fishes

MDPI

Article

Antibacterial Activity of *Bacillus* Strains against Acute Hepatopancreatic Necrosis Disease-Causing *Vibrio campbellii* in Pacific White Leg Shrimp

Hye Jin Jeon [1,†], Jae Won Song [2,†], Chorong Lee [1], Bumkeun Kim [1], Seon Young Park [3], Ji Hyung Kim [4,*], Jee Eun Han [1,*] and Jae Hak Park [2,*]

[1] College of Veterinary Medicine, Kyungpook' National University, Daegu 41566, Korea
[2] Department of Laboratory Animal Medicine, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul 08826, Korea
[3] Division of Animal and Dairy Sciences, College of Agriculture and Life Science, Chungnam National University, Daejeon 34134, Korea
[4] Department of Food Science and Biotechnology, Gachon University, Seongnam 13120, Korea
* Correspondence: kzh81@gachon.ac.kr (J.H.K.); jehan@knu.ac.kr (J.E.H.); pjhak@snu.ac.kr (J.H.P.)
† These authors contributed equally to this work.

Abstract: Acute hepatopancreatic necrosis disease (AHPND) is a bacterial disease caused by *Vibrio parahaemolyticus*. Currently, various *Vibrio* strains, including *V. campbellii*, *V. owensii*, and *V. harveyi*, have been reported as causative pathogens. Thus, controlling AHPND to maintain high production in shrimp aquaculture is difficult. We evaluated the antimicrobial activity of five *Bacillus* strains (B1, B3, B5, B7, and B8)—isolated from seawater in Jeju, South Korea—against 12 *Vibrio* strains (10 AHPND strains and 2 non-AHPND strains). All tested *Bacillus* strains inhibited the growth of at least one of the tested *Vibrio* strains in the dot-spot method. Among them, B1 and B3, the most effective *Bacillus* strains against the *Vibrio* strains, particularly against AHPND-causing *V. campbellii* (Vc_{AHPND}), were further used in a challenge test. After 48–60 h of Vc_{AHPND} immersion, a significantly higher survival rate was observed in the B1-treated group (100%) than in the non-*Bacillus*-treated group (64.3%). Based on the qPCR analysis of AHPND, the cycle threshold values were 31.63 ± 0.2 (B1-treated group) and 38.04 ± 0.58 (B3-treated group), versus 28.70 ± 0.42 in the control group. Genome sequencing and phylogenetic analysis revealed that B1 and B3 were classified as *B. velezensis*. The 16S rRNA sequences and complete genome sequences of B1 and B3 were deposited in GenBank.

Keywords: acute hepatopancreatic necrosis disease; antibacterial; aquaculture; *Penaeus vannamei*; probiotics; shrimp; *Vibrio campbellii*; *Vibrio parahaemolyticus*

Citation: Jeon, H.J.; Song, J.W.; Lee, C.; Kim, B.; Park, S.Y.; Kim, J.H.; Han, J.E.; Park, J.H. Antibacterial Activity of *Bacillus* Strains against Acute Hepatopancreatic Necrosis Disease-Causing *Vibrio campbellii* in Pacific White Leg Shrimp. *Fishes* **2022**, *7*, 287. https://doi.org/10.3390/fishes7050287

Academic Editor: Bernardo Baldisserotto

Received: 17 August 2022
Accepted: 14 October 2022
Published: 15 October 2022

1. Introduction

Acute hepatopancreatic necrosis disease (AHPND) is a bacterial disease caused by *Vibrio* spp. carrying toxin genes (*pir*A and *pir*B) located in a large plasmid (69 kb). AHPND affects the digestive tract of shrimp and the tubular cells of the hepatopancreas, disturbing digestion and resulting in mass mortality. *V. parahaemolyticus* is primarily associated with AHPND (Vp_{AHPND}), but other *Vibrio* species that carry binary toxin genes, including *V. campbellii* (Vc_{AHPND}), *V. owensii* (Vo_{AHPND}), and *V. harveyi* (Vh_{AHPND}), have been reported recently [1–4]. AHPND was first reported in China (2009), and it spread to several countries, including Vietnam (2010), Malaysia (2011), Thailand (2012), Mexico (2013), the Philippines (2015), the USA (2019), and South Korea (2020) [1,5–10]. This disease is known to cause tremendous economic losses in the shrimp aquaculture industry. Shrimp production has considerably decreased following the outbreak of AHPND, and the economic damage is estimated to exceed 1 billion dollars per year in Asia [11].

Although antibiotics have been extensively used to treat bacterial diseases in aquaculture for decades [12], their utilization particularly in the form of overuse or misuse has

resulted in antibiotic resistance [13–18]. As antibiotic alternatives, probiotics have been frequently used in aquaculture to control bacterial diseases, especially against pathogenic *Vibrio* infections and AHPND. In a previous report, shrimp treated with *Bacillus* probiotics in the form of dietary supplements showed a higher survival rate following challenge with Vp_{AHPND} [19,20]. In addition to their antimicrobial activity, probiotics have various advantages in aquaculture such as promoting growth, strengthening immunity, and restoring water quality [21,22]. Meanwhile, spore-forming *Bacillus* spp. are resistant to heat and pressure and are widely used as feed additives [23].

Besides the use of probiotics, various methods have been utilized in shrimp aquaculture such as immunostimulant therapy, quorum sensing control of bacterial virulence, phage therapy, and herbal medicine [18,24–26]. Paopradit et al. [27] reported the reduced virulence and decreased mortality of Vp_{AHPND} following treatment with quorum sensing inhibitors such as indole or indole-containing compounds. In addition, previous studies [28,29] have confirmed the control of both growth and infection of Vp_{AHPND} using bacteriophages in double-layer agar culture and a series of bioassays, respectively.

Although *V. parahaemolyticus* is the cause of most cases of AHPND, other *Vibrio* spp., such as *V. campbellii*, *V. harveyi*, and *V. owensii*, are also known to cause this disease in fields, thereby resulting in substantial economic losses on farms. However, preventative methods and studies on AHPND have mainly focused on Vp_{AHPND}, and the antimicrobial activity against Vc_{AHPND}, Vh_{AHPND}, and Vo_{AHPND} has been poorly studied [25,30]. In this study, we isolated five *Bacillus* strains and evaluated their antimicrobial activity against ten AHPND-causing *Vibrio* strains and two non-AHPND *Vibrio* strains using a dot-spot test (in vitro). In addition, a challenge test against Vc_{AHPND} was performed using two *Bacillus* strains (B1 and B3) that strongly inhibited Vc_{AHPND} among five *Bacillus* strains in the dot-spot test. The findings revealed that B1 and B3 treatment groups significantly suppressed Vc_{AHPND} growth compared with the effect of the non-*Bacillus* treatment group. Finally, the genomic sequences of these two *Bacillus* strains were completely analyzed, and both strains were classified as *B. velezensis*. Their 16S rRNA sequences and complete genome sequences were deposited in GenBank. The results of this study provide useful and practical strategies that can be applied in the shrimp culture industry, which is currently experiencing declines in shrimp production because of harmful shrimp diseases, including AHPND caused by Vp_{AHPND}, and Vc_{AHPND}.

