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Probiotic effects of *Bacillus* spp. from Pacific white shrimp *(Litopenaeus vannamei)* on water quality and shrimp growth, immune responses, and resistance to *Vibrio parahaemolyticus* (AHPND strains)

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ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND), a serious disease caused by some *Vibrio* spp., impacts the Pacific white shrimp industry worldwide, especially in Thailand. To effectively overcome this problem, efficacious probiotic candidates were isolated from shrimp farms near coastal areas. The isolated *Bacillus* probiotics were screened for their ability to control pathogenic *Vibrio* spp. and various *V. parahaemolyticus* AHPND (VP_{AHPND}) strains. Among the obtained probiotics, *Bacillus subtilis* AQAHBS001, which broadly inhibited various strains of VP_{AHPND}, was evaluated on a laboratory scale in water-soluble and feed applications of viable probiotic. The water addition of 1×10^3–1×10^5 CFU/mL of this probiotic effectively reduced total ammonia but did not improve shrimp growth and resistance to VP_{AHPND}. However, feed supplemented with the selected probiotic at 1×10^7 and 1×10^9 CFU/kg diet and provided to shrimp continuously for 5 weeks efficiently improved growth, as indicated by significant final weight gain, average daily growth, specific growth rates and feed conversion ratios. Additionally, this probiotic significantly elevated immune responses through phagocytic activity and clearance efficiency and enhanced the expression of the *prophenoloxidase*, *lysozyme*, and *anti-lipopolysaccharide factor* genes. Furthermore, *B. subtilis* AQAHBS001 obviously improved midgut characteristics by increasing microvilli and intestinal wall thickness. Finally, this probiotic evidently improved resistance to VP_{AHPND}.

1. Introduction

Pacific white shrimp, *Litopenaeus vannamei*, is an economically important species of the aquaculture industry worldwide, especially in Thailand. In 2011, the annual shrimp production in Thailand flourished and reached a peak at approximately 600,000 tons [1]. In recent years, however, shrimp farming in Thailand has suffered serious losses and currently continues to suffer losses from a serious disease outbreak caused by acute hepatopancreatic necrosis disease (AHPND) [2]. The cause of AHPND is *Vibrio parahaemolyticus* (VP_{AHPND}), which has a plasmid containing two *Photorhabdus* insect-related (Pir A and B) toxin genes. These toxin genes were later found in all VP_{AHPND}, *V. campbellii* and *V. harveyi* strains but were absent in nonpathogenic strains [1,3]. According to the annual report by the Food and Agriculture Organization (FAO), shrimp production in Thailand dropped from its peak in 2011 to less than 300,000 tons in 2014 [2], as a result of the AHPND outbreak that continues to date.

To effectively overcome serious diseases in shrimp culture, several chemicals and drugs, particularly antibiotics, are typically applied to control diseases caused by harmful bacteria [4]. Although these applications control diseases, they often further create other serious problems due to excessive use or misuse, resulting in residues in shrimp and shrimp products and leading to the emergence of resistant bacteria with increased virulence, especially *Vibrio* species [1,5]. Thus, to mitigate the negative impacts from both diseases and antibiotics, other alternatives, such as the use of probiotics in shrimp aquaculture, have been widely applied not only to control infectious diseases but also to promote growth performance, immunity and disease resistance in aquaculture animals [6]. Probiotics are well recognized as a popular health supplement for humans and are used in animal husbandry. In aquaculture, probiotics have been widely applied to inhibit the growth of opportunistic pathogens and reduce the prevalence of viruses [7,8]. In addition, probiotics may improve nutrients, enzyme activities, immune responses and water quality [9–11]. *Bacillus* species are bacteria that have been commonly selected as probiotics for humans and have also been applied in the aquaculture industry. Normally, *Bacillus* species

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can be found in soil and water and can also adhere and survive in the digestive tract with the acid and bile salts of shrimp [12]. This bacterium is a Gram-positive spore-forming bacterium that can create spore coats within vegetative cells and remain in a dormant state for long periods. In addition, *Bacillus* species are able to produce a wide range of extracellular substances, such as trypsin, lipase, amylase and antimicrobial peptides, against a variety of pathogenicity factors [13,14]. Based on the results of *Bacillus* application in many studies, this species is widely applied as a probiotic, and it has been proven to improve growth performance, immune system function and disease management in shrimp aquaculture [15].

The current research aimed to isolate and identify efficacious probiotic *Bacillus* spp. from shrimp farms and to test their efficacy against *Vibrio* spp., especially VP_{AHPND}, and ability to enhance properties regarding immunity, growth, and water quality in shrimp farming under *in vitro* and *in vivo* conditions. The selected probiotic *Bacillus subtilis* AQAHBS001 obtained in the present study exhibits strong potential as a probiotic candidate, as it effectively improved shrimp growth, water quality, shrimp immune responses and disease resistance against shrimp pathogens. The results from this study provide useful and practical strategies that can be applied in the shrimp culture industry, which is now declining from harmful shrimp diseases, especially from AHPND.

2. Materials and methods

2.1. Isolation of candidate probiotic bacteria

MYP agar (Himedia, India) was used to isolate bacteria from five shrimp farms located in Chantaburi, Rayong and Trat provinces, Eastern Thailand. These shrimp farms were certified by the Department of Fisheries of Thailand as Good Aquaculture Practice (GAP) farms that had few antibiotic applications recorded. Pacific white shrimp (15–20 g; 10 pieces per farm) were collected, and 0.1 g was sampled from the midgut of each shrimp. At the same time, 1 mL of culture water (20–30 ppt) from each shrimp pond was also collected. Midgut and water samples were subjected to serial ten-fold dilution, spread on MYP agar and incubated at 30 °C overnight [16]. All bacterial colonies that appeared on the agar plates were randomly subcultured based on morphological characteristic differences. All obtained bacterial colonies were further tested for their efficiency in controlling shrimp pathogenic bacteria using dot-spot methods as described by Spelhaug and Harlander [17].

2.2. Preliminary screening of probiotic candidates for their ability to control pathogenic bacteria

In this experiment, candidate bacteria from section 2.1 and 4 bacteria pathogenic to shrimp, *Vibrio alginolyticus* (VA_{AQH1}), *V. harveyi* (VH_{AQH1}), *V. parahaemolyticus* (VP_{AQH1}) and *V. vulnificus* (VV_{AQH1}), that were obtained from Laboratory of Aquatic Animal Health Management, Department of Aquaculture, Faculty of Fisheries, Kasetsart University were grown in 10 mL trypticase soy broth (TSB) (Merck, Germany) supplemented with 1.5% NaCl for 24 h and centrifuged at 2500 rpm for 10 min, and the supernatants were discarded. Bacterial pellets were washed 2 times with 1.5% NaCl under the previous conditions. Bacterial suspensions of each strain were normalized with 1.5% NaCl to an absorbance of 1.0 at 600 nm to obtain a final concentration of approximately 5×10^8 CFU/mL using a spectrophotometer (Milton Roy, PA, USA). A bacterial suspension of pathogenic bacteria was swabbed on a TSA plate supplemented with 1.5% NaCl, and candidate probiotics were dot-spotted on the surface of agar overlaid with pathogenic bacterial suspension. The plates were incubated at 30 °C overnight, and clear zones that occurred around each probiotic dot were recorded.

2.3. Microbiological characteristics of candidate probiotics

The effects of temperature, pH and salinity on the growth of candidate probiotics from section 2.2 were tested under various conditions as described by Duc et al., 2004 [18]. Briefly, candidate probiotics were prepared as previously described. Each candidate bacterium was inoculated into 15 mL test tubes containing 10 mL TSB supplemented with 1.5% NaCl to a final concentration of 1×10^5 CFU/mL with 3 replicates. For temperature, each probiotic bacterium was inoculated and incubated at different temperatures, 25, 30 and 40 °C. For pH conditions, candidate bacterial solutions were cultured in TSB supplemented with 1.5% NaCl and pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 using 0.02 N HCl for acidic and 0.02 N NaOH for alkaline pH values. For salinity, the candidate probiotic bacteria were grown in TSB containing 0, 1, 2, 3, 4 and 5% NaCl. All test tubes were incubated in a shaking incubator at 30 °C (except temperature test) for 120 h. The growth of each test condition was determined by an iMARK™ Microplate reader (Bio-Rad, USA) at 600 nm at 0, 12, 24, 48, 96 and 120 h.

2.4. Identification of candidate probiotics

2.4.1. Biochemical properties of probiotic bacteria

All of the candidate bacteria were cultured on TSA supplemented with 1.5% NaCl and incubated at 30 °C overnight. A single colony of each bacterial strain was selected to determine the colony and cell morphology, Gram strain, catalase and oxidase tests based on the methods of Holt et al. [19]. The bacterial suspension of each strain was prepared and tested for biochemical properties using the API 50CHB E Kit (BioMerieux, USA) with the methods recommended by the company instructions, and analyses for species identification were performed with API software (BioMerieux APIWEBTM, USA).

2.4.2. Molecular-based methods

Single colonies of each candidate probiotic from section 2.4.1 were selected, and genomic DNA was extracted using DNAzol® (Thermo Fisher Scientific, USA) and quantified according to the protocol provided by the company. The 16S rRNA gene of the *Bacillus* group was amplified using a PCR assay with the specific forward primers BS001qPCR_F and BS001qPCR_R (Table 1) and PCR conditions based on the methods previously described by Giffel et al. [20]. PCR products were purified and cloned into the pGEM-T Easy vector using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and pGEM-T® Easy Vector (Promega, USA), respectively, following the recommended protocol from each company. Positive clones containing target DNA inserts were selected using blue-white colony screening methods, and plasmid DNAs were extracted with the Plasmid DNA Extraction Mini Kit (Thermo Fisher Scientific, USA). Sequencing of the extracted plasmid was carried out with the Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham Research Biotech) (Macrogen, Korea), and sequence analyses of nucleotides and amino acids were conducted using Genetyx version 7.0. Full-length sequences were compared with information available in the GenBank database in National Center for Biotechnology Information (NCBI) using the BLASTX and BLASTN algorithm programs (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (see Table 2).

2.5. Efficiency of candidate probiotics at inhibiting pathogenic bacteria in vitro

2.5.1. Dot-spot technique

All of the candidate probiotic bacteria were used to analyze their inhibitory activity against shrimp pathogenic bacteria, including strains VA_{AQH1}, VH_{AQH1}, VP_{AQH1}, VV_{AQH1} and the additional VP_{AHPND} strains $VP_{AHPNDAQH1.2}$, $VP_{AHPNDAQH3.2}$, $VP_{AHPNDAQS1}$, $VP_{AHPNDAQS2}$ and $VP_{AHPNDAQS3}$, by the dot-spot technique using the previous methods described in section 2.2. After incubation at 30 °C for 72 h, the diameter

Table 1
Primers used in this study.

Gene	Primer name	Sequence 5′-3′	Amplicon	Purposes	Accession number
16s rRNA	BS001qPCR_F	ATCGGCCACACTGGGACTGAGACA	175 bp	qRT-PCR analysis	[63]
	BS001qPCR_R	ACCGCCCTATTCGAACGGTACTTG			
ALF (LvALF1)	ALF-F	CATTCGGCCTTGACTTCG	259 bp	qRT-PCR analysis	EW713395
	ALF-R	ATCCAGGACACCACATCCTG			
Lysozyme	Liva Lysozyme qPCR F	GGACTACGGCATCTTCCAGA	97 bp	qRT-PCR analysis	AY170126
	Liva Lysozyme qPCR R	ATCGGACATCAGATCGGAAC			
Prophenoloxidase	ProPO-F	CGGTGACAAAGTTCCTCTTC	122bp	qRT-PCR analysis	AY723296
	ProPO-R	GCGGGTCGCCGTAGTAAG			
Superoxide dismutase	SOD-F	TCATGCTTTGCCACCTCT C	143 bp	qRT-PCR analysis	AY486424
	SOD-R	CCGCTTCAACCAACTTCTTC			
b-actin	b-actin-F	TCCACGAGACCACCTACAACTC	140 bp	qRT-PCR analysis	AF300705
	b-actin-R	GAGGGCAGTGATTTCCTTCTG			

of the inhibitory clear zone was measured with a Vernier caliper.

