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Quorum Quenching Bacteria *Bacillus velezensis* DH82 on Biological Control of *Vibrio parahaemolyticus* for Sustainable Aquaculture of *Litopenaeus vannamei*

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Aquatic pathogens such as *Vibrio parahaemolyticus* cause a bacterial infection that reduces the economic benefits of aquaculture and affects the food quality and safety of human beings. Quorum quenching (QQ) is considered a novel strategy of microbial antagonism that inhibits pathogens and reduces the abuse of antibiotics. This study investigates a QQ bacterial strain, *Bacillus velezensis* DH82 from the deep sea Yap trench, *in vitro* to examine the effects of DH82 and its functional products against *V. parahaemolyticus*, focusing on the Quorum sensing (QS) regulation and the inhibition of pathogenicity and bacterial growth. The study also conducted *in vivo* investigation in the aquaculture of *Litopenaeus vannamei* challenged with *V. parahaemolyticus* by immersion and injection challenge. The results of the QS regulator transcription level demonstrated the multiple QQ enzymes in DH82 regulated the pathogenicity but could not fully control the biofilm formation; the effective antibacterial activity of extracellular peptides on microbial antagonism verified the inhibition on bacterial growth of *V. parahaemolyticus*. The *in vivo* experiment in aquaria demonstrated the effective enrichment of DH82 and inhibition of *Vibrio* in both the aquatic system and the shrimp intestine. The dietary DH82 relieved the negative effect of *Vibrio* on the activity of enzyme acid phosphatase (ACP), alkaline phosphatase (AKP), superoxide dismutase (SOD) under challenge of *Vibrio* pathogens, and was not harmful to host according to lysozyme (LZM) activity. DH82 also ameliorated the damage to the intestine and muscles induced by *V. parahaemolyticus* infection according to tissue imaging. Though DH82 did present some dose-dependent adverse effects to the host, the findings revealed the effective QQ and antibacterial activity of DH82 on emerging biocontrol against *V. parahaemolyticus*, therefore indicating the potential application of DH82 as a biological control reagent in the sustainable and green production of aquaculture.

Keywords: *Bacillus velezensis*, microbial antagonism, biological control, sustainable aquaculture, *Litopenaeus vannamei*, *Vibrio* pathogens, quorum quenching (QQ)

INTRODUCTION

As one of the most important economic activities for food production, marine aquaculture provides high-quality protein resources and therefore is required with high yield and food safety. Though high stocking density aquaculture could maximize the commercial profits on aquatic animal production, the farming conditions would inevitably lead to the frequent occurrence of aquatic diseases (Long et al., 2019), among which, *Vibrio parahaemolyticus* is a common pathogenic bacterium causing diseases to fish, shrimp, and other aquatic animals (Fuente et al., 2015; Jin et al., 2017). Besides, *V. parahaemolyticus* is also the primary food-borne pathogen causing infection in humans (Baker-Austin et al., 2018). *V. parahaemolyticus* could invade aquatic animals via a wound and form a biofilm in the host (Sun et al., 2007), causing a bacterial infection through the released virulence factors under the regulation of quorum sensing (QS) (Chang and Lee, 2018), or intermediate bacterial resistance against antibiotics through the regulation of outer membrane protein and efflux pump protein (Zhao et al., 2018).

There is a lack of effective solutions for dealing with the problems of bacterial infection caused by pathogenic *Vibrio*. Farmers in less developed regions use an abundance of antibiotics when facing outbreaks of *Vibrio* to reduce their losses. Due to the abuse of antibiotics, there is now widespread drug resistance in *V. parahaemolyticus* from aquatic products and aquatic environment (Elmahdi et al., 2016), which not only increases the difficulty of prevention and treatment of *V. parahaemolyticus*, but also makes it more difficult for scientific researchers to develop novel antibiotics. With the probiotics and immune stimulators being applied as feed additives in aquaculture (Ganguly and Mukhopadhayay, 2010), microbial antagonism based on QS inhibition is regarded as an effective alternative to replacing antibiotics (Defoirdt, 2013) on the biological control of aquatic pathogens.

