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Effects of *Bacillus aryabhattai* TBRC8450 on vibriosis resistance and immune enhancement in Pacific white shrimp, *Litopenaeus vannamei*

Surapun Tepaamorndech[a,*], Kanittha Chantarasakha[a], Yutthana Kingcha[a], Sage Chaiyapechara[b], Metavee Phromson[c], Malinee Sriariyanun[d], Catherine P. Kirschke[e], Liping Huang[e], Wonnop Visessanguan[a]

[a] Food Biotechnology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Phahonyothin Rd., Pathumthani, 12120, Thailand
[b] Aquatic Molecular Genetics and Biotechnology Laboratory, BIOTEC, 113 Phahonyothin Rd., Pathumthani, 12120, Thailand
[c] Aquatic Product Development and Service Laboratory, BIOTEC, 113 Phahonyothin Rd., Pathumthani, 12120, Thailand
[d] Department of Mechanical and Process Engineering, The Sirindhorn International Thai-German Graduate School of Engineering, King Mongkut's University of Technology North Bangkok, Bangkok, 10800, Thailand
[e] Obesity and Metabolism Research Unit, USDA/ARS/Western Human Nutrition Research Center, 430 West Health Sciences Drive, Davis, CA, 95616, USA

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ABSTRACT

The use of probiotics in aquaculture is a practical alternative to promote animal health and disease prevention. Meanwhile, this practice can also reduce the use of prophylactic antibiotics. The purpose of this study was to identify candidate probiotics that could control pathogen populations in host's gastrointestinal (GI) tract and stimulate host immunity in shrimp aquaculture. *Bacillus aryabhattai* TBRC8450, a bacterial strain isolated from the environment in a shrimp farm, has an antimicrobial activity against many pathogenic strains of *Vibrio harveyi* and *V. parahaemolyticus*. Supplementation of *B. aryabhattai* to Pacific white shrimp (*Litopenaeus vannamei*) not only decreased the abundance of *Vibrio* populations, but also shifted the bacterial community in the shrimp GI tract. We found that supplementation of *B. aryabhattai* triggered shrimp innate immunity and antioxidant activities. mRNA expression of genes encoding microbial peptides and antioxidant enzymes, including C-type lectin, penaeidin-3, heat shock protein 60, thioredoxin, and ferritin, was significantly upregulated in the hepatopancreas of shrimp fed *B. aryabhattai.* Furthermore, phenoloxidase activity in the hemocytes and the total antioxidant activity in the plasma were increased, indicating enhanced immune and antioxidant responses at the systemic level. In contrast, supplementation of *B. aryabhattai* had no effect on the total hemocyte count and superoxide dismutase activity in the plasma and hepatopancreas. Importantly, a pathogen challenge test using *V. harveyi* 1562 showed a significant increase in survival rates of shrimp fed *B. aryabhattai* compared to the control group. Our findings suggest that *B. aryabhattai* TBRC8450 can likely be used as a probiotic to reduce the population of *V. harveyi* in the shrimp GI tract and to enhance shrimp innate immunity and antioxidant capacity for vibriosis resistance in shrimp aquaculture.

1. Introduction

Shrimp aquaculture is a rapidly growing industry with a total production in million metric tons each year [1]. *L. vannamei*, known as Pacific white shrimp, is the most dominant species in shrimp world productions [1]. Pacific white shrimp are predominantly produced from aquaculture in China and Southeast Asian countries [1]. *L. vannamei* originally cultivated in the Pacific coast of the Central and South America was introduced into Asia due to its high growth performance and disease resistance [2]. However, after a few decades of massive cultivation, Pacific white shrimp became susceptible to many disease outbreaks [3–5]. As the result, the production of Pacific white shrimp has decreased dramatically, leading to massive decline in economic incomes.

Infectious diseases resulting from *Vibrio* spp. have been a major burden in shrimp aquaculture worldwide [6–8]. Although *Vibrio* spp. are considered as commensal bacteria in marine animals. Some strains of *V. harveyi* and *V. parahaemolyticus* have been known to cause serious shrimp outbreaks [9–12]. Infection of *V. harveyi* causes luminous vibriosis, white tail disease, and bright-red syndrome, leading to massive mortality in shrimp hatcheries [13]. On the other hand, *V. parahaemolyticus* is a causative pathogen of acute hepatopancreatic necrosis

* Corresponding author.
E-mail address: surapun.tep@biotec.or.th (S. Tepaamorndech).

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syndrome which has severely damaged shrimp productions and economic incomes [14,15]. The emergence of *Vibrio*-associated diseases seems more prevalent. It has been postulated that climate change and warming ocean water increase pathogenic *Vibrio* levels in the environment [16,17]. Effective strategies, such as the use of biosecurity or biocontrol agents aimed at controlling *Vibrio*-associated outbreaks in shrimp aquaculture, are required.

The widespread and uncontrolled use of prophylactic antibiotics is often seen in aquaculture to prevent pathogen infections and disease outbreaks, especially in developing countries [18,19]. As a result, significant amounts of antibiotic residues remain in the environment and become a main determinant for a rise of antibiotic resistance bacteria [18]. The prevalence of antibiotic resistant *Vibrio* bacteria has been reported in aquaculture areas [20–22]. It has been found that *V. cholera* acquires its antibiotic resistant genes from non-*cholera Vibrio* in shrimp farms. This may directly link to cholera epidemics due to multiple antibiotic resistances to *V. cholera* [23]. The increase in risk of antibiotic resistance in bacteria has imposed a need to restrict use of antibiotics in aquaculture productions.

