A Dissertation for the Degree of Doctor of Philosophy

Use of Probiotics to Improve Productivity of Pacific White Leg Shrimp (*Penaeus vannamei*) Aquaculture

흰다리새우(*Penaeus vannamei*)의 생산성 향상을 위한 프로바이오틱스 활용 연구

By

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February 2023

College of Veterinary Medicine

Department of Veterinary Pathobiology and Preventive Medicine

(Laboratory Animal Medicine)

Graduate School of Seoul National University

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Jae Won Song, D.V.M.

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1. ABSTRACT

The global shrimp market is expected to grow 4.2 percent annually to reach USD 23.4 billion by 2026. The aquatic infectious disease outbreaks that occur in shrimp aquaculture, including white spot syndrome virus (WSSV) and acute hepatopancreatic necrosis disease (AHPND), are causing huge economic losses to the shrimp culture industry. Various substances, including antibiotics, are used to prevent and treat infectious diseases. In this study, the functional use of probiotics was studied as one of the ways to improve productivity and reduce antibiotic use in shrimp aquaculture. We investigated the effect of a *Bacillus* spp. strain, a representative functional probiotic, on the growth, feed efficiency, and non-specific immune response in Pacific white leg shrimp (*Penaeus vannamei*), and the possibility of utilizing it as an antibiotic substitute. In Chapter 1, the shrimp feed diet supplemented with *Bacillus* spp. (*Bacillus subtilis*, *Bacillus velezensis*, and *Bacillus pumilus* mixed in equal ratios) was evaluated to determine the improvement effect on growth, feed efficiency, non-specific immune response, water quality, and survival rate. In Chapter 2, a study was conducted focusing on evaluating antibacterial activity to control aquatic infectious disease using the *Bacillus* strain.

In Chapter 1, to evaluate the functionality of the *Bacillus* spp. mixture as a feed supplement, four experimental feeds were prepared by adding a mixture of three *Bacillus* spp. strains (*B. subtilis*, *B. velezensis*, and *B. pumilus*) to the basal diet of Pacific white leg shrimp at different levels (0%, 0.05%, 0.1%, and 0.2%). The Pacific white leg shrimp (initial average body weight: 0.45g) were randomly assigned to 500

L composite tanks (filled with 400 L of brackish water) at a stocking rate of 70 shrimp $tank^{-1}$ for 10 weeks. There were five replicates per dietary treatment. The results showed that the diet containing 0.1% *Bacillus* spp. mixture significantly increased the growth, protein efficiency ratio, feed conversion ratio, and survival rate compared to that of the control. Diets containing 0.05% and 0.1% *Bacillus* spp. mixture significantly increased the digestive enzymes compared to that of the control. Furthermore, the diet containing 0.1% *Bacillus* spp. mixture significantly improved hematological and immune profiles. The total *Vibrio* spp. count was lowest in the diet supplemented with 0.1% of *Bacillus* spp. mixture; accordingly, the bacterial clearance efficiency was higher in this diet. The Pacific white leg shrimp fed the *Bacillus* spp. mixture diets (at 0.05%, 0.1%, and 0.2%) had lower cumulative mortality than the control after being challenged with *Vibrio parahaemolyticus*. Our results suggest that dietary supplementation with 0.1% *Bacillus* spp. mixture is the most effective in improving the performance and immunity of Pacific white leg shrimp.

In Chapter 2, we evaluated the antibacterial activity of *Bacillus* strains and the possibility of controlling AHPND occurring during shrimp aquaculture. This study evaluated the antibacterial 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. Based on genome sequencing and phylogenetic analysis, B1 and B3 were classified as *B. velezensis*.

Therefore, the long−term supply of *Bacillus* spp. strains during the aquaculture period is expected to improve the growth performance and increase the resistance to diseases such as AHPND, thereby increasing the productivity of the Pacific white leg shrimp aquaculture industry.

Keywords: Pacific white leg shrimp, probiotics, *Bacillus* spp. mixture, *B. velezensis*, feed additives, growth performance, AHPND, *Vibrio campbellii*, antibacterial activity

Student number: 2012−21541

2. GENERAL INTRODUCTION

Pacific white leg shrimp

The Pacific white leg shrimp, *Penaeus vannamei* (formerly *Litopenaeus vannamei*) (Boone, 1931), is a tropical species with a natural distribution along the western Pacific coast from Mexico to Peru between the 20 ℃ isotherms. It was introduced into Asia experimentally in the late 1970s but commercially in Taiwan and China in 1996 (1) and subsequently to several countries in southeast and south Asia, such as Thailand, Indonesia, and Vietnam. Shrimp culture is one of the fastest-growing industries worldwide. Pacific white leg shrimp is among the most widely aquacultured species, especially in Asia, mainly due to its wide consumer demands, ease of cultivation, rapid growth rate, and high economic value (2). According to FAO, Pacific white leg shrimp accounted for 51.7% of crustaceans' production in 2020 (3). With the growing size of the culture industry, disease outbreaks have been a major problem in the shrimp culture industry in the past decades.

Various factors reduce productivity in shrimp aquaculture. Pathogenic (bacteria, viruses, parasites, and fungi) and/or non-pathogenic (environmental parameters, nutritional deficiency, and algal toxins) diseases are very significant issues, and the outbreaks result in the highest economic losses in the industry (4). As the industrial scale grows, the shrimp culture industry has developed high-density aquaculture techniques and systems to increase economic efficiency by maximizing productivity. However, high-density aquaculture of aquatic animals has caused the deterioration of the aquatic farm environment. Mortality and reduced productivity of shrimp

aquaculture due to frequent disease outbreaks are major problems (5). Hence, dietary supplementation of immunostimulants has been recommended to control the diseases by improving the innate immunity of the shrimp and the aquaculture environment (6).

Dietary supplements in aquaculture

Optimizing growth rates and yields while minimizing the extent and impact of these diseases without the adverse effects associated with chemotherapeutic interventions is, therefore, a primary concern. Suggestions to minimize the risk of disease outbreaks in Pacific white leg shrimp include biosecurity, developing disease-resistant strains, and applying antibiotics. In addition, using feed additives such as seaweed, herbal extracts, essential oils, organic acids, prebiotics, probiotics, or synbiotics, has also been recommended. However, there is some debate over the cost and difficulty of developing genetically based, pathogen-specific host resistance (7-13). Moreover, the overuse of antibiotics has resulted in antibiotic-resistant microbes and environmental pollution with antibiotic residues (14). Thus, using dietary supplements appears to be a more cost-effective and environmentally sustainable option for managing shrimp health in aquaculture (15).

Immunostimulants in aquaculture

An immunostimulant is defined as a substance that enhances the innate or non-specific immune response by interacting directly with cells of the system, activating them (16). Immunostimulants can be divided into several groups depending on their sources: chemical agents, bacterial preparations, polysaccharides, animal or plant

extracts, nutritional factors, and cytokines (17). Recently, polysaccharides from seaweeds have also been tested as immunostimulants for shrimp (18). An immunostimulant has a broad efficacy range and multiple functions.

Probiotic use in aquaculture

The FAO/WHO defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." In various species, including humans, probiotics are used for health benefits. Like the general definition of probiotics, probiotics in aquaculture are defined as the supplementation of microbes in a living form that leads to modulation of microbial population in the hosts for advantageous effects on growth, immunity, and disease resistance (19). Using probiotics is considered one of the most effective therapeutic ways in shrimp aquaculture; this application can reduce the use of antibiotics, and effects such as increased feed efficiency and improved water quality in aquaculture are expected (20).

In shrimp aquaculture, intensive shrimp culture systems with higher stocking density to improve productivity were trending with the expansion of world aquaculture in the past decades. In an intensive culture system, the aquaculture environment is more likely to have water contamination from various factors. Shrimp in these environments is susceptible to bacterial or viral disease. The shrimp culture industry has suffered severe economic losses because of the frequent outbreaks of diseases such as early mortality syndrome (EMS) and WSSV (21–25). Chemical disinfectants and antibiotics were widely used in intensive shrimp culture for disease prevention, and overuse of antibiotics led to resistance genes among opportunistic pathogens

such as *Vibrio* species (26–28). The accumulation of antibiotic residuals and the emergence of antibiotic–resistant bacteria adversely affect the food safety of aquaculture products and may have been the leading cause of foodborne outbreaks, leading to a significant threat to human health worldwide (29–31). Additionally, the invertebrate immune system highly depends on innate mechanisms and, in theory, is incapable of responding to specific vaccines (32). The development of vaccines for shrimp infectious diseases had limited success and information that has resulted from these studies (33). Therefore, seeking an alternative way to solve this threat is critical (12).

Probiotics are an alternative remedy to reduce antibiotic use in shrimp aquaculture (20). Probiotics have been examined to control infectious diseases in shrimp aquaculture and other farmed aquatic species (34–38). They play important roles in removing pathogenic organisms, increasing beneficial microbial balance in the host intestine, assisting in enzymatic digestion, and stimulating innate immune functions (39, 40). If a probiotic strain could colonize and become well–established inside the host intestine, it would be more beneficial because they could change the gut microflora into a beneficial flora more quickly (41).

Probiotics have various mechanisms. They can produce anti–pathogenic substances to enhance the immune function, strength of the epithelial shield, mucosal adhesion of the intestine, and the competitive removal or reduction of pathogenic adhesions (42). Many beneficial effects of several probiotics, such as the species of *Bacillus*, *Dunaliella*, *Enterobacter*, *Lactobacillus*, *Pseudomonas,* and mixed cultures, have been demonstrated; these are successfully used in shrimp diets (43–57).

Bacillus species

Most *Bacillus* spp. are not harmful and are beneficial to aquatic animals. *B. subtilis*, *B. licheniformis*, and *B. pumilus* have been shown to positively affect feed digestibility, growth performance, and the prevention of microbial diseases in shrimp (5, 58, 59). In addition, recent studies show that *B. velezensis* has the same effect as other useful *Bacillus* spp. strains (60, 61). Based on previous research, multi-strain probiotics consist of a mixture of two or more strains or species of bacteria that have been demonstrated to be more beneficial to the host than a single strain or species alone (56).

One of the important properties of *Bacillus* spp. is to form spores for the outside environment. It is one of the reasons for using *Bacillus* spp. as a probiotic in the aquaculture industry. Especially, *Bacillus* spores remain viable for long periods and are tolerant to temperature alternations, making them ideal for industrial uses (62). Antibiotics from *Bacillus* spp. could easily penetrate through the protective slime layer of gram-negative bacteria because the *Bacillus* spp. itself secretes several slime and biofilm degrading enzymes (63). *Bacillus* spp. has been proven to have an immunostimulatory effect by producing antimicrobial peptides (64, 65). In the case of shrimp culture, several *Bacillus* spp. are reported to have great potential as a probiotic when they are supplemented in shrimp diets or rearing water (45, 66, 67). Enzymatic activities of *Bacillus* spp. and their beneficial effects on the growth performance of cultured shrimp have been well documented. Moreover, it is stated that *Bacillus*-based probiotics can release various enzymes that could improve the digestibility of indigestible nutrients in the host gut when they are germinated. However, information on the digestibility of probiotics in shrimp is lacking (68, 69).

B. subtilis, *B. pumilus*, and *B. velezensis* species isolated from prawn intestines, seawater, and fermented soybean are reported to have highly active digestive enzymes such as lipase, trypsin, amylase, protease, and high antimicrobial activity against pathogens such as *V. harvei*, *Streptococcus iniae*, *Aeromonas salmonicida*, *Edwardsiella tarda*, and *Staphylococcus aureus* (26, 51, 69–71). Additionally, the effects of *Bacillus* spp. as an immunostimulatory agent for various diseases have been documented (21, 65, 72–74).

Acute hepatopancreatic disease (AHPND)

AHPND (also known as EMS) is a bacterial disease caused by *Vibrio* spp. carrying toxin genes (*pir*A and *pir*B) in a large plasmid (69 kb) (75, 76). 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}). However, 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 (77–79). AHPND has caused losses in shrimp production in Asia and Central America (80, 81). The pathology of AHPND has two distinct phases. In the acute phase, the infected hepatopancreas shows a detachment of tubule epithelial cells from the basement membrane and tubule epithelial degeneration in the absence of bacterial cells. In the terminal stage, the hepatopancreas shows extensive intra-tubular hemocytic infiltration and the development of massive secondary bacterial infection. Generally, AHPND affects shrimp production at the post-larvae stage and causes low immunity at the larval

stage, making developing shrimp susceptible to infection by various pathogens and leading to mass mortality (82-84).