2. Materials and Methods

2.1. Bacillus and Vibrio Candidate Isolation and Polymerase Chain Reaction (PCR)

For the isolation of *Bacillus* spp., seawater samples were collected from six different sites in Jeju Island, South Korea. These seawater samples were subjected to a serial dilution process, and dilutions were spread onto tryptic soy agar (TSA; Difco, Becton Dickinson, Franklin Lakes, NJ, USA) plates supplemented with 2% NaCl (TSA+). The plates were incubated at 28 °C for 24–48 h. Subsequently, we picked the bacterial colonies displaying sporulated shapes on the agar plates based on morphology, and the colonies were sub-cultured for pure culture isolation. Isolates were preserved in tryptic soy broth (TSB; Difco, Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 2% NaCl (TSB+) containing 25% glycerol at −80 °C until further analysis. Each isolate was grown in TSB+ (28 °C, 200 rpm, 24–48 h) and DNA was extracted using the protocol of the modified DNeasy® Blood & Tissue Kit (Qiagen, Germany). For *Bacillus* identification, PCR was performed using the extracted DNA, and the BacF/R primers, as described by Solichova et al. [31] (Table 1).

For the isolation of *Vibrio* spp., seawater and hepatopancreas samples were collected from Mexico, Vietnam, Thailand, South Korea, and the USA. These samples were serially diluted and spread on thiosulfate citrate bile salts sucrose (TCBS) (MB Cell, South Korea) agar plates, which were incubated at 28 °C for 24–48 h. Next, we picked green and yellow colonies from the TCBS plates, and the colonies were sub-cultured for pure culture isolation. Isolates were preserved in TSB+ containing 25% glycerol at −80 °C until further analysis.

Each isolate was grown in TSB+ (28 °C, 200 rpm, 24–48 h) and used for DNA extraction using the boiling method described by Dashti et al. [32]. For *Vibrio* identification, PCR was conducted using the extracted DNA and the primer sets (Tox R-F/R, Vc.fts.z-F/R, and Vh.topA-F/R) described by Kim et al. [33] and Cano-Gomez et al. [34] (Table 1). To identify AHPND virulence genes, PCR targeting AHPND toxin genes (*pir*A and *pir*B) was conducted using the method described by Han et al. [35] (Table 1).

Table 1. Primers for *Bacillus*, AHPND toxin genes (*pir*A and *pir*B), and *Vibrio* species.

Target	Primers	Sequence (5′–3′)	Amplicon Size (bp)	Reference
Bacillus	BacF BacR	GCTGGTTAGAGCGCACGCCTGATA CATCCACCGTGCGCCCTTTCTAAC	263	[31]
AHPND toxin	VpPirA-284F VpPirA-284R	TGACTATTCTCACGATTGGACTG CACGACTAGCGCCATTGTTA	284	[35]
	VpPirA-392F VpPirA-392R	TGATGAAGTGATGGGTGCTC TGTAAGCGCCGTTTAACTCA	392	
V. parahaemolyticus	Tox R-F Tox R-R	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	368	[33]
V. campbellii	Vc.fts.z-F Vc.fts.z-R	AAGACAGAGATAGACTTAAAGAT CTTCTAGCAGCGTTACAC	294	[34]
V. harveyi	Vh.topA-F Vh.topA-R	TGGCGCAGCGTCTATACG TATTTGTCACCGAACTCAGAACC	121	

2.2. Antimicrobial Activity Test (In Vitro)

For antimicrobial activity testing, the *Bacillus* strains that were obtained were further tested for their ability to inhibit the growth of *Vibrio* strains using the dot-spot method described by Spelhaug and Harlander [36]. *Vibrio* strains were grown in TSB+ with shaking at 200 rpm and 28 °C for 24 h, and bacterial suspensions of each strain were normalized with 2.5% NaCl to obtain a final concentration of approximately 5×10^6 colony forming units (CFU) mL^{-1}. *Bacillus* strains were grown in TSB+ with shaking at 200 rpm and 28 °C for 24–48 h to obtain a final concentration of approximately 5×10^8 CFU mL^{-1}. Then, 100 µL of each *Vibrio* strain suspension was inoculated into 5 mL of soft agar and poured onto TSA+ plates. Ten-microliter aliquots of *Bacillus* strain suspensions were dot-spotted on the surface of *Vibrio*-overlaid agar. The plates were incubated at 28 °C for 12–24 h, and the clear zones around each *Bacillus* colony were recorded.

B. velezensis CR-502^T (=NRRL B-41580^T) was obtained from the Korean Collection for Type Cultures (KCTC) and set as the reference strain in this experiment. The experiments were also conducted using the same methods.

2.3. Antimicrobial Activity Test (Challenge Test)

Bacillus strains that showed the strongest inhibitory effects in the dot-spot test were further subjected to the challenge test. As experimental shrimp, the Pacific white leg shrimp (*Penaeus vannamei*) at the post-larval stage (stages PL_{15}–PL_{16}) were purchased from a local shrimp farm (Jeju Province, South Korea) and transported to the Laboratory of Aquatic Biomedicine, College of Veterinary Medicine, Kyungpook National University in South Korea. Shrimp were fed a commercial diet twice daily in a 700 L acrylic tank for 35 days to be acclimated to the experimental conditions and facilities. Then, the shrimp (average weight of 0.2 ± 0.05 g) were randomly distributed into 22 L acrylic tanks with 18 L of aerated artificial seawater. For the antimicrobial activity test (challenge test), experimental shrimp (N = 56) were divided into four groups with duplicates.

In group 1, the experimental shrimp (N = 14) were exposed to a suspension of *Bacillus* (B1) for 14 days via immersion at a concentration of 1.0×10^6 CFU mL^{-1} water. Then, the shrimp were challenged with a Vc_{AHPND} (16-904/1) suspension via immersion at a concentration of 2.0×10^6 CFU mL^{-1} water. In group 2, the experimental shrimp (N = 14)

were exposed to *Bacillus* (B3) suspension for 14 days via immersion at a concentration of 1.0×10^6 CFU mL^{-1} water. Then, the shrimp were challenged with a Vc_{AHPND} (16-904/1) suspension via immersion at a concentration of 2.0×10^6 CFU mL^{-1} water. In group 3, the experimental shrimp (N = 14) were exposed to the same amount of fresh broth (TSB+) without *Bacillus* strains (B1 and B3) for 14 days via immersion. Then, they were challenged with a Vc_{AHPND} (16-904/1) suspension via immersion at a concentration of 2.0×10^6 CFU mL^{-1} water. In group 4, the experimental shrimp (N = 14) were exposed to the same amount of fresh broth (TSB+) without *Bacillus* for 14 days, and then they were not challenged Vc_{AHPND} (16-904/1). The experiment was started at the same time and under the same conditions for all groups. The tanks were filled with aerated artificial seawater and maintained without water change for 28 days. The water temperature, dissolved oxygen level, pH, and salinity were maintained at 25–28 °C, 6.39–7.21 ppm, 6.48–7.10, and 23–25 ppt, respectively. Shrimp were fed shrimp feed three times a day at 5% of their body weight and monitored for 28 days.

To confirm the presence of AHPND, dead shrimp were collected and tested using the PCR method previously described by Han et al. [35]. To quantify AHPND, surviving shrimp were randomly sampled on the day of termination (day 14). The hepatopancreas of each shrimp was collected aseptically; next, 30 mg of the hepatopancreas tissue was used for DNA extraction using the DNeasy® Blood & Tissue Kit. Using the extracted DNA, quantitative PCR was performed to quantify the AHPND toxin gene *pir*A in the hepatopancreas in the groups using the method described by Han et al. [37].