Bacillus is one of the main antagonistic bacteria widely used for biological control in agriculture (Jiang et al., 2018) and aquaculture (Wang C. et al., 2019). Under the regulation of QS (Bareia et al., 2018), *Bacillus* can produce lipopeptides, polyketones, and other metabolites with antibacterial, antiviral, or antitumor activities (Wu et al., 2019). In addition, the acyl-homoserine lactonase AiiA (Dong et al., 2000) widely exists in *Bacillus*, is one of the famous quorum quenching (QQ) enzymes that could recognize and degrade AHLs signal molecules produced by Gram-negative bacteria, and achieve QS inhibition by intervening in the biofilm formation and toxin release of AHLs/Lux mediated pathogens. Nevertheless, the application of QQ enzymes is limited by the enzyme activity and stability under complex environmental conditions of aquaculture and feed production, therefore the *in vivo* study of QQ is more focused on the utilization of *Bacillus* strain. Though *Bacillus* had been verified to modulate gut microbiota and control pathogens as probiotics in shrimp aquaculture (Olmos Soto, 2021), the effects of probiotics with QQ capacity on outbreaks of *Vibrio* pathogens and the host response still need to be verified by *in vivo* experiments, especially under the synergistic effect of the antibacterial metabolites.

In previous work, targeting the prevention and disease control of aquatic pathogens, a marine bacterium, *Bacillus velezensis* DH82 strain, was isolated from underlying seawater of the Western Pacific Yap trench at a depth of 6,000 m (Wang Q. H. et al., 2019) and identified as a QQ bacteria with multiple functional products for microbial antagonism, including the verified extracellular secreted anti-bacterial peptides (Wang et al., 2020) and intracellular products QQ enzymes of AiiA (Liu et al., 2020) and YtnP (Sun et al., 2021). Aiming to qualify the efficiency of DH82 on *Vibrio* control for sustainable aquaculture, especially under the circumstance of *Vibrio* outbreaks, DH82 is *in vitro* investigated against *V. parahaemolyticus* to assess the antibacterial and QQ ability of its functional products and is also used as biocontrol reagents to culture white shrimp *Litopenaeus vannamei*, to investigate the probiotic properties and *in vivo* effects on the host against the infection of *V. parahaemolyticus* by bath and injection challenge under high stocking density.

MATERIALS AND METHODS

Bacteria, Plasmids, and Reagents

Bacillus velezensis DH82 strain (GenBank accession no. MK203035) was isolated from the seawater of the Western Pacific Yap trench at the depth of 6,000 m and was kindly provided by the Third Institute of Oceanography (Xiamen, China). The engineered Escherichia *coli* BL21 (DE3) strains respectively carry the expression clone of AiiA and YtnP from DH82, *E. coli:*pET28a/AiiA (Liu et al., 2020) and *E. coli:*pET28a/YtnP (Sun et al., 2021), which were constructed in this lab in previous work. *V. parahaemolyticus* 17SZ strain was isolated from the infected shrimp sample in the *Vibrio* breakout case at Xiamen in 2017 and was provided by the Center for Disease Control of Siming District, Xiamen, China. The AHLs, C6-HSL (Cat. 56395), 3-O-C6-HSL (Cat. K3255), 3-O-C10-HSL (Cat. O9014), and 3-O-C12-HSL (Cat. O9139), were purchased from Sigma-Aldrich.

