reaction		+	+	+	+
Grown on NaCl medium:					
	0.5%	+	+	+	+
	1%	+	+	+	+
	2%	+	+	+	+
	4%	+	+	+	+
	6%	+	+	+	+
	8%	+	+	+	+
	10%	-	-	-	-
Grown on pH medium:					
	2	-	-	-	-
	4	+	+	+	+
	6	+	+	+	+
	8	+	+	+	+
	10	+	+	+	+
	12	-	-	-	-
Grown on temperature:					
	10°C	-	-	-	-
	20°C	+	+	+	+
	30°C	+	+	+	+
	40°C	+	+	+	+
	50°C	+	+	+	+
	60°C	+	+	+	+
ONPG		-	-	-	-
Arginine		+	+	+	+
Lysine		-	-	-	-
Ornithine		-	-	-	-
Citrate		+	+	+	+
H_2S production		-	-	-	-
Urease		-	-	-	-
Tryptophane		+	+	+	+
Indole		-	-	-	-
Voges-Proskauer		+	+	+	+
Gelatin		+	+	+	+
Glucose		-	-	-	-
Mannitol		-	-	-	-
Inositol		-	-	-	-
Sorbitol		-	-	-	-
Rhamnose		-	-	-	-
Saccharose		-	-	-	-
Melibiose		-	-	-	-
Amygdalin		-	-	-	-
Arabinose		-	-	-	-

Note: +: positive; -: negative

The phylogenetic tree (Figure 7) shows that the two strains, BLN7 and STNC, cluster within the same group as other *Bacillus* species on GenBank. The high bootstrap values indicate robust support for the clustering of these isolates with known *Bacillus* species. Overall, the combined use of PCR amplification, sequencing, and phylogenetic analysis, along with traditional biochemical and morphological methods, provides comprehensive and reliable identification of the *Bacillus* strains. These findings underscore the potential application of these *Bacillus* isolates as probiotics in aquaculture, particularly for managing bacterial diseases in shrimp farming.

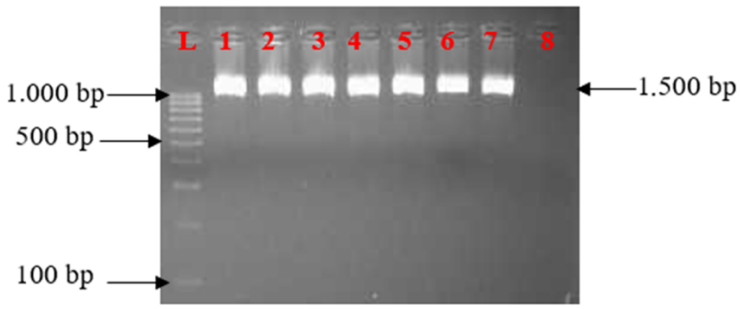

Figure 6 Amplification results of the 16S rRNA gene segment of *Bacillus*. *M: 100 bp standard ladder; Lane 1-7: bacterial strains* ST8, L1, BA, BLN7, STNC, BLB7, and BLN8*; Lane 8: Negative control.*