2.4. Genome Sequencing and Phylogenetic Analysis of the Selected Bacillus Strains

The genomes of two selected *Bacillus* strains (B1 and B3) were sequenced using a hybrid approach on a PacBio RS II system (Pacific Biosciences Inc., Menlo Park, CA, USA) by constructing a 20 kb SMRTbellTM template library and on the HiSeq X-10 platform (Illumina Inc., San Diego, CA, USA) by preparing a DNA library using the TruSeq Nano DNA Library Prep Kit (Illumina). Genome assembly of the filtered PacBio reads was performed using the HGAP (v3.0) pipeline, the 150-bp Illumina paired-end reads were mapped using BWA-MEM (v0.7.15), and errors were corrected using Pilon (v1.21) using the default parameters. Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/books/NBK174280/ (accessed on August 2022) [38]. The regions and clusters of secondary metabolites present in the genomes of both strains were predicted using antibiotics & Secondary Metabolite Analysis Shell (anti-SMASH) v.6.1.1 [39]. The phylogenetic trees of the two *Bacillus* strains based on the 16S ribosomal RNA (rRNA) genes and whole-genome sequences were constructed using selected 20-type species of the genus *Bacillus*. First, the 16S rRNA sequences of the two isolates were aligned with 20 representative species of the genus *Bacillus* using Clustal X (ver. 2.0) [40] and BioEdit (ver. 7.0) [41], and the maximum-likelihood phylogenetic tree based on the concatenated sequences was generated using MEGA X [42] with 1000 bootstrap replicates. Second, the whole genome-based phylogenetic tree was generated using the Type (Strain) Genome Server and inferred with FastME 2.1.6.1 [43] from Genome BLAST Distance phylogeny approach (GBDP) distances calculated using genome sequences. The branch lengths were scaled in terms of GBDP distance formula D5, and the numbers above the branches were GBDP pseudo-bootstrap support values >60% from 100 replications. The regions and clusters of secondary metabolites present in the genomes of both the B1 and B3 strains and *B. velezensis* CR-502^T (=NRRL B-41580^T) were predicted using antibiotics & Secondary Metabolite Analysis Shell (anti-SMASH) v.6.1.1 [42] and compared.

2.5. Accession Numbers of Nucleotide Sequences and Strain Deposition

The 16S rRNA sequences of *Bacillus* B1 and B3 were deposited in GenBank under the accession numbers OP364972 and OP364977, respectively. The complete genome sequences of B1 and B3 were deposited in GenBank under the accession numbers CP100040 and CP100041, respectively.

2.6. Statistical Analysis

Survival data in the challenge test were analyzed via one-way analysis of variance (ANOVA) using SPSS version 24.0 (SPSS Inc., Chicago, IL, USA). The mean differences were compared using Duncan's multiple range test when a significant difference was identified using ANOVA. For the comparison of means, the significance level was set at $p < 0.05$. Data are presented as the mean ± SD, and the percentage data were arcsine-transformed before the comparisons.

3. Results

3.1. Identification of Bacillus and Vibrio Strains

In total, five *Bacillus* strains were obtained from seawater samples collected from Jeju Island, South Korea. Using PCR with primers specific for the genus *Bacillus*, all five strains (B1, B3, B5, B7, and B8) were confirmed to be *Bacillus* spp., as presented in Table 2 and Figure S1.

Table 2. *Bacillus* and *Vibrio* strains and their identification using PCR.

Strain	Origin	Source	Isolation Year	PCR Identification	Accession No [a]
Bacillus strains					
B1	South Korea	Seawater	2019	*Bacillus* spp.	OP364972
B3	South Korea	Seawater	2019	*Bacillus* spp.	OP364977
B5	South Korea	Seawater	2019	*Bacillus* spp.	-
B7	South Korea	Seawater	2019	*Bacillus* spp.	-
B8	South Korea	Seawater	2019	*Bacillus* spp.	-
Vibrio strains					
16-904/1	Mexico	Shrimp	2016	AHPND *Vibrio campbellii*	-
13-028/A3	Vietnam	Shrimp	2015	AHPND *V. parahaemolyticus*	KM067908
15-250/20	Latin America	Shrimp	2015	AHPND *V. parahaemolyticus*	-
CH49	Thailand	Seawater	2019	AHPND *V. parahaemolyticus*	-
CH50	Thailand	Seawater	2019	AHPND *V. parahaemolyticus*	-
CH51	Thailand	Seawater	2019	AHPND *V. parahaemolyticus*	-
CH52	Thailand	Seawater	2019	AHPND *V. parahaemolyticus*	-
CH53	Thailand	Seawater	2019	AHPND *V. parahaemolyticus*	-
19-021D1	South Korea	Seawater	2019	AHPND *V. parahaemolyticus*	MN631018, MN631020
19-022A1	South Korea	Seawater	2019	AHPND *V. parahaemolyticus*	MN631019, MN631021
NSU116	Latin America	Shrimp	2016	Non-AHPND *V. parahaemolyticus*	-
LB4	USA	Seawater	2017	Non-AHPND *V. harveyi*	-

[a]: Accession number of 16S rRNA sequences.

Twelve *Vibrio* strains were obtained from seawater and hepatopancreas tissue samples from shrimp. Using PCR with primers specific for *V. parahaemolyticus*, *V. campbellii*, and *V. harveyi*, one strain was identified as *V. campbellii* (16-904/1), 10 strains were identified as *V. parahaemolyticus* (13-028/A3, 15-250/20, CH49, CH50, CH51, CH52, CH53, 19-021D1, 19-022A1, and NSU116), and one strain was identified as *V. harveyi* (LB4). Using PCR targeting the AHPND toxin genes, ten strains (16-904/1, 15-250/20, 13-028/A3, CH49, CH50, CH51, CH52, CH53, 19-021D1, and 19-022A1) were identified as AHPND strains, and two strains (NSU116 and LB4) were identified as non-AHPND strains, as presented in Table 2 and Figure S1.

3.2. Antimicrobial Activity Test (In Vitro)

Using the dot-spot method, five *Bacillus* strains (B1, B3, B5, B7, and B8) were demonstrated to inhibit the growth of at least one of the tested *Vibrio* strains in shrimp, including Vc_{AHPND} (16-904/1), Vp_{AHPND} (13-028/A3, 15-250/20, CH49, CH50, CH51, CH52, CH53, 19-021D1, and 19-022A1), non-AHPND *V. parahaemolyticus* (NSU116), and non-AHPND *V. harveyi* (LB4), as evidenced by a clear zone around the *Bacillus* colonies (Tables 3 and S1).

In particular, B1 and B3 exhibited stronger inhibitory effects on Vc_{AHPND} than the other *Bacillus* strains (type strain, B5, B7, and B8), as presented in Tables 3 and S2.

Table 3. Inhibitory effects of *Bacillus* strains against *Vibrio* strains (dot-spot test).

Vibrio Strains	*Bacillus* Strains					
	Type Strain [a] (*B. velezensis*)	B1	B3	B5	B7	B8
16-904/1	+	++	++	−	+	+
13-028/A3	++	−	+	−	−	−
15-250/20	+	−	+	+	−	+
CH49	+	+	+	−	−	−
CH50	+	−	+	−	−	+
CH51	−	−	+	++	−	−
CH52	+	−	+	−	−	−
CH53	−	++	++	++	+	+
19-021D1	+	++	++	++	+	+
19-022A1	+	++	++	++	+	+
NSU116	+	++	++	+	+	+
LB4	−	+	+	+	−	−

[a]: CR-502^T (= NRRL B-41580^T). +: clear zone smaller than 1 mm, ++: clear zone between 1 and 2 mm in size. −: No clear zones were observed.