Bacterial Culture and Preparation of Functional Products

The QQ enzyme expressing strain was cultured in Luria–Bertani (LB) media containing 50 mg/L kanamycin with shaking at 180 rpm at 37°C, 0.2 mM IPTG was inoculated after 2-h incubation of bacterial culture to induce the protein expression. The overnight cultured bacteria were washed and concentrated 5:1 with PBS (pH 7.0), performed ultrasonic breaking (operate for 3 s at 300 W then break for 6 s, and repeat for 60 times) on ice and centrifuged at 4°C at 8,000 g for 15 min to harvest the crude extract in the supernatant. The supernatant containing crude enzyme was loaded to the high affinity NI-NTA chromatography, washed using a lysis buffer (300 mM NaCl and 50 mM NaH_2PO_4, pH 7.4), then washed with an imidazole elution buffer (300 mM NaCl, 200 mM imidazole, and 50 mM NaH_2PO_4, pH 7.4) to purify the His-tagged enzyme. The purified enzyme was sterilized by filtration using 0.22 μm syringe filter (Millipore, Cat. SLGP033R) and analyzed by SDS-PAGE. The concentration of protein was qualified using the Bradford assay.

The overnight culture of DH82 was inoculated 1% (v/v) in 1 L fresh LB broth (10 g/L Tryptone, 5 g/L Yeast extract, and 10 g/L NaCl, pH7.0) and cultured with shaking at 180 rpm at 37°C for 24 h. The bacterial culture was centrifuged at 10,000 rpm at 4°C for 10 min to harvest supernatant, then filter sterilized with 0.22 μm membrane. The filtered supernatant was added slowly with $(NH_4)_2SO_4$ to 70% of saturability, standing overnight at 4°C, and was centrifuged at 10,000 rpm at 4°C for 20 min to collect the salting-out proteins. The salting-out proteins were re-dissolved in 10 mL PBS (pH 7.0) to harvest crude proteins. The crude proteins were then desalted using Solarbio MD44 dialysis membrane (Cat. YA1078) by soaking in distilled water for 18 h and stored for further study.

DH82 were additionally prepared in a 200 L fermentation tank (15 g/L glucose, 20 g/L bean pulp, 10 g/L peptone, 5 g/L corn flour, 0.5 g/L $MgSO_4$, 1 g/L K_2HPO_4, 2 g/L NaCl, 3 g/L $CaCO_3$, pH 7.0), attached with corn starch and dried for granulation to produce bacterial powder, at a final concentration of 10^{11} CFU/g, for further *in vivo* study.

Agar Diffusion Assay for Antimicrobial Activity Against *Vibrio parahaemolyticus*

A 1.5 mL overnight culture of 17SZ in LB broth was mixed with 150 mL fresh LB medium (1.5% agar) maintained at 50°C. The agar-culture solution was immediately poured as 20 mL portions in petri dishes. Each 2 mL DH82 overnight culture in LB broth was centrifuged at 6,000 g for 5 min, then filtered using 0.22 μm membrane to collect the supernatant. The pellets were resuspended in PBS and then treated with 1 μg/mL lysozyme at 37°C for 30 min, then centrifuged at 8,000 g for 5 min to harvest the bacterial lysate from the supernatant. 50 μL of above prepared supernatant, cell pellets, and cell lysate were respectively spotted in wells punched in the solidified agar, compared with 50 μL of salting-out antibacterial protein as a positive control. The plates were incubated at 30°C for 24 h for examination of the bacterial inhibition zone.

Assessment of Quorum Quenching Activity Against *Vibrio parahaemolyticus* by RT-qPCR

Bacterial Growth of *Vibrio parahaemolyticus* Under Quorum Quenching Regulation

The overnight culture of 17SZ was diluted to OD_{600} value at 0.1, then 1:100 (v/v) inoculated in 40 mL fresh LB broth for incubation with shaking at 30°C for 12 h, with additional QQ enzyme at a final concentration of 50 μg/mL for treatment, and the same volume of sterile water as the negative control. The bacterial density at OD_{600} was measured to obtain the growth curve.