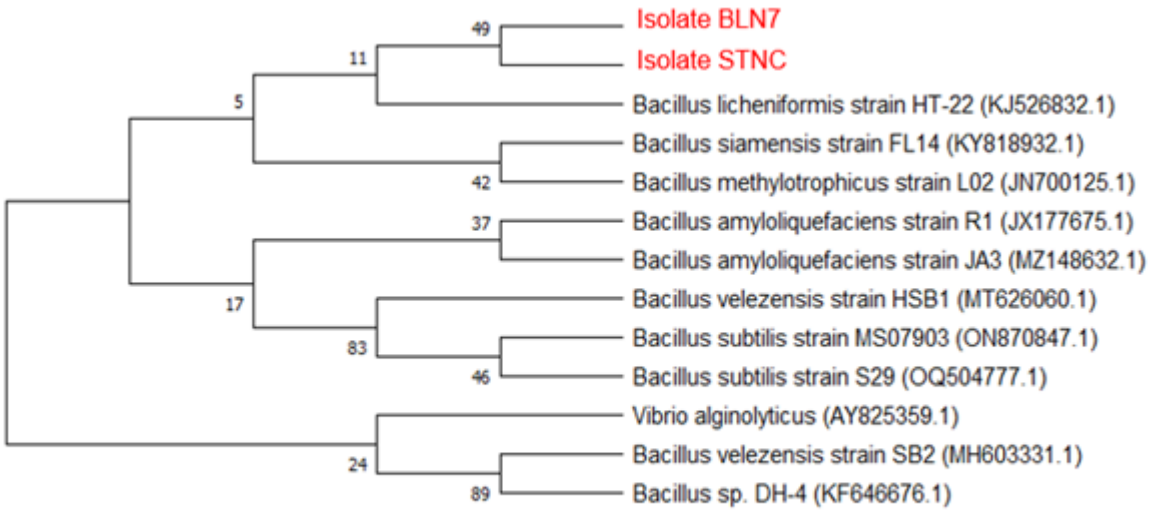

Figure 7 The phylogenetic tree illustrating the relationship between *Bacillus* isolates using a bootstrap value of 1,000 replicates (*V. alginolyticus* (AY825359.1) used as an outgroup).

DISCUSSION

Bacillus is a versatile group of bacteria widely utilized in microbial products for aquaculture, particularly shrimp farming, due to their ability to improve water quality and inhibit a broad spectrum of pathogenic microbes (Kuebutornye et al., 2019; Hlordzi et al., 2020; Purivirojkul and Areechon, 2007). In this study, a total of 42 *Bacillus* strains were isolated from the intestines of shrimp, pond water, and pond mud in super-intensive shrimp ponds in Bac Lieu province. This finding aligns with previous research indicating that *Bacillus* can be isolated from similar sources in intensive shrimp farming environments (Duc et al., 2022).

The isolated *Bacillus* strains exhibited characteristics typical of the genus: they were gram-positive, endospore-forming, motile, and catalase-positive. They also showed positive reactions for arginine, citrate, tryptophane, sodium pyruvate, and gelatin, consistent with previous descriptions of Bacillus bacteria (Dao et al., 2015; Peng et al., 2019). Similar to the findings of Truong et al. (2021), the colonies of *Bacillus* isolated from white-leg shrimp ponds were round, creamy white, and had entire or irregular margins. These isolates were confirmed as *Bacillus* through the use of the API 20E kit and 16S rRNA gene sequencing. However, this study did not identify the isolates at the species level.

Bacillus bacteria possess spores that enhance their survival across various environments, making them advantageous for probiotic production in aquaculture

(Saggese et al., 2021). Moreover, the isolated *Bacillus* strains demonstrated the ability to survive in low pH environments (pH 3) and high salt concentrations (6% NaCl). These characteristics suggest that the *Bacillus* isolates can thrive in the shrimp digestive system and the high-salinity conditions typical of shrimp ponds. These characteristics show that the isolated *Bacilus* isolates can survive in the shrimp pond environment with water temperature, salinity, and pH recorded as 26-30°C, 10-20‰, and 6.5-7.2, respectively. Therefore, the *Bacillus* strains identified in this study show significant potential for developing biological products to prevent Early Mortality Syndrome (EMS) in white-leg shrimp.

The effectiveness of probiotic products is evident when they are introduced into the digestive tract, as they enhance food metabolism, boost immunity, and inhibit the growth of pathogenic microorganisms. This leads to a balanced intestinal microflora and a reduction in gastrointestinal diseases (Amara and Shibl, 2013). The present study demonstrated that 20 out of 40 *Bacillus* strains exhibited antagonistic activity against *V. parahaemolyticus*. Among these, four strains (BLN7, STNC, BLB7, and BLN8) showed the strongest antagonistic activity, with inhibition zones of 24 ± 0.00 mm, 12.03 ± 0.06 mm, 11.97 ± 0.12 mm, and 11.93 ± 0.12 mm, respectively. This aligns with findings by Truong et al. (2021), who reported inhibition zones of 13.05 ± 0.35 mm, 12.5 ± 0.3 mm, and 9.90 ± 0.2 mm for *Bacillus* strains CM3.1, CM2.2, and TV1.2 against Vp AHPND. Similarly, Minh et al. (2019) found that nine out of twenty-five *Bacillus* strains showed resistance to *V. parahaemolyticus* NT7 within 24 hours, with inhibition zones ranging from 10.33 mm to 18.50 mm. Furthermore, Balcazar et al. (2007) identified that B. subtilis UTM 126 antagonized pathogenic *Vibrio* strains, including *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*. Phuong et al. (2018) also demonstrated that *B. licheniformis* (B1) isolated from the intestine of Mullet fish inhibited *V. parahaemolyticus* with a clear zone of 15 mm.