3.3. Antimicrobial Activity Test (Challenge Test)

Based on the dot-spot test result, we selected the B1 and B3 strains with a strong inhibitory effect against Vc_{AHPND}, and their antimicrobial activities against Vc_{AHPND} were evaluated using a challenge test. Rapid mortality was observed between 48 and 60 h. After 60 h of Vc_{AHPND} immersion, a significantly higher survival rate was observed in the B1 treatment group (group 1, 100%) than in the non-*Bacillus* treatment group (group 3, 64.3%) (Table 4). At the end of the challenge test, shrimp in group 1 (Vc_{AHPND} immersion after B1 treatment) and in group 2 (Vc_{AHPND} immersion after B3 treatment) had numerically higher cumulative survival rates than in group 3 (Vc_{AHPND} immersion without B1 and B3 treatment) (Figure 1 and Table 4). During the challenge test, 16 shrimp were dead (5 in group 1, 5 in group 2, and 6 in group 3), and 11 shrimp (3 in group 1, 2 in group 2, and 6 in group 3) were further examined for PCR (Figure S2).

The cycle threshold (C_t) values of the *pir*A toxin gene in the hepatopancreas tissue of shrimp were 31.63 ± 0.20 in group 1, 38.04 ± 0.58 in group 2, and 28.70 ± 0.42 in group 3. The *pir*A toxin gene was not detected in any tested samples in group 4.

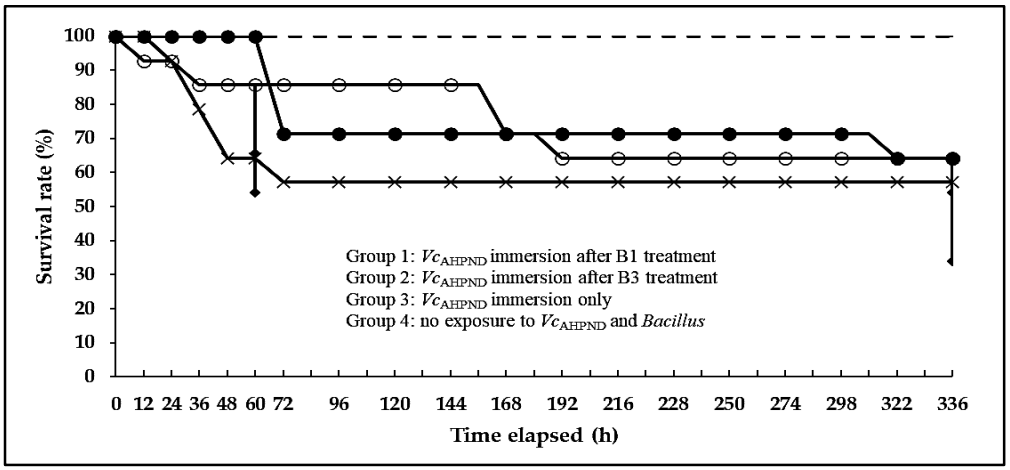

Figure 1. The survival rates (%) of Pacific white leg shrimp challenged with Vc_{AHPND} (16-904/1).

Table 4. The survival rates (%) of Pacific white leg shrimp at 60 and 336 h after Vc_{AHPND} (16-904/1) immersion.

Survival (%)	Treatments			
	Group 1	**Group 2**	**Group 3**	**Group 4**
60 h	100 ± 0.0 [a]	85.7 ± 20.2 [ab]	64.3 ± 10.1 [b]	100 ± 0.0 [a]
336 h	64.3 ± 10.1	64.3 ± 30.3	57.1 ± 0.0	100 ± 0.0

Values are presented as mean ± SD of duplicate groups. Values with different superscript letters in the same row are significantly different ($p < 0.05$). Values without superscript letters are not significantly different.

3.4. Genome Sequencing and Phylogenetic Analysis of the Selected Bacillus Strains

Two *Bacillus* strains (B1 and B3) were selected and further analyzed for genomic investigations. The genomes of B1 and B3 consisted of circular double-stranded DNA having a length of 3,929,791 bp and 3,929,788 bp, respectively, with 46.50% G+C content, and both genomes encoded 3750 protein-coding genes, 86 transfer RNAs, and 27 rRNAs (Table 5). Direct comparison of the 16S rRNA sequences of the B1 and B3 strains against the GenBank database revealed that the two *Bacillus* isolates were most similar to *B. siamensis* KCTC 13613^T (NR_117274.1; 99.1% and 99.1%) and *B. velezensis* CR-502^T (AY603658.1; 99.6% and 99.7%). However, the resultant phylogeny did not clearly differentiate the two strains at the species level (Figure 2a). Therefore, we conducted a whole genome-based phylogenetic analysis to confirm the exact taxonomical position of the strains, and the resultant phylogeny revealed that the two isolates were clustered together with *B. velezensis* NRRL B-41580^T (LLZC00000000.1) (Figure 2b). Based on these results, B1 and B3 were finally classified as *B. velezensis*, one of the recently classified species in the operational group *B. amyloliquefaciens* [44].

Table 5. Features of the B1 and B3 genomes.

Features	Strains	
	B1	**B3**
Size (bp)	3,929,791	3,929,788
G+C content (%)	46.50	46.50
Contigs	1	1
Chromosomes	1	1
Plasmids	0	0
tRNAs	86	86
rRNAs	27	27
Protein-coding genes	3750	3750
GenBank accession number	CP100040	CP100041

During the in silico search for biosynthetic gene clusters (BCGs) for the production of potential antibiotics and/or secondary metabolites, four types of BCGs, including non-ribosomal peptide, ribosomally synthesized and post-translationally modified peptide, polyketide, and lipopeptide gene clusters, were detected in B1 and B3 genomes. A more thorough analysis revealed that these BCGs were detected in seven of 12 predicted regions of the two genomes, and a total of 54 substances related to secondary metabolites were detected. When limited to the cutoff similarity value of 80% for substances that have been identified till date, fourteens substances in total, namely bacillibactin, amylocyclicin, paenibactin, difficidin, fengycin, plipastatin, bacillomycin D, mycosubtilin, iturin, paenilarvins, bacillaene, macrolactin (H, B, 1c, and E), surfactin, and bacilysin, were identified from the B1 and B3 strains (Tables 6 and 7). Although a comparison of the substances detected in the type strains of *B. velezensis* used in this study with the B1 and B3 genomes (cutoff > 80%) indicated that they were mostly similar, differences in the three substances (mersacidin, plipastatin, and surfactin) were found (Table S3). First, mersacidin which was detected in the genome of *B. velezensis* NRRL B-41580^T was not identified in the genomes of the B1 and

B3 isolates. Second, plipastatin and surfactin were only detected in the two *Bacillus* isolates obtained in this study. Additional detailed information on the other five predicted regions of the *Bacillus* isolates B1 and B3 is presented in Table S4.