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AHPND: acute hepatopancreatic necrosis disease

anti-SMASH: antibiotics & Secondary Metabolite Analysis Shell

BGCs: biosynthetic gene clusters

BOD: biological oxygen demand

BP: *Bacillus pumilus*

BS: *Bacillus subtilis*

BV: *Bacillus velezensis*

CFU: colony forming unit

COD: chemical oxygen demand

DHC: differential hemocyte count

DO: dissolved oxygen

DWG: daily weight gain

EMS: early mortality syndrome

FAO: Food and Agriculture Organization of the UN

FCR: feed conversion ratio

FI: feed intake

GBDP: Genome BLAST Distance phylogeny approach

LD_{50}: lethal dose 50 percent

L-DOPA: L-Dihydroxyphenylalanine

MCP: monocalcium phosphate

NRP: non-ribosomal peptide

PA: phagocytic activity

PAMPs: pathogen-associated molecular patterns

PBS: phosphate buffered saline

PCR: polymerases chain reaction

PER: protein efficiency ratio

PI: percentage inhibition

PO: phenoloxidase activity

PR: phagocytosis rate

PRPs: pattern-recognition proteins

RiPP: ribosomally synthesized and post-translationally modified peptide

SCFAs: short-chain fatty acids

SOD: superoxide dismutase activity

SR: survival rate

TAN: total ammonia nitrogen

THC: total hemocyte count

TSS: total suspended solids

Wf: final weight

Wg: weight gain

Wi: initial weight

WSSV: white spot syndrome disease

7. CHAPTER 1

Dietary Supplement with Probiotics in Pacific White Leg Shrimp

; Effect of probiotics (a mixture of *Bacillus subtilis*, *Bacillus velezensis*, *Bacillus pumilus*) on growth performance, feed utilization and immune response of Pacific white leg shrimp (*Penaeus vannamei*)

7.1. INTRODUCTION

According to the 'The State of World Fisheries and Aquaculture' released by the Food and Agriculture Organization of the United Nations (3), in 2022, global aquaculture production reached 122.6 million tons, with a total value of USD 281.5 billion. At the species level, Pacific white leg shrimp (*Penaeus vannamei*) was the top species produced in 2020, with 5.8 million tons (3). In recent years, the shrimp culture industry has suffered great losses due to deteriorating water quality and environmental stress, leading to serious outbreaks of viral diseases like WSSV and bacterial diseases caused by vibriosis, the most severe being AHPND caused by *V. parahaemolyticus* (85, 86).

Optimizing growth rates and yields while minimizing the extent and impact of these diseases without the adverse effects associated with chemotherapeutic interventions is, therefore, a primary concern. Suggestions to minimize the risk of disease outbreaks in Pacific white leg shrimp include biosecurity, developing disease-resistant strains, and applying antibiotics. In addition, using feed additives such as seaweed, herbal extracts, essential oils, organic acids, prebiotics, probiotics, or synbiotics, has also been recommended (7-13). However, there is some debate over the cost and difficulty of developing genetically based, pathogen-specific host resistance. Moreover, the overuse of antibiotics has resulted in antibiotic-resistant microbes and environmental pollution with antibiotic residues. Thus, using feed additives appears to be a more cost-effective and environmentally sustainable option for managing shrimp health in aquaculture.

Of the many suggestions for beneficial feed additives, probiotics seem to be one of the most promising options (10, 87). Most evidence suggests that cell wall components of probiotic bacteria, such as β-glucan and lipopolysaccharides, have immunostimulatory effects through the presence of foreign molecules with pathogen-associated molecular patterns (PAMPs) that are recognized and bound by pattern-recognition proteins (PRPs) (88). In addition, probiotics may promote the growth of shrimp by releasing extracellular bacterial enzymes and bioactive products from probiotic metabolic processes. These compounds may activate precursors of the host's digestive enzymes and augment the nutritional absorption ability that contributes to the efficacy of food utilization (11, 89). Probiotics stimulate the proliferation and degranulation of shrimp hemocytes for immune activation due to the presence of bacterial cell walls. Pathogen-associated molecular patterns are subsequently recognized and bound by specific pattern-recognition proteins, triggering melanization and phagocytosis processes (12, 89). Hence, probiotics may be considered a sound strategy for improving the growth performance and health status of cultured shrimp.

Presently, a diverse range of beneficial bacteria has been used as probiotics in aquaculture, especially shrimp aquaculture. These include *Bacillus*, *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Carnobacterium*, *Lactococcus*, *Bifidobacterium*, *Streptococcus*, *Thiobacillus*, *Nitrobacter*, *Nitrosomonas*, and *Photorhodobacterium* (90). Among these, the genus *Bacillus* constitutes a diverse group of rod-shaped, gram-positive bacteria characterized by their ability to produce robust spores. Most *Bacillus* species are not harmful and are beneficial to aquatic animals. *B. subtilis*, *B. velezensis,* and *B. pumilus* have been

shown to positively affect feed digestibility, growth performance, and the prevention of microbial diseases in shrimp (5, 38, 58). Based on previous research, multi-strain probiotics consist of a mixture of two or more strains or species of bacteria that have been demonstrated to be more beneficial to the host than a single strain or species alone (56). In addition, feeding a commercial probiotic containing *B. subtilis* and *B. velezensis* significantly improved the survival rate of the Nile tilapia after being challenged with *Streptococcus agalactiae* (91). Most observations have focused on growth performance or immune parameters, while evidence of alterations in the intestinal bacterial community, digestive enzyme excretion, immune responses, and disease resistance in Pacific white leg shrimp is still sparse. Therefore, this study aimed to examine the effects of a *Bacillus* spp. mixture (*B. subtilis*, *B. velezensis,* and *B. pumilus*) on Pacific white leg shrimp's growth performance and immune response and its resistance to AHPND.

7.2. MATERIALS AND METHODS

Probiotics bacteria

The tested probiotic was prepared and provided by the Animal Health Solution Team, CJ BIO, CJ CheilJedang Corp. (Seoul, South Korea). A probiotic produced by Biotopia Corp. (Chuncheon, South Korea). The *Bacillus* spp. mixture consisted of *Bacillus subtilis* (BS) ($\geq 3.0 \times 10^8$ CFU g^{-1}), *Bacillus velezensis* (BV) ($\geq 3.0 \times 10^8$ CFU g^{-1}) and *Bacillus pumilus* (BP) ($\geq 3.0 \times 10^8$ CFU g^{-1}).

Experimental diets and design

A basal diet with an iso-nitrogenous content of 45.4% crude protein (Van Wyk et al., 1999) and iso-calorific content of 19.9 KJ g^{-1} was prepared (Table 1). Experimental diets included a control (without probiotic supplementation) and three treatment diets that included a *Bacillus* spp. mixture at different levels, i.e., 0.05%, 0.1%, and 0.2% w/w (Table 2).

All ingredients in the basal diet were ground and mixed to prepare the test diets. The *Bacillus* spp. mixture was thoroughly dispersed in water (15% w/w) and then added to the ground and mixed basal diet ingredients. The mixture was then pelleted using a pelleting machine with a mesh size of 1.5 mm. The diets were steamed at 100 ℃ for 10 min, dried at 50 ℃ for 10 h to reach 10% moisture, and stored at −20 ℃ until used.

Table 1. The formulation and chemical composition of the basal diets (dry matter basis) before adding the probiotic supplement.

Ingredients	Experimental diet (%)
Fish meal Peru[a]	12
Fish meal Ca Mau[b]	18
Wheat gluten[c]	3
Defatted soybean meal[d]	28
Squid liver powder[e]	5
Wheat flour	25
Fish oil	3
Lecithin	1
Binder (GG)[f]	0.5
Cholesterol	0.1
Choline chloride 60%	0.6
MCP	0.3
Premix[g]	2.0
Vitamin C	0.1
Gelatin	1.0
Lysine	0.1
Meth	0.3
Total (%)	100
Chemical composition (%)	Ratio (%)
Crude Protein	45.44
Crude fat	7.82
Carbohydrate	34.45
Ash	10.51
Gross energy (KJ g^{-1})	19.89

[a] Peruvian fishmeal, Pesquera Exalmar (CP 65%).

[b] Eco-Fish Ca Mau Viet Nam (CP 60%).

[c] VMC Group Viet Nam.

[d] Maharashtra Solvent extraction LTD India.

[e] An Giang Agriculture and foods import-export joint stock company.

[f] Binder was provide (Guar gum) was imported from Pakistan and supplied by Hoa chat Can Tho Comp, Vietnam.

[g] Premix was provided by DSM, Germany.

Table 2. Bacterial properties of the four test diets, prepared by adding *Bacillus* spp. mixture to the vasal diet in Table 1.

Sources	Treatment	Viable count (CFU g^{-1})	Endospore count (CFU g^{-1})
Stock	*Bacillus* spp. mixture*	1.84 x 10^{10}	9.5 x 10^{9}
Formulated pellet (experimental diets)	B0	Control	
	B1 (0.05% *Bacillus* spp. mixture)	1.83 x 10^{5}	0.69 x 10^{5}
	B2 (0.1% *Bacillus* spp. mixture)	2.67 x 10^{5}	1.55 x 10^{5}
	B3 (0.2% *Bacillus* spp. mixture)	8.01 x 10^{5}	4.38 x 10^{5}

* The *Bacillus* spp. mixture consisting of BS (≥3.0 x 10^{8} CFU g^{-1}), BV (≥3.0 x 10^{8} CFU g^{-1}), and BP (≥3.0 x 10^{8} CFU g^{-1}).

Shrimp and feeding trial

Healthy juvenile Pacific white leg shrimp (PL_{15}, N = 1400, average body weight: 0.45 g) were obtained from the shrimp hatchery of the College of Aquaculture and Fisheries, Can Tho University, Vietnam. The post−larvae were nursed in a 4 m^3 composite tank for 4 weeks under normal laboratory conditions (temperature: 30 ℃, DO: 5.0 mg L^{-1}, pH: 7.5 and aerated) and fed a commercial diet (45% protein) four times a day (at 7:00, 11:00, 16:00, and 21:00) to satiation.

The feeding trial was carried out in a 500 L composite tank (filled with 400 L of brackish water with a salinity of 13.4–13.5 mg L^{-1}) in an open system at the Wet-Lab of the College of Aquaculture and Fisheries, Can Tho University. At the beginning of the experiment, the shrimp were randomly assigned to 20 tanks (5 for each diet) at a density of 70 shrimp per tank. Shrimp were fed to satiation (about 5–6% of body weight day^{-1}) four times a day at 7:00, 11:00, 16:00, and 21:00. The amount of feed consumed in each tank was recorded daily by removing and weighing (dry weight) excess feed to ascertain intake. Water quality parameters were recorded twice daily during the feeding trial period (10 weeks (Table 3). Dead shrimp were removed daily, and their number was recorded. All shrimp in each tank were weighed at the end of the feeding trial. The shrimp were then used for a bacterial challenge experiment.

Growth performance parameters

At the end of the feeding trial, growth performance parameters were calculated as follows:

Weight gain (Wg, g) = Wf – Wi

Daily weight gain (DWG, g day^{-1}) = (Wf – Wi) / 70 days

Feed intake (FI, % $shrimp^{-1}day^{-1}$) = 100 x consumed feed / [(Wi + Wf)/2 x T]

Feed conversion ratio (FCR) = (consumed feed) / (weight gain)

Protein efficiency ratio (92) = (Wf – Wi) / (dry protein intake)

Survival rate (SR, %) = {(Final no-of shrimp) / (Initial no-of shrimp} x 100

where, the initial weight (Wi, g) was determined before the experiment, and the final weight (Wf, g) was determined after the 10-week experiment.

Digestive enzyme activity

For enzyme extraction, a tube with the pooled intestines of five shrimp $tank^{-1}$ was homogenized with 1000 μL of ice-cold buffer (20 mM Tris-HCl, 1 mM EDTA, 10 mM $CaCl_2$, pH 7.5), using a shaking machine (92, 93). Each treatment was replicated five times. The tubes were then centrifuged for 30 min at 1700 × *g* at 4 °C. The supernatant was transferred into new 1.5 mL tubes, stored at −80 °C, and used as the crude enzyme extract in protein content and enzymatic assay analysis later. Protein content was determined using the Bradford method (94).

Trypsin activity was assayed using N-benzoyl L-arginine-*p*-nitroanilide as the substrate (95, 96). Briefly, the reaction mixture was prepared by combining 240 μL of 100 mM Tris buffer (pH 8.5, containing 20 mM $CaCl_2$), 100 μL 2.4 mM L-BAPA, and 50 μL enzyme extract. Production of *p*-nitroaniline (*p*NA) was

measured by monitoring the increase in absorbance at 405 nm $minute^{-1}$ for 7 min at 37 ℃. One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1 absorbance unit at 405 nm in one min.

Chymotrypsin activity was measured according to Murashita et al. using N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPFNA, Sigma−Aldrich Inc., St. Louis, MO, USA) as a substrate (97). A 50 μL volume of the crude enzyme was added to 240 μL of 100 mM Tris buffer (pH 8.5, containing 20 mM $CaCl_2$) and 100 μL 2.4 mM SAPFNA, then incubated for 7 min at 37 ℃. Production of *p*NA was measured at 405 nm. One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1 absorbance unit at 405 nm in one min.