Probiotic bacteria, on the other hand, are live microorganisms conferring health benefits to host when consumed in adequate amounts [24]. An application of probiotics as an alternative method to decrease prophylactic uses of antibiotics has been a great interest for the aquaculture industry [25]. *Bacillus* spp. have been widely used as probiotics in aquacultures due to their ability to grow in the marine environment and produce a variety of bioactive compounds [26,27]. Supplementation of *Bacillus* spp. decreases shrimp mortality exposed to pathogenic *Vibrio* [28–34]. Studies have also demonstrated that *Bacillus* spp. exert the beneficial effect by their antimicrobial or immune stimulating function. However, whether an application of a single *Bacillus* strain in shrimp aquaculture could achieve both antimicrobial and immune stimulation activities was largely unexplored.

The present study aimed to identify novel probiotics with antimicrobial activities against pathogenic *Vibrio* and immune stimulant ability to enhance resistance to pathogenic *Vibrio* infections in Pacific white shrimp. Bacterial isolates were obtained from shrimp farms. Single bacterial colonies were collected and screened for their antimicrobial activities. We investigated the beneficial effects of the selected bacterial strain on the *Vibrio* population in the GI tract, immune and antioxidant responses, and *Vibrio* infection-related death rates in Pacific white shrimp. Here we provide evidence demonstrating for the first time that *B. aryabhattai* TBRC8450 could reduce *Vibrio* population in the GI tract. Meanwhile, it is also able to promote immune and antioxidant functions, leading to increased shrimp survival during *Vibrio* infection. Our findings show strong scientific evidence that the use of a single beneficial bacterial strain, such as *B. aryabhattai* as a probiotic is feasible in shrimp aquaculture.

2. Materials and methods

2.1. Isolation of Bacillus and analysis of antimicrobial and hemolytic activities

The sediment samples were collected from shrimp farms in Chachoengsao and Rayong, Thailand. *Bacillus* spp. were isolated as previously described with some modifications [35]. Briefly, the samples were heated at 80 °C for 15 min, plated on Luria-Bertani (LB) containing 2% NaCl, and incubated at 30 °C for 24 h. Bacterial isolates were then collected for antimicrobial activity assays. Antagonistic activities against shrimp pathogens, including *V. harveyi* and *V. parahaemolyticus*, were analyzed using a spot-on-the-lawn technique [36,37]. Hemolytic activity was examined using sheep blood agar plates [38]. The plates were incubated at 30 °C for 3 days. Levels of hemolytic activity in the bacterial samples were evaluated by comparing the sample to that of *B. cereus*, a positive control for the assay. The bacterial species were identified using 16S rDNA sequencing. DNA was isolated using a Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. 16S rDNA was amplified using PCR with a 27f-CM primer (5′-AGAGTTTGATCMTGGCTCAG-3′) and a 1492r primer (5′-TACGGYTACCTTGTTACGACTT-3′) [39]. PCR fragments were purified in agarose gel and were subject to DNA sequencing. Bacterial 16S rRNA sequence similarities were analyzed using the Ribosomal Database Project (RDP) [40]. DNA sequence alignments were illustrated using a Phylogeny.fr program [41].

2.2. Shrimp, supplementation, and tissue collection

Pacific white shrimp postlarvae were obtained from a commercial hatchery farm in Chachoengsao, Thailand. Shrimp were acclimated in a 2000 L tank and screened for viral infections, including White Spot Syndrome Virus, Yellow Head Virus, and Infectious Hypodermal and Hematopoietic Virus as described previously [42]. Shrimp at an average body weight of 0.9 ± 0.1 g were randomly transferred to 220 L tanks (n = 8, 100 shrimp/tank) and cultivated using a biofloc system [43]. The control group (n = 4 tanks) was fed a commercial diet (Wave, Inteqc) containing 38% of crude protein four times a day, while the study group (n = 4) was fed the same diet supplemented with *B. aryabhattai* TBRC8450. The bacteria were obtained by culturing in LB as described above, centrifuged at 2500 g for 10 min, and washed in phosphate-buffered saline (PBS). The supplemental diet was top-coated with *B. aryabhattai* TBRC8450 at 1×10^8 CFU/g diet and stored at 4 °C until use. Salinity was controlled at 15 g/L. Carbon to nitrogen ratio was maintained at 15. Molasses was added as an organic carbon source [44]. Aeration was provided using air blowers. Temperature, dissolved oxygen, pH were monitored every other day. Alkalinity and inorganic nitrogen, including total ammonium nitrogen, nitrate, and nitrite were assessed every week. Shrimp were grown in the tanks for 6 weeks and anesthetized on ice before tissue collections. Hemolymph was collected from the ventral sinus as described previously [45]. Hemocytes were removed by centrifugation at 1000*g* for 5 min and the collected plasma samples were stored at −80 °C. The hepatopancreas and the GI tract containing the midgut and hindgut were harvested, snap-frozen in liquid nitrogen, and stored at −80 °C until use. All animal experiments were conducted in accordance with National Institutes of Health Guidelines for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee of BIOTEC.

2.3. DNA isolation and automated ribosomal intergenic spacer analysis (ARISA)

Bacterial genomic DNA was isolated from the GI tract using a Quick-DNA Fecal/Soil Microbe kit (Zymo Research) according to the manufacturer's protocol. Isolated DNA samples were stored at −20 °C until use. Community structure of shrimp gut bacteria was analyzed using ARISA [46,47]. Briefly, 100 ng DNA was amplified using an ITSF primer (5′-GTCGTAACAAGGTAGCCGTA-3′) on which FAM was 5′ end-labeled and an ITSeub primer (5′-GCCAAGGCATCCACC-3′) [48]. ITS amplicons were subject to fragment analysis using a 3730xl DNA analyzer (ThermoFisher Scientific). The fragment length was called based on a GeneScan™ 1200 LIZ™ dye size standard (ThermoFisher Scientific) using Peak Studio software [49]. To compensate for size variations in different samples, size-calling parameters were assigned as described previously [50]. Community structure of the bacteria was then illustrated using Non-metric multidimensional scaling (nMDS) based on a Bray-Curtis similarity matrix.