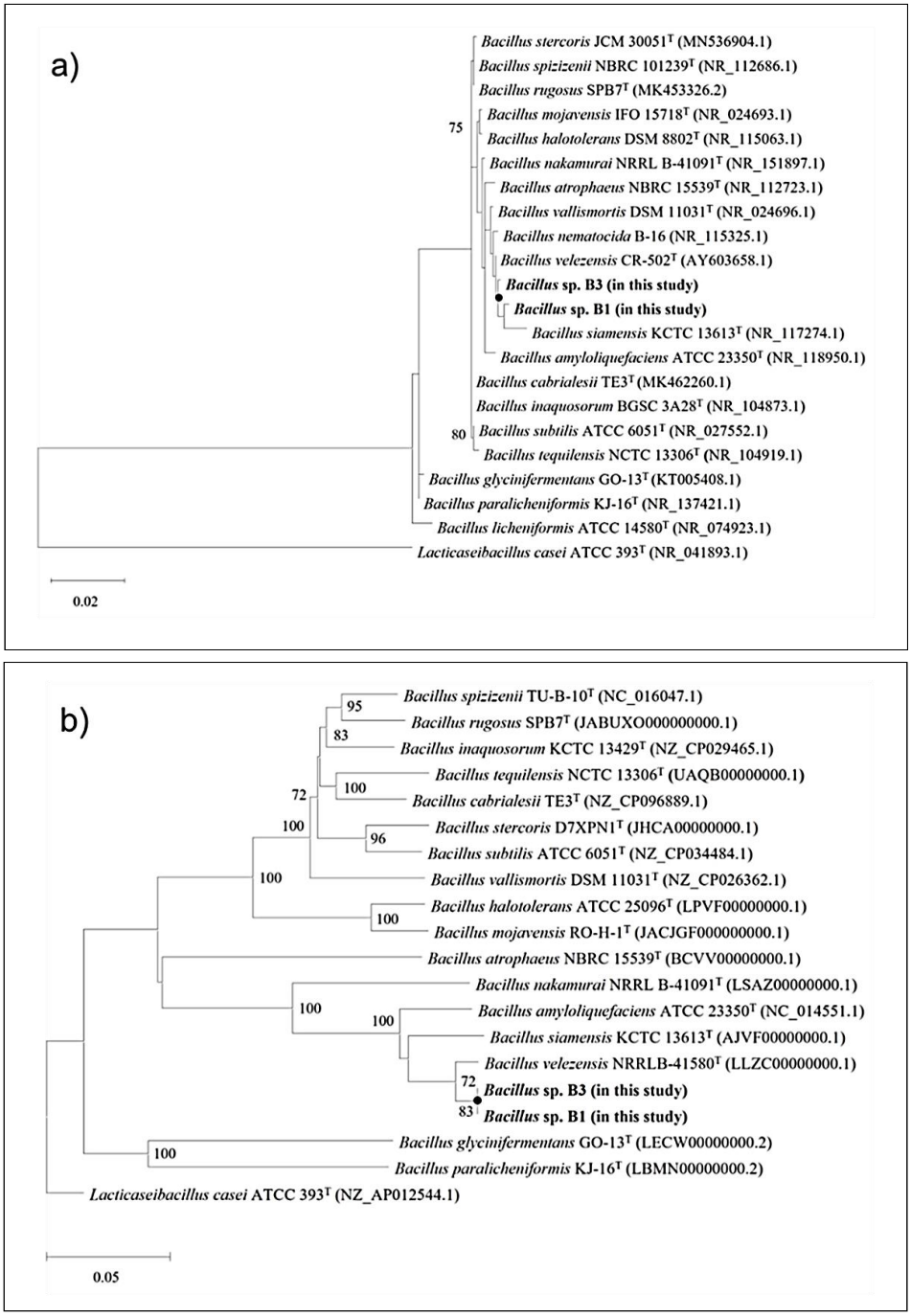

Figure 2. Phylogenetic tree based on the 16S rRNA gene sequences (**a**) and whole-genome sequences (**b**) detailing the relationships of *Bacillus* isolates B1 and B3 with 20 type strains of *Bacillus* spp. and the outgroup *Lactobacillus casei* ATCC 393^{T}. The bootstrapping values are indicated at the branches using 1000 and 100 replicates, and only bootstrap values >70 are presented. The scale bar represents 0.02 or 0.05 nucleotide substitutions per site.

Table 6. The secondary metabolite gene clusters in the isolate B1 obtained using anti-SMASH.

Region	Position		Biosynthetic Gene Clusters	Substance	Similarity (%)
	From	To			
1	127,555	178,059	NRP [1]	Bacillibactin	100
			RiPP:head-to-tail cyclized peptide [2]	Amylocyclicin	100
			NRP	Paenibactin	100
			NRP:NRP siderophore	Bacillibactin	100
2	804,233	896,592	Polyketide + NRP	Difficidin	100
5	1,180,156	1,314,466	NRP	Fengycin	100
			NRP	Plipastatin	100
			Polyketide + NRP:lipopeptide	Bacillomycin D	100
			Polyketide + NRP	Mycosubtilin	100
			Polyketide + NRP	Iturin	88
			NRP	Paenilarvins	100
6	1,388,208	1,488,773	Polyketide + NRP	Bacillaene	100
7	1,707,961	1,796,194	Polyketide	Macrolactin H	100
			Polyketide	Macrolactin H/ macrolactin B/macrolactin 1c/macrolactin E	100
11	2,792,616	2,858,023	NRP:lipopeptide	Surfactin	82
12	3,479,618	3,521,036	Other	Bacilysin	100
			Other	Bacilysin	100

[1] NRP, non-ribosomal peptide. [2] RiPP, ribosomally synthesized and post-translationally modified peptide.

Table 7. The secondary metabolite gene clusters in the isolate B3 obtained using anti-SMASH.

Region	Position		Biosynthetic Gene Clusters	Substance	Similarity (%)
	from	to			
2	117,650	251,960	NRP [1]	Fengycin	100
			NRP	Plipastatin	100
			Polyketide + NRP:lipopeptide	Bacillomycin D	100
			Polyketide + NRP	Mycosubtilin	100
			Polyketide + NRP	Iturin	88
			NRP	Paenilarvins	100
3	325,702	426,267	Polyketide + NRP	Bacillaene	100
4	645,796	733,631	Polyketide	Macrolactin H	100
			Polyketide	Macrolactin H/ macrolactin B/ macrolactin 1c/ macrolactin E	100
8	1,730,328	1,794,305	NRP:Lipopeptide	Surfactin	82
9	2,417,108	2,458,526	Other	Bacilysin	100
			Other	Bacilysin	100
10	2,994,836	3,046,627	NRP	Bacillibactin	100
			RiPP:head-to-tail cyclized peptide [2]	Amylocyclicin	100
			NRP	Paenibactin	100
			NRP:NRP siderophore	Bacillibactin	100
11	3,671,331	3,765,123	Polyketide + NRP	Difficidin	100

[1] NRP, non-ribosomal peptide. [2] RiPP, ribosomally synthesized and post-translationally modified peptide.

4. Discussion

In this study, we evaluated the antimicrobial activity of five *Bacillus* isolates against 12 shrimp *Vibrio* strains (10 AHPND *Vibrio* strains [9 *V. parahaemolyticus* and 1 *V. campbellii*] and 2 non-AHPND *Vibrio* strains [1 *V. parahaemolyticus* and 1 *V. harveyi*]). *Bacillus* spp. are usually isolated from soil, fermented soybean paste (cheonggukjang), plants, and pond water, and are incubated at 30–37 °C [45–47]. The *Bacillus* strains described in this study were isolated from seawater and were found to grow well at 28–37 °C. Additionally, all *Bacillus* strains exhibited growth in both TSA and TSA+ (supplemented with 2% NaCl), indicating that these strains could be applied to water with wide ranges of salinity.

In the dot-spot test, B1, B3, B5, B7, and B8 exerted inhibitory effects on at least one of tested *Vibrio* strain. In addition, these strains showed inhibitory effects against isolates from both South Korea and several other countries (Mexico, Vietnam, Latin America, Thailand, and the USA). This indicates that the *Bacillus* strains used in this study can be used globally in various shrimp-farming countries to control AHPND. Management of AHPND, a disease which results in extensive mortality in shrimp, could increase shrimp production and decrease economic losses in shrimp farming.