Bacillus bacteria exhibit antagonistic abilities by producing various antibacterial substances (Caulier et al., 2019; Shleeva et al., 2023). Previous research has shown that *Bacillus* species secrete antimicrobial compounds, including peptide and lipopeptide antibiotics like bacteriocin, subtilin, subtilomycin, iturins, cerecidin, polymyxins, haloduracin, and bacteriocin-like inhibitory substances (Stoica et al., 2019). These substances exhibit antagonistic activity against a broad spectrum of bacterial agents in fish, such as *Shigella*, *Vibrio*, *Photobacterium*, *Tenacibaculum*, *Aeromonas*, and *Edwardsiella* (Chalasani et al., 2015; Santos et al., 2021). The inhibitory activity of Bacillus bacteria depends on the bacterial strain and culture conditions, highlighting the need for further studies to determine the factors of temperature, time, bacterial population, and concentration that affect their antibacterial activity against *V. parahaemolyticus.*

Extracellular enzymes play a crucial role in supporting food digestion, facilitating nutrient absorption, and promoting weight gain in animals. Therefore, the ability to produce extracellular enzymes is a key criterion for selecting bacterial strains as probiotics. In this study, all four strains (BLN7, STNC, BLB7, and BLN8) with the strongest antagonistic activity against *V. parahaemolyticus* also exhibited amylase, cellulase, and protease activities. This observation is consistent with Jamilah et al. (2009), who found that more than 90% of *Bacillus* isolates from shrimp ponds displayed both proteolytic and amylolytic activity. Similarly, Wang et al. (2020) reported that *Bacillus* strains derived from the digestive tract of *P. monodon* produced multiple hydrolytic enzymes, including protease, amylase, lipase, and cellulases. In Vietnam, Dat et al. (2019) identified *Bacillus* sp. W12 from water and sediment samples of fish ponds in Thua Thien Hue province, which produced cellulase, protease, and amylase enzymes capable of breaking down organic materials and cellulose.

The primary components of shrimp feed are protein and starch. Overfeeding can lead to the accumulation of these substances, reducing water quality in commercial shrimp ponds. The extracellular protease and amylase produced by the isolated *Bacillus* strains in this study suggest their potential for maintaining

water quality by reducing the total suspended solids in shrimp pond water columns (Monier et al., 2023). Truong et al. (2021) also demonstrated that water treated with *B. subtilis* CM3.1, which exhibited high amylase, protease, and cellulase activities, significantly reduced TAN (total ammonia nitrogen) and NO_2-N (nitrite nitrogen) concentrations, thereby enhancing shrimp survival rates and specific growth rates.

Overall, these findings underscore the potential application of *Bacillus* strains in developing probiotic products for shrimp farming, aiming to improve health, water quality, and disease resistance in aquaculture systems.

CONCLUSIONS

This study highlights the antibacterial activity of *Bacillus* isolates against *V. parahaemolyticus*, a key pathogen in shrimp farming. From 36 samples collected in intensive white-leg shrimp ponds in Bac Lieu province, 42 *Bacillus* strains were isolated. Among these, 20 exhibited inhibitory effects against *V. parahaemolyticus*, with strains BLN7, STNC, BLB7, and BLN8 showing the strongest activity, as evidenced by inhibition zones up to 24 mm. These strains also produced enzymes like amylase, cellulase, and protease. Morphological, biochemical, and 16S rRNA gene sequencing confirmed the identity of these *Bacillus* strains, with BLN7 and STNC showing over 98% similarity to known beneficial *Bacillus* species. This research is novel in focusing on indigenous *Bacillus* strains, leveraging local microbial biodiversity, and aligning with sustainable aquaculture practices. The findings provide a foundation for developing biological products to prevent Early Mortality Syndrome (EMS) in white-leg shrimp, thereby improving health and reducing mortality rates. Future research should explore the mechanisms of antibacterial action, environmental factors affecting probiotic efficacy, and practical applications in large-scale shrimp farming. Such studies will validate these probiotics, contributing to sustainable and resilient aquaculture practices and advancing shrimp farming with beneficial *Bacillus* strains.

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