2.5.2. Coculture system

Suspensions of candidate probiotic and pathogenic bacteria (VP_{AHPND}) were prepared with methods similar to those described in section 2.3, and 1×10^5 CFU of each bacterium was inoculated under cocultured conditions in 10 mL of 1% TSB supplemented with 1.5% NaCl in a shaking incubator at 30 °C. Control groups of each candidate probiotic and pathogenic bacterium were separately set with similar conditions. A number of bacteria were evaluated, and the results were recorded with the same protocol described in section 2.1 at 0, 24, 48, 72, 96 and 120 h by using MYP agar for *Bacillus* spp. and TCBS agar for *Vibrio* spp.

2.6. Application of a selectively probiotic bacterium to improve water quality, growth and disease resistance in Pacific white shrimp by the water supplement method

Pacific white shrimp (12.03 ± 2.76 g) were purchased from a private shrimp farm in Eastern Thailand and quarantined in a 3000-L fiberglass tank for 7 days. The rearing conditions were approximately 28 °C, 20 ppt salinity, 5 mg/L DO, pH 7.5 and $CaCO_3$ alkalinity of 80 mg/L. After quarantine, 25 shrimp were randomly transferred into one of 20,250-L fiberglass tanks containing 150 L of water at 20 ppt salinity for further acclimatization for 7 days. During this period, shrimp were fed daily with a commercial diet at 3% body weight, and approximately 50% of the water volume was exchanged every 3 days.

A probiotic candidate AQAHBS001 (B12) was freshly prepared in TSB supplemented with 1.5% NaCl at 30 °C for 24 h. Bacterial cells were harvested and prepared as bacterial stocks at 1×10^6, 1×10^8, 1×10^9 and 1×10^{10} CFU/mL with the methods described in section 2.2. On the first day of the trial, 1.5 mL of each bacterial stock (in the previous section) was added into 5 different shrimp tanks prepared previously to generate 4 replicates of 4 treatments containing 1×10^2, 1×10^3, 1×10^4 and 1×10^5 CFU/mL, respectively. The other 4 tanks were placed in 1.5 mL of 1.5% NaCl to serve as the control group. During this period, 20% of water in each tank was exchanged, and the same concentrations of probiotic bacteria were also applied every 5 days.

During the trial, experimental shrimp in each treatment were similarly fed a commercial diet at 3% body weight. Shrimp weight was measured weekly, and the feed provided was recorded daily until week 5 of the experiment. Growth parameters such as initial weight, final weight, WG, ADG, SGR, FCR and survival rate (%) were calculated based on a method previously described by Graaf and Prein [21]. Water quality, such as temperature, pH, and DO, was determined daily using YSI pH100A and YSI550A, respectively. Alkalinity, total ammonia (NH_3-N) and nitrite (NO_2-N) were quantified with the standard methods of Apha et al. [22] every 5 days. Total bacterial, *Bacillus* and *Vibrio* counts in culture water were analyzed every 5 days using similar procedures as described above.

In this experiment, one shrimp from each tank of each treatment was collected every week. DNA samples were extracted from the gills, swimming legs and midgut using the same protocol as in section 2.4.2. The copy number of the applied probiotic bacterium was analyzed

Table 2
Effects of probiotic AQAHBS001 on growth rate during application in cultured water and feed supplementation trials. The letters indicate differences among treatment groups at days 15 or 30 of the trial. The different letters for each parameter in the same row indicate significant differences at $P < 0.05$.

Parameters	Water application trial				
	Control	TW1	TW2	TW3	TW4
Initial weight (g)	9.08 ± 0.27a	9.08 ± 0.27a	9.08 ± 0.27a	9.08 ± 0.27a	9.08 ± 0.27a
Final weight (g)	17.98 ± 1.12a	18.44 ± 1.50a	19.22 ± 0.57a	19.18 ± 0.85a	19.65 ± 1.10a
WG (g)	8.90 ± 1.12a	9.36 ± 1.50a	10.14 ± 0.57a	10.10 ± 0.85a	10.58 ± 1.10a
ADG (g/day)	0.25 ± 0.03a	0.27 ± 0.04a	0.29 ± 0.02a	0.29 ± 0.02a	0.30 ± 0.03a
SGR (%/day)	1.95 ± 0.18a	2.03 ± 0.23a	2.14 ± 0.09a	2.14 ± 0.13a	2.21 ± 0.16a
FCR	1.34 ± 0.19a	1.27 ± 0.22a	1.17 ± 0.07a	1.18 ± 0.10a	1.13 ± 0.13a
Survival rate (%)	72.4 ± 1.26a	78.7 ± 1.26a	77.5 ± 0.96a	80.6 ± 0.58a	77.5 ± 0.96a
Feed supplementation trial					
	Control	TF1	TF2	TF3	
Initial weight (g)	12.03 ± 2.76a	12.03 ± 2.76a	12.03 ± 2.75a	12.03 ± 2.76a	
Final weight (g)	18.25 ± 0.84a	18.72 ± 2.76 ab	19.52 ± 1.82 ab	21.12 ± 2.08b	
WG (g)	6.23 ± 0.84a	6.70 ± 2.76a	7.50 ± 1.83 ab	9.10 ± 2.08b	
ADG (g/d)	0.18 ± 0.02a	0.19 ± 0.08a	0.21 ± 0.05 ab	0.26 ± 0.06b	
SGR (%/d)	1.19 ± 0.14a	1.27 ± 0.44a	1.38 ± 0.27 ab	1.61 ± 0.28b	
FCR	2.03 ± 0.33b	1.88 ± 1.30 ab	1.68 ± 0.47a	1.38 ± 0.35a	
Survival rate (%)	73.75 ± 3.54a	80.00 ± 2.98a	74.00 ± 7.64a	76.00 ± 5.70a	

using qPCR analysis. Briefly, 100 ng DNA from each organ of a shrimp sample was subjected to qPCR amplification in a total volume of 20 μL including QuantiTect® SYBR® Green PCR Master Mix Kit, HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTP mix, the fluorescent dye SYBR Green I (Qiagen, MA, USA), and RNase-free water was mixed with synthesized cDNA and 1 μL of each primer (Table 1). The specific primer pair was designed based on the 16S RNA sequence of the above section. The reaction was run at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s and extension at 72 °C for 60 s in an Mx Pro™ 3005P QPCR system (Stratagene, USA). The absolute DNA copy number of probiotic bacteria at each week was analyzed with MxPro™ QPCR version 4.0 based on the standard curve methods described by Whelan et al. [23].

At the end of the probiotic application, 10 shrimp from each tank of each treatment were intramuscularly injected with 100 μL containing 1×10^7 CFU/mL of $VP_{AHPNDAQH1.2}$, which was prepared under the same conditions described in section 2.2. Moreover, one additional group of untreated shrimp was intramuscularly injected with 0.85% NaCl to serve as a negative control. Mortality was recorded daily for up to 10 days. Moribund shrimp were collected to analyze bacterial infections in the hepatopancreas by the loop isolation method on TCBS agar and the histopathological process described below.

2.7. Effect of probiotic-supplemented feed on water quality, growth, immune parameters, immune-related gene expression and disease resistance in Pacific white shrimp

The same batch of Pacific white shrimp was used and prepared according to the same methods described in section 2.6. Twenty-five fiberglass tanks (250 L) containing 200 L of fully aerated water at 20 ppt salinity were prepared. Twenty shrimp were randomly placed into each tank to set up 5 replicates of 5 treatments for the following experiments.

Probiotic *Bacillus* AQAHBS001 was cultured using the same procedures as described in section 2.6. Shrimp feed (Betagro, Thailand) was used and top-dressed with probiotic bacterium to produce the experimental feed. Four different formulated feeds were prepared every 3 days, feed formulas TF1, TF2 and TF3 were sprayed with 200 mL of 0.85% NaCl containing 1×10^5, 1×10^7 and 1×10^9 CFU probiotic/kg diet, respectively, and another feed was mixed with 200 mL of 0.85% NaCl to serve as a control group. All formulated feeds were air dried for 30 min, coated with squid oil at 20 mL/kg diet and dried under shelter for 20 min before being stored at 4°C.

The shrimp in each of the different treatment groups were fed five different tested diets. Group 1 and group 2 were fed with a C diet to serve as the positive and negative control groups, and the other 3 groups were fed daily with TF1, TF2 and TF3 formula feed 3 times at 3% body weight for 5 weeks. During this experiment, the water was exchanged every 3 days at 20% of the volume, and the water temperature, pH, DO and alkalinity were maintained at 28–30 °C, pH 7.5–8.3, 4–5 mg/L and 80–120 mg/L $CaCO_3$, respectively. Throughout the experiment, 5 shrimp in each tank of each treatment were randomly weighed, and feed uptake was recorded. Growth parameters were calculated according to the same methods described in section 2.6.

During the trial, one shrimp of each replicate was randomly selected at weeks 0, 1, 3 and 5, and 0.5 mL of hemolymph was withdrawn using 1 mL syringe (with a 23G needle) containing 0.5 mL anticoagulant (10% sodium citrate in 0.85% NaCl). Ten microliters of diluted hemolymph was counted using a hemocytometer and calculated as described in Vargas-Albores et al. [24]. Diluted hemolymph was further put into a 1.5 mL microtube, centrifuged at 2500 rpm for 10 min, and serum was separated. Living cells on the pellets were washed 2 times with RPMI medium using the same methods described by Caroline et al. [25]. The remaining hemolymph was counted for total number, and this component was provided for further experiments in the below sections.

The hemocytes from the above section of each shrimp were diluted to a final concentration of 5×10^6 cells/mL with RPMI medium. Two hundred microliters of diluted hemocytes was loaded onto 22×22 mm^3 cover glass to allow all living cells to adhere to the glass surface for 120 min. Unattached cells were washed 3 times with RPMI medium, and 200 μL containing 1×10^7 latex beads (Sigma-Aldrich, USA)/mL was added. After 90 min, the cover glass was washed 3 times with RPMI medium, and the cells were stained with the Dip-quick Staining Kit (MPimpex, Thailand) following the manufacturer's protocol. Phagocytosed cells of 200 counted cells were observed under compound microscope (40 ×), and phagocytic activity expressed by percent phagocytosis (PP) and phagocytic index were calculated following the methods described by Rengpipat et al. [26].

Clearance efficiency of shrimp hemolymph was carried out at only week 5 with the modified method from Martin et al. [27]. Briefly, a suspension of the pathogenic bacteria $VP_{AHPNDAQH1.2}$ was prepared under the same conditions as in section 2.2. The bacterial concentration was brought to 2×10^6 CFU/mL, and 0.5 mL was placed in a 1.5 mL Eppendorf tube containing 0.5 mL of the cell-free hemolymph of each shrimp from the above section and completely mixed. Tubes were incubated in a shaking incubator at 30 °C, and at 0, 3, 6 and 12 h, 100 μL of bacterial solution was serially diluted ten-fold (10^{-1} to 10^{-3}) with 0.9 mL 1.5% NaCl. One hundred microliters of bacterial solution was plated on TCBS agar and incubated at 30 °C for 24 h. Colonies were counted to calculate the remaining number. One shrimp from each tank of each group was collected every week. The midgut of each shrimp was used for genomic DNA extraction, and then the bacterial DNA copy number of the applied probiotic bacterium was estimated by qPCR as described above.