In the challenge test, the B1 treatment group (100%) exhibited a significantly higher survival rate than the non-*Bacillus* treatment group (64.3%) at 60 h. In a previous study by Han et al. [48], Vc_{AHPND} was highly pathogenic to shrimp, similar to Vp_{AHPND}, and the accumulative mortality in shrimp was as high as 100% within 2 days of Vc_{AHPND} laboratory infection. In this study, two *Bacillus* strains (B1 and B3) displayed prominent antimicrobial effects within 2–3 days (48–60 h) of Vc_{AHPND} infection compared with the findings in the positive control group (Vc_{AHPND} immersion without B1 and B3 treatment); thus, both strains are expected to emerge as alternatives to antibiotics for controlling Vc_{AHPND}. Moreover, among the live shrimp collected on the day of experiment termination, the C_t value was higher in samples of the *Bacillus*-treated groups than in the positive control group. Therefore, these results suggested that the two *Bacillus* strains identified in this study exhibited antimicrobial activity against pathogenic Vc_{AHPND}. Additionally, the histopathology of the hepatopancreas was examined after exposure to *Bacillus* spp. for 14 days in our preliminary study. The structure of the hepatopancreas was found to be similar between the *Bacillus* treatment groups and the control group (not exposed to Vc_{AHPND} and *Bacillus*), indicating that *Bacillus* strains are harmless to shrimp.

The two strains (B1 and B3), which showed antimicrobial activity using the dot-spot test (in vitro) and challenge test, were finally classified as *B. velezensis* based on their whole genome-based phylogeny. Several studies have examined the probiotic effects of *B. velezensis* in various organisms. For example, Chauyod et al. [49] demonstrated that *B. velezensis* significantly inhibited the growth of *Vibrio* spp. isolated from shrimp, including Vp_{AHPND}, using the disk diffusion test. Li et al. [50] reported that the expression of immune-related genes such as IL-8 and IgM was upregulated in the hybrid grouper fed a feed supplemented with *B. velezensis* (1×10^7 CFU g^{-1}) compared with the findings in the control group, and the former also exhibited increased resistance to *V. harveyi*. Other studies described the antibacterial activity of *B. velezensis* against *V. parahaemolyticus* isolated from shrimp [51] and *V. anguillarum* isolated from seabass [52]. Although the predicted secondary metabolites derived from the B1 and B3 *Bacillus* strains were relatively similar to those previously reported from related *Bacillus* species [39], plipastatin and surfactin were only found in the two isolates, and they were not detected during our in silico analysis of the type strain of *B. velezensis*. These results suggest that the newly isolated B1 and B3 strains will have additional advantageous characteristics in terms of their potential use in the aquaculture industry. Till date, most previously reported secondary metabolites produced by *Bacillus* spp. were known to have surfactant and antibiotic activity [53]. In particular, the potential presence of surfactin, which was previously reportedly associated with antibacterial activity against multidrug-resistant *Vibrio* spp. [54] in the two *Bacillus* strains might explain their antimicrobial activity against pathogenic Vc_{AHPND} in this study; however, further studies are warranted regarding the predicted presence of surfactin in

the isolates because of its relatively low similarity with previously reported compounds. Moreover, the potential presence of iturin and fengycin, which have been associated with the antifungal activity of some *Bacillus* strains [55], may contribute to the potential usability of the *Bacillus* strains identified in this study.

5. Conclusions

In summary, two *Bacillus* strains isolated from seawater in Korea were shown to have antimicrobial activity against *Vibrio* strains in shrimp using dot-spot and challenge test, and secondary metabolites derived from the B1 and B3 strains were more various than those previously reported for *Bacillus* spp., indicating that both strains can be used as potential candidates for the management of vibriosis and AHPND, including Vc_{AHPND}, in shrimp aquaculture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes7050287/s1, Figure S1: (a) PCR analysis for *Bacillus* identification (263 bp). Lane M: 100-bp ladder, Lane N: Negative control (DDH2O), Lane 1: B1, Lane 2: B3, Lane 3: B5, Lane 4: B7, and Lane 5: B8. (b) PCR analysis for *Vibrio* spp. identification (*V. parahaemolyticus*: 349 bp, *V. campbellii*: 294 bp, *V. harveyi*: 121 bp). Lane M: 100-bp ladder, Lane N: Negative control (DDH2O), Lane 1: 13-028/A3, Lane 2: 15-250/20, Lane 3: CH49, Lane 4: CH50, Lane 5: CH51, Lane 6: CH52, Lane 7: CH53, Lane 8: 19-021D1, Lane 9: 19-022A1, Lane 10: NSU116, Lane 11: 16-904/1, and Lane 12: LB4; Figure S2: PCR analysis was performed to identify AHPND toxin genes (*pir*A: 284 bp, *pir*B: 392 bp) in dead shrimp; Table S1: Clear zone diameter (mm) illustrating the antibacterial activity of *Bacillus* strains used in this study against 12 *Vibrio* strains; Table S2: Clear zone images of *Bacillus* strains (type strain, B1 and B3) against the representative *Vibrio* strains (16-904/1, 19-021D1, and 19-022A1); Table S3: The predicted secondary metabolite gene clusters in *Bacillus velezensis* NRRL B-41580$^{\mathrm{T}}$ using anti-SMASH; Table S4: The additional secondary metabolite gene clusters in *Bacillus* isolates B1 and B3 using anti-SMASH.

Author Contributions: Writing—original draft preparation, H.J.J. and J.W.S.; formal analysis, C.L.; B.K. and S.Y.P. writing—review and editing and supervision, J.H.K.; J.E.H. and J.H.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF2019R1C1C1006212, NRF2021R1I1A1A01040303 and NRF-2022R1I1A3066435). This research was also supported by Development of technology for biomaterialization of marine fisheries by-products of Korea institute of Marine Science & Technology Promotion (KIMST) funded by the Ministry of Oceans and Fisheries (KIMST-20220128).

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of Kyungpook National University (KNU 2020-0052 and 23 April 2020).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Tran, L.; Nunan, L.; Redman, R.M.; Mohney, L.L.; Pantoja, C.R.; Fitzsimmons, K.; Lightner, D.V. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Dis. Aquat. Org.* **2013**, *105*, 45–55. [CrossRef] [PubMed]
2. Kondo, H.; Van, P.T.; Dang, L.T.; Hirono, I. Draft genome sequence of non-*Vibrio parahaemolyticus* acute hepatopancreatic necrosis disease strain KC13. 17.5, isolated from diseased shrimp in Vietnam. *Genome Announc.* **2015**, *3*, e00978-15. [CrossRef] [PubMed]
3. Dong, X.; Wang, H.; Zou, P.; Chen, J.; Liu, Z.; Wang, X.; Huang, J. Complete genome sequence of *Vibrio campbellii* strain 20130629003S01 isolated from shrimp with acute hepatopancreatic necrosis disease. *Gut Pathog.* **2017**, *9*, 31. [CrossRef] [PubMed]
4. Liu, F.; Li, S.; Yu, Y.; Yuan, J.; Yu, K.; Li, F. Pathogenicity of a *Vibrio owensii* strain isolated from *Fenneropenaeus chinensis* carrying *pir*AB genes and causing AHPND. *Aquaculture* **2021**, *530*, 735747. [CrossRef]