Analysis of Quorum Quenching Regulation by RT-qPCR

Each 1 mL of 17SZ culture was sampled at 1, 2, 4, 6, and 12 h during growth, centrifuged at 6,000 g for 5 min, and resuspended with the same volume of sterile water to harvest the bacterial pellets. RNA samples were respectively extracted from the pellets using Trizol RNA extraction kit (Takara, Dalian) and then used as a template for reverse transcription by PrimeScriptRT reagent Kit with gDNA Eraser (Takara) to construct cDNA for the further qPCR program. The primers listed in **Table 1** were respectively used to amplify the sequence of 16S rRNA, *aphA*, *opaR*, *tlh* by qPCR using Bio-Rad CFX Connect. The transcription levels of key regulators on QS in *V. parahaemolyticus* were analyzed, compared with that of 16S rRNA, to determine the activity of QQ enzymes on bacterial inhibition against *V. parahaemolyticus.*

Microplate Biofilm Assay by Crystal Violet

One microliter of 17SZ overnight culture was inoculated in 200 μL of Biofilm medium [BM, filter sterilized tap water supplemented with 5 mM sodium citrate, 0.5% casamino acids (Difco) and 0.5% v/v standard brain heart infusion broth (Difco)] in 96 well microplates, with additional 50 μg/mL QQ enzyme for treatment in triplicate, compared with sterilized water as a negative control. Bacterial cultures were grown statically at 30°C for 3 days when a luxuriant biofilm was apparent. After this time the wells were subsequently washed with phosphate buffered saline (PBS). Two hundred microliters of 0.1% crystal violet solution was then added to each well and left for 15 min at room temperature, after which the crystal violet was removed prior to three washes with PBS. Two hundred microliters of ethanol was then added to each well to dissolve any crystal violet bound to the well and any remaining biofilm. After 15 min at room temperature, the absorbance of the wells was measured at 580 nm using Tecan Infinite M200 Pro.

In vitro Assessment of DH82 and the Fractions on Pathogen Inhibition

Each 100 μL DH82 overnight culture in LB broth was centrifuged at 6,000 g for 5 min to collect the supernatant and cell pellets. The pellets were resuspended in PBS and then treated with 1 μg/mL lysozyme at 37°C for 30 min, then centrifuged at 8,000 g for 5 min to harvest the bacterial lysate from the supernatant. Each 100 μL of DH82 supernatant and lysate, 0.1, 1, and 10 μL DH82 overnight culture, 0.1, 1 and 10 μL crude QQ enzyme as well, were respectively mixed with 1 μL of 17SZ overnight culture in 200 BM medium in 96 well microplates, to assess the effect of different fractions of DH82 on the inhibition of biofilm formation and planktonic bacterial amount of 17SZ, compared with PBS as a negative control.

TABLE 1 | List of primers for RT-qPCR.

Primer	Sequence (5′–3′)	References
16S rRNA-F	GAGGGTGACTCTGCGG	This study
16S rRNA-R	CGGCATCCGTCATGATG	This study
aphA-F	AGCATCGGTTACTTCTGGAAAG	Zhang et al., 2016
aphA-R	GTTGAACAGCACAAGCCATAAG	Zhang et al., 2016
opaR-F	TGTCTACCAACCGCACTAACC	Zhang et al., 2016
opaR-R	GCTCTTTC AACTCGGCTTCAC	Zhang et al., 2016
tlh-F	ACTCAACACAAGAAGAGATCGACAA	Niu et al., 2018
tlh-R	GATGAGCGGTTGATGTCCAA	Niu et al., 2018

The above mixtures were incubated in BM at 30°C for 3 days to form biofilms. The biofilms were analyzed by crystal violet assay, and the supernatants of the biofilm cultures were spread on LB agar plates and incubated at 37°C for 24 h to calculate the planktonic bacterial amount by plate counting method.