2.4. Quantification of intestinal vibrio abundance

Quantitative PCR (qPCR) was performed in a SYBR Green-based PCR assay using a SsoAdvanced SYBR® Green supermix (Biorad). Ten-nanogram DNA isolated from the GI tract was used. Specific primers

and PCR conditions for detecting 16S rRNA genes of *Vibrio* and total bacteria were described previously [51,52]. qPCR was used to estimate the levels of *Vibrio* population as this method has been shown to be able to rapidly and accurately detect and quantify both culturable and nonculturable *Vibrio* spp. [53]. qPCR was performed in duplicate on a CFX384 Real-time System (Biorad). All primers were confirmed with melting curve analyses. The Cycle threshold (Ct) of 16S rRNA genes specific to *Vibrio* spp. and total marine bacteria was reported. The Ct levels are inversely proportional to the amount of the bacterial population.

2.5. Gene expression analysis

Hepatopancreas was homogenized in TRIzol (Thermo Fisher Scientific) using Dounce tissue grinders (Sigma-Aldrich). Total RNA was isolated according to the manufacturer's protocol. cDNA was generated using an iScript™ cDNA synthesis kit (Biorad) and diluted with double-distilled water before use. qPCR was performed in a SsoAdvanced SYBR® Green supermix (Biorad) on a CFX384 Real-time System (Biorad). Gene expression was performed in duplicate. Specific primers used in this study were adopted from previous studies [54–57]. The expression of the target genes was normalized to the expression of *Actb*. Fold changes were calculated using a $2^{-\Delta\Delta CT}$ method [58].

2.6. Analysis of innate immunity

Hemolymph was collected into a syringe containing an anticoagulant (10% formalin in 0.45 M NaCl). Total hemocyte count (THC) was performed as described previously [45]. The formalin-fixed hemocytes were stained with a rose bengal dye (1.2% rose bengal in 50% ethanol) and incubated at room temperature (RT) for 10 min. THC was done on a hemocytometer using an inverted phase-contrast microscope (Eclipse Ts-2i, Nikon). The remaining formalin-fixed hemocytes were centrifuged at 100 g at 4 °C for 10 min. The cell pellet was washed with cacodylate-citrate (CAC) buffer, and resuspended in the same buffer, and frozen in liquid nitrogen until use. The samples were then thawed at RT and centrifuged at 5000 g at 4 °C for 20 min. Hemocyte lyses were collected and used to determine phenoloxidase (PO) activity [59]. The lyse was mixed with 0.1% trypsin in CAC buffer, incubated at RT for 10 min followed by addition of 0.3% L-3,4-dihydroxyphenylalanine (Sigma Aldrich) in CAC buffer. One unit of the PO activity was defined as an increased absorbance of 0.001/min/mg protein. The other non-specific immune parameters, including superoxide dismutase (SOD) and antioxidant, were analyzed in the plasma and hepatopancreas. SOD and antioxidant assay kits (Cayman Chemical) were used according to the manufacturer's instructions. Optical density was measured at 490 nm using a microplate reader (Spark™ 10M, TECAN). The PO, SOD and antioxidant activity were normalized to the total proteins in the sample. Protein concentrations were measured using a BCA protein assay kit (ThermoFisher Scientific).

2.7. Susceptibility of shrimp to V. harveyi

After 4 weeks on the control or the supplemental diet, shrimp were distributed into 70 L tanks (n = 20 shrimp) and acclimated for 5 days before pathogen challenge tests. The same control and supplemental diets were provided for the groups during acclimation and challenge. *V. harveyi* 1562, a pathogenic strain that can cause shrimp death, was cultured in a marine broth at 30 °C with shaking at 200 rpm for 18 h. Bacterial samples were centrifuged at 2500 g for 10 min, washed with PBS, and resuspended in the salt water. Shrimp in both control and supplemental groups were immersed in the pathogen tanks each containing *V. harveyi* at 1×10^8 CFU/ml [60]. Unchallenged shrimp were used as the controls for the treatment. The mortality was monitored at 12 h and every 24 h for 7 days.

Table 1
The antimicrobial activity against shrimp pathogens using a spot-on-the-lawn technique.

Shrimp pathogens	Strain	Inhibition area (mm)
V. harveyi	Vh1	3.60 ± 0.13
	639	1.21 ± 0.17
	1526	1.85 ± 0.14
V. parahaemolyticus	Vp1	2.51 ± 0.37
	1681	1.72 ± 0.13
	1691	0.54 ± 0.06

B. aryabhattai TBRC8450 was seeded on the soft thiosulfate-citrate-bile salts-sucrose agar inoculated by *V. harveyi* and *V. parahaemolyticus*. The antimicrobial activity was indicated by clear zone (mm). Values are the mean ± S.E., n = 6 per group.

2.8. Statistic analysis

The results were presented as the mean ± S.E. Student's *t*-test was used in comparison of the control and supplemental groups. ANOSIM and Incidence Rate Ratio (IRR) were performed to determine differences in the structural community and the rate of mortality, respectively. Incidence rates were calculated by the number of survivals divided by the person-time during *Vibrio* challenge. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Identification of B. aryabhattai and in vitro antimicrobial activity

A few hundred isolates were grown from the samples collected from the environment in shrimp farms. We found one isolate showing an antimicrobial activity against many pathogenic *Vibrio* spp. including *V. harveyi* Vh1, 639, and 1526 as well as *V. parahaemolyticus* Vp1, 1681, and 1691. The clear zone of the bacterial isolate and the pathogenic *Vibrio* ranged between 0.5 and 3.5 mm (Table 1). Based on the results of 16S rDNA sequencing and the phylogenetic tree analysis, the bacterial isolate with the antimicrobial activity was mostly close to *B. aryabhattai* B8W2. (Fig. 1A). Thus, the bacterial isolate was identified as *B. aryabhattai* TBRC8450 and deposited in the Thailand Bioresource Research Center (TBRC). In addition, we found that the newly identified *B. aryabhattai* strain could not lyse red blood cells on the sheep blood agar, indicating that this bacterial strain is nonpathogenic potentially safe for use as a probiotic bacterial strain (Fig. 1B).