Amylase activity was evaluated according to Murashita et al. (97). A 1% starch solution was used as a substrate. Briefly, a 50 μL volume of enzyme extract was added to 25 μL of 20 mM sodium phosphate buffer (containing 6.0 mM NaCl, pH 6.9) and 25 μL of the substrate the solution. The mixture was incubated at 37 ℃ for 60 min. Following the addition of 50 μL dinitrosalicylic acid reagent (1% dinitrosalicylic acid and 30% sodium potassium tartrate in 0.4 M NaOH), the mixture was incubated for 5 min in 100 ℃ water. Production of maltose released was measured at 540 nm.

Hemolymph collection and analysis

Total hemocyte count (THC) was evaluated for shrimp in the feeding experiment, with 10 shrimp/treatment. A volume of approximately 100 μL of hemolymph was withdrawn from the pleopod base of the first abdominal segment of the shrimp with a sterile 1 mL syringe and gently mixed with 900 μL of sterile anticoagulant solution (trisodium citrate 30mM, NaCl 338 mM,

glucose 115 mM, EDTA 10mM, pH 7). Ten microliters of the diluted hemolymph were observed under a light microscope at 40X magnification. This was repeated twice (98). The THC was calculated as THC (cell mm^{-3}) = Hemocyte count of 5 cells × 10 × 10.

Differential hemocyte count (DHC) was determined for shrimp in the feeding experiment, with 10 shrimp/experiment. The diluted hemolymph was centrifuged at 5000 rpm for 5 min at 4 °C, washed, and resuspended in 200 μL of formalin-AS pH 4.6 (1:10). A volume of 50 μL of the suspension was spread onto a glass slide and the hemocytes fixed using ethanol for 5 minutes. This glass slide was stained with Giemsa for 30 minutes and washed in acetone and xylene (99). The stained glass slide was used to identify and count hyaline and granular cells under a light microscope at 100X magnification using a Fas the density of each hemocyte (cell mm^{-3}) = (number of each hemocyte × THC)/200.

Non-specific immune response

Phenoloxidase activity (PO) was evaluated for shrimp in the feeding experiment, with 10 shrimp/treatment. Phenoloxidase activity was determined by using L-Dihydroxyphenylalanine (L-DOPA) (100). Briefly, hemolymph (200 μL) was collected from the ventral sinus and mixed with 900 μL of sterile anticoagulant, centrifuged at 2500 rpm for 20 min at 4 °C and the supernatant removed. Then, 1 mL cacodylate citrate buffer solution (pH 7.0) was added and centrifuged at 2500 rpm for 20 min at 4 °C. The supernatant was removed, and a further 200 μL buffer solution (pH 7.0) was added. A volume of 100 μL of the suspension was either added to 50 μL cacodylate buffer solution (control tube) or 50 μL Trypsin solution. Fifty microliters of L-DOPA were added, followed by 800 μL

of Cacodylate buffer solution. The optical density was measured using a spectrophotometer at 490 nm.

Phagocytic activity (PA) was determined on 10 shrimp/treatment. The method for the measurements of phagocytic activity was performed as previously described (101). In brief, experimental shrimp were injected with 20 μL of bacterial suspension (1×10^8 CFU mL^{-1}). After 1 h, a total of 100 μL of hemolymph was collected and mixed with 100 μL of sterile anticoagulant. Diluted hemolymph was fixed with 200 μL of 0.1% paraformaldehyde for 30 min at 4 ℃, washed, and resuspended in 400 μL of sterile phosphate buffered saline (PBS). A 50 μL sample of the suspension was spread on a glass slide, air-dried, stained with Giemsa, and observed under a light microscope. Phagocytic activity, defined as phagocytosis rate (PR), defined as PR (%) = (phagocytic hemocytes / total hemocytes) x 100.

Superoxide dismutase (SOD) activity was measured in a subsample of 10 shrimp/treatment. SOD activity was determined according to the method described by Beauchamp and Fridovich (1971) using nitroblue tetrazolium (NBT) in the presence of riboflavin (102). Briefly, 100 μL of hemolymph was diluted in 500 μL PBS buffer and then centrifuged at 5000 × *g* for 5 min at 4 ℃. The supernatant was then incubated at 65 ℃ for 5 min to acquire crude SOD extract. Then, 150 μL of SOD crude extract was added to 50 μL of nitroblue tetrazolium (NBT) reagent (0.1 mM EDTA, 13 μM methionine, 0.75 mM NBT and 20 μM riboflavin in 50 mM phosphate buffer, pH 7.8), and incubated for 2 min. The optical density was measured using a spectrophotometer at 560 nm.

Bacterial clearance efficiency was evaluated in a subsample of 10 shrimp/treatment. For this, experimental shrimp were injected with 20 μL of

bacterial suspension ($1x10^8$ CFU mL^{-1}). After 1 h, a 200 μL volume of diluted hemolymph was further diluted to 20 mL with saline solution (103). A volume of 50 μL of diluted hemolymph sample was spread on separate TSA plates and incubated at 28 °C for 24 h. The number of colonies on the plates was then counted. The clearance efficiency, defined as percentage inhibition (PI), was calculated using the following formula: PI (%) = 100 – (CFU in the test group – CFU in the control group) x 100.

Total *Vibrio* spp. count in hepatopancreas

The density of *Vibrio* in shrimp hepatopancreas was individually measured for 10 shrimp/treatment using a standard plate count method. The hepatopancreas (HP) were dissected and homogenized in 1.0 mL of sterile saline solution (SSS) (0.85% NaCl). Serial ten-fold dilutions with the saline solution were performed. A total volume of 0.1 mL of the dilution was streaked onto TCBS agar (HiMedia Laboratories Pvt Ltd, Mumbai, India). The plates were incubated at 28 °C for 24 hours. The results are reported as CFU g^{-1}.

Histology of the mid-gut

At the end of the 10-week experiment, the intestine of five shrimp per treatment was fixed in standard Davidson solution for 24 h and then transferred to 70% alcohol solution. Afterward, the samples were processed with the standard histological procedure involving dehydration, diaphanization, and paraffin embedding. Paraffin blocks 5 μm in thickness were cut with a microtome for subsequent staining with hematoxylin and eosin (104). The mid-gut slides

were observed, and the villus height was measured under a light microscope according to a previously described method (105).

Determination of Median Lethal Dose (LD_{50}) of *Vibrio parahaemolyticus*

To determine the LD_{50} of *V. parahaemolyticus*, four treatments (three bacterial doses and one control) were replicated three times each. The treatments were allocated randomly in 12 buckets (80 L). An overnight culture of bacteria in tryptone soya broth (HiMedia Laboratories Pvt Ltd, Mumbai, India) was centrifuged at 5000 rpm for 10 minutes. Bacterial cells were washed twice with 0.85% NaCl and then resuspended in the same solution to obtain a bacterial suspension with a concentration of 10^8 CFU mL^{-1}. The bacterial suspension was subjected to ten-fold serial dilutions to obtain concentrations ranging from 10^8 to 10^6 CFU mL^{-1}. Shrimp was immersed in different bacteria concentrations (10^8, 10^7, and 10^6 CFU mL^{-1}) and saline water (control treatment). Moribund or dead shrimp were removed from the experiment tanks daily for 14 days. The LD_{50} value was calculated using a previously described method (106).

Challenge experiment

A strain of *V. parahaemolyticus* was cultured on nutrient agar (HiMedia Laboratories Pvt Ltd, Mumbai, India) supplemented with 1.5% NaCl for 24 h at 28 ℃ and transferred into 10 mL nutrient broth (HiMedia Laboratories Pvt Ltd, Mumbai, India) supplemented with 1.5% NaCl for 24 h at 28 ℃. The bacteria stock was centrifuged at 8000 rpm for 15 min at 4 ℃. The resulting bacterial pellet was resuspended in PBS, and the concentration of the bacterial suspension was measured using a spectrophotometer at 600 nm.

At the end of the 10-week feeding experiment, a bacterial challenge experiment was carried out using the immersion method (76). The challenge experiment was conducted with five treatments and three replicates. Thirty shrimp from each treatment of the feeding experiment were challenged with *V. parahaemolyticus*, by immersion in the bacterial solution at 5.4 x 10^7 CFU mL^{-1} (dose of LD_{50}), prepared as described above, for 15 min. Shrimp in the negative control group were immersed in sterile saline. After immersion, shrimp were transferred directly into experimental tanks containing clean seawater. Experimental shrimp (10 shrimp $tank^{-1}$) were kept in a 100 L glass tank containing 60 L of 15‰ seawater at 27.5 ± 1 ℃. Each glass tank was provided with continuous aeration, and water was renewed every three days during the challenge test. Shrimp were fed with experimental diets twice daily. Clinical signs and the mortality rate were recorded for 14 days. The moribund shrimp were tested for the presence of *V. parahaemolyticus* using the PCR method (107).

PCR method for detection of *V. parahaemolyticus*

The PCR was carried out using specific primers to amplify *ToxA* and *ToxB* genes (107). PCR products were analyzed by 2% agarose gel electrophoresis in 0.5X TAE buffer and stained with Ethidium bromide. A 100 bp DNA ladder was used as a size marker. Expected amplicons were 1269 bp (1^{st} PCR product) and 230 bp (2^{nd} PCR product).

Data analysis

All data were presented as mean ± SD. The mean difference of parameters among treatments was tested by one-way ANOVA followed by Duncan's multiple range tests (using MBI SPSS Statistics Version 21). The differences were considered significant at $p < 0.05$.

7.3. RESULTS

Water quality parameters

Water temperature in the range 28.1–28.2 ℃ in the morning and 29.5–29.9 ℃ in the afternoon, pH values ranged from 7.9 to 8.1. DO concentrations were maintained at 6.4–6.5 mg L^{-1}, and salinities (13.4–13.5‰) and alkalinity (174–179 mg $CaCO_3$ L^{-1}) were stable throughout the feeding experiment (Table 3). In general, temperature, pH, DO, salinity, and alkalinity concentrations were not significantly different between treatments ($p > 0.05$) and were in acceptable ranges for growth of the Pacific white leg shrimp. However, the concentrations of total ammonia nitrogen (TAN), NO_2^--N and NO_3^--N were relatively high, but there were no statistical differences between treatments (Table 3). The highest TAN was detected in the B1 treatment group, followed by B2 treatment group, whereas the lowest one was B3 treatment group. High nutrient loading level and low water exchange rate could have been responsible for high levels of nitrogenous waste products such as NH_4^+, NH_3, NO_2^-, and NO_3^- in the present study.

Growth performance, survival rate, and feed utilization

After 10 weeks of culture, staring at about 0.45 g $shrimp^{-1}$, the final mean weight (Wf) of the experiment was the lowest (15.5 g/shrimp) in the B0 treatment group, and significant differences ($p < 0.05$) with all *Bacillus* spp. mixture treatment groups were observed. The weight gain (Wg) and daily weight gain (DWG) of experimental shrimp showed a similar pattern to the final mean weight. However, the highest survival rate (83.8%) was found in the B1

treatment group, and it was significantly different from the other treatments, except for the B3 treatment group. The lowest survival rate was found in the B0 treatment group (65.0%), which was significantly lower than in the rest of the treatments. The probiotic supplementation in the diet reduced the feed intake of experimental shrimp (Table 4). The highest feed intake (4.21% shrimp^{-1} day^{-1}) was found in the B0 treatment group, and it was significantly different ($p < 0.05$) between probiotic supplementation treatments. The feed conversion ratio (FCR) was the lowest in the B1 treatment group (1.23) and the highest in the B0 treatment group (1.57). There were significant differences ($p < 0.05$) between the groups with and without probiotic addition. The optimum protein efficiency ratio (92) was in the B1 treatment group (2.04), and the lowest PER was in the B0 treatment group (1.60). Also, there was a significant difference ($p < 0.05$) between the groups with and without probiotic addition in PER. As a result of the high growth and the highest survival rate, the yield observed in the B1 treatment group (3.06 kg^{-1} m^3) was significantly higher than that of the other groups, whereas the significantly lowest yield was found in the B0 treatment group (2.01 kg^{-1} m^3).

Digestive enzyme activity

Digestive enzyme activities after 10 weeks were higher than those after 5 weeks (Figure 1). Higher levels of amylase, trypsin and chymotrypsin were found in shrimps fed the diets with probiotics supplementation compared to the shrimps fed the diet without probiotic addition. Shrimps fed B1 diet (0.05% *Bacillus* spp. mixture) had the highest levels of digestive enzymes, and digestive enzyme levels then progressively decreased with increase of probiotic

concentration in the diets. Shrimps fed the dietary probiotics supplementation had higher digestive enzyme activities than those in the control. Shrimps fed the B1 treatment had a higher chymotrypsin activity than the other treatments.