At week 5 of the supplemented-feeding period, experimental infection proceeded with the same methods as described in section 2.6. During this trial, shrimp in each treatment were further fed with their tested diets as previously described. The mortality was recorded daily for up to 10 days, and the histopathology and bacterial infection in the hepatopancreas of moribund shrimp were analyzed by loop isolation methods as described above.

2.8. Effect of probiotic-supplemented feed on relative mRNA expression of immune-related genes

Shrimp randomly selected from those in section 2.7 at each time point were used to extract total RNA from the hemocytes, gills and hepatopancreas. The samples (approximately 50 mg) of each tissue were preserved in 1 mL of NucleoZOL® (Machery-Nagel, Germany) and homogenized using (MP FastPrep-24™, U.K.) for total RNA extraction based on the manufacturer's protocol. Reverse transcription was used to synthesize first-strand cDNA using the RevertAid™ 1st Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the recommended protocol from the company.

Four specific primers (Table 1) of immune-related genes, including *ALF*, *LSZ*, *proPO* and *SOD*, and one reference *β-actin* gene were used to molecularly quantify shrimp immune responses. One microliter of first-strand DNA from the protocol in the above section was used. The qPCR reaction followed the methods described by Livak and Schmittgen (2001) [28] with 95 °C for 10 min followed by 40 cycles of 30 s of denaturation at 95 °C, 60 s of annealing at 58 °C and 60 s of extension at 72 °C. Each sample from each treatment was analyzed with 3 replicates.

2.9. Histological analysis

The same shrimp samples from section 2.7 were preserved in Davidson's fixative and histological examinations of the hepatopancreas and midguts were performed following the methods described by Bell and Lightner (1988) [29]. Briefly, after fixation, hepatopancreas and midguts were dissected using sterilized surgical scissors and histological sections were serially prepared. Samples were dehydrated, stained and paraffin embedded. Tissue sections (0.5–0.7 μm) were cut using a microtome and stained with hematoxylin and eosin (H&E). Histological

sections were observed using a compound microscope at 10× and 40 × magnification.

2.10. Statistical analysis

The log bacterial numbers from the coculture experiment in section 2.5.3 were compared with t-tests. Other data, such as the dimeter clear zone in section 2.5.1; the growth parameters, water quality and number of colonies in section 2.6; the immune parameters and mortality in section 2.7; and the qPCR analysis in sections 2.6, 2.7 and 2.8, were subjected to one-way analysis of variance (ANOVA), and multiple comparisons were performed with Duncan's new multiple range test (DMART) using SPSS 17.0 software. A survival analysis of the challenge test in each group in sections 2.6 and 2.7 was performed using the Kaplan-Meier method with the same software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation and characterization of probiotic candidates

A total of 12 *Bacillus* isolates (B1-B12) were collected and preliminarily screened from healthy Pacific white shrimp using mannitol egg yolk polymyxin (MYP) agar. All isolates were Gram-positive bacilli, spore-forming, catalase positive and oxidase positive (Table S1), with some different characteristics in colony morphology. During the secondary screening, only 3 isolates, B4, B6 and B12, exhibited effective colonization and inhibitory effects against the tested pathogens (data not shown). In this experiment, candidate probiotics were further tested for their capability for growth and endurance *in vitro*. Experimentally, these three *Bacillus* isolates grew at temperatures ranging from 25 to 40 °C (Figs. 1a, 2a and 3a), B4 grew at pH values ranging from 4 to 10, B6 grew at pH 5 and 7 and B12 grew at pH values ranging from 5 to 9 (Figs. 1b, 2b and 3b). All three *Bacillus* species grew at salinities between 0 and 5% NaCl except B6, which did not grow at 5% NaCl (Figs. 1c, 2c and 3c).

The biochemical properties of each isolate were tested by an API 50 CHB E assay, which showed 99.9% identity with *Bacillus pumilus* for B4, 97.6% identity with *Bacillus lentus* for B6 and 99.9% identity with *Bacillus subtilis* for B12 (Table S1). Additionally, PCR analysis using universal primers for the bacterial 16S rRNA gene demonstrated a positive band of approximately 1400 bp in all 3 *Bacillus* isolates. After these DNA fragments were sequenced, BLAST searches of the nucleotide sequences from the isolates B4, B6 and B12 showed identical homology to *B. pumilus*, *B. amyloliquefaciens* and *B. subtilis,* with high scores of 99.8, 99.6 and 99.9%, respectively. Additionally, phylogenetic analysis of the 16S rRNA gene clearly confirmed that isolate B4 was located in the *B. pumilus* cluster, B6 was grouped with *B. amyloliquefaciens* and B12 was located in the *B. subtilis* cluster (Fig. S1).

3.2. Inhibitory effects of candidate probiotics on pathogenic bacteria

The inhibition zones from the dot-spot technique on tryptic soy agar (TSA) plates supplemented with 1.5% NaCl are shown in Fig. 2. B4 showed inhibition zones of 1.30 ± 0.58, 2.2 ± 0.29, 8.00 ± 2.00 and 1.70 ± 0.58 mm against *Vibrio alginolyticus* (VA_{AQH1}), *V. harveyi* (VH_{AQH1}), *V. parahaemolyticus* (VP_{AQH1}) and $VP_{AHPNDAQS2}$ (Fig. 2a), respectively. B6 exhibited an inhibitory effect only on *V. parahaemolyticus*, with an inhibition zone of 1.78 ± 0.85 mm (Fig. 2b). Interestingly, B12 clearly demonstrated its efficacy against VP_{AQH1}, $VP_{AHPNDAQH1.2}$, $VP_{AHPNDAQS1}$, $VP_{AHPNDAQS2}$ and $VP_{AHPNDAQH3}$ with inhibition zones of 28.3 ± 1.53, 5.7 ± 1.15, 6.3 ± 1.15, 1.7 ± 1.15 and 3.3 ± 1.53 mm, respectively (Fig. 2c). Additionally, when these candidate probiotics were evaluated under coculture conditions (Fig. 3a-c), only B12 could effectively inhibit the target pathogen $VP_{AHPNDAQH1.2}$. The number of B12 colonies was significantly higher than that of $VP_{AHPNDAQH1.2}$ colonies from 48 to 120 h at the end of the experiment ($P < 0.05$) (Fig. 3). Based on the results from these studies, the isolate B12 was chosen, named *Bacillus subtilis* AQAHBS001, and further used for application in laboratory culture trials.

3.3. Effects of probiotic AQAHBS001 on growth performance, water quality, and disease resistance in Pacific white shrimp via water-supplemented administration

The growth performance of shrimp was calculated at week 5 of the experiment. No significant difference in all growth parameters was detected ($P > 0.05$), even though the lowest values were always detected in the control group for all parameters. The final weight, weight gain (WG), average daily growth (ADG), % specific growth rate (SGR) and feed conversion ratio (FCR) of all shrimp treatment groups ranged from 17.98 ± 1.12–19.65 ± 1.10 g, 8.90 ± 1.12–10.58 ± 1.10 g, 0.25 ± 0.03–0.30 ± 0.03 g/day, 1.95 ± 0.18–2.21 ± 0.16%/day and 1.34 ± 0.19–1.13 ± 0.13, respectively (Table S2). Likewise, survival rates among the control and treatment groups were not significant ($P < 0.05$), with a range of 72.4 ± 1.26–80.6 ± 0.58%.

In this experiment, the water temperature, pH and dissolved oxygen (DO) at different time courses were not significantly different among the control and probiotic-supplemented groups and ranged from 27.6 to 28.5 °C, 7.89–7.99 and 6.62–7.0 mg/L, respectively. The alkalinity among the tested groups tended to increase during the trial as a result of water exchange, but no significant differences were observed among groups at different time intervals. However, at weeks 3–5, the total ammonia in the probiotic-supplemented groups TW2, TW3 and TW4 (1×10^3, 1×10^4 and 1×10^5 CFU/mL, respectively) trended to decrease from the highest peak at week 2 to lower than the total ammonia in the control and TW1 groups until the end of the experiment (Table S2). Clear significant differences were observed at week 5, and the control and TW1 groups contained higher concentrations of total ammonia than the TW2, TW3 and TW4 groups, with 0.76 ± 0.12, 0.83 ± 0.28, 0.38 ± 0.09, 0.28 ± 0.16 and 0.42 ± 0.27 mg NH_3-N/L ($P < 0.05$), respectively. Within treatments, nitrite in every tested group significantly increased from weeks 3–5, and no significant difference among groups was noted at all times evaluated, except in week 3 (Table S2). Finally, the total bacteria count in water did not fluctuate much during the experimental periods. Significant differences in this parameter were recorded at weeks 2 and 5, especially at week 5, where the TW4 group had the highest total bacteria count, which was significantly different from the bacteria counts of the other groups, with log CFU/mL values of 4.13 ± 0.24, 4.38 ± 0.34, 4.45 ± 0.35, 4.78 ± 0.50 and 5.19 ± 0.66 for the control, TW1, TW2, TW3, and TW4 groups, respectively ($P < 0.05$).

During the trial, the total *Vibrio* count was measured, and the number of *Vibrio* spp. slightly increased, but there were no significant differences ($P > 0.05$) at any week with a log CFU/mL of 3.86–4.00. For the *Bacillus* levels in water, at week 5, only treatment TW4 was significantly different from the other groups ($P < 0.05$), with log CFU/ml values of 4.06 ± 0.25, 4.29 ± 0.35, 4.42 ± 0.36, 4.75 ± 0.61 and 5.18 ± 0.70 CFU/mL for the control, TW1, TW2, TW3, and TW4 groups, respectively (Table S2).