5. Kua, B.C.; Iar, A.; Siti Zahrah, A.; Irene, J.; Norazila, J.; Nik Haiha, N.Y.; Fadzilah, Y.; Mohammed, M.; Siti Rokhaiya, B.; Omar, M.; et al. Current status of acute hepatopancreatic necrosis disease (AHPND) of farmed shrimp in Malaysia. In Proceedings of the Addressing Acute Hepatopancreatic Necrosis Disease (AHPND) and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia, Makati City, Philippines, 22–24 February 2016; pp. 55–59.
6. Joshi, J.; Srisala, J.; Truong, V.H.; Chen, I.T.; Nuangsaeng, B.; Suthienkul, O.; Lo, C.F.; Flegel, T.W.; Sritunyalucksana, K.; Thitamadee, S. Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture* **2014**, *428*, 297–302. [CrossRef]
7. Nunan, L.; Lightner, D.; Pantoja, C.; Gomez-Jimenez, S. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. *Dis. Aquat. Org.* **2014**, *111*, 81–86. [CrossRef]
8. Leobert, D.; Cabillon, N.A.R.; Catedral, D.D.; Amar, E.C.; Usero, R.C.; Monotilla, W.D.; Calpe, A.T.; Fernandez, D.D.G.; Saloma, C.P. Acute hepatopancreatic necrosis disease (AHPND) outbreaks in *Penaeus vannamei* and *P. monodon* cultured in the Philippines. *Dis. Aquat. Org.* **2015**, *116*, 251–254.
9. Dhar, A.K.; Piamsomboon, P.; Caro, L.F.A.; Kanrar, S.; Adami, R., Jr.; Juan, Y.S. First report of acute hepatopancreatic necrosis disease (AHPND) occurring in the USA. *Dis. Aquat. Org.* **2019**, *132*, 241–247. [CrossRef]
10. Han, J.E.; Choi, S.K.; Han, S.H.; Lee, S.C.; Jeon, H.J.; Lee, C.; Kim, K.Y.; Lee, Y.S.; Park, S.C.; Rhee, G.; et al. Genomic and histopathological characteristics of *Vibrio parahaemolyticus* isolated from an acute hepatopancreatic necrosis disease outbreak in Pacific white shrimp (*Penaeus vannamei*) cultured in Korea. *Aquaculture* **2020**, *524*, 735284. [CrossRef]
11. Lee, C.T.; Chen, I.T.; Yang, Y.T.; Ko, T.P.; Huang, Y.T.; Huang, J.Y.; Huang, M.F.; Lin, S.J.; Chen, C.Y.; Lin, S.S.; et al. The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 10798–10803. [CrossRef]
12. Dawood, M.A.; Koshio, S.; Esteban, M.Á. Beneficial roles of feed additives as immunostimulants in aquaculture: A review. *Rev. Aquac.* **2018**, *10*, 950–974. [CrossRef]
13. Rekiel, A.; Wiecek, J.; Bielecki, W.; Gajewska, J.; Cichowicz, M.; Kulisiewicz, J.; Batorska, M.; Roszkowski, T.; Beyga, K. Effect of addition of feed antibiotic flavomycin or prebiotic BIO-MOS on production results of fatteners, blood biochemical parameters, morphometric indices of intestine and composition of microflora. *Archi. Tierz. Dummerstorf* **2007**, *50*, 172–180.
14. Defoirdt, T.; Sorgeloos, P.; Bossier, P. Alternatives to antibiotics for the control of bacterial disease in aquaculture. *COMICR* **2011**, *14*, 251–258. [CrossRef]
15. Capita, R.; Alonso-Calleja, C. Antibiotic-resistant bacteria: A challenge for the food industry. *Crit. Rev. Food Sci. Nutr.* **2013**, *53*, 11–48. [CrossRef]
16. Hai, N.V. The use of probiotics in aquaculture. *J. Appl. Microbiol.* **2015**, *119*, 917–935. [CrossRef]
17. Lulijwa, R.; Rupia, E.J.; Alfaro, A.C. Antibiotic use in aquaculture, policies and regulation, health and environmental risks: A review of the top 15 major producers. *Rev. Aquac.* **2020**, *12*, 640–663. [CrossRef]
18. Zhu, F. A review on the application of herbal medicines in the disease control of aquatic animals. *Aquaculture* **2020**, *526*, 735422. [CrossRef]
19. Vogeley, J.L.; Interaminense, J.A.; Buarque, D.S.; da Silva, S.M.B.C.; Coimbra, M.R.M.; Peixoto, S.M.; Soares, R.B. Growth and immune gene expression of *Litopenaeus vannamei* fed *Bacillus subtilis* and *Bacillus circulans* supplemented diets and challenged with *Vibrio parahaemolyticus*. *Aquac. Int* **2019**, *27*, 1451–1464. [CrossRef]
20. Amoah, K.; Dong, X.H.; Tan, B.P.; Zhang, S.; Chi, S.Y.; Yang, Q.H.; Liu, H.; Yang, Y.; Zhang, H.T. Administration of probiotic *Bacillus licheniformis* induces growth, immune and antioxidant enzyme activities, gut microbiota assembly and resistance to *Vibrio parahaemolyticus* in *Litopenaeus vannamei*. *Aquac. Nutr.* **2020**, *26*, 1604–1622. [CrossRef]
21. Chauhan, A.; Singh, R. Probiotics in aquaculture: A promising emerging alternative approach. *Symbiosis* **2019**, *77*, 99–113. [CrossRef]
22. Khademzade, O.; Zakeri, M.; Haghi, M.; Mousavi, S.M. The effects of water additive *Bacillus cereus* and *Pediococcus acidilactici* on water quality, growth performances, economic benefits, immunohematology, and bacterial flora of whiteleg shrimp (*Penaeus vannamei* Boone, 1931) reared in earthen ponds. *Aquac. Res.* **2020**, *51*, 1759–1770. [CrossRef]
23. Nithya, V.; Halami, P.M. Evaluation of the probiotic characteristics of *Bacillus* species isolated from different food sources. *Ann. Microbiol.* **2013**, *63*, 129–137. [CrossRef]
24. Flegel, T.W.; Lightner, D.V.; Lo, C.F.; Owens, L. *Shrimp Disease Control: Past, Present, and Future*; Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M., Subasinghe, R.P., Eds.; Diseases in Asian Aquaculture VI. Fish Health Section; Asian Fisheries Society: Manila, Philippines, 2008; Volume 505, pp. 355–378.
25. Kewcharoen, W.; Srisapoome, P. 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 Immunol.* **2019**, *94*, 175–189. [CrossRef]
26. Galaviz-Silva, L.; Cázares-Jaramillo, G.E.; Ibarra-Gámez, J.C.; Molina-Garza, V.M.; Sánchez-Díaz, R.; Molina-Garza, Z.J. Assessment of probiotic bacteria from marine coasts against *Vibrio parahaemolyticus* (AHPND strains) in *Litopenaeus vannamei*. *Aquac. Res.* **2021**, *52*, 6396–6409. [CrossRef]
27. Paopradit, P.; Aksonkird, T.; Mittraparp-arthorn, P. Indole inhibits quorum sensing-dependent phenotypes and virulence of acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus*. *Aquac. Res.* **2022**, *53*, 3586–3597. [CrossRef]