Non-specific immune parameters

Hematological parameters of shrimp fed with different concentrations of probiotics are shown in Figure 2. After 10 weeks of feeding, the number of hyaline cells (HC) and granular cells (GC) in all dietary probiotics treatment groups (B1, B2, and B3) were higher than those fed the control treatment group (B0). Granular cells (GC) were significantly higher in the B2 treatment group than in other treatment groups ($p < 0.05$). There was a significant increase in total hemocyte count (THC) in shrimp fed with different concentrations of probiotics compared to the control diet group ($p < 0.05$). After 10 weeks, PO activity of experimental shrimp was significantly higher in the B2 treatment group and significantly lower in the B0 group ($p < 0.05$) (Figure 3). Likewise, the phagocytic activity and SOD activity were significantly higher ($p < 0.05$) in the B2 treatment group and lowest in the B0 treatment group.

After 10 weeks of feeding, there was a higher *V. parahaemolyticus* clearance efficiency in the B2 (60.6%) and B3 (56.3%) treatment groups compared to the B1 treatment group (53%) (Figure 4). Total *Vibrio* count was lower in all dietary *Bacillus* spp. mixture treatment groups than in the control group, the B2 treatment group treatment had a lower ($p < 0.05$) *Vibrio* count than either the B1 or B3 treatment groups (Figure 4). The strongest immune responses were found in the B2 treatment group.

Intestine histology

Intestinal morphological parameters of Pacific white leg shrimp fed with different concentrations of *Bacillus* spp. mixtures are shown in Figures 5 and 6. The B2 and B3 treatment groups had greater villus height compared to the control treatment group (29.1 μm and 25.5 μm vs. 18.8 μm, respectively). The crypt depth of shrimps fed the B2 treatment was significantly deeper than that of the shrimps fed the control treatment. There was no significant probiotic effect ($p > 0.05$) on villus width (Figure 5).

Bacterial challenge test

When challenged by artificial inoculation with *V. parahaemolyticus*, shrimps fed the diets containing probiotics had significantly lower rate of mortality than those fed the control diet without probiotics supplementation (Figure 7). Cumulative mortality of the B1, B2, and B3 treatment groups (50–60%) was significantly lower than that of the B0 treatment group (76%). Mortality rate was inversely related to the levels of probiotics in the diets with the lowest mortality rate (50%) in B3 treatment group it was followed by the B2 treatment group with 54% mortality, the B1 treatment group with 58%, and the B0 treatment group with 76% (Figure 7). Nested-PCR results showed that the moribund shrimp were infected with *V. parahaemolyticus*.

Table 3. Water quality parameters in experiment tanks (mean ± SD).

Parameters		Treatments			
		B0	B1	B2	B3
Temp (℃)	AM	28.2 ± 0.06	28.2 ± 0.16	28.2 ± 0.06	28.1 ± 0.13
	PM	29.9 ± 0.10	29.9 ± 0.09	29.9 ± 0.08	29.5 ± 0.80
pH	AM	7.9 ± 0.02	7.9 ± 0.01	7.9 ± 0.01	7.9 ± 0.02
	PM	8.1 ± 0.03	8.1 ± 0.03	8.1 ± 0.03	8.1± 0.02
DO (mg/L)		6.5 ± 0.06	6.4 ± 0.10	6.4 ± 0.07	6.4 ± 0.03
Salinity (mg/L)		13.4 ± 0.20	13.5 ± 0.13	13.5 ± 0.14	13.5 ± 0.17
Alkalinity (mg/L)		179 ± 4.83	178 ± 4.59	177 ± 3.30	175 ± 6.45
TAN (mg/L)		3.07 ± 0.76	4.28 ± 2.17	3.63 ± 0.29	3.01 ± 0.44
NH_3 (mg/L)		0.35 ± 0.11	0.61 ± 0.50	0.43 ± 0.08	0.35 ± 0.03
NO_2^--N (mg/L)		3.18 ± 0.32	3.28 ± 0.36	3.34 ± 0.14	3.45 ± 0.35
NO_3^--N (mg/L)		41.8 ± 6.83	45.4 ± 3.24	46.6 ± 2.97	46.1 ± 1.68

Table 4. Growth parameters, survival rate, and nutrient utilization of Pacific white leg shrimp for 10 weeks.

Parameters	Treatments			
	B0	B1	B2	B3
Wi (g)	0.45 ± 0.00	0.45 ± 0.00	0.45 ± 0.00	0.45 ± 0.00
Wf (g)	15.5 ± 0.91^{b}	18.2 ± 1.16^{a}	19.1 ± 0.76^{a}	17.6 ± 1.18^{a}
Wg (g)	15.0 ± 0.91^{b}	17.8 ± 1.16^{a}	18.7 ± 0.76^{a}	17.1 ± 1.19^{a}
DWG (g day^{-1})	0.21 ± 0.01^{b}	0.25 ± 0.02^{a}	0.27 ± 0.01^{a}	0.24 ± 0.02^{a}
FI (% shrimp^{-1} day^{-1})	4.21 ± 0.27^{a}	3.33 ± 0.05^{b}	3.34 ± 0.06^{b}	3.36 ± 0.07^{b}
PER	1.60 ± 0.10^{b}	2.04 ± 0.03^{a}	1.99 ± 0.04^{a}	2.02 ± 0.04^{a}
FCR	1.57 ± 0.09^{a}	1.23 ± 0.02^{b}	1.26 ± 0.03^{b}	1.24 ± 0.03^{b}
SR (%)	65.0 ± 2.34^{c}	83.8 ± 2.79^{a}	75.3 ± 2.40^{b}	78.0 ± 3.60^{ab}

Values are means of three replicate group's ± SD. Within a row, value with the same letters is not significantly different ($p > 0.05$). Wi: initial mean weight, Wf: final mean weight, Wg: mean weight gain, DWG: Daily weight gain, FI: feed intake, PER: protein efficiency ratio, FCR: feed conversion ratio, SR: survival rat

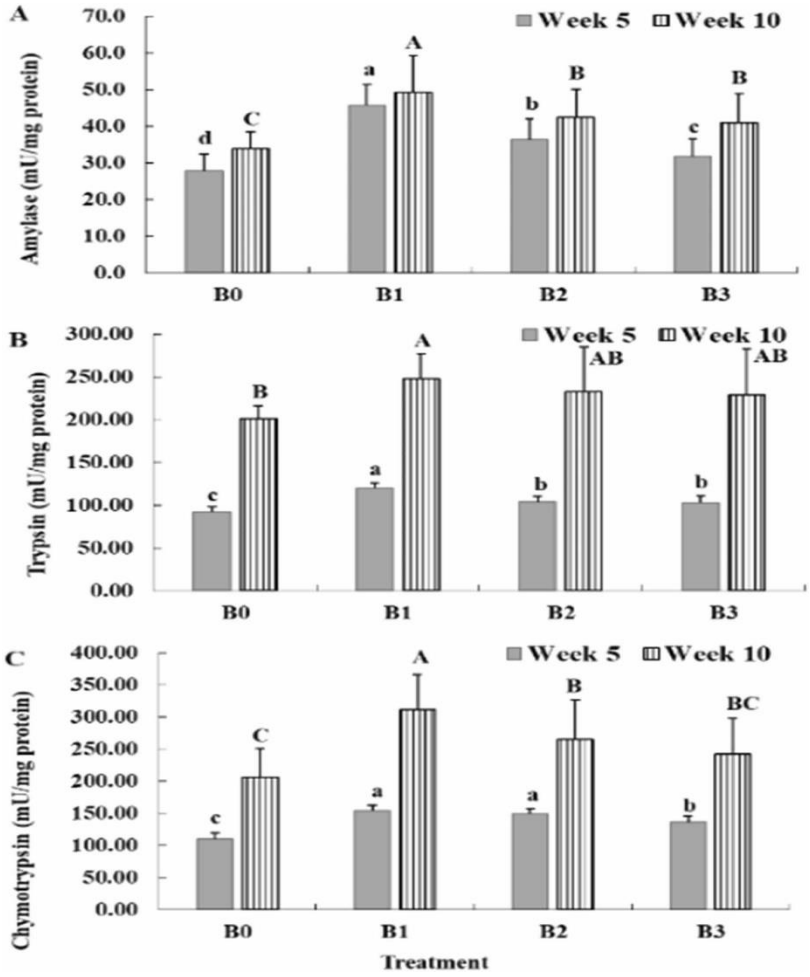


Figure 1. Digestive enzyme activity in the intestine of shrimp fed different levels of the *Bacillus* spp. mixture after 5 and 10 weeks of culture. Values are mean ± SD. Means with the same letters (a or A) were not significantly different ($p > 0.05$).

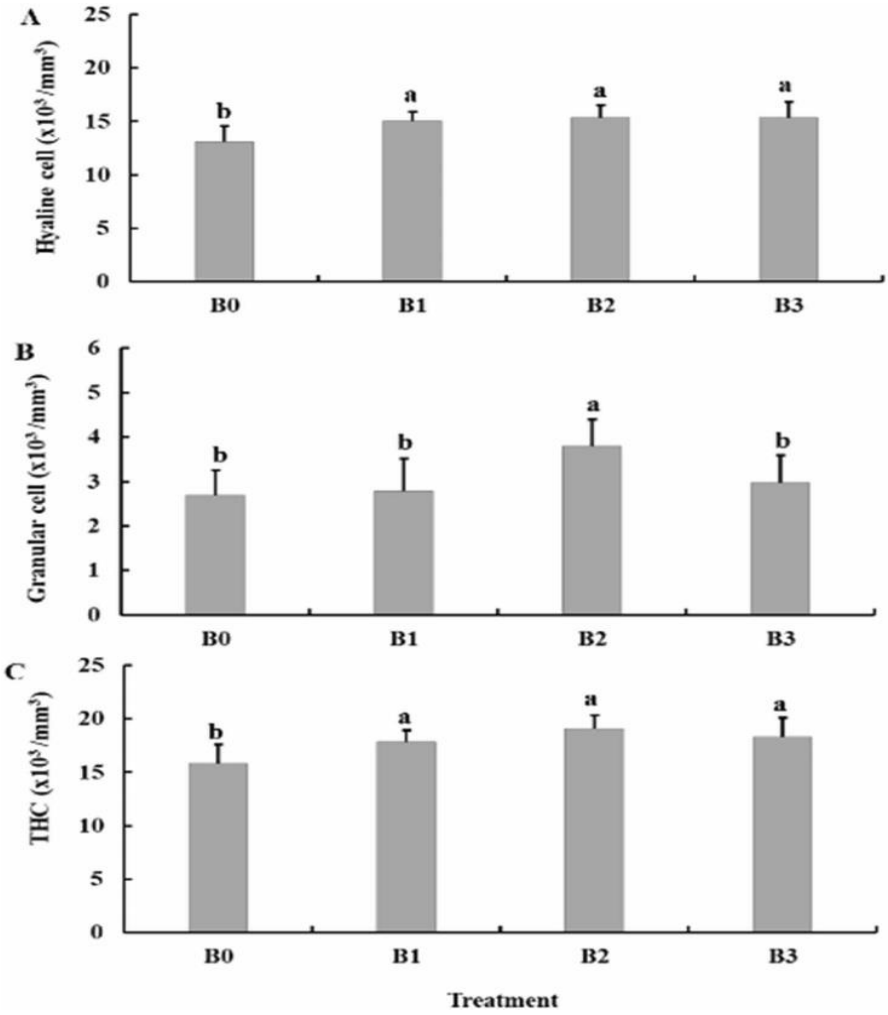


Figure 2. Counts of hyaline cells (A), granular cells (B) and hemocyte (C) in shrimp fed diets with different levels of the *Bacillus* spp. mixture for 10 weeks. Data are means ± SD for ten shrimp in each treatment. Means with the same letters were not significantly different ($p > 0.05$).