In vivo Assessment on the *Vibrio* Control of DH82

The trial experiments of shrimp farming were performed in aquaria located at Zhaoan, Zhangzhou, Fujian Province, China (longitude 117.17501, latitude 23.71148). White shrimp (*L. vannamei*), with a length of 10.0 ± 1.0 cm and the weight of 13.3 ± 0.8 g, were grown in an aquarium (length × width × depth = 96 mm × 74 mm × 66 mm) containing 300 L fresh seawater and continuous aeration under high stocking density. The cultures in each aquarium were absorbed by siphon to remove excreta of shrimps and pumped in 30 L fresh seawater to refill the aquarium at noon every day. The aquaria were placed indoors with good air circulation.

The shrimps were fed using non-antibiotic commercial feed thrice daily (6 a.m., 12 a.m., and 6 p.m.) for 1 week to acclimatize to the diet before use. The experiments were approved by the institutional research ethics committee of Huaqiao University and carried out in accordance with the PRC National Standard GB T 35823-2018.

In vivo Assessment Under Bath Challenge

150 shrimps per aquaria were randomly allocated for five treatments in duplicate for 28 days, respectively treated with 100 mL of fresh LB broth (blank) as control (group A), same volume of 17SZ (group B), DH82 (group C), continuous daily dietary of DH82 with the same volume (group D), and the challenge with the same volume of 17SZ on day 1 along with daily dietary of DH82 (group E). The bacterial culture of 17SZ ($OD_{600\,nm}$ 0.6, 10^8 CFU/mL) was directly poured into the aquaria to simulate the emerging out-breaking of *V. parahaemolyticus*, whilst the 0.3 g pre-made DH82 powder was activated in 300 mL of 100 g/L brown sugar water and mixed with feeding stuff to feed the shrimps, to achieve the bacterial inoculation with a final concentration of 10^5 CFU/mL (Ottaviani et al., 2020) to both 17SZ and DH82 after seawater exchanging.

The water samples in each group were collected and spread on an LB agar medium to calculate the planktonic bacterial number, the Sodium Chloride Polymyxin Base (SCPB) medium containing 250 U/mL Polymyxin B was used to calculate the bacterial number of *Vibrio* sp.

DNA Extraction and High Throughput Sequencing

Total genomic DNA of microbial community were extracted from the intestine samples of the shrimps in blank groups (int_12), treated with DH82 in the first week (int_13), infected with 17SZ (int_20), and those treated with continuous dietary DH82 after 17SZ infection (int_15), using an MP FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, United States) according to the manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Shanghai) and used as template DNA to amplify the hypervariable region V3-V4 of the bacterial 16s rRNA with the primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by PCR using Phusion® High-Fidelity PCR Master Mix kit (NEB). The PCR amplification of 16s rRNA was performed as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturing at 98°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 15 s, and single extension at 72°C for 5 min, and end at 4°C. The PCR products were extracted from 2% agarose gel and purified using a DNA Gel Extraction Kit (Omega Bio-tek, China) according to the manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, Beijing).

The purified amplicons were pooled in equimolar and paired-end sequenced on Illumina MiSeq PE300 platform/NovaSeqPE250 platform (Illumina, San Diego, CA, United States) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 (Chen et al., 2018), and merged by FLASH version 1.2.7 (Magoč and Salzberg, 2011) with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region is 0.2. Reads that could not be assembled were discarded; (iii) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted with exact barcode matching or a maximum two nucleotide mismatch in primer matching.

Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using the UPARSE version7.1 (Edgar, 2013), and the chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 (Wang et al., 2007) according to the SILVA ribosomal RNA database using a confidence threshold of 0.7. Alpha diversity index including observed OTUs, richness estimators, such as Ace, Chao, Shannon, and Simpson index, were calculated based on the frequency of OTUs and genera in the assigned sequence collections after rare sequences were removed. The OTUs of int_15 and int_20 were analyzed by Fisher's exact test to compare the difference of the abundance on genus level.