During the above experiment, the number of probiotic AQAHBS001 in the gills, swimming legs and midgut of all shrimp groups was quantified (Fig. 4a-c). Surprisingly, the copy number of the target AQAHBS001 probiotic was initially detected at low concentrations in every organ of all shrimp groups, including the control. Furthermore, the copy number of the target bacterium in these organs at different time points fluctuated greatly during weeks 1–4 after probiotic application and was increasingly detected in all organs and at all times in the control shrimp. However, compared to the copy number of AQAHBS001 at all other time periods, the copy number of AQAHBS001 at week 5 in all organs of all shrimp in all groups increased and reached the highest copy number, and the copy number of AQAHBS001 in TW1-

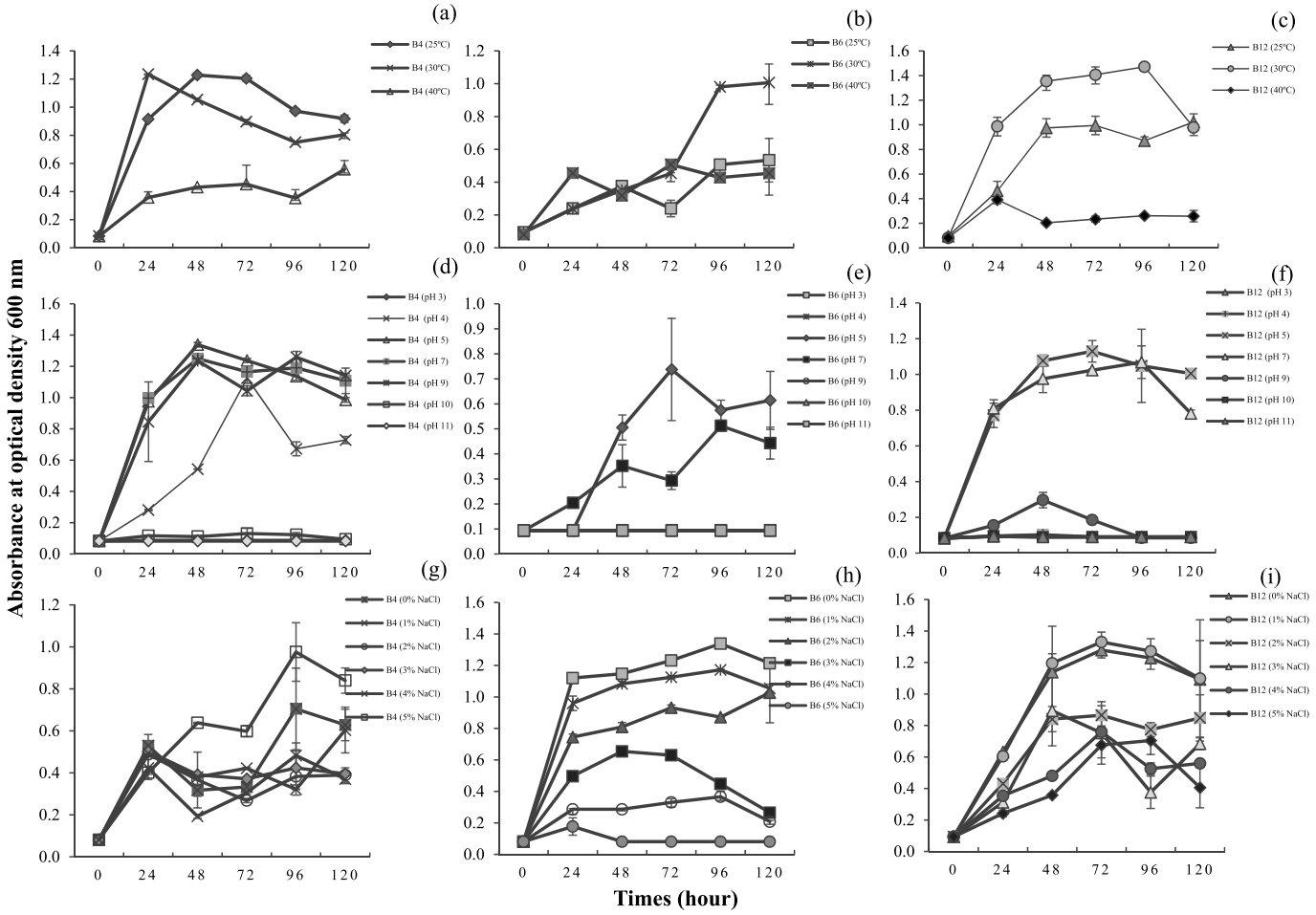

Fig. 1. Effects of various conditions on the growth of isolated *Bacillus* spp., pH (a–c), temperature (d–f) and salinity (g–i).

TW4 was significantly higher than that in the control in all tested organs (Fig. 4a-c). Especially in the midgut, the copy number of the target bacterium was proportionally and significantly increased ($P < 0.05$) depending on the concentration applied, with values of 3.12 ± 0.10, 3.36 ± 0.08, 3.52 ± 0.07 and 3.68 ± 0.08 log copy number/1000 ng DNA for TW1, TW2, TW3 and TW4, respectively, which were also significantly higher than that of the control, with a value of 1.96 ± 0.12 ($P < 0.05$) (Fig. 4b).

At the end of the water application, shrimp of all groups were infected with a lethal dose of $VP_{AHPNDAQH1.2}$. The cumulative mortality of the control, TW1 and TW2 groups rapidly reached 100% at 24 h, while TW3 and TW4 showed cumulative mortalities of 86.67 ± 3.85% and 93.30 ± 5.44%, respectively (Fig. 5). However, the cumulative mortality among groups was not significant ($P > 0.05$).

3.4. Effects of AQAHBS001-supplemented feed on water quality, growth, immune parameters, expression of immune-related genes and disease resistance

In this experiment, the water temperature, pH, DO, alkalinity, ammonia, nitrite, and total *Vibrio* count in each group were in a good range for shrimp survival and slightly different among the probiotic-treated and control groups from the beginning until the end of the feeding experiment, but these values were not significantly different at any time interval ($P > 0.05$), except for the total bacterial count of the TF2 group (1×10^7 CFU/kg diet), which was significantly higher than that of the other groups only at week 4 ($P < 0.05$) (Table S3).

The effect of probiotic application in feed on shrimp growth was measured at week 5 of the experimental period. The final weight, WG, ADG and %SGR were consistently significant in the TF3 treatment (1×10^9 CFU/kg diet), which differed from the other groups, except for group TF2. Additionally, the FCRs in groups TF2 and TF3 (1.68 ± 0.47 and 1.38 ± 0.35) were significantly greater than those in the control (2.03 ± 0.33) ($P < 0.05$) but not in the TF1 (1×10^5 CFU/kg diet) group (1.88 ± 1.30) ($P > 0.05$). However, there was no significant difference in survival rate among all treatments that exhibited survival ranges of 73.75 ± 3.54–80.0 ± 2.98 ($P > 0.05$) (Table 1).

Prior to proceeding with the feed experiment, the copy number of AQAHBS001 probiotic had the lowest value of approximately 0.43 ± 0.20 log copy number/1000 ng DNA. However, at week 1, the log copy number in every group was more highly expressed than that in other weeks. During this period, TF2 and TF3 contained the highest concentration of bacterial DNA with log 7.06 ± 0.72 and 7.91 ± 0.71, which were significantly higher than those in the control and TF1, with log 2.39 ± 0.29 and 5.71 ± 0.25, respectively ($P < 0.05$) (Fig. 6). The copy numbers in all groups decreased later at weeks 3 and 5; however, TF3 still significantly expressed a higher AQAHBS001 log copy number than the other groups, with 3.19 ± 0.80 and 2.18 ± 0.09 at weeks 3 and 5, respectively ($P < 0.05$) (Fig. 6).

The total hemocytes count and clearance efficacy of hemolymph were first reported. No significant difference among groups was observed during the feeding trial ($P > 0.05$) (Fig. 7a). However, the clearance efficacy of hemolymph in TF1, TF2 and TF3 exhibited a significant inhibitory effect against $VP_{AHPNDAQH1.2}$ at 6 and 12 h after exposure ($P < 0.05$) (Fig. 7b).

The percent phagocytic activity (PA) was determined to be significant at weeks 3 and 5 ($P < 0.05$). Particularly at week 3, the phagocytic activities of TF1, TF2 and TF3 were significantly higher than

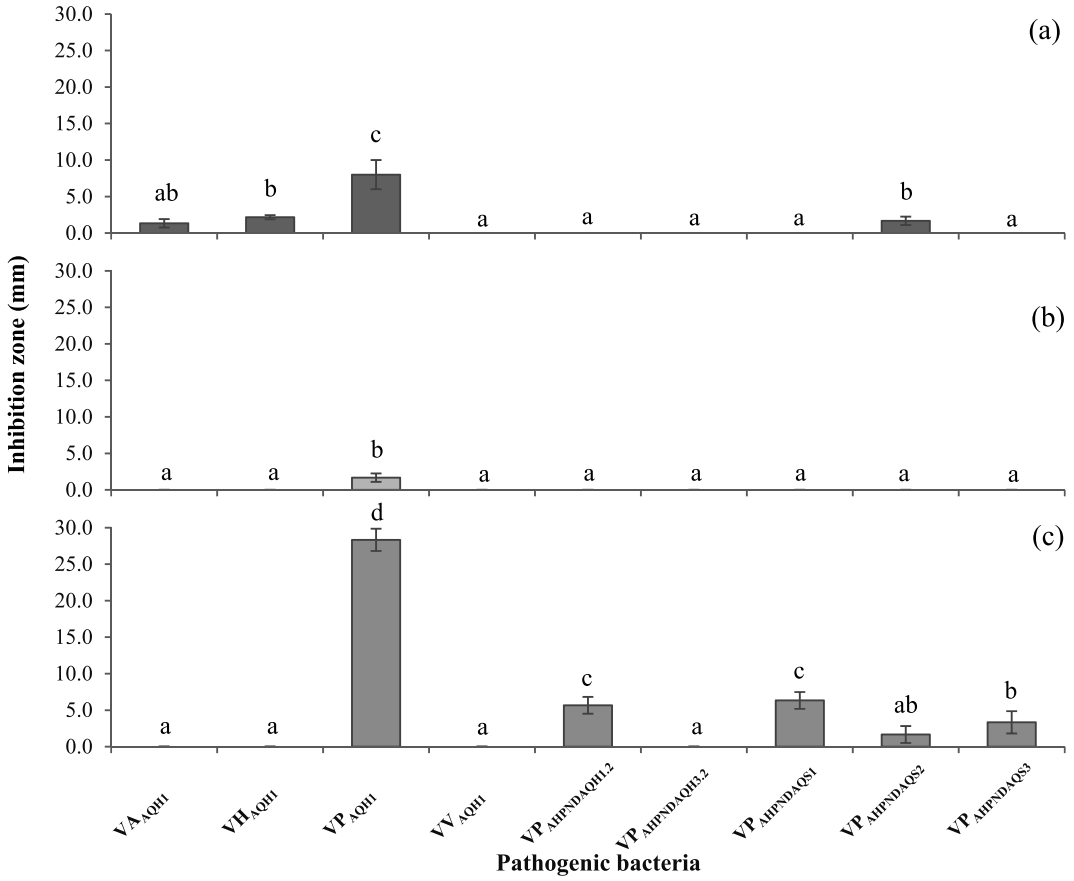

Fig. 2. Efficacy of the inhibitory ability of candidate probiotics against shrimp pathogenic bacteria indicated by inhibitory clear zones of the dot-spot technique. B4 (a), B6 (b) and B12 (c). The different letters on each bar indicate significant differences ($P < 0.05$).

that of the control, with values of 34.8 ± 2.47, 33.8 ± 2.33, 37.25 ± 0.87 and 30.36 ± 3.06, respectively ($P < 0.05$). However, at week 5, only the phagocytic activities of TF2 and TF3 were significantly higher than those of the other groups ($P < 0.05$) (Fig. 7c). However, in the case of the phagocytic index (PI), only at week 3 was the phagocytic index of the control significantly lower than that of the TF1, TF2, and TF3 groups, with 2.32 ± 0.27, 2.92 ± 0.37, 2.74 ± 0.34 and 3.00 ± 0.22, respectively ($P < 0.05$); no significant difference among the treated groups was observed ($P > 0.05$) (Fig. 7d).

The expression of the *anti-lipopolysaccharide factor* (*ALF*), *lysosome* (*LSZ*), *prophenoloxidase* (*proPO*) and *superoxide dismutase* (*SOD*) genes was measured in the gills, hemocytes and hepatopancreas by real-time RT-PCR.