3.2. Effect of B. aryabhattai on vibrio abundance and the microbial population in the shrimp gut

To confirm our *in vitro* result of antimicrobial activities of *B. aryabhattai* TBRC8450, we next investigated whether we could use *B. aryabhattai* TBRC8450 as a probiotic supplement to decrease *Vibrio* abundance in shrimp GI tract. We used qPCR to quantify overall populations of *Vibrio* spp. in the shrimp gut in both *B. aryabhattai* TBRC8450-supplemented and control groups [61]. As shown in Fig. 2A, the Ct values of the 16S rRNA gene specific to *Vibrio* spp. were significantly higher in shrimp fed the supplemental diet than that of the control, indicating reduced abundances of *Vibrio* spp. in the GI tract of shrimp fed a supplemental diet. In contrast, the Ct levels of the 16S rDNA gene specific to marine bacterial species were not different between the supplemental and control groups. These data suggested that *B. aryabhattai* supplementation resulted in decreased abundances of *Vibrio*, while the populations of overall marine bacterial species remained unchanged in the shrimp GI tract. We then investigated different bacterial communities in the GI tract of shrimp from both groups using ARISA. As shown in Fig. 2B, a nMDS plot illustrated a distinct

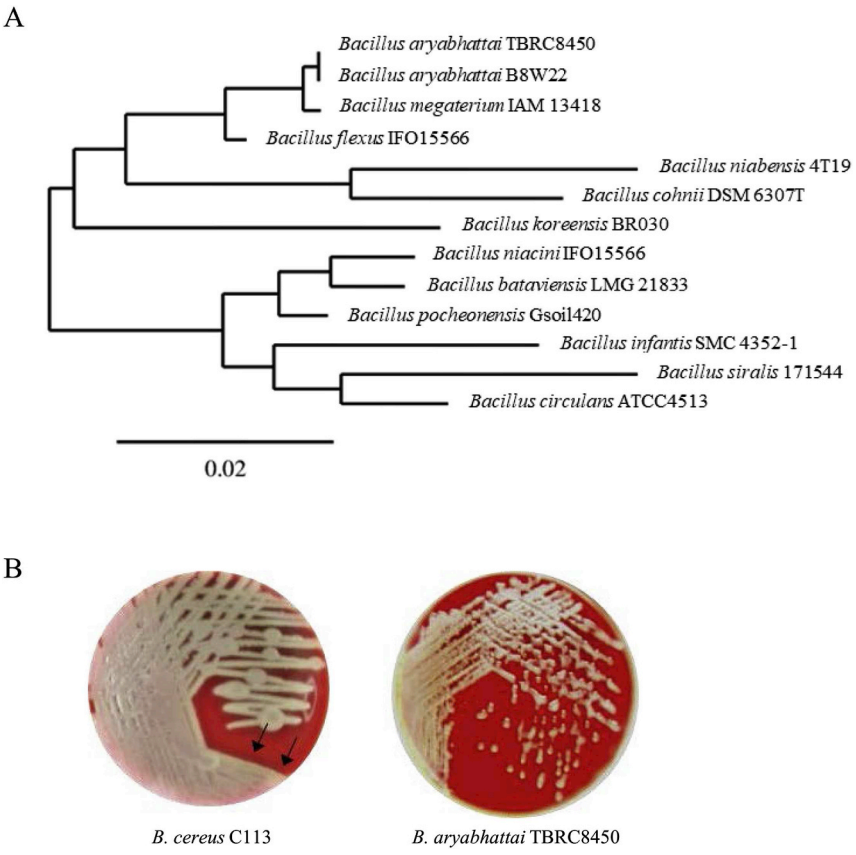

Fig. 1. The phylogenetic relationship and hemolytic activity of *B. aryabhattai* TBRC8450. A. The cladogram of *B. aryabhattai* TBRC8450 with type strains of *Bacillus* spp. based on the 16S rRNA gene sequences. Sequence similarity of *B. aryabhattai* TBRC8450 was analyzed using a Ribosomal Database Project (RDP). The phylogenetic tree was constructed using Phylogeny.fr with approximate likelihood ratio test for branches. B. Growth of *B. aryabhattai* TBRC8450 on sheep blood agar. *B. cereus* showing a β-hemolytic activity (arrows) was used as a positive control. The bacterial cultures were grown on sheep blood agar at 37 °C for 3 days.

separation of the bacterial community in shrimp supplemented *B. aryabhattai* compared to the control group. This significant difference revealed by the ARISA profiles was further confirmed by ANOSIM ($R = 0.8083$, $p < 0.01$). Taken together, we showed that supplementation of *B. aryabhattai* could not only decrease the abundance of *Vibrio* spp., but also change the bacterial diversity in the shrimp GI tract.

3.3. Effects of B. aryabhattai on shrimp immune responses

We investigated whether *B. aryabhattai* supplementation altered the expression of immune-related genes in the shrimp hepatopancreas, a key organ in immune responses [54,62]. Gene expression of C-type lectin (*lec*), prophenoloxidase (*ppo*), lipopolysaccharide- and β-1,3-glucan-binding protein (*lgβp*), serine proteinase (*sp*), penaeidin3 (*pen3*), and heat shock protein 60 (*hsp60*) has been used as the indicators for immune responses in shrimp [54–57]. As shown in Fig. 3A, the mRNA expression of *lec*, *pen3a,* and *hsp60* was significantly upregulated in the hepatopancreas from the supplemental group compared to that from the control group ($p < 0.05$). We also observed an upward trend in *ppo* mRNA expression in the shrimp fed supplemental diet ($p = 0.07$). On the other hand, the *lgβp* mRNA expression showed an opposite pattern with significantly lower expression in the supplemental group than the control group ($p < 0.05$). In addition, we analyzed immune function by measuring THC and enzymatic activities of PO in hemocytes. As shown in Fig. 3B, no differences in THC were found between two groups. However, supplementation of *B. aryabhattai* remarkably increased the PO activity in shrimp hemocytes (Fig. 3C). Together, our data demonstrated that *B. aryabhattai* supplementation was able to regulate immune-related gene expression in the hepatopancreas and the PO activity in the hemocytes, leading to increased shrimp immune response.