28. Jun, J.W.; Han, J.E.; Tang, K.F.; Lightner, D.V.; Kim, J.; Seo, S.W.; Park, S.C. Potential application of bacteriophage pVp-1: Agent combating *Vibrio parahaemolyticus* strains associated with acute hepatopancreatic necrosis disease (AHPND) in shrimp. *Aquaculture* **2016**, *457*, 100–103. [CrossRef]
29. Jun, J.W.; Han, J.E.; Giri, S.S.; Tang, K.F.; Zhou, X.; Aranguren, L.F.; Kim, H.J.; Yun, S.; Chi, C.; Kim, S.G.; et al. Phage application for the protection from acute hepatopancreatic necrosis disease (AHPND) in *Penaeus vannamei*. *Indian J. Microbiol.* **2018**, *58*, 114–117. [CrossRef]
30. Nguyen, N.D.; Pande, G.S.J.; Kashem, M.A.; Baruah, K.; Bossier, P. Acute hepatopancreatic necrosis disease (AHPND) toxin degradation by *Bacillus subtilis* DSM33018. *Aquaculture* **2021**, *540*, 736634. [CrossRef]
31. Solichová, K.; Němečková, I.; Šviráková, E.; Horáčková, Š. Novel identification methods including a species-specific PCR for hazardous *Bacillus* species. *Acta Aliment.* **2019**, *48*, 415–422. [CrossRef]
32. Dashti, A.A.; Jadaon, M.M.; Abdulsamad, A.M.; Dashti, H.M. Heat treatment of bacteria: A simple method of DNA extraction for molecular techniques. *Kuwait Med. J.* **2009**, *41*, 117–122.
33. Kim, Y.B.; Okuda, J.U.N.; Matsumoto, C.; Takahashi, N.; Hashimoto, S.; Nishibuchi, M. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the toxR gene. *J. Clin. Microbiol.* **1999**, *37*, 1173–1177. [CrossRef] [PubMed]
34. Cano-Gomez, A.; Høj, L.; Owens, L.; Baillie, B.K.; Andreakis, N. A multiplex PCR-based protocol for identification and quantification of *Vibrio harveyi*-related species. *Aquaculture* **2015**, *437*, 195–200. [CrossRef]
35. Han, J.E.; Tang, K.F.; Tran, L.H.; Lightner, D.V. Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Dis. Aquat. Org.* **2015**, *113*, 33–40. [CrossRef] [PubMed]
36. Spelhaug, S.R.; Harlander, S.K. Inhibition of foodborne bacterial pathogens by bacteriocins from *Lactococcus lactis* and *Pediococcus pentosaceous*. *J. Food Prot.* **1989**, *52*, 856–862. [CrossRef]
37. Han, J.E.; Tang, K.F.; Pantoja, C.R.; White, B.L.; Lightner, D.V. qPCR assay for detecting and quantifying a virulence plasmid in acute hepatopancreatic necrosis disease (AHPND) due to pathogenic *Vibrio parahaemolyticus*. *Aquaculture* **2015**, *442*, 12–15. [CrossRef]
38. Silva, F.D.J.; Ferreira, L.C.; Campos, V.P.; Cruz-Magalhães, V.; Barros, A.F.; Andrade, J.P.; Roberts, D.P.; de Souza, J.T. Complete genome sequence of the biocontrol agent *Bacillus velezensis* UFLA258 and its comparison with related species: Diversity within the commons. *GBE* **2019**, *11*, 2818–2823. [CrossRef]
39. Blin, K.; Shaw, S.; Kloosterman, A.M.; Charlop-Powers, Z.; Van Wezel, G.P.; Medema, M.H.; Weber, T. antiSMASH 6.0: Improving cluster detection and comparison capabilities. *Nucleic Acids Res.* **2021**, *49*, W29–W35. [CrossRef]
40. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947–2948. [CrossRef]
41. Hall, T.; Biosciences, I.; Carlsbad, C. BioEdit: An important software for molecular biology. *GERF Bull. Biosci.* **2011**, *2*, 60–61.
42. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Bio. Evol.* **2018**, *35*, 1547. [CrossRef]
43. Lefort, V.; Desper, R.; Gascuel, O.F. 2.0: A comprehensive, accurate, and fast 684 distance-based phylogeny inference program. *Mol. Biol. Evol.* **2015**, *685*, 32.
44. Fan, B.; Blom, J.; Klenk, H.P.; Borriss, R. *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus siamensis* form an "operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex. *Front. Microbiol.* **2017**, *8*, 22. [CrossRef]
45. Amin, M.; Rakhisi, Z.; Ahmady, A.Z. Isolation and identification of *Bacillus* species from soil and evaluation of their antibacterial properties. *AJCMI* **2015**, *2*, 23233. [CrossRef]
46. Jeon, H.L.; Yang, S.J.; Son, S.H.; Kim, W.S.; Lee, N.K.; Paik, H.D. Evaluation of probiotic *Bacillus subtilis* P229 isolated from cheonggukjang and its application in soybean fermentation. *LWT* **2018**, *97*, 94–99. [CrossRef]
47. Mohamed, E.A.; Farag, A.G.; Youssef, S.A. Phosphate solubilization by *Bacillus subtilis* and *Serratia marcescens* isolated from tomato plant rhizosphere. *J. Environ. Prot. Sci.* **2018**, *9*, 266. [CrossRef]
48. Han, J.E.; Tang, K.F.J.; Aranguren, L.F.; Piamsomboon, P. Characterization and pathogenicity of acute hepatopancreatic necrosis disease natural mutants, pirABvp (−) *V. parahaemolyticus*, and pirABvp (+) *V. campbellii* strains. *Aquaculture* **2017**, *470*, 84–90. [CrossRef]
49. Chauyod, K.; Rattanavarin, S.; Sarapukdee, P.; Porntheeraphat, S.; Sritunyalucksana, K.; Khemthongcharoen, N. *Bacillus velezensis* suppression on the growth of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease in marine shrimp. *J. Appl. Aquac.* **2022**, 1–15. [CrossRef]
50. Li, J.; Wu, Z.B.; Zhang, Z.; Zha, J.W.; Qu, S.Y.; Qi, X.Z.; Wang, G.X.; Ling, F. Effects of potential probiotic *Bacillus velezensis* K2 on growth, immunity and resistance to *Vibrio harveyi* infection of hybrid grouper (*Epinephelus lanceolatus*♂ × *E. fuscoguttatus*♀). *Fish Shellfish. Immunol.* **2019**, *93*, 1047–1055. [CrossRef]
51. Li, X.; Gao, X.; Zhang, S.; Jiang, Z.; Yang, H.; Liu, X.; Jiang, Q.; Zhang, X. Characterization of a *Bacillus velezensis* with antibacterial activity and inhibitory effect on common aquatic pathogens. *Aquaculture* **2020**, *523*, 735165. [CrossRef]
52. Monzón-Atienza, L.; Bravo, J.; Torrecillas, S.; Montero, D.; Canales, A.F.G.D.; de la Banda, I.; Galindo-Villegas, J.; Ramos-Vivas, J.; Acosta, F. Isolation and Characterization of a *Bacillus velezensis* D-18 Strain, as a Potential Probiotic in European Seabass Aquaculture. *Probiotics Antimicrob. Proteins* **2021**, *13*, 1404–1412. [CrossRef]

53. Sumi, C.D.; Yang, B.W.; Yeo, I.C.; Hahm, Y.T. Antimicrobial peptides of the genus *Bacillus*: A new era for antibiotics. *Can. J. Microbiol.* **2015**, *61*, 93–103. [CrossRef]
54. Xu, H.M.; Rong, Y.J.; Zhao, M.X.; Song, B.; Chi, Z.M. Antibacterial activity of the lipopetides produced by *Bacillus amyloliquefaciens* M1 against multidrug-resistant *Vibrio* spp. isolated from diseased marine animals. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 127–136. [CrossRef]
55. Devi, S.; Kiesewalter, H.T.; Kovács, R.; Frisvad, J.C.; Weber, T.; Larsen, T.O.; Kovacs, A.T.; Ding, L. Depiction of secondary metabolites and antifungal activity of *Bacillus velezensis* DTU001. *Synth. Syst. Biotechnol.* **2019**, *4*, 142–149. [CrossRef]