In the gills, *ALF* clearly showed significant upregulation at weeks 3 and 5 ($P < 0.05$). At week 3, the relative expression levels of *ALF* in shrimp in groups TF2 and TF3 (12.02 ± 3.33 and 12.54 ± 3.62) were higher than those in the control and TF1 groups, with fold changes of 3.53 ± 1.07 and 3.71 ± 1.33, respectively (Fig. 8a). At week 5, the control shrimp exhibited significantly lower expression levels than the shrimp in all probiotic-treated groups, with 2.82 ± 0.95-, 5.85 ± 0.63-, 8.16 ± 1.74- and 9.39 ± 1.85-fold changes, respectively ($P < 0.05$). The *lysozyme* gene was highly upregulated in all groups at weeks 1 and 5 (Fig. 8b); in week 1, the TF1 and TF3 treatments showed significant expression with fold changes of 322.47 ± 111.32 and 399.56 ± 79.68, respectively, which were higher than the 169.01 ± 35.36-fold change ($P < 0.05$) in the control group. At week 5, the expression of the *lysozyme* gene was increased only in TF3 compared to the other groups (399.56 ± 79.68 $P < 0.05$). The *proPO* gene was highly upregulated only in week 3 in all shrimp groups; however, only shrimp in TF2 significantly expressed this gene with a 427.46 ± 104.16-fold change (Fig. 8c). Finally, *SOD* gene expression was not significantly different at any time point ($P > 0.05$) (Fig. 8d).

In hemocytes, the *ALF* gene was significantly upregulated only at week 3, particularly in TF2 and TF3 shrimp, which had higher expression levels (366.63 ± 20.86 and 413.25 ± 51.17) than the control (236.15 ± 50.49; Fig. 8e). The *LSZ* gene in all groups was significantly increased after probiotic application at week 3 and week 5 (Fig. 8f). The *proPO* gene was clearly upregulated only in week 3, and the expression of the *proPO* gene was significantly increased only in the TF2 group compared with the other groups ($P < 0.05$) (Fig. 8g). *SOD* gene expression was upregulated in probiotic-fed shrimp at weeks 1 and 3. However, in week 3, the expression levels in the control group were clearly lower than those in the TF1-TF3 groups, with fold changes of 0.89 ± 0.22, 4.77 ± 0.95, 5.10 ± 0.48, and 3.18 ± 0.56, respectively ($P < 0.05$) (Fig. 8h).

In the hepatopancreas, at weeks 1–5, *ALF*, *LSZ* and *SOD* gene expression levels in the TF2 and TF3 groups tended to be significantly higher than those in the other groups ($P < 0.05$) (Fig. 8i, j and l). The expression of the *proPO* gene was obviously significantly upregulated only in week 3. However, the expression levels of the *proPO* gene were significantly increased only in groups TF2 and TF3 compared with the other groups, with fold changes of 64.68 ± 12.70 and 40.30 ± 16.51, respectively (Fig. 8k).

After the dietary supplementation period, at week 5, all shrimp in the control and probiotic-treated TF1-TF3 groups were intramuscularly infected with $VP_{AHPNDAQH1.2}$. The mortality of the shrimp in every group clearly was rapid from 24 to 48 h, trended to slow down from 72 to 120 h and was then stable until the end of the trial at 168 h. During different exposure times, shrimp in groups TF2 and TF3 clearly showed lower mortality than the other groups. At the end of the challenge test,

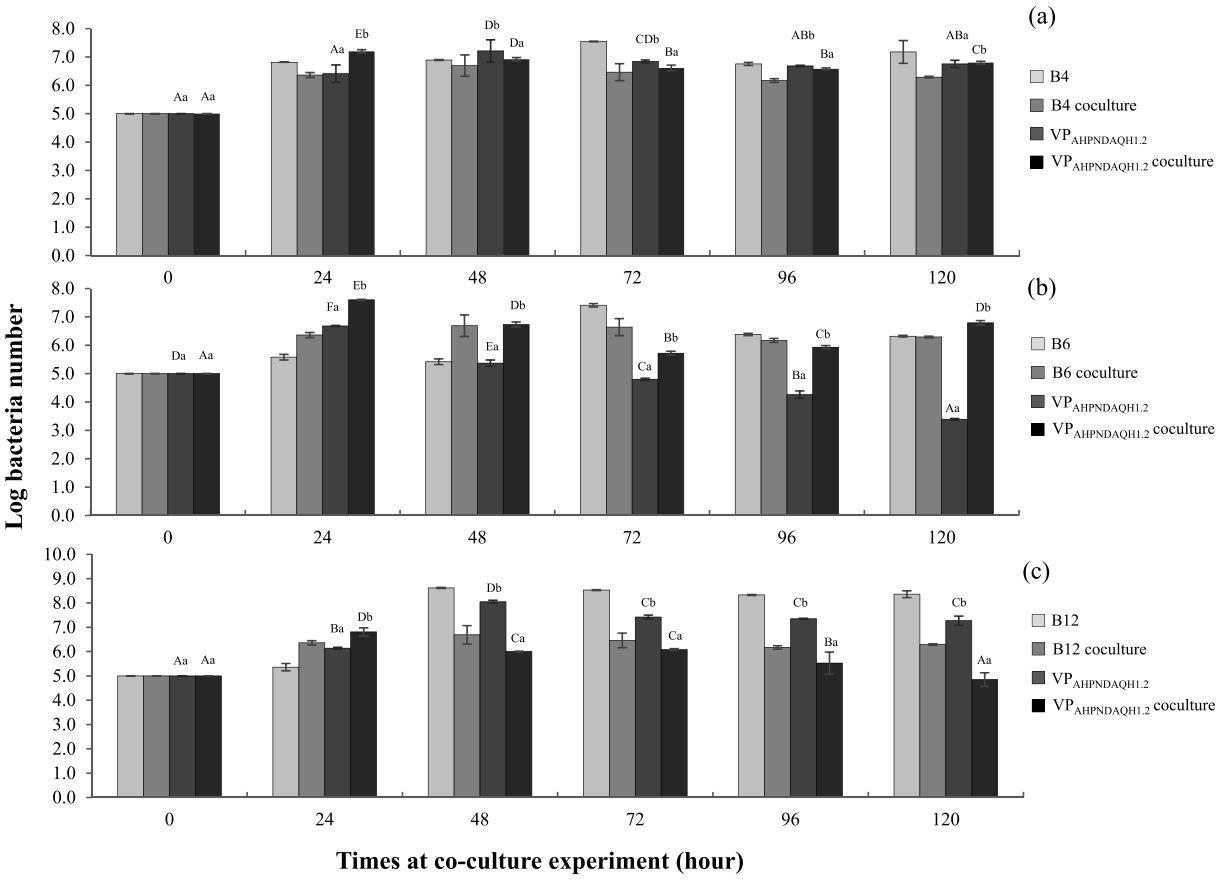

Fig. 3. Efficacy of the inhibitory ability of candidate probiotics against shrimp pathogenic bacteria by coculture conditions in broth medium. The different capital and lowercase letters on each bar indicate significant differences between each treatment in different weeks and among treatments during each week, respectively ($P < 0.05$). This description is also provided for Figs. 4 and 6–8.

the TF2 and TF3 groups exhibited significant differences, with 50.0 ± 0.0% and 33.3 ± 5.7% mortality, respectively, which strongly differed from the control, with a mortality of 69.99 ± 10.0% ($P < 0.05$) (Fig. 9). The negative control clearly showed no mortality during the trial. Severely moribund shrimp obviously showed alterations in the hepatopancreas, and many green colonies were clearly isolated on thiosulfate-citrate-bile salts-sucrose (TCBS) agar from this organ (data not shown).

3.5. Histological studies

After probiotic supplementation for 5 weeks, histological measurements of the hepatopancreas and midgut were investigated. The effect of the dietary supplementation of *Bacillus* AQAHBS001 on these target organs was clear. The hepatopancreas of shrimp in all groups distinctly showed bulkiness containing clear complete cells, which comprised many B, R and F cells with no significant differences (data not shown). However, the midgut of shrimp in groups TF2 and TF3 contained increased villus areas, villus thickness, mucosa thickness, and intestinal wall thickness compared with those of the TF1 and control groups (Fig. 10A and B).

4. Discussion

Currently, several studies have recorded the role of probiotics in preventing the colonization and overgrowth of pathogens [30]. In addition, using probiotics also helps immunity, improves growth and promotes water quality [31]. Dietary administration of probiotics has been reported to control various pathogens during animal culture. Probiotics are also environmentally friendly, which is a characteristic unlike those of antibiotics and chemicals, as antibiotic and chemical drugs are cost effective but likely toxic. Moreover, the overuse or misuse of these materials also causes antibiotic resistance in many pathogenic bacteria, including *Vibrio* spp [32]. Therefore, efforts are being made to develop new suitable probiotics from several aquatic animal species and to prevent and relieve disease outbreaks in shrimp and other aquatic species [33].

In the current experiment, only 3 isolates, B4, B6 and B12, were successfully obtained, and they showed inhibitory effects against various *Vibrio* spp. The candidates B4, B6 and B12 were then identified according to API 50 CHB E and 16S rRNA sequencing, resulting in their identification as *B. pumilus*, *B. amyloliquefaciens* and *B. subtilis*, respectively. It was shown that those obtained *Bacillus* spp. could survive in a wide range of environments, which enables their broad application in shrimp growth conditions, where pH, salinity and temperature frequently fluctuate [34].

The efficiency of these isolates in controlling pathogens, which is a key factor in selecting appropriate bacteria as probiotics, was evaluated [12]. Based on *in vitro* laboratory results, B4, B6 and B12 inhibited *V. parahaemolyticus*; however, only *B. subtilis* AQHPS001 (B12) showed the highest antagonistic property against VP_{AHPND} strains. However, among the VP_{AHPND} strains, there were different sizes of the inhibitory clear zone, and $VP_{AHPNDAQH3.2}$ was the only strain that resisted B12. This suggests that there are varieties of VP_{AHPND} and that each strain may employ different mechanisms in response to the target B12. Previous reports found that *Bacillus* spp. could produce many kinds of bacteriocins, such as subtilin, subtilosin, coagulin, megacin, bacillin, bacillomycin, mycosubtilin, toximycin and xanthobacidin, which could reduce pathogen colonization by directly inhibiting pathogens while having no resulting effects on the virulence resistance genes of