3.4. Effects of B. aryabhattai on antioxidant activities

The effect of dietary supplementation of *B. aryabhattai* TBRC8450 on the antioxidant capacity was analyzed using qPCR. We quantified the mRNA expression levels of antioxidant-related genes, including catalase (*cat*), superoxide dismutase (*sod*), glutathione s-transferase (*gst*), glutathione peroxidase (*gpx*), thioredoxin (*trx*), and ferritin (*fer*) in the hepatopancreas. As shown in Fig. 4A, the expression levels of *trx* and *fer* were significantly increased in the supplemental group compared to the control group ($p < 0.05$). There were no differences in the expression of *cat*, *sod*, *gst*, and *gpx*. In addition, an elevation of the total antioxidant activity was found in the hepatopancreas as well as the plasma isolated from the supplemental shrimp compared to the controls (Fig. 4B and C, $p < 0.05$ and $p < 0.01$, respectively). Nevertheless, we did not found any difference in the SOD activities between the supplemental and control groups in both hepatopancreas and plasma (Fig. 4D and E). These findings suggest that *B. aryabhattai* supplementation may improve antioxidant status and could promote a defense mechanism against pathogen infections in shrimp.

3.5. The effect of B. aryabhattai on the survival rates of shrimp challenged by V. harveyi

We hypothesized that *B. aryabhattai* TBRC8450 supplementation could improve disease resistance indicated by increased survivals during *Vibrio* infections. Shrimp fed the supplemental and control diet were challenged with *V. harveyi* 1562, a known pathogen to cause shrimp death. As shown in Fig. 5, the survival rates of the challenged shrimp were significantly decreased in both dietary groups within 12 h.

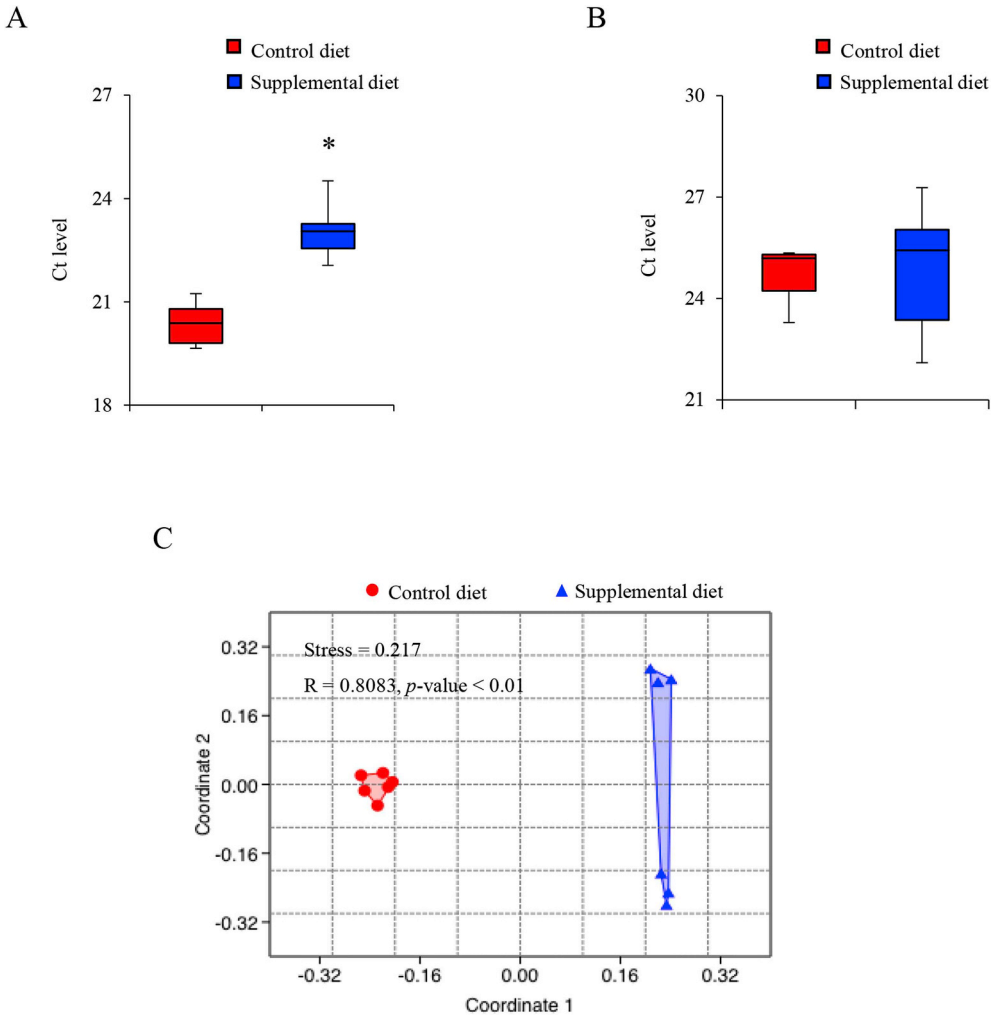

Fig. 2. Bacterial quantification and community analysis in the shrimp GI tract. The GI tract was collected from the shrimp fed a control or *B. aryabhattai* TBRC8450-supplemented diet for 6 weeks. Total genomic DNA was isolated and used for PCR. A. The Ct levels of the 16S rRNA amplicons specific to *Vibrio* spp. B. The Ct levels of the 16S rRNA amplicons specific to the total bacterial populations. Values are the mean ± S.E., n = 6 per group. *, $p < 0.05$. C. nMDS plots using Bray-Curtis dissimilarities based on ASIRA. ARISA profiles were constructed from ITS amplicons. The red dots and blue triangles represented bacterial community isolated from each shrimp group (control or *B. aryabhattai* TBRC8450 supplementation), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

However, the cumulative survival rate was higher in the supplemented group than the control group at all time points. IRR showed significant differences in the survival rate between the challenged groups fed the supplemental and control diet ($p < 0.05$). In the unchallenged groups (both supplemental and control diet), all shrimp survived. Taken together, *B. aryabhattai* supplementation in shrimp improved survival rate during *Vibrio* infections.