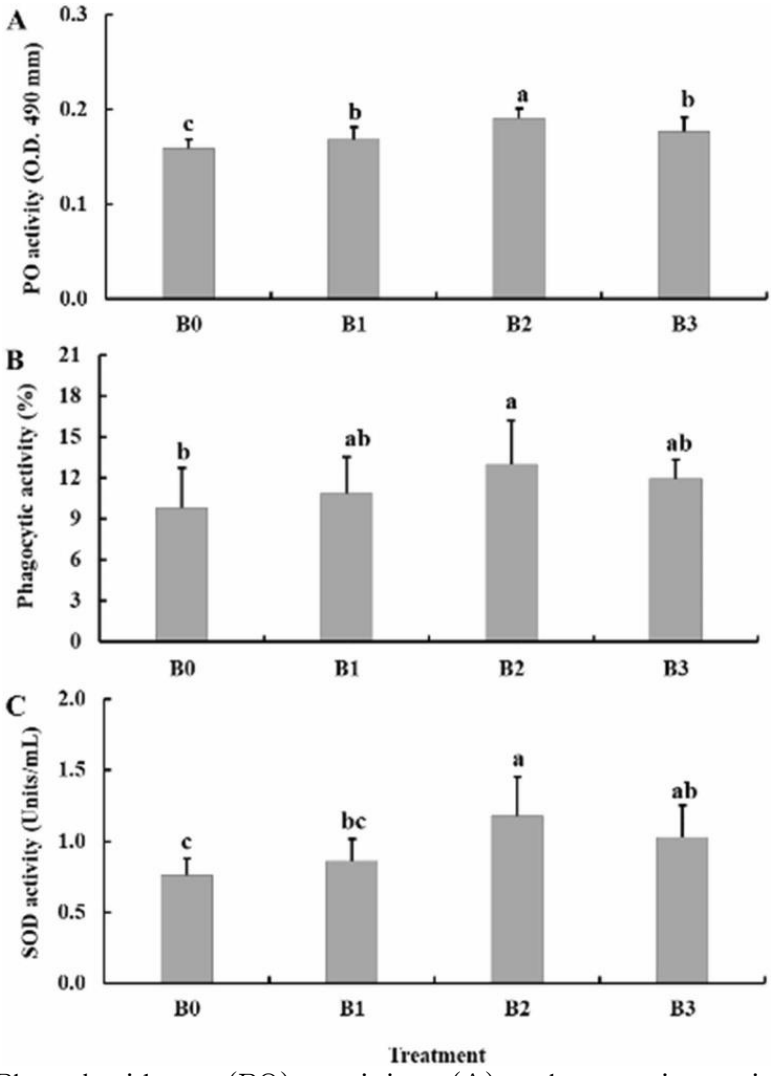


Figure 3. Phenoloxidase (PO) activity (A), phagocytic activity (B) and superoxide dismutase (SOD) activity (C) in shrimp fed diets with different levels of the *Bacillus* spp. mixture for 10 weeks. Values are means ± SD for ten shrimp in each treatment. Means with the same letters were not significantly different ($p > 0.05$).

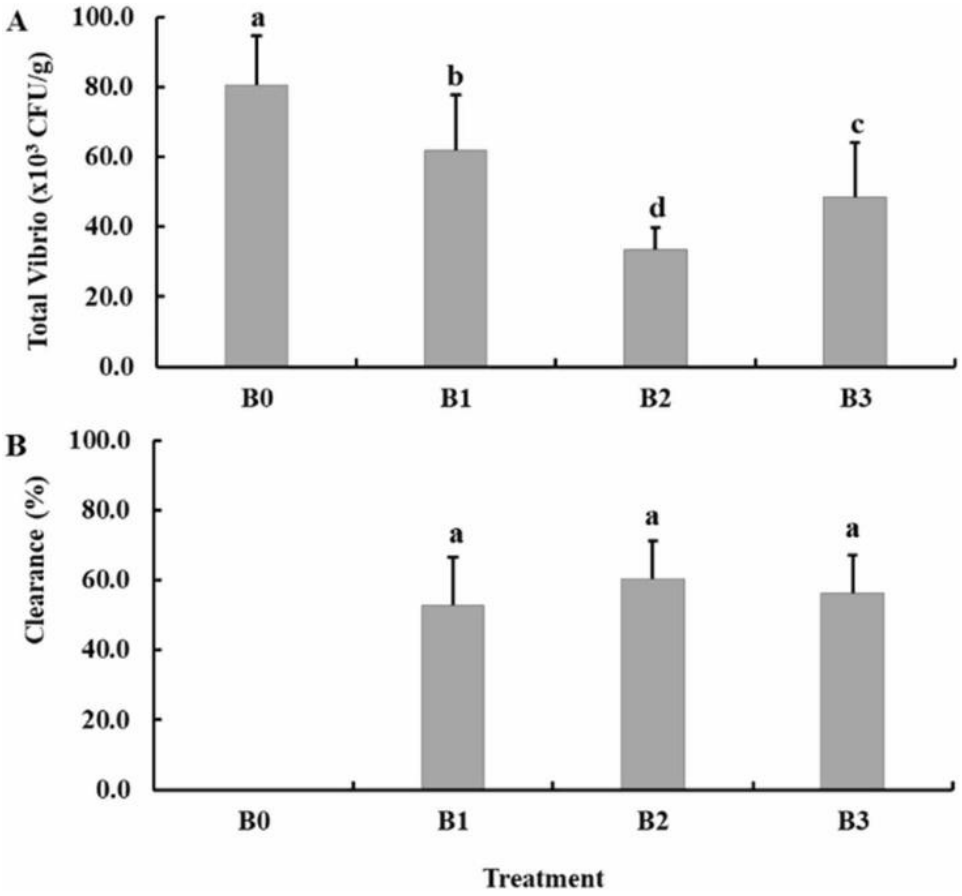


Figure 4. Total *Vibrio* count in hepatopancreas (A), and clearance efficiency of *Vibrio* (B) in the hemolymph of shrimp fed diet with different levels of the *Bacillus* spp. mixture for 10 weeks. Values are means ± SD for ten shrimp in each treatment. Means with the same letters were nor significantly different ($p > 0.05$).

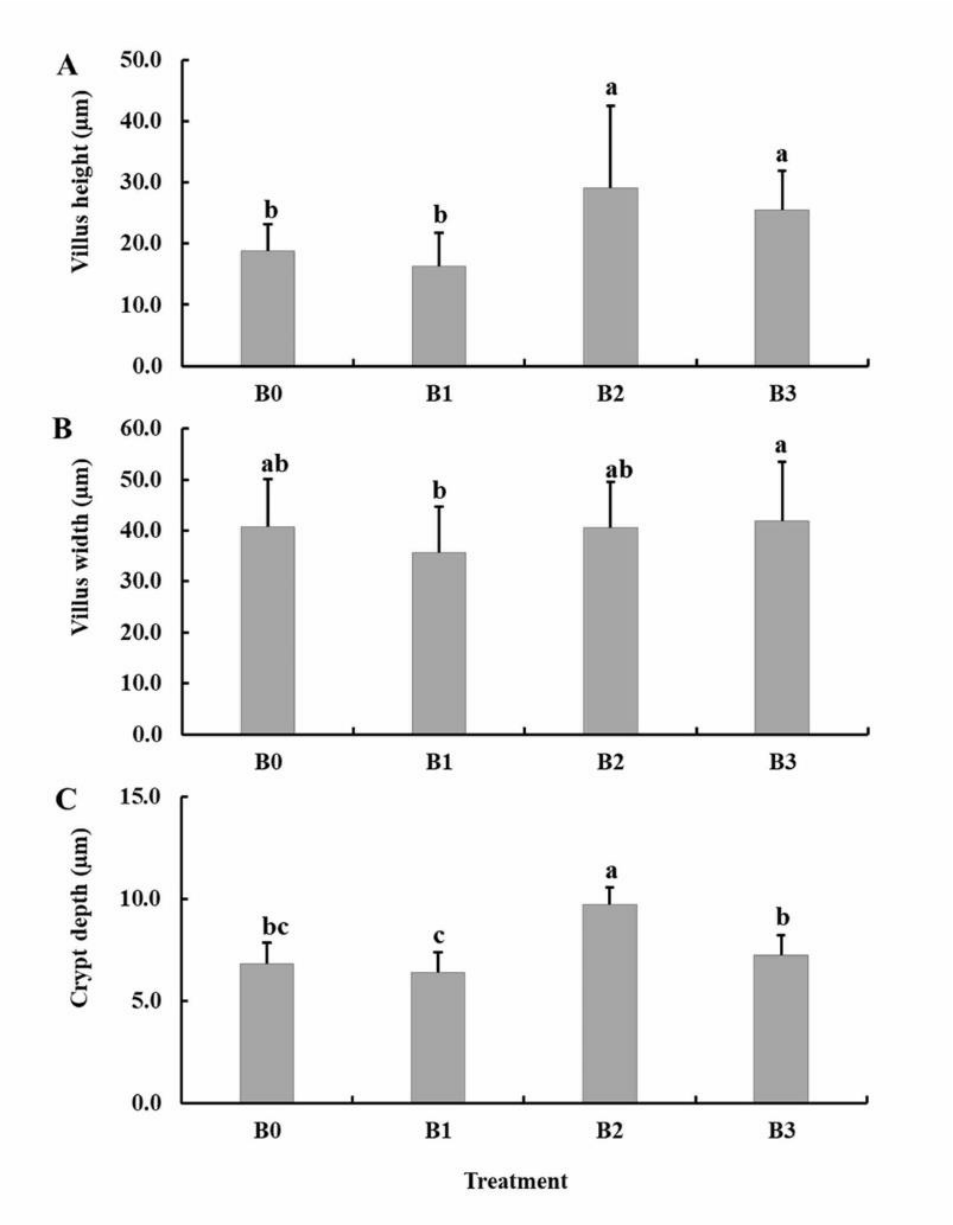


Figure 5. Villus height (A), villus width (B), and crypt depth (C) of the epithelial layer in the intestine of Pacific white leg shrimp fed diet with different levels of the *Bacillus* spp. mixture, for 10 weeks. Values are means ± SD for ten shrimp in each treatment. Means with the same letters were nor significantly different ($p > 0.05$).

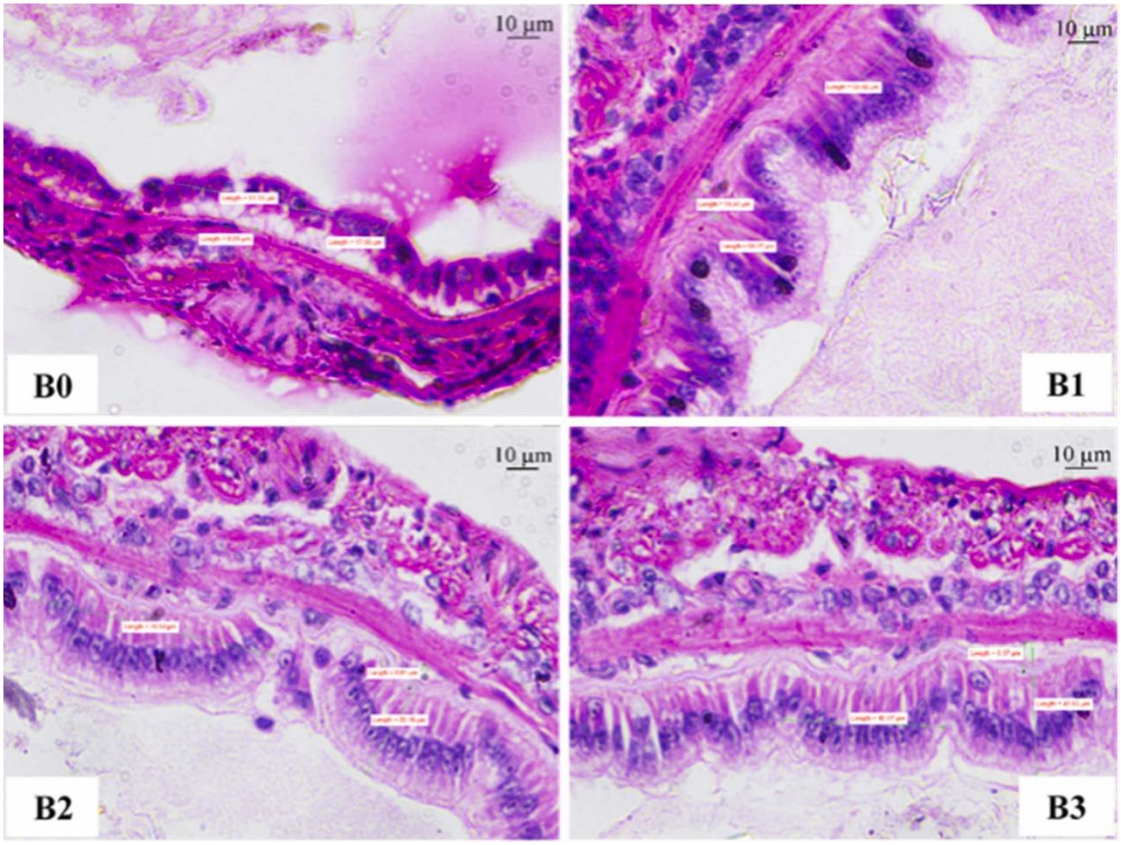


Figure 6. Histology of the intestines of Pacific white leg shrimp fed diet with different levels of the *Bacillus* spp. mixture for 10 weeks.