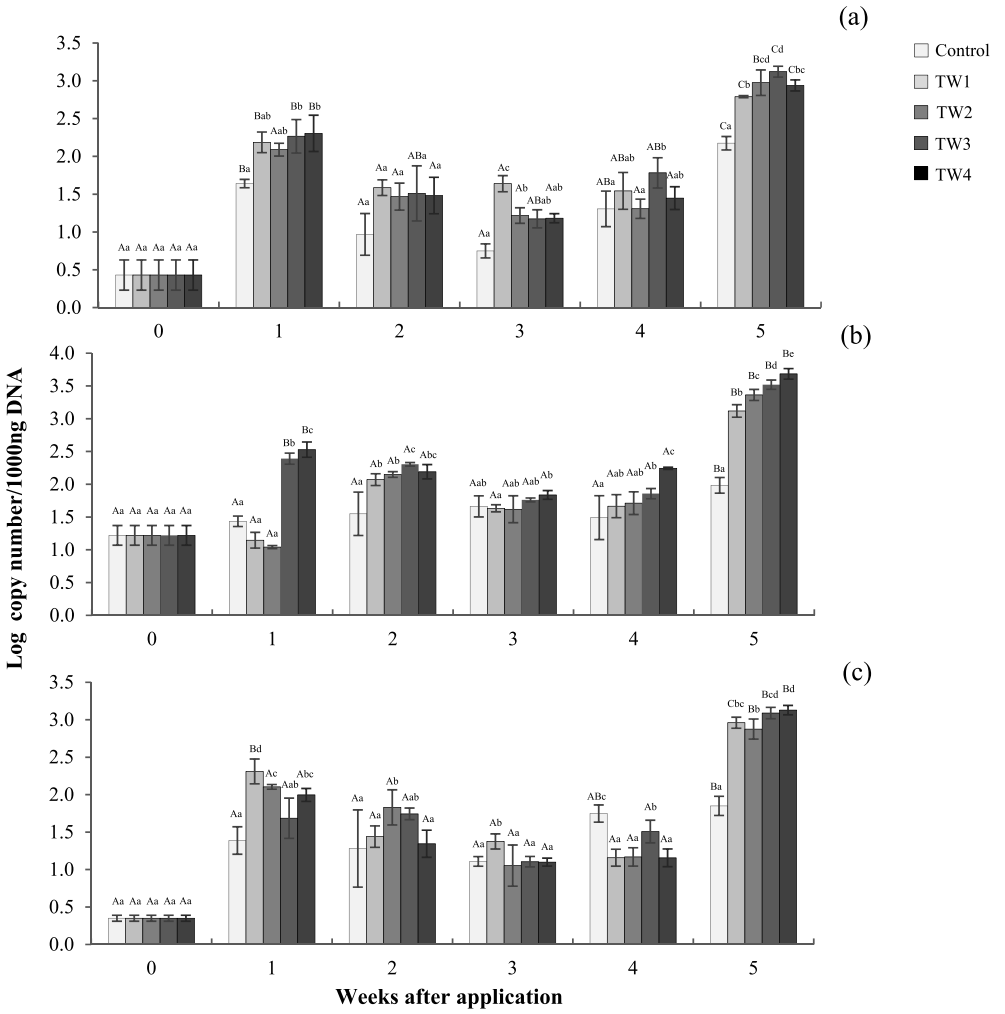

Fig. 4. Log copy number analysis of AQAHBS001 DNA in the gills (a), midgut (b) and swimming legs (c) of shrimp during probiotic water application using qPCR detection.

pathogenic bacteria [8,35,36]. Zhao et al. [37] also reported that *Bacillus* spp. could secrete quorum-quenching enzymes, which are expected to be quorum-sensing blockers to reduce disease infection. These results suggest that *B. subtilis* AQAHBS001 possesses more effective characteristics that are important for controlling the various harmful VP_{AHPND} strains than other candidates. For these reasons, it was further chosen to study its application on a laboratory scale.

In water-supplemented conditions, the effect of *B. subtilis* AQAHBS001 application was investigated. This practice did not have an effect on any shrimp growth parameters or survival, but compared with the untreated control, treatment with 1×10^3–1×10^5 CFU/mL of this probiotic significantly reduced only the levels of total ammonia in water at weeks 3 and 5 after application. These results suggest that *B. subtilis* AQAHBS001 could support the growth and activity of nitrifying bacteria, which in turn contributes to the effective conversion of ammonia nitrogen into nitrate [38,39]. However, the results clearly suggested that the application of the selected probiotic *via* water addition was not effective in protecting the treated shrimp from lethal VP_{AHPND}.

In contrast, the application of *Bacillus* AQAHBS001 by feed supplementation in TF3 (1×10^9 CFU/kg diet) clearly enhanced growth performance effectively, and the growth performance in TF3 was significantly greater than that in the control group and almost TF1 (1×10^5 CFU/kg diet) but did not differ significantly from that in TF2 (1×10^7 CFU/kg diet) (Table 1). The application of feed supplemented with probiotics, especially *B. subtilis*, for growth improvement has been studied in several ways and has been shown to benefit animal yield [9,40,41]. Although the mechanism of *B. subtilis* activity on the host is not well understood, certain activities have been suggested to increase digestive enzyme activity, help breakdown large units of food during the digestion process, and enhance absorption surface area, particularly microvilli structure, which in turn contributes to the increased absorption of nutrients [42–44]. In addition, there are multiple ways to improve the digestive activity of probiotics, such as providing crucial enzymes, organic compounds, vitamins and amino acids. These mechanisms were described in a study by Zokaeifar et al. [40], who reported that *B. subtilis* strains L10 and G1 could produce digestive enzymes, including protease and amylase.

According to the above results, histological alterations in the intestinal tracts and hepatopancreas were observed. The results strongly supported that using the probiotic *B. subtilis* AQAHBS001 effectively improved the morphological characteristics of the midgut, especially the mucosal structure, which plays a role in digestive activity, the absorption of nutrients and the immune system by forming defensive barriers to prevent the colonization of opportunistic pathogens [45,46]. These results suggested that high doses of *B. subtilis* AQAHBS001 provided positive effects on intestinal villi by increasing the height and width of the intestinal epithelial cells and villi, which contributes to increasing the vast absorptive surface to aid the host in absorbing more nutrients [47].

Based on the present study, qPCR analysis clearly showed that *B. subtilis* AQAHBS001 applied *via* both water and feed supplementation could significantly increase in viable number in various target organs.

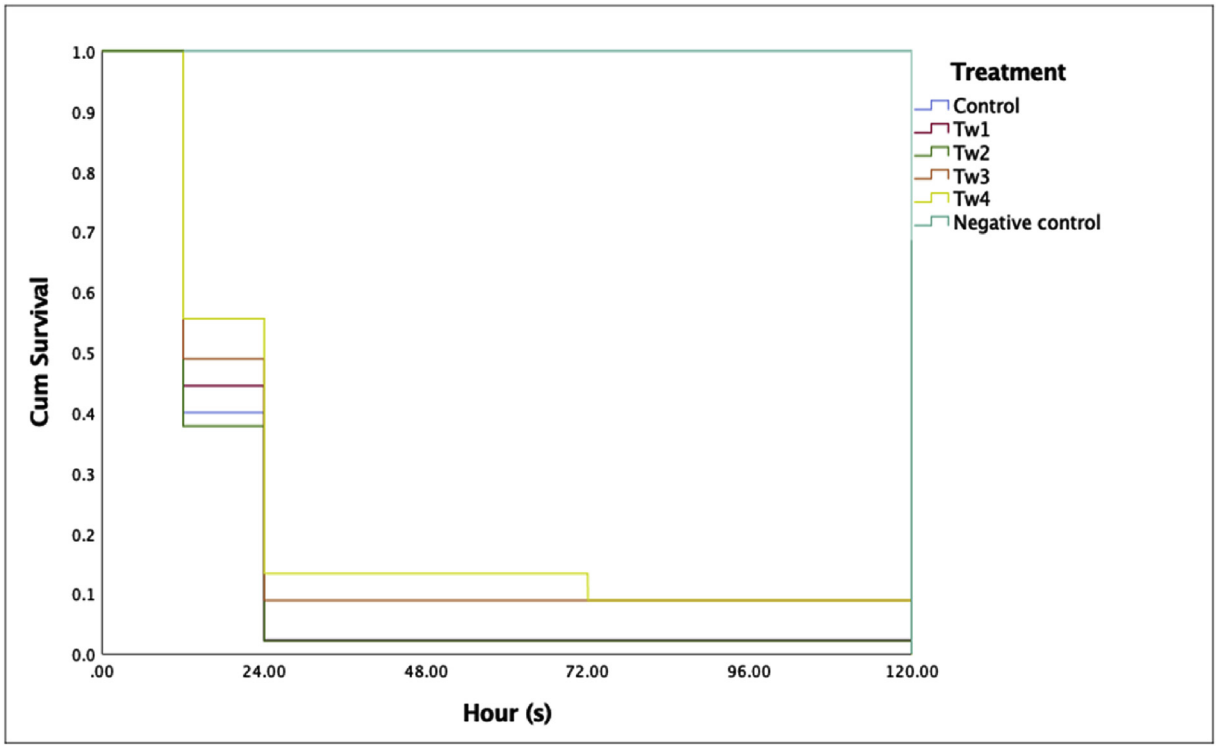

Fig. 5. Effects of AQAHBS001 application in culture water on disease resistance against $VP_{AHPNDAQH1.2}$. The survival analysis indicated no significant differences among the test groups ($P > 0.05$).

These results suggest that *B. subtilis* AQAHBS001 has the ability to adhere, colonize and survive in outer surface areas, the surrounding water and the midgut of shrimp. In the water supplementation trial, the target DNA of the probiotic was clearly detected in the gills, swimming legs and midgut of all shrimp groups, with the highest concentrations at the low levels of 2–3 log copy number/1000 ng DNA at week 5, even in the control shrimp. These results suggest that *B. subtilis* may be a normal flora in the shrimp culture environment. However, the levels of probiotic in all treatment groups strongly increased and were significantly higher than those in the control group, suggesting that the application of a viable form of the probiotic significantly affected the number of *B. subtilis* AQAHBS001 in this experiment.

In the trials with supplemented feed, the *B. subtilis* AQAHBS001 number in the midgut of probiotic-treated shrimp significantly increased at week 1 after application with a high log copy number of 6–8 and immediately decreased to 2–3 log at weeks 3 and 5, respectively, but a significant number was still found in the TF2 and TF3 treatments. This phenomenon may be due to microbiota balance and microbiological optimization in the gastrointestinal tracts of shrimp. Adhesion and colonization of probiotics within the gastrointestinal tract or intestinal mucus are important to support the response activities of the host, such as reducing and eliminating the colonization of opportunistic pathogens and improving the digestive system [12,48,49].

Improvement in immune responses and disease resistance is one of the most important targets of probiotic application. In the water-treated experiment, it was clear that the water application of *B. subtilis* AQAHBS001 at 1×10^2-$1x10^5$ CFU/mL for 5 weeks was inadequate to elevate immune responses and disease resistance against a virulent VP_{AHPND} strain. However, in feed additive trials, feeding shrimp *B. subtilis* AQAHBS001 at 1×10^5–1×10^9 CFU/kg diet was very effective in enhancing the clearance efficiency of shrimp hemolymph and the phagocytic activity of shrimp hemocytes. qRT-PCR analysis also indicated that these probiotic levels strongly elevated mRNA expression levels of *ALF*, *LSZ*, *proPO* and *SOD* genes in the gills, hepatopancreas and hemocytes of tested shrimp more effectively. Additionally, when experimental shrimp were challenged with a virulent VP_{AHPND} strain by

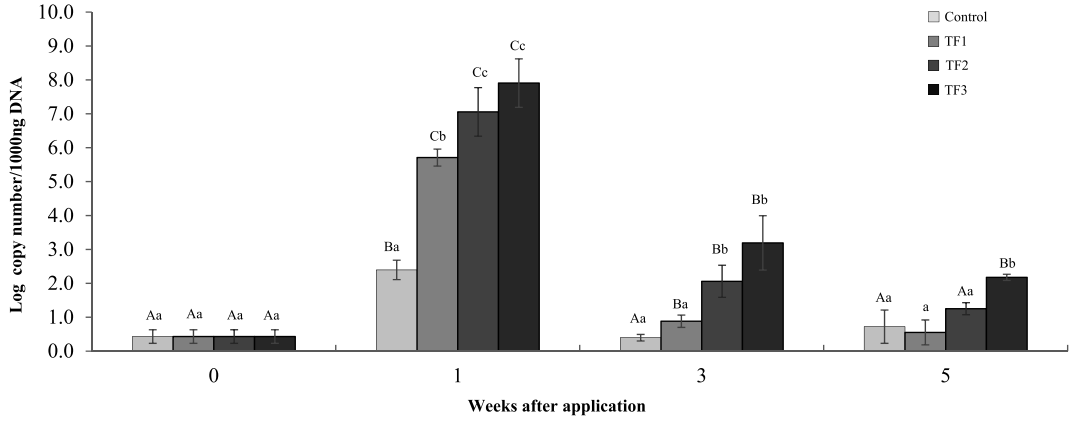

Fig. 6. Copy number analysis of AQAHBS001 DNA in the intestine of shrimp during the application of feed supplemented with AQAHBS001 using qPCR detection.