4. Discussion

Prophylactic use of antibiotics in aquaculture has raised a serious concern of antibiotic resistance to marine pathogens [63]. An increase in bacterial isolates with multiple antibiotic resistance was found in shrimp farms where antimicrobial compounds were applied [22]. Development of probiotics is an alternative strategy to decrease antibiotic use in aquacultures [64]. *Bacillus* spp. has been previously used as probiotics in shrimp cultivation [30–34]. However, we have shown, for the first time, that *B. aryabhattai*, could be used as a potential probiotic for shrimp cultivation. *B. aryabhattai* TBRC8450 possesses an antimicrobial activity against many members of pathogenic *Vibrio* spp. It also has immune and antioxidant stimulant capability. As a result, shrimp fed *B. aryabhattai* showed resistance to pathogen infections. Our data suggest that shrimp farms could use *B. aryabhattai* as a probiotic to avoid prophylactic antibiotic use in aquacultures.

The members of *Bacillus* spp. have been known to produce a wide range of antimicrobial peptides [65–67]. We postulate that the antimicrobial activity of *B. aryabhattai* may have resulted from antimicrobial peptides produced from the bacterium as evidenced in the spot-on lawn assay. It is worth noting that the antimicrobial activity of *B. aryabhattai* was only detected in the presence of *Vibrio*, suggesting a prerequisite for specific inducers [68]. Thus, we examined the peptide profiles of *B. aryabhattai* co-cultured with *V. harveyi* using a MALDI Biotyper system [69]. An inducible peptide at 6.4 kDa was identified (supplemental data, Fig. S1). This peptide of *B. aryabhattai* was not detected in the absence of *V. harveyi*. These observations suggest that the peptide produced in the presence of a pathogenic bacterial species may possess an antimicrobial activity. However, further studies are warranted.

Similar to that of the territorial animals, the health of marine animals is associated with the microbial community in the GI tract [70]. Infections from pathogenic species not only weaken the host's immunity, but also change the microbiota in the host's digestive system [61,71]. Manipulations of the gut microbiota have been proposed as an alternative method to strengthen the host immunity and to prevent pathogen infections in aquaculture [72]. We showed that supplementation of *B. aryabhattai* could result in a bacterial community shift in the shrimp GI tract. Our results support the notion that the shift in the gut microbiota may be associated with the improved innate