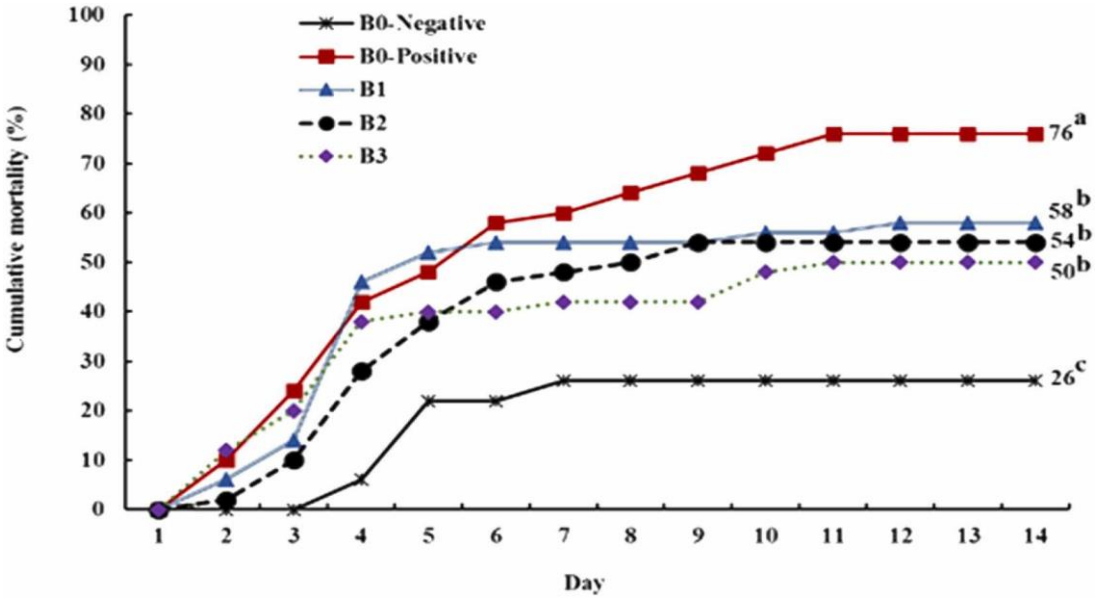


Figure 7. Cumulative mortality of Pacific white leg shrimp fed diets containing different levels of the *Bacillus* spp. mixture, after immersion in a solution containing *V. parahaemolyticus* over a period 14 days. Treatment B0−Positive, B1, B2, and B3 were exposed to the pathogen; Treatment B0−Negative was a control without *Bacillus* spp. mixture and without exposure to *V. parahaemolyticus*. Curves with same letters were not significantly different ($p > 0.05$).

7.4. DISCUSSION

The advantage of probiotics in Pacific white leg shrimp aquaculture have been reviewed by several researchers since the last decade. To date, a diverse range of beneficial bacteria, such as *Bacillus*, *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Carnobacterium*, *Lactococcus*, *Bifidobacterium*, *Streptococcus*, *Paracoccus*, *Thiobacillus*, *Nitrobacter*, *Nitrosomonas*, and *Photorhodobacterium* has been used as probiotics in aquacultures (90, 108, 109). The bioproducts containing single or multi-strain probiotic bacteria have been widely used to improve growth and health status of Pacific white leg shrimp (91, 120). In this study, *Bacillus* was used as a probiotic for promoting the growth performance and health status of the Pacific white leg shrimp. The probiotic product comprised three *Bacillus* strains, i.e., *B. subtilis*, *B. velezensis*, and *B. pumilus*. The probiotic bacteria are served as the exoenzyme providers in the present study. Probiotic bacteria directly or indirectly affect digestive enzymatic activities in the intestines of aquatic animals. In fact, most researchers agree that probiotic metabolisms directly activate enzyme biosynthesis in the pancreas or hepatopancreas and induce the secretion of digestive enzymes into the intestines of shrimp. As to indirect effects, probiotic bacteria can release extracellular enzymes involved in the digestion of proteins, carbohydrates, and fats. In this study, shrimp being fed *Bacillus* spp. had higher digestive enzyme activity, including α-amylase, trypsin, and chymotrypsin, than those of the control shrimp, in which the B1 treatment group had the highest chymotrypsin activity as compared to the control, B2, and B3 treatment groups. Interestingly,

as the same *Bacillus* spp. mixture supplement, feeding at the highest dose, i.e., B3 (0.2% *Bacillus* spp. mixture), showed a decrease in enzyme activity at both sampling times (5 and 10 weeks). Further advanced molecular research is needed to elucidate this phenomenon.

In probiotic bacteria, "zymogens," known as inactive precursors of proteolytic enzymes, are converted to their active forms, and secreted into the environment. Regulation of bacterial protease activity depends on transcription and translation protease gene expressions (89). Feeding activity is closely related to appetite, which determines the amount of food intake. In this study, feeding probiotics induced higher digestive enzyme activity, including trypsin, chymotrypsin, and α−amylase, resulting in decreased FI (compared to the control treatment) and PER, thereby improving feed efficiency in all probiotic treatments. Interestingly, the growth performance, WG, and DWG dramatically increased in shrimp fed the diet with probiotics supplementation compared to the control diet without probiotic administration.

Administration of *Bacillus* spp. mixture in the digestive tract can serve as a provider of exogenous enzymes and assist the process of simplifying feed macromolecules into micromolecules that can be used as sources of energy or as precursors for the synthesis of cellular components. In the present study, no significant difference in growth performance was observed among the *Bacillus* spp. mixture treatments (B1, 0.05%; B2, 0.1%; and B3, 0,2% of *Bacillus* spp. mixture). However, the B1 treatment group showed a higher survival rate, resulting in a higher yield (kg/m^3) as compared to the B2 treatment group, but not reaching a statistical difference. Based on the enzyme activity, protein efficiency ratio, feed efficiency, survival rate, and growth performance data,

feeding with a *Bacillus* spp. mixture at 0.05–0.1% (denoted as the B1 and B2 treatments group in this study) could be more suitable for the Pacific white leg shrimp.

Numerous studies assumed that probiotics improve the nutrient absorption ability of a host's intestinal tract. According to Huynh et al.89, free amino acids are produced during microbial metabolism processes. Then, amino acids are further utilized by both intestinal bacteria and the host. However, substantial amounts of free amino acids seem to escape assimilation and participate in the synthesis of short-chain fatty acids (SCFAs). Among SCFAs, butyrate acts as a signal transduction molecule responsible for developing and augmenting the barrier function of the colonic epithelium. In addition, lactate is a metabolite of glucose and an energy source for many tissues. SCFAs stimulate the physiological pattern of proliferation and permeability of colonic epithelial cells that improve the gut's nutrient absorption ability. However, the concentrations of SCFAs released, such as butyrate, lactate, or propionate, are unknown in this study. The intestine histology showed that shrimp in the B2 treatment group had higher villus height and crypt depth than that of other treatments. This could be because the *Bacillus* spp. mixture treatment was able to improve the barrier function of the colonic epithelium and maintain the normal cell numbers in the intestine of the host, as reviewed by Huynh et al. (89).

Huynh et al. proposed a possible mode of action through which probiotics can modulate nonspecific cellular and humoral defense mechanisms, among which the mucosal immune response is considered the first line of defense against invading pathogens (89). In fact, mucous membranes are in direct contact with the outside environment, and the intestinal epithelium is known as a natural

barrier of the digestive tract providing defense against extrinsic invasions. During probiotic dietary administration, many probiotic bacteria colonize mucus membranes and prevent pathogens from adhering by competing for substrates and places of adhesion. Previous studies found that probiotics could trigger encapsulation and phagocytosis processes in shrimp. The activation of immune responses and disease resistance was established in this study. Shrimp fed the diet containing the *Bacillus* spp. mixture at 0.1% (B2 treatment group) showed significant increases in THC, PO, SOD, and bacterial clearance either before or after being challenged with pathogenic bacteria, thereby suppressing the cumulative mortality after 14 days of challenge with *V. parahaemolyticus* causing AHPND. Of interest, the cumulative mortalities of shrimp in *Bacillus* spp. mixture treatments (B1, B2, and B3) were not significantly different. However, the cumulative mortalities of shrimp in the B1, B2, and B3 treatment groups were significantly lower than those in the control group. As discussed above, shrimp in the B1 treatment group showed better survival rate, enzyme activity, intestinal microbiota, and growth performance, while shrimp in the B2 treatment group had better immune responses and disease resistance to bacterial infection. Therefore, it could be referred that *Bacillus* spp. mixture should be used at doses in a range of 0.05–0.1% to improve growth and the health status of the white leg shrimp.

TAN, NO_2^--N, and NO_3^--N concentrations in treatments varied highly, and differences did not reach statistical significance among treatments over 28 days of culture. It is not clear whether the dietary administration of *Bacillus* spp. mixtures significantly improved water quality based on the concentrations of TAN, NO_2^--N, and NO_3^--N in water. Therefore, further studies should

investigate the positive effect of probiotic mixtures on water quality and which probiotic mixtures could be used as water-based additives. Additionally, several crucial water quality parameters, such as turbidity, total suspended solids (TSS), chemical oxygen demand (COD), and biological oxygen demand (BOD), should also be assessed in future research.

The results of this study demonstrated that *Bacillus* spp. mixture (BS, BV and BP mixture) could enhance the growth performance survival rate, immune responses and disease resistance of Pacific white leg shrimp. Diet containing *Bacillus* spp. mixture at levels of 0.05–0.1% had the highest growth rate and survival rate, and the greatest improvement in immune response and survival rate after being challenged with *V. parahaemolyticus*. These results indicated that *Bacillus* spp. mixture has potential as a probiotic for practical application in the aquaculture of Pacific white leg shrimp.

8. CHAPTER 2

Antimicrobial Activity of Probiotics against Disease in Pacific White Leg Shrimp

; Antibacterial activity of *Bacillus* strains against acute hepatopancreatic necrosis disease-causing *Vibrio campbellii* in Pacific white leg shrimp (*Penaeus vannamei*)

8.1. 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. *Vibrio 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 (75, 76, 78, 79). 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) (75, 76, 78, 79, 110, 111). 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 annually in Asia (112).

Although antibiotics have been extensively used to treat bacterial diseases in aquaculture for decades (113), their overuse or misuse has resulted in antibiotic resistance (114–119). 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 a challenge with Vp_{AHPND} (120, 121). In addition to their antimicrobial activity, probiotics have various advantages in aquaculture, such

as promoting growth, strengthening immunity, and restoring water quality (122, 123). Meanwhile, spore-forming *Bacillus* spp. are resistant to heat and pressure and are widely used as feed additives (124).

Besides probiotics, various methods such as immunostimulant therapy, quorum sensing control of bacterial virulence, phage therapy, and herbal medicine have been utilized in shrimp aquaculture (114, 125-127). Paopradit et al. (128) reported 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 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 (129, 130).

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 the field, 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 (126, 131). In this study, we isolated five *Bacillus* strains and evaluated their antibacterial 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 complete genomic sequences of these two *Bacillus* strains were 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}.

8.2. MATERIALS AND METHODS

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 subcultured 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 DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) protocol with slight modifications. For *Bacillus* identification, PCR was performed using the extracted DNA and the BacF/R primers, as described by Solichova et al. (132) (Table 5).

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, KisanBio, Seoul, 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 subcultured 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. (133). 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. (134) and Cano−Gomez et al. (135) (Table 1). PCR targeting AHPND toxin genes (*pir*A and *pir*B) was conducted using the method described by Han et al. (136) to identify AHPND virulence genes (Table 5).

Antimicrobial activity test (In Vitro)

For antimicrobial activity testing, the obtained *Bacillus* strains were further tested for their ability to inhibit the growth of *Vibrio* strains using the dot−spot method described by Spelhaug and Harlander (137). *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.

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 body weight 0.2 ± 0.05 g) were randomly distributed into 22 L acrylic tanks containing 18 L of aerated artificial seawater. For the antimicrobial activity test (challenge test), experimental shrimp (N = 56) were divided into four groups. The experiment was conducted in duplicate.

In group 1, the experimental shrimp (N = 14) were exposed to a suspension of *Bacillus* (B1) at a concentration of 1.0×10^6 CFU mL^{-1} water for 14 days via immersion. 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 a *Bacillus* (B3) suspension at a concentration of 1.0×10^6 CFU mL^{-1} water for 14 days via immersion. 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 simultaneously 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 ℃, 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.

Dead shrimp were collected and tested using the PCR method previously described by Han et al. (136) to confirm the presence of AHPND. Surviving shrimp were randomly sampled on the day of termination (day 14) to quantify AHPND. 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. (138).

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) (139). 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 (140). 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) (141) and BioEdit (ver. 7.0) (142). The maximum-likelihood phylogenetic tree was generated based on the concatenated sequences using MEGA X (143) with 1,000 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 (144) using the 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-502T (=NRRL B-41580T) were predicted using anti-SMASH v.6.1.1 (143) and compared.

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.

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.

Table 5. 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	GCTGGTTAGAGCGCACGCCTGATA	263	[31]
	BacR	CATCCACCGTGCGCCCTTTCTAAC		
AHPND toxin	VpPirA-284F	TGACTATTCTCACGATTGGACTG	284	[35]
	VpPirA-284R	CACGACTAGCGCCATTGTTA		
	VpPirB-392F	TGATGAAGTGATGGGTGCTC	392	
	VpPirB-392R	TGTAAGCGCCGTTTAACTCA		
V. parahaemolyticus	Tox R-F	GTCTTCTGACGCAATCGTTG	368	[33]
	Tox R-R	ATACGAGTGGTTGCTGTCATG		
V. campbellii	Vc.fts.z-F	AAGACAGAGATAGACTTAAAGAT	294	[34]
	Vc.fts.z-R	CTTCTAGCAGCGTTACAC		
V. harveyi	Vh.topA-F	TGGCGCAGCGTCTATACG	121	
	Vh.topA-R	TATTTGTCACCGAACTCAGAACC		

8.3. RESULTS

Identification of *Bacillus* and *Vibrio* strain

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 6 and Figure 9.