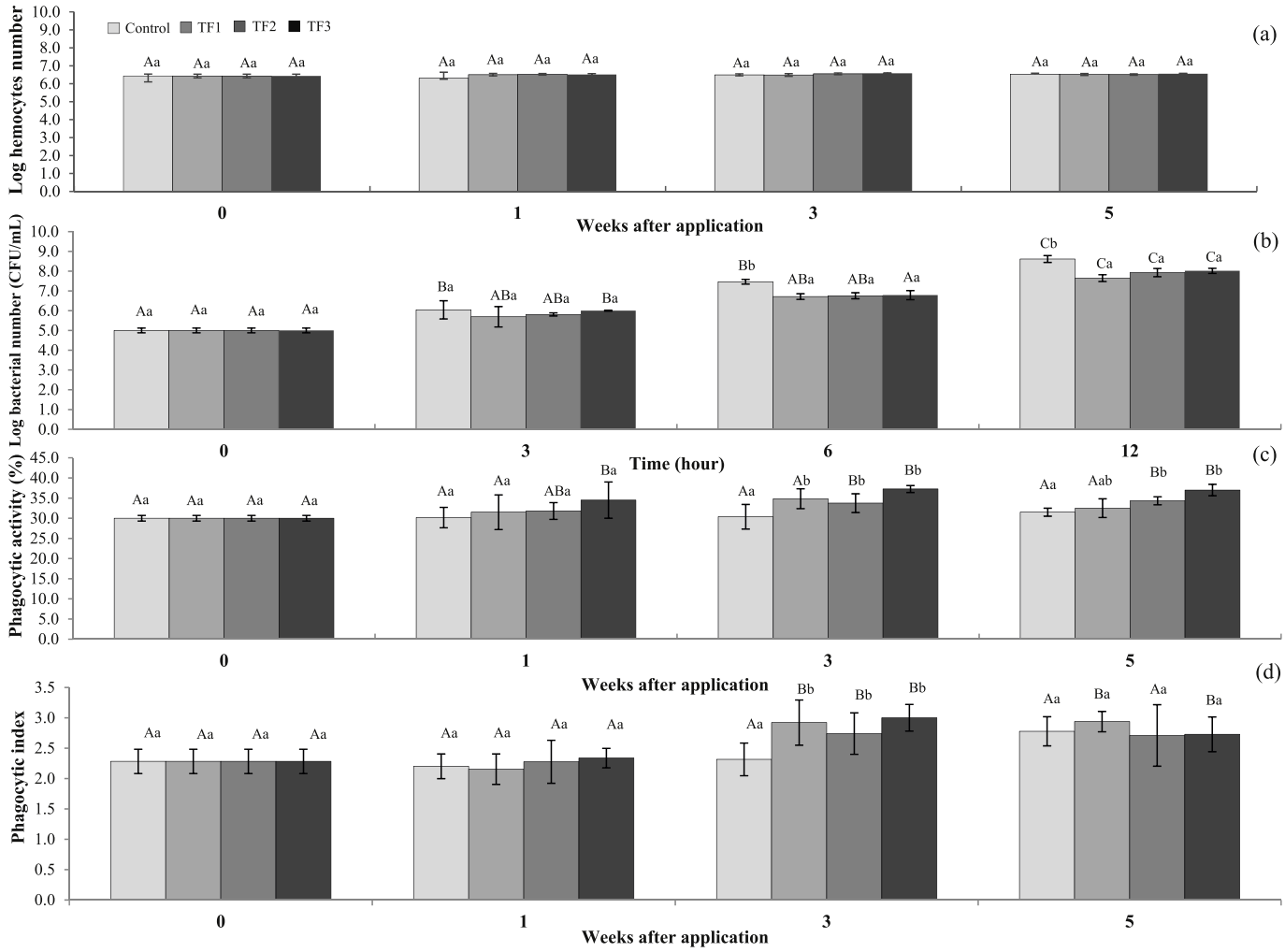

Fig. 7. Effects of probiotic AQAHBS001-supplemented feed on the immune responses of shrimp. Total hemocyte count (a), clearance efficiency (b), phagocytic activity (c) and phagocytic index (d).

intramuscular injection, shrimp in the TF2 and TF3 groups clearly demonstrated more effective disease resistance against VP_{AHPND} than the control groups. Taken together, these obtained data indicate that feed supplemented with the viable form of *B. subtilis* AQAHBS001 is an applicable practice to overcome the harmful disease caused by virulent VP_{AHPND}.

According to many previous reports, it is believed that invertebrates have efficient innate immunity only to prevent foreign antigens such as bacteria, fungi, viruses and parasites, relying on the chemical and physical activities of hemocytes [50,51]. In accordance with this study, the total hemocyte count was not significantly different among treatments in this experiment. This is consistent with previous reports that also showed no significant effect of *B. subtilis* application on total hemocyte count [52]. However, the application of probiotic *B. subtilis* AQAHBS001 was demonstrated to effectively enhance the percent phagocytic activity and phagocytic index to levels higher than those of the control group. Previous studies found that feed supplemented with the probiotic *B. subtilis* enhanced phagocytosis by configuration of peptidoglycan of used probiotics [53]. In addition, application of the probiotic *Bacillus subtilis* WB600 improved phagocytosis of viral proteins in WSSV infection in *L. vannamei* [7,54].

Generally, pathogen-associated molecular patterns (PAMPs) act as key stimulators of the immune system, consisting of molecular structures, such as glucan and laminarin from fungal cell walls, viral unmethylated DNA, single-strand and double-strand RNA from viruses, lipopolysaccharide (LPS) and peptidoglycan (PG), which is a major component of the cell walls of both Gram-negative and Gram-positive bacteria [55,56]. Most likely, *B. subtilis* AQAHBS001 may provide such key components, mainly peptidoglycan, lipoteichoic acid or any immune-triggered molecules important for enhancing and improving the shrimp immune system.

In the present experiment, the expression of the *ALF*, *LSZ*, *proPO* and *SOD* genes was investigated in the gills, hemocytes and hepatopancreas by qRT-PCR. These genes were significantly upregulated after probiotic feeding in the TF2 and TF3 groups, especially at weeks 3 and 5.

In accordance with the results of phagocytosis, the expression of the *SOD* gene increased. SOD activity can be used to indicate the immunomodulation of shrimp because when the phagocytic process is initiated, reactive oxygen species (ROS) are produced. Then, superoxide anions, hydrogen peroxide and hydroxyl radicals will be produced to destroy pathogens [57,58]. These ROS might be affected by the host itself; therefore, the host must effectively protect cells against oxidative damage caused by ROS by reducing or relying on antioxidant enzymes such as SOD, catalase and glutathione peroxidase [59].

In the present study, *ALF* expression was detected at elevated levels in hemocytes, gills and hepatopancreas; this finding suggested that *ALFs* are induced upon application of the target probiotic in a dose-dependent manner and use some components to trigger the Toll and Imd signaling pathways, which are involved in the regulation of PAMPs [60].

The effect of *B. subtilis* AQAHBS001 application on immune responses was supported by Amoah et al. [45] and Wongsasak et al. [46],

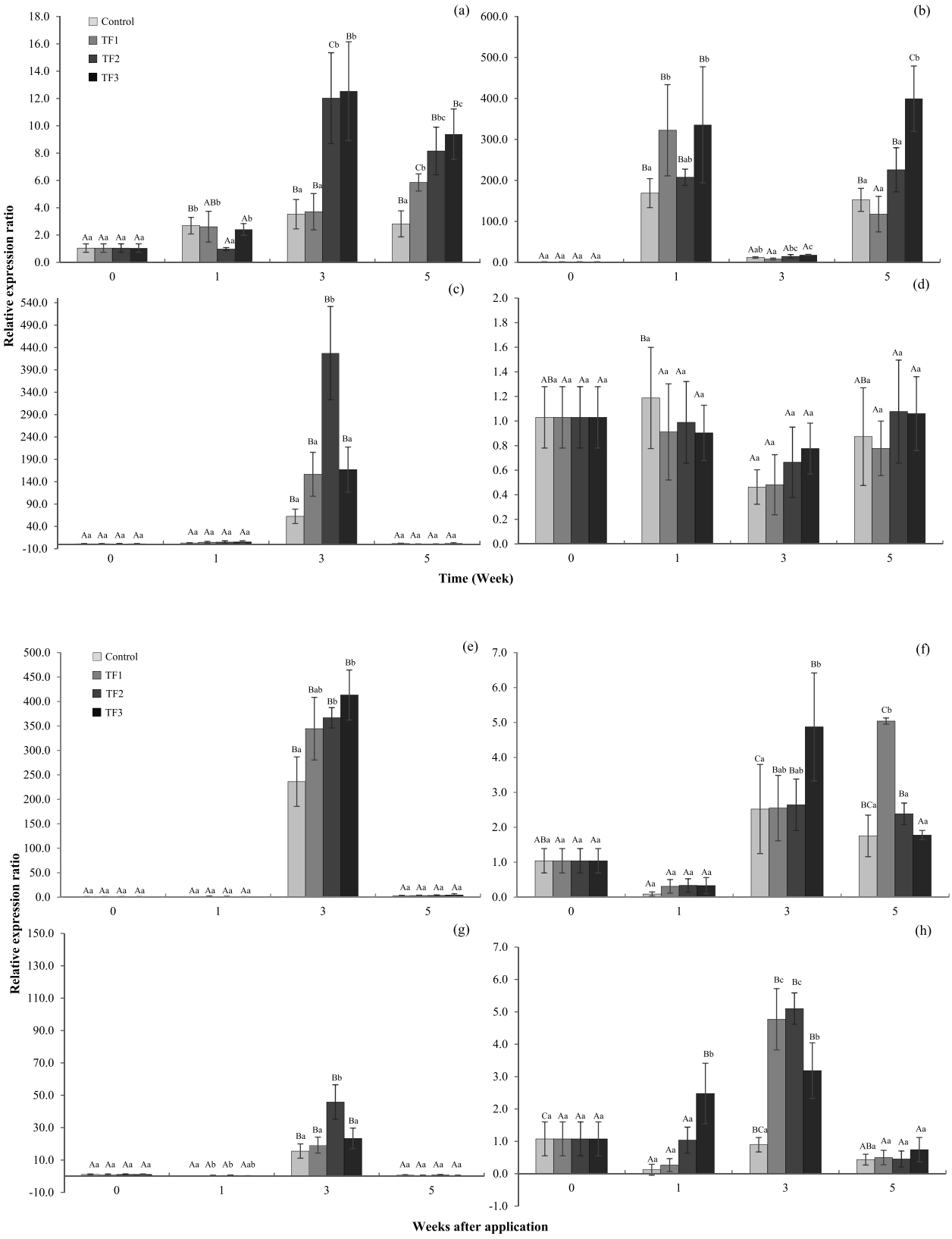

Fig. 8. Effects of probiotic AQAHBS001-supplemented feed on the expression of immune-related genes in the gills (a–d), hemocytes (e–h) and hepatopancreas (i–l) of shrimp. *ALF* (a, e and i), *LSZ* (b, f and j), *proPO* (c, g and k) and *SOD* (d, h and l) genes.

who discovered that prebiotics and probiotics can enhance the immune response of crustaceans, such as antibacterial peptide, proPO and lysozyme activities. The upregulation of *LSZ* expression was expected to be due to degranulation after exposure to PAMPs, and *LSZ* expression could also induce lysozyme activity [61], leading to bacterial killing [57]. In the present study, *LSZ* showed the highest upregulation in the gills after weeks 1 and 5 after feeding with the probiotics, suggesting that *LSZ* activity may be specifically induced by *B. subtilis* AQAHBS001