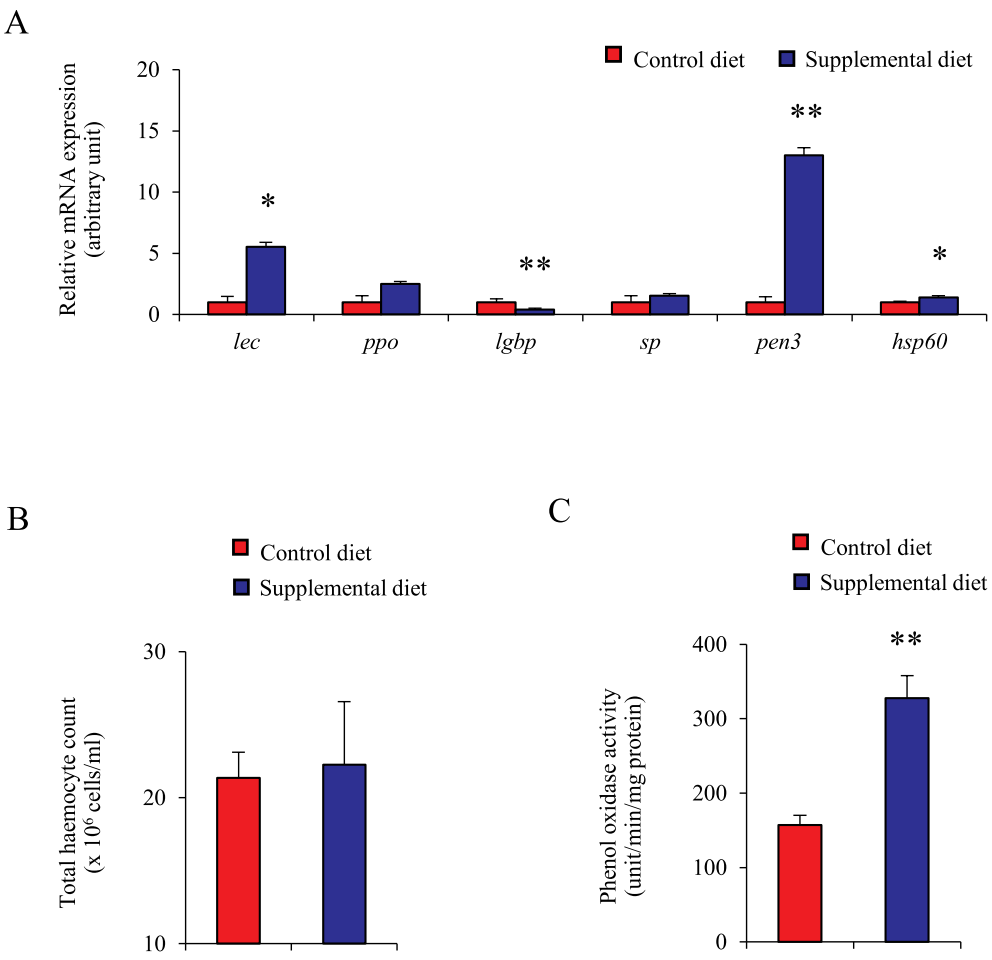

Fig. 3. The immune-associated activities in Pacific white shrimp. The hepatopancreas and hemolymph were collected from shrimp fed a control or *B. aryabhattai* TBRC8450 supplemented-diet for 6 weeks. Hemocytes were isolated and used for biochemical assays. A. Expression of*lec*, *ppo*, *lgβp*, *sp*, *pen3a*, and *hsp60* mRNAs in the shrimp hepatopancreas. The amount of target mRNA was measured by a SYBR-based qPCR. *Actb* was used as the internal reference. Four independent experiments, each with duplicate, were performed. B. Total hemocyte count (THC) in the hemolymph. C. PO activities in the hemocyte. Values are the mean ± S.E., n = 6 per group. *, $p < 0.05$; **, $p < 0.01$.

immunity in shrimp. In addition, we showed that the mRNA levels of the immune function-related as well as oxidative status-related genes were significantly increased in shrimp fed *B. aryabhattai*. Upregulation of *lec*, *pen3,* and *hsp60* expression was detected in the hepatopancreas. It is known that Lec recognizes microbial components and triggers expression of anti-microbial peptides [73]. Knockdown of *lec* in shrimp can lead to bacterial proliferations in the hemolymph causing shrimp death [73]. *Pen3* encodes class 3 penaeidin, which exerts a broad antimicrobial activity spectrum [74]. Pen3 is commonly stored in cells and released to response to pathogen infections [75,76]. Hsp60 is a chaperonin involved in stress responses and the innate immunity [77,78]. Upregulation of *Hsp60* was detected when shrimp were challenged by Gram-positive and Gram-negative bacteria [79]. Moreover, we demonstrated an increase in the haemocyte PO activity when shrimp were supplemented *B. aryabhattai*. PO is a rate-limiting enzyme in the pro-phenoloxidase (PPO) cascade [80]. During infections, the PPO cascade was activated by the pathogen-associated molecular pattern [81]. As a result, PPO zymogen is converted to the active PO, leading to a melanin production [81]. Melanin binds to the bacterial membrane and subsequently elevates hemocyte adhesion in the shrimp immunity [80]. These findings support that *B. aryabhattai* functions as an immunostimulant, which promotes the shrimp innate immunity.

The antioxidant activity has been known to play a key role in the host defense mechanism [82–84]. During pathogen infections, reactive oxygen species (ROS) was generated to eliminate invading pathogens [85,86]. After pathogen clearance, ROS must be promptly removed by the antioxidant system [86]. Without proper ROS elimination, ROS can induce cell apoptosis and tissue injury [87]. Inactivation of antioxidant enzymes can cause sudden death of shrimp after pathogen infections [82]. We demonstrated that supplementation of *B. aryabhattai* TBRC8450 elevated the total antioxidant activity in the shrimp hepatopancreas and plasma. An increase in the antioxidant activity may have resulted from enzymatic and non-enzymatic reactions. Our results showed upregulation of antioxidant-related genes including *fer* and *trx* in the hepatopancreas [54]. Fer, an iron storage protein, functions in detoxification and immunity [88]. Fer administration stimulates innate immunity and increases survival rates of shrimp challenged with a white spot syndrome virus [89]. Trx is a disulfide reductase, which serves as an electron donor in the antioxidant system and functions in the cell defense pathway against oxidative stress and pathogen infections [90–92]. These results demonstrated that *B. aryabhattai* supplementation may at least increase the enzymatic antioxidant activity by upregulation of *fer* and *trx* expression. However, the effect of *B. aryabhattai* supplementation on the non-enzymatic antioxidant remains to be elucidated.

Pathogenic *Vibrio* have been shown to infect shrimp in the GI tract [93]. The pathogen colonizes in the early part of the midgut, releases virulent factors, and invades into the rest of shrimp cells [93,94]. It appears that the use of probiotic supplementation may prevent pathogen infections in aquaculture via different mechanisms, including productions of inhibitory substances, activations of the host immunity, and competitive exclusion [27,95,96]. As expected, supplementation of