Twelve *Vibrio* strains were obtained from seawater and shrimp hepatopancreas an*campbellii*, and *V. harveyi*, 1 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 1 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 6 and Figure 9.

Antimicrobial activity test (In Vitro)

Using the dot-spot method, the five *Bacillus* strains (B1, B3, B5, B7, and B8) inhibited 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 7). In particular, B1 and B3

exhibited stronger inhibitory effects on *Vc*$_{AHPND}$ than the other *Bacillus* strains (B5, B7, and B8), as presented in Tables 7 and 8.

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*$_{AHPND}$. 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 9). 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 that of group 3 (*Vc*$_{AHPND}$ immersion without B1 and B3 treatment) (Figure 8 and Table 9). During the challenge test, 16 shrimp died (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 using PCR (Figure 10). The cycle threshold (Ct) 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.

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 with a length of 3,929,791 bp and 3,929,788 bp,

respectively. Both had a G+C content of 46.50%, and both genomes contained 3750 protein-coding genes, 86 tRNAs, 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 13613T (NR_117274.1; 99.1% and 99.1%) and *B. velezensis* CR-502T (AY603658.1; 99.6% and 99.7%). However, the resultant phylogeny did not clearly differentiate the two strains at the species level (Figure 11a). Therefore, we conducted a whole genome-based phylogenetic analysis to confirm the exact taxonomical position of the strains. The resultant phylogeny revealed that the two isolates were clustered together with *B. velezensis* NRRL B-41580T (LLZC00000000.1) (Figure 11b). 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* (145).

During the in silico search for biosynthetic gene clusters (BGCs) to produce potential antibiotics and/or secondary metabolites, four types of BGCs, 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 BGCs were detected in 7 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 to date, 14 substances in total were identified from the B1 and B3 strains, namely bacillibactin, amylocyclicin, paenibactin, difficidin, fengycin, plipastatin, bacillomycin D, mycosubtilin, iturin, paenilarvins, bacillaene, macrolactin (H, B, 1c, and E), surfactin, and bacilysin, (Tables 11 and 12). 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 three substances (mersacidin, plipastatin, and surfactin) were found (Table 13). First, mersacidin which was detected in the genome of *B. velezensis* NRRL B-41580T 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.

Table 6. *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 *V. 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.

Table 7. Inhibitory effects of *Bacillus* strains against *Vibrio* strains (dot-spot test).

Vibrio strains	*Bacillus* strains					
	Type strain[a]	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]: *B. velezensis* 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.

Table 8. Clear zone images of *Bacillus* strains (type strain, B1, and B3) against the representative *Vibrio* strains (16-904/1, 19-021D1, and 19-022A1).

Vibrio strains	*Bacillus* strains		
	Type strain[a]	B1	B3
16-904/1 (*V. campbellii*)			
19-021D1 (*V. parahaemolyticus*)			
19-022A1 (*V. parahaemolyticus*)			

[a]: *B. velezensis* CR-502^T (=NRRL B-41580^T)

Table 9. The survival rate (%) 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.

Table 10. 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

Table 11. 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 12. 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.

Table 13. The predicted secondary metabolite gene clusters in *Bacillus velezensis* NRRL B-41580^T using anti-SMASH.

Region	Position		Type	Biosynthetic gene clusters	Substance	Similarity (%)
	from	to				
11	557,919	651,711	transAT-PKS	Polyketide + NRP[1]	Difficidin	100
15	316,967	368,757	RiPP-like	RiPP:head-to-tail cyclized peptide[2]	Amylocyclicin	100
			NRP	NRP	Bacillibactin	100
					Paenibactin	100
				NRP:NRP siderophore	Bacillibactin	100
16	1	66,223	transAT-PKS	Polyketide + NRP	Bacillaene	85
	291,097	379,327	transAT-PKS	Polyketide	Macrolactin H	100
					Macrolactin H / B / 1c / E	100
17	242,499	283,917	Other	Other	Bacilysin	100
	440,952	464,140	Lanthipeptide-class-ii	RiPP:lanthipeptide	Mersacidin	100
18	2	100,307	transAT-PKS	Polyketide + NRP:lipopeptide	Bacillomycin D	100
				NRP + Polyketide	Mycosubtilin	100
					Iturin	88
			NRPS	NRP	Paenilarvins	100
					Fengycin	86

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

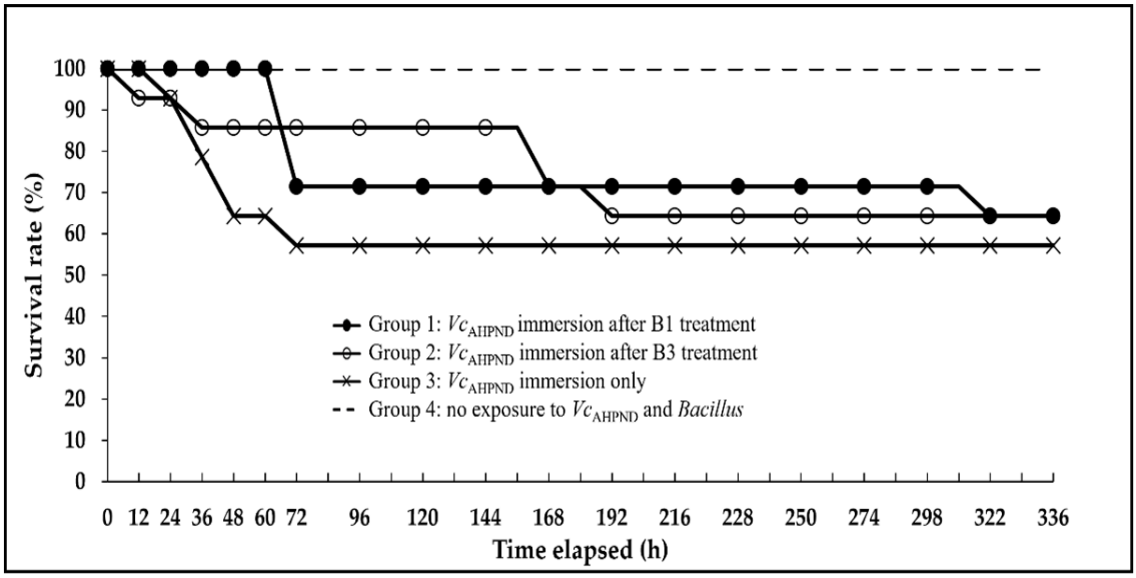
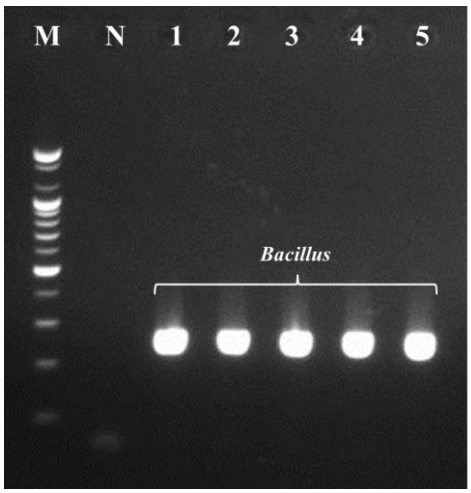
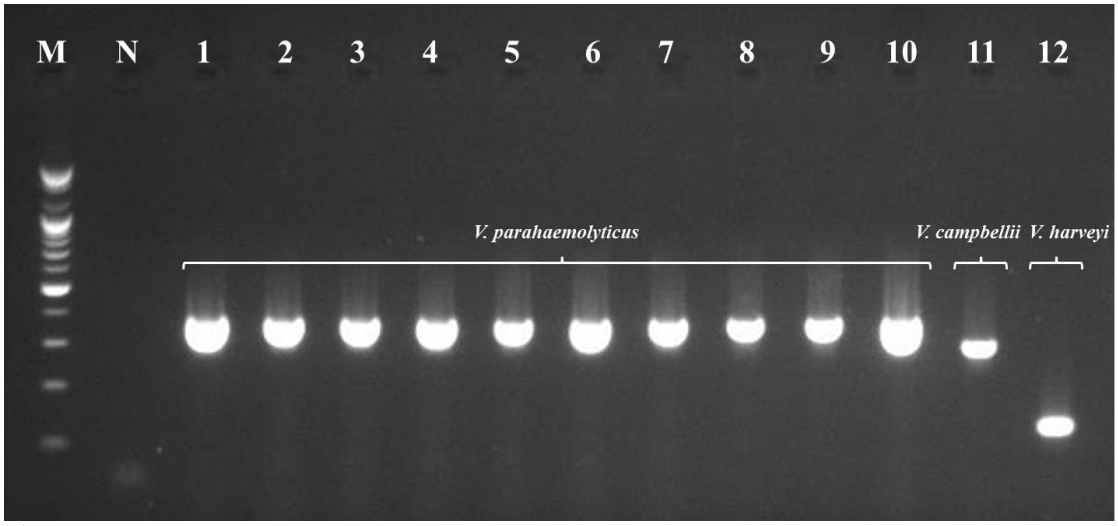


Figure 8. The survival rate (%) of Pacific white leg shrimp challenged with Vc_{AHPND} (16-904/1).



a)



b)

Figure 9. PCR analysis for *Bacillus* and *Vibrio* identification. **a)** PCR analysis for *Bacillus* identification (263 bp). Lane M: 100-bp ladder, Lane N: Negative control (DDH_2O), 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 (DDH_2O), 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.

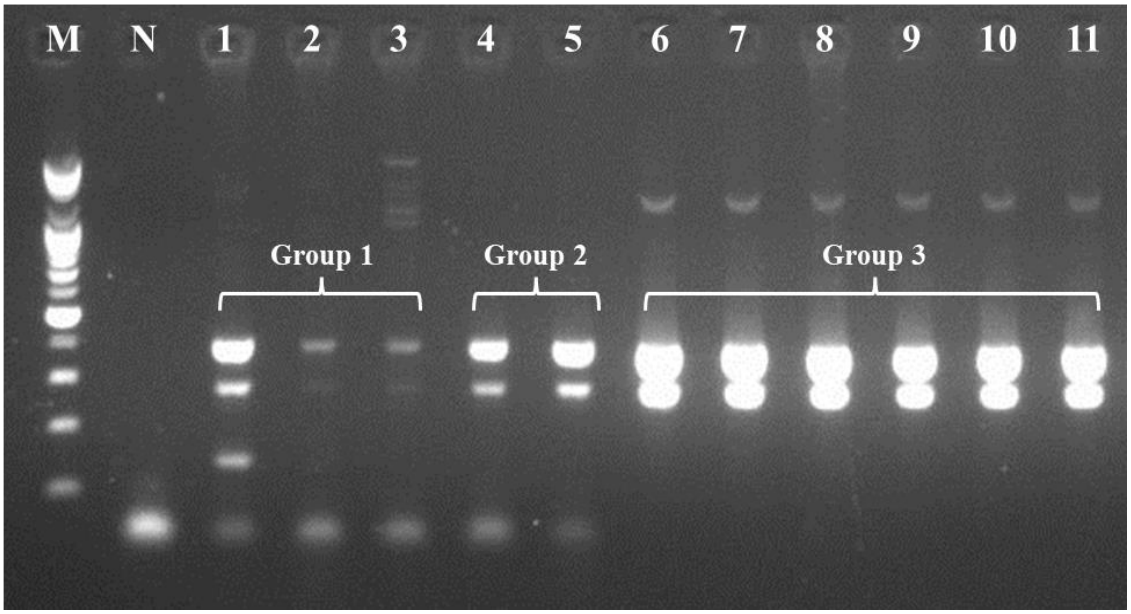


Figure 10. PCR analysis was performed to identify the AHPND toxin genes (*pir*A: 284 bp, *pir*B: 392 bp) in dead shrimp. Lane M: 100−bp ladder, Lane N: Negative control (DDH_2O), Lane 1−3: dead shrimp in group 1 (Vc_{AHPND} immersion after B1 treatment), Lane 4−5: dead shrimp in group 2 (Vc_{AHPND} immersion after B3 treatment), and Lane 6−11: dead shrimp in group 3 (Vc_{AHPND} immersion without B1 and B3 treatment).