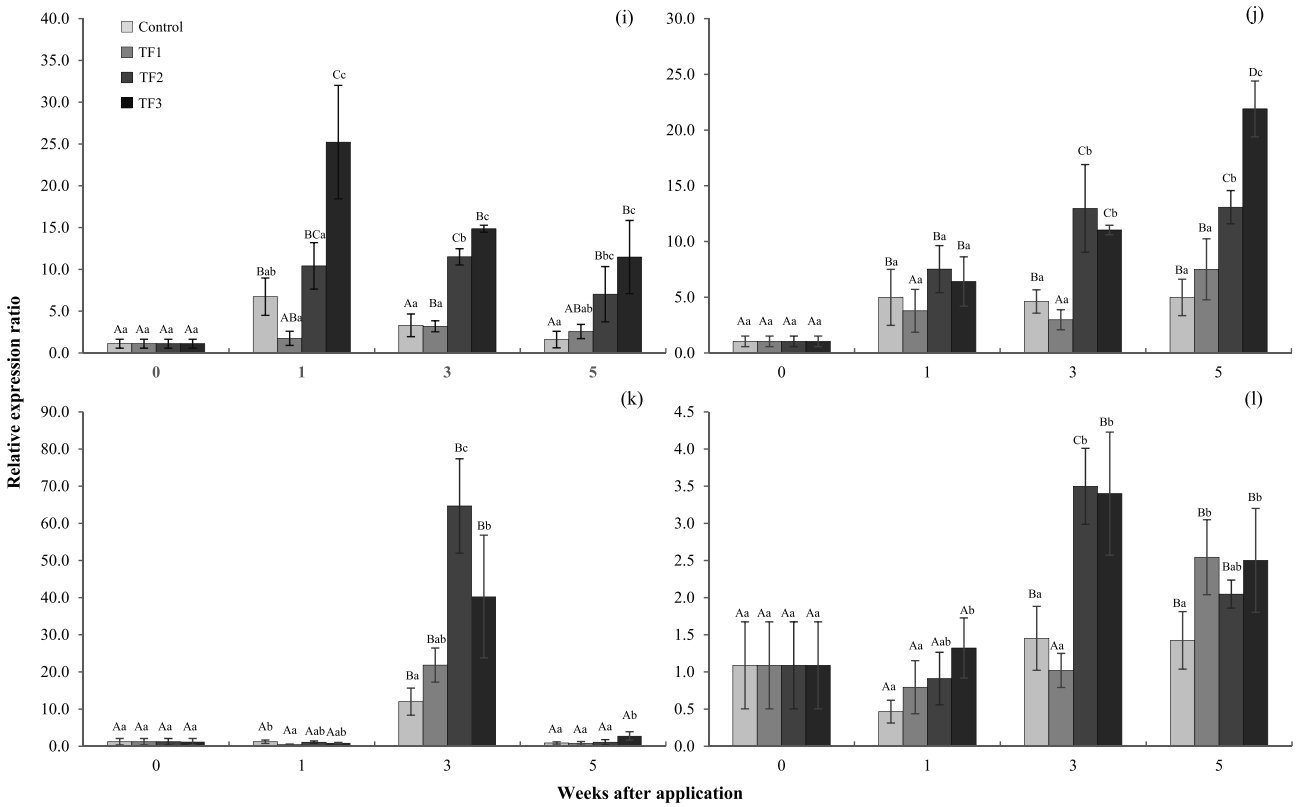

Fig. 8. (*continued*)

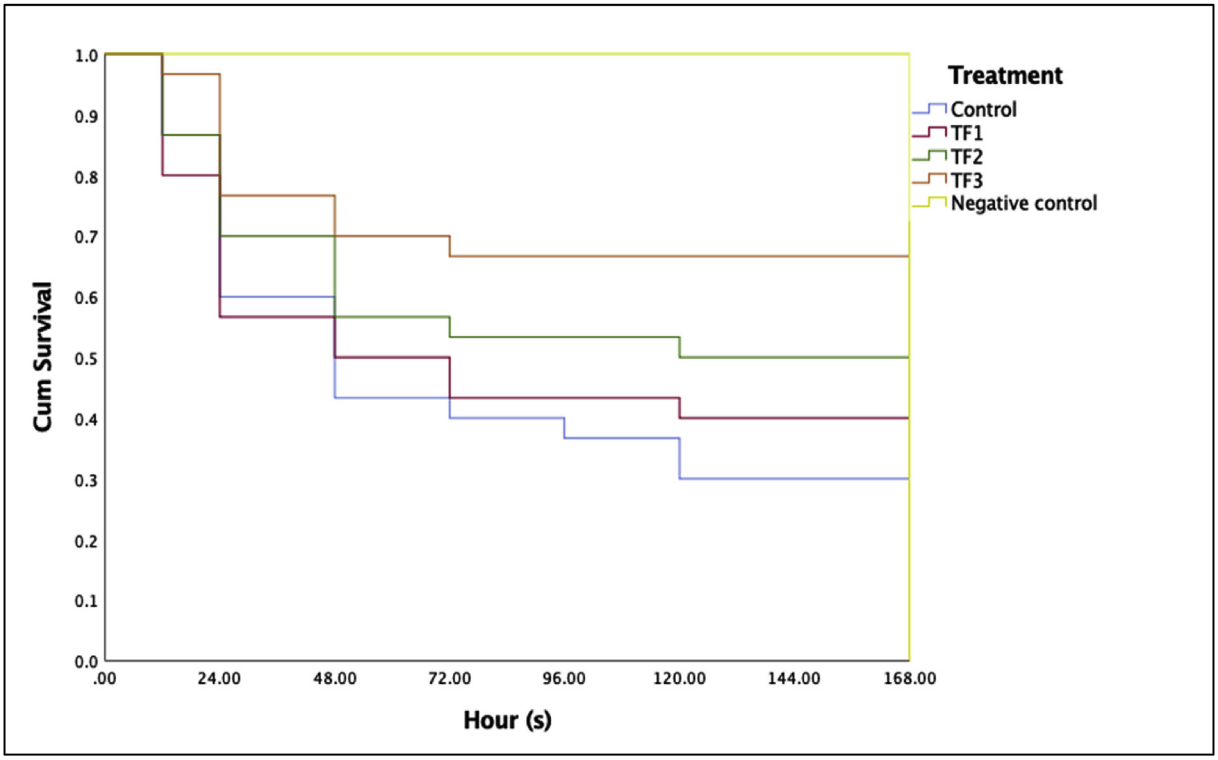

Fig. 9. Effects of probiotic AQAHBS001-supplemented feed on disease resistance against $VP_{AHPNDAQH1.2}$. The survival analysis indicated significant differences among the test groups ($P < 0.05$).

in the gills.

As in previous studies, the proPO system can be stimulated by PAMPs; in the current experiment, it was clear that the highest expression of the *proPO* gene was significantly observed in all tested tissues at week 3, and at this time, TF2 and TF3 seemingly showed significant levels of *proPO* mRNA. Similar results were examined in the application of *B. aryabhattai TBRC8450* and *B. subtilis* E20, which resulted in an increase in the expression of the *proPO* gene [36,62]. It has

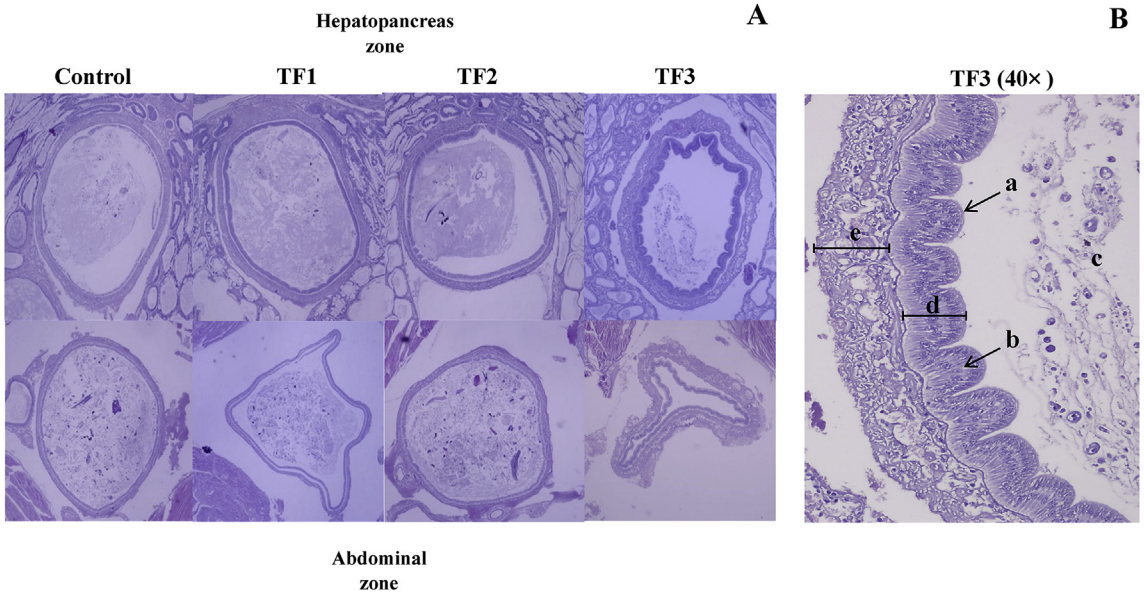

Fig. 10. A. Effects of the probiotic AQAHBS001-supplemented feed on the histology of the midgut. Control, TF1, TF2 and TF3 are a control group, shrimp groups fed 1×10^5, 1×10^7 and 1×10^9 CFU of probiotics/kg diet, respectively. Microscopic observation was conducted at 4 × magnification. B. 40 × magnification of intestinal characteristics of shrimp in the TF3 treatment; (a) mucosa brush border, (b) intestine epithelium, (c) intestine lumen, (d) epithelium height and (e) intestinal wall thickness.

been reported that hemocytes are responsible for proPO production upon being triggered by PAMPs and increase the conversion performance of inactive proPO to the active form [61]. However, high expression levels of this gene found in the gills and hepatopancreas suggested functional circulating and stimulating hemocytes located in these tested organs.

5. Conclusions

In conclusion, according to the results of the *in vitro* and *in vivo* experiments, the candidate probiotic *B. subtilis* AQAHBS001 isolated from the midgut of shrimp collected from shrimp farms had the clear ability to survive in harsh environmental conditions, such as a wide range of temperatures, pH values and salinities. This isolate also effectively inhibited *Vibrio* spp. *in vitro*, especially VP_{AHPND}, which has caused significant losses in shrimp production worldwide. *B. subtilis* AQAHBS001 supplemented in feed proliferated and colonized the shrimp intestinal tract and demonstrated enhanced results in growth and immune parameters, including immune gene expression. Additionally, shrimp fed this probiotic showed significantly increased disease resistance against VP_{AHPND}, which is very important for application in shrimp farms. Therefore, the suitable forms and application of *B. subtilis* AQAHBS001 at both laboratory and farm scales should be further evaluated to confirm its effectiveness and achieve its potential application.

Author contributions

Conceptualization, P.S.; methodology, P.S.; validation, P.S. and W.K.; formal analysis, W.K.; investigation, W.K.; data curation, P.S.; writing-original draft preparation, W.K.; writing-review and editing, P.S.; supervision, P.S.; project administration, P.S.; and funding acquisition, P.S.

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.013.

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