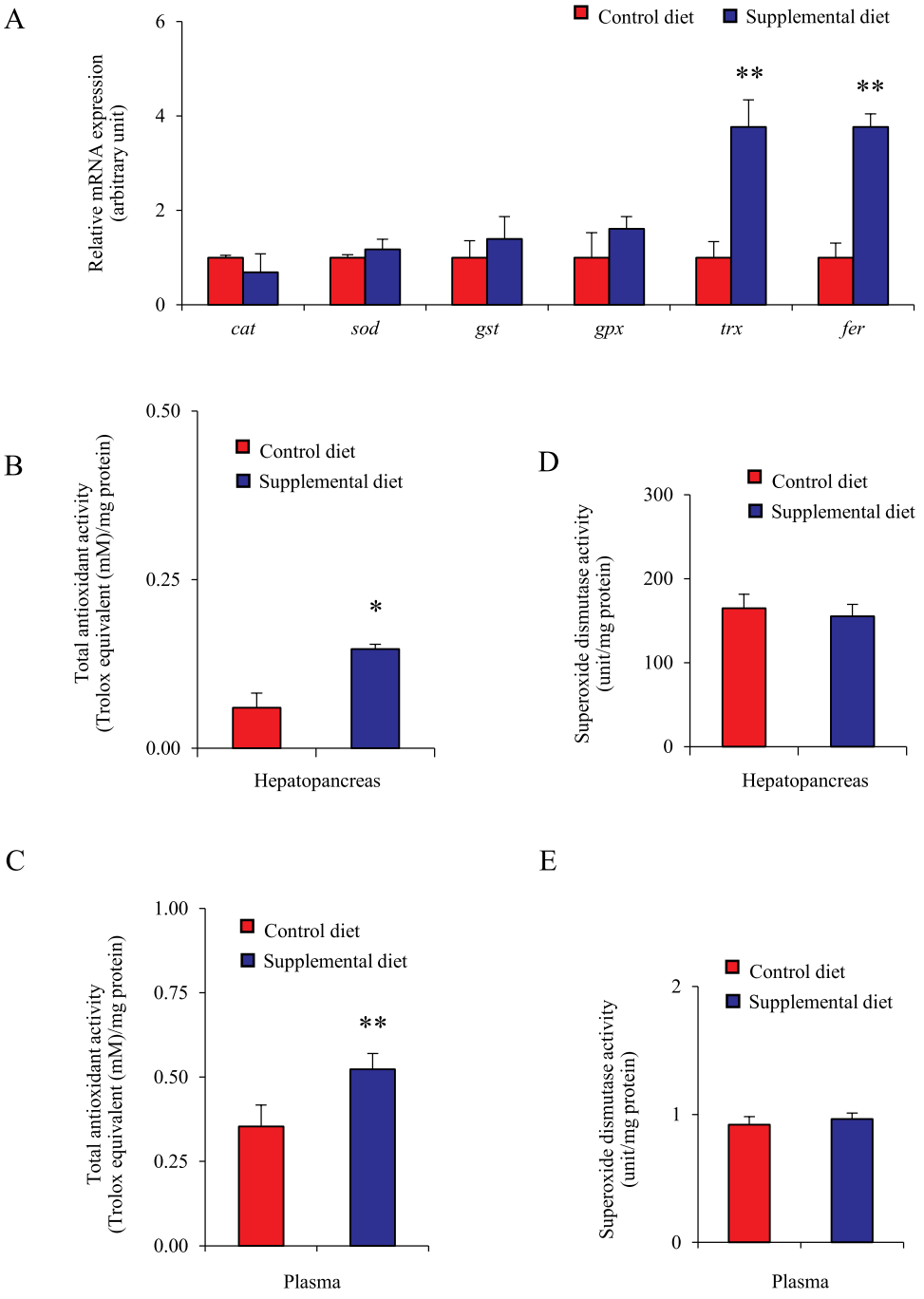

Fig. 4. The antioxidant associated activities in Pacific white shrimp. The hepatopancreas and hemolymph were collected from shrimp fed a control or *B. aryabhattai* TBRC8450 supplemented diet for 6 weeks. Plasma was isolated and used for the biochemical assays. A. Expression of *cat*, *mnsod*, *gst*, *gpx*, *trx*, and *fer* mRNAs. The amount of target mRNA was measured by a SYBR-based qPCR. *Actb* was used as the internal reference. Four independent experiments, each with duplicate, were performed. The total antioxidant activity in the hepatopancreas (B) and plasma (C). Superoxide dismutase (SOD) activities in the hepatopancreas (D) and plasma (E). Values are the mean ± S.E., n = 4–6 per group. *, $p < 0.05$; **, $p < 0.01$.

B. aryabhattai TBRC8450 showed a decrease in the shrimp mortality from *V. harveyi* infections. We speculate that *B. aryabhattai* supplementation enhances pathogen clearance due to its antimicrobial and immune stimulant activities. It is highly possible that colonization of *V. harveyi* in the GI tract was reduced from the antimicrobial activity of *B. aryabhattai*. Enhancement of immune and antioxidant activities of shrimp may be another mechanism contributing to *Vibrio* resistance in *B. aryabhattai* fed shrimp.

In conclusion, *B. aryabhattai* TBRC8450 isolated from the sediment in a shrimp farm possessed an antimicrobial activity against a group of pathogenic *V. harveyi* and *V. parahaemolyticus*. Administration of *B. aryabhattai* resulted in an alteration in bacterial community and decreased *Vibrio* populations in the shrimp GI tract. *B. aryabhattai* supplementation also upregulated immune and antioxidant activities. Shrimp fed *B. aryabhattai* showed an increase in survivability when challenged with *V. harveyi*. These findings suggest that *B. aryabhattai* supplementation is an alternative method to replace some prophylactic antibiotics used for disease prevention and to increase shrimp survival rate during *Vibrio* infections in Pacific white shrimp.

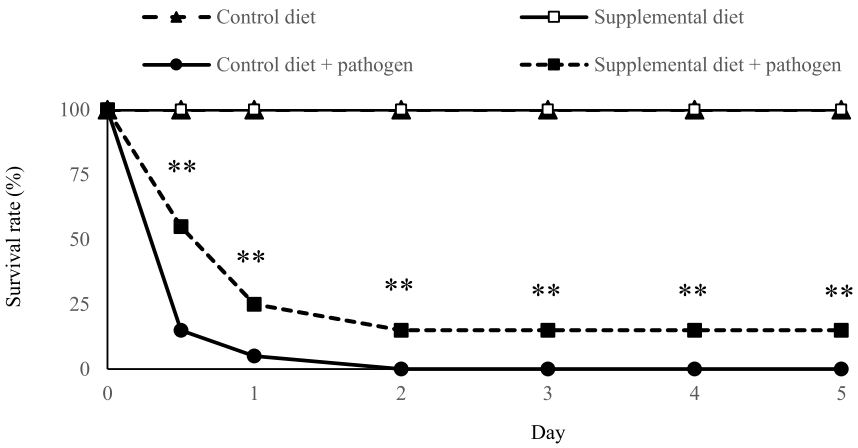

Fig. 5. The survival rate (%) of Pacific white shrimp challenged by *V. harveyi* 1562. Shrimp were fed a control or *B. aryabhattai* TBRC8450 supplemented diet for 4 weeks and challenged with the pathogenic *Vibrio* sp. n = 20 per group. Mortality was monitored at 12 h, and every 24 h for 5 days. *, $p < 0.05$; **, $p < 0.01$.

Conflicts of interest

No conflict of interest is claimed.

Acknowledgements

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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.2018.11.010.

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