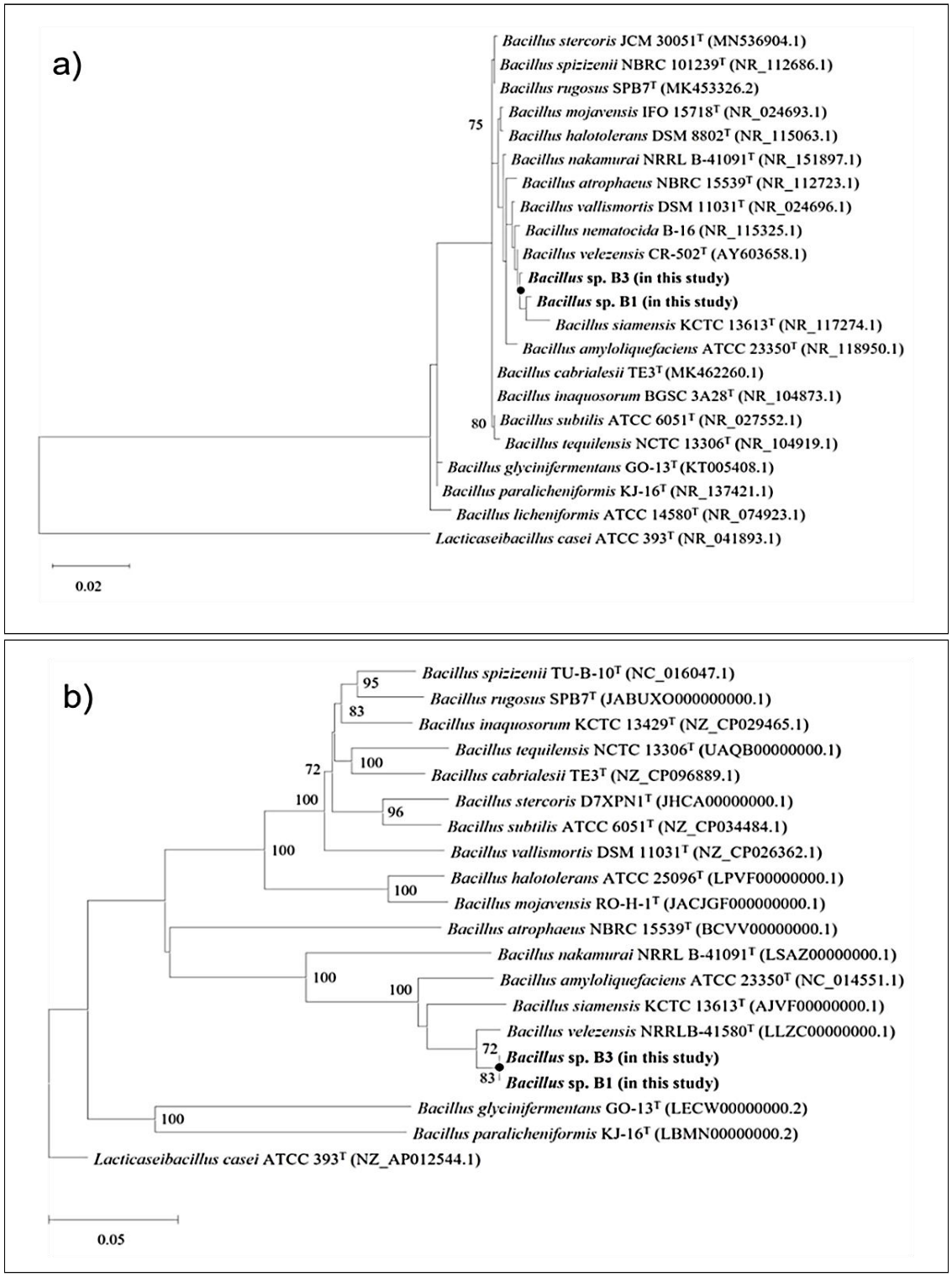


Figure 11. Phylogenetic tree based on the 16S rRNA gene sequences a) and whole-genome sequences b) detailing the relationships of *Bacillus* isolates B1 and B3 with 20 type strains of *Bacillus* spp. and the outgroup *Lactobacillus casei* ATCC 393^{T}. The bootstrapping values are indicated at the branches 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.

8.4. DISCUSSION

In this study, we evaluated the antibacterial activity of 5 *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 (146-148). 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 salinity ranges. In the dot-spot test, B1, B3, B5, B7, and B8 displayed inhibitory effects on at least one of the tested *Vibrio* strains. 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 that 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. (149), 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 significant antibacterial 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 trains are expected to emerge as alternatives to antibiotics for controlling *Vc*$_{AHPND}$. Moreover, among the live shrimp collected on the termination day, the Ct value was higher in samples of the *Bacillus*–treated groups than in samples of the positive control group. Therefore, these results suggested that the two *Bacillus* strains identified in this study exhibited antibacterial 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 hepatopancreas structure was 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 antibacterial activity using the dot–spot test (in vitro) and challenge test, were finally classified as *B. velezensis* based on their whole genome phylogeny. Several studies have examined the probiotic effects of *B. velezensis* in various organisms. For example, Chauyod et al. (150) demonstrated that *B. velezensis* significantly inhibited the growth of *Vibrio* spp. isolated from shrimp, including *Vp*$_{AHPND}$, using a disk diffusion test. Li et al. (151) reported that the expression of immune–related genes such as IL–8 and IgM was upregulated in 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 (152) and *V. anguillarum* isolated from seabass (153).

Although the predicted secondary metabolites derived from the B1 and B3 *Bacillus* strains were relatively similar to those previously reported from related *Bacillus* species (140), plipastatin and surfactin were only found in the two isolates. 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. To date, most previously reported secondary metabolites produced by *Bacillus* spp. have surfactant and antibiotic activities (154). In particular, the potential presence of surfactin, which was previously reportedly associated with antibacterial activity against multidrug-resistant *Vibrio* spp. (155) in the two *Bacillus* strains might explain their antibacterial activity against pathogenic *Vc*$_{AHPND}$ in this study. However, further studies regarding the predicted presence of surfactin in the isolates are warranted 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 (156), may contribute to the potential usability of the *Bacillus* strains identified in this study. 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 B1 and B3 strains were more various than those previously reported for *Bacillus* spp., indicating that both strains can be used as potential candidate for the management of vibriosis and AHPND, including *Vc*$_{AHPND}$, in shrimp aquaculture.

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10. 국문 초록

2026 년까지 전세계 새우 시장 규모는 연평균 4.2% 성장해 234 억 달러에 달할 것으로 예상되는 가운데, 양식 새우에서 발생하는 대표적인 수산생물 전염병인 흰반점 바이러스, 급성간췌장괴사병(Acute hepatopancreatic necrosis disease, AHPND)등은 새우 양식 산업에 막대한 경제적 손실을 끼치고 있다. 전염성 질병의 예방과 치료를 위해 항생제를 포함한 다양한 물질들이 사용되고 있다. 본 연구에서는 현재 새우 양식에서 가장 많이 양식되고 있는 흰다리새우의 양식 과정에서 생산성을 향상시키고 항생제 사용을 줄이기 위한 방안의 하나로 프로바이틱스의 기능적 활용에 대해 연구하였다. 대표적인 기능성 프로바이오틱스인 *Bacillus* spp. 균주를 사용하여 흰다리새우의 성장, 사료 효율, 비특이적 면역 반응에 미치는 연구와 항생제 대체제로서의 가능성에 대하여 연구하였다. Chapter 1 에서는 *Bacillus* spp. 균주 3 종(*Bacillus subtilis*, *Bacillus velezensis*, *Bacillus pumilus*)을 동일한 비율로 혼합한 사료 첨가물을 흰다리새우 사료에 혼합 첨가하여 성장, 사료 효율, 비특이적 면역 반응, 수질, 생존률에 대한 개선 효과를 평가하였다. Chapter 2 에서는 *B. velezensis* 를 활용한 AHPND 의 병원체에 대한 항균 활성에 초점을 두어 연구를 진행하였다.

Chapter 1 에서는 *Bacillus* spp. 균주 복합물의 사료 첨가물로써 기능성을 평가하기 위해 흰다리새우의 기본 사료에 *Bacillus* spp. 균주 3 종 복합물을 각각 다른 수준으로(0%, 0.05%, 0.1%, and 0.25%)첨가하여 4 가지 실험 사료를 제작하였다. 흰다리새우(초기평균무게: 0.45g)는 총 20 개의 수조에 각 70 마리씩 5 반복으로 배치하였고, 사양실험은 10 주간 진행되었다. *Bacillus* spp. 균주 3 종 복합물을 사료 내 0.1% 첨가한 실험구에서 대조구에 비해 유의적으로 높은 성장률, 단백질 효율, 사료전환율과 생존율이 관찰되었다. 0.05%와 0.1% *Bacillus* spp. 균주 3 종

복합물을 첨가한 실험구는 대조구에 비해 소화 효소를 유의하게 증가시켰다. 또한, 0.1% *Bacillus* spp. 균주 3 종 복합물을 첨가한 실험구는 혈액 및 면역 프로파일을 개선했을 뿐만 아니라 가장 높은 박테리아 제거 효율을 보였다. *Bacillus* spp. 균주 3 종 복합물을 첨가한 모든 실험구는 *V. parahaemolyticus* 를 투여한 공격 실험에서 대조구보다 누적 폐사율이 낮게 나타났다. 본 실험결과로 미루어 볼 때. 흰다리새우를 양식하는 과정에서 사료에 *Bacillus* spp. 균주 복합물을 첨가하여 공급하는 것은 흰다리새우의 성장성과 면역반응을 높이는데 효과적이라는 것을 보여주며, 0.1% 농도로 *Bacillus* spp. 균주 3 종 복합물을 첨가하였을 때 가장 효과적인 것으로 판단된다.

Chapter 2 에서는 *Bacillus* spp. 균주의 항균 활성을 평가하고 이를 활용해서 흰다리새우 양식 과정에서 발생하는 AHPND 의 제어 가능성에 대해 실험하였다. 제주 해수에서 분리한 5 개의 *Bacillus* spp. 균주(B1, B3, B5, B7, B8)를 총 12 개의 *Vibrio* 균주(AHPND 균주 10 개 및 비 AHPND 균주 2 개)에 대해 항균 활성을 평가하였다. 시험에 사용된 5 개의 *Bacillus* spp. 균주는 dot-spot test 에서 하나 이상의 *Vibrio* spp. 균주에 대해 성장을 억제하였다. 이 *Vibrio* spp. 균주 중, AHPND 를 유발하는 *V. campbellii*(Vc_{AHPND})에 대해 가장 효과적인 *Bacillus* spp. 균주인 B1 과 B3 균주를 추가로 공격 실험에 사용하였다. 공격 실험에서 Vc_{AHPND} 를 흰다리새우에 노출시킨 결과, 48-60 시간이 지난 초기 생존율의 경우 B1 *Bacillus* spp. 균주 처리구(100%)에서 Vc_{AHPND} 만 노출시킨 대조구(64.3%)보다 유의하게 더 높은 생존율이 관찰되었다. 공격 실험의 종료된 후, B1 *Bacillus* spp. 균주 처리구와 B3 *Bacillus* spp. 균주 처리구는 Vc_{AHPND} 만 노출시킨 대조구보다 높은 누적 생존율을 보였다. 공격 실험에 사용된 흰다리새우의 간췌장조직에서 *pir*A 유전자를 qPCR 분석하여 확인한 Cycle Threshold(CT)값은 31.63 ± 0.2(B1 *Bacillus* spp. 균주 처리구) 및 38.04 ± 0.58(B3 *Bacillus* spp. 균주 처리구)인 반면 대조구(Vc_{AHPND} 처리구)에서는 28.70 ± 0.42 였다. 유전자 분석 및

계통발생학적 분석 결과, 연구에 사용된 *Bacillus* spp. 균주 B1 과 B3 는 *B. velezensis* 로 확인되었다.

이상의 결과에서, 흰다리새우 양식 기간 동안 *Bacillus* spp. 균주 복합물(*Bacillus subtilis*, *Bacillus velezensis*, *Bacillus pumilus*)을 사료에 첨가하여 장기간 공급하는 것은 흰다리새우의 성장, 사료 효율, 사료 소화율, 면역 반응과 생존율을 효과적으로 개선시킬 수 있는 것으로 판단된다. 또한, *B. velezensis* 의 자체 보유 유전자를 통한 항균 활성으로 AHPND 와 같은 흰다리새우 양식 과정에서 발생할 수 있는 대규모 폐사 유발 질병에 대한 저항성을 높일 수 있는 것으로 판단된다. 이는 Vc_{AHPND} 와 같이 AHPND 의 새로운 원인 병원체가 계속해서 확인되는 가운데 예방 및 치료를 위한 효과적인 사료첨가제로 사용될 수 있다. 따라서, 흰다리새우 양식 산업에서 *Bacillus spp.*를 활용하는 것은 흰다리새우 양식의 생산성을 향상시킬 수 있다고 판단된다.

주요어 : 흰다리새우, 사료첨가제, 프로바이오틱스, 바실러스, 생산성, 비브리오 캠벨리, 급성간췌장괴사병, 항균 활성

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