In vivo Assessment Under Injection Challenge

50 shrimps were randomly allocated for 10 treatments for 7 days (five replicates per treatment), respectively injected on day 1 by syringe with 1 mL of 17SZ and DH82 mixture (1:1) at high dose ($OD_{600\ nm}$ 0.6, 10^8 CFU/mL), medium dose ($OD_{600\ nm}$ 0.06, 10^7 CFU/mL) and low dose ($OD_{600\ nm}$ 0.006, 10^6 CFU/mL), compared with those individually injected with the same volume of PBS (pH 7.0), 17SZ and DH82 at the same dose (1:1 mixed with PBS at pH 7.0) as control. The injections were performed on the muscle of the shrimp between the 5th and 6th uromere. The shrimps under injection challenge in each group were partitioned

by nets in the aquarium and continuously fed with non-antibiotic feed as normal. The survival number and elapsed days were recorded and analyzed using GraphPad Prism 6 software.

Analysis of Non-specific Immunity

Approximate 1 mL of hemolymph were sampled from the ventral sinus of each shrimp on day 28 after the bath challenge, incubated at 4°C overnight to separate the serum. The serums were centrifuged at 1,000 g for 15 min at 4°C to harvest the supernatants. The enzyme activities of alkaline phosphatase (AKP), acid phosphatase (ACP), superoxide dismutase (SOD), catalase (CAT), and lysozyme (LZM) in the supernatants were respectively assessed using commercial detection kits (Nanjing Jiancheng Bioengineering Institute) and measured using MD microplate reader (SpectraMax M5).

Analysis of Infected Tissues by Hematoxylin-Eosin (H&E) Staining Assay

The infected tissues of white shrimps (intestines from bath challenge and muscles from injection challenge) were immersed in Bouin's Fixative for 4 h and transferred to 70% ethanol. The individual lobes of infected tissue biopsy material were placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in paraffin wax blocks. The tissue sections were dewaxed twice in xylene for 20 min, rehydrated through decreasing concentrations of ethanol from 100% to 75% for 5 min in each step, and washed in sterile water. The sections were then stained with hematoxylin for 5 min, rinse with sterile water, followed with increasing concentration of ethanol from 85, 95% for 5 min in each step, and stained with eosin for 5 min. After staining, the sections were dehydrated through 100% ethanol thrice and xylene twice for 5 min in each step and sealed with neutral gum. The tissue sections were imaged using a brightfield microscope (Nikon Eclipse E100) and an imaging system (Nikon DS-U3).

Statistical Analysis

The experimental data were analyzed by the software GraphPad Prism 6. P value from T-test were used to determine the difference between each experimental group.

RESULTS

Functionals of Bacterial Products From DH82 on Antagonism

The recombinant QQ enzymes were investigated on the *in vitro* effect of QQ against *V. parahaemolyticus*, as shown in **Figure 1**, the treatment of AiiA and YtnP both present a tendency of depression on the gene expression level of key regulatory proteins AphA and OpaR during bacterial growth. AiiA significant functioned at 2 h (*P*-value was 0.00027) and 12 h (*P*-value was 0.029) on *aphA* and significantly depressed on *opaR* at 4 h (*P*-value was 0.018); by contrast, YtnP was observed with higher activity on the down-regulation, significantly depressed *aphA* at early stage of 1 h (*P*-value was 5.6E-05), 2 h (*P*-value was 3.9E-07) and 4 h (*P*-value was 0.049), and down-regulated *opaR* at 4 h (*P*-value was 0.013), which indicated the potential cooperation of AiiA and YtnP, and possibly involving other unidentified QQ enzymes in DH82, on the inhibition against pathogens at different growth stages.

As shown in **Figure 1C**, the presence of QQ enzymes both remarkably down-regulated the expression level of virulence factors, thermolabile hemolysin (TLH), that the treatment of AiiA present significant difference during the whole test period of 12 h (*P*-value at 1, 2, 4, and 6 h were all lower than 0.001, and was 0.022 at 12 h), and YtnP functioned with an extremely significant difference at 1, 2, 4, and 12 h (*P*-value all lower than 0.001).

The effects of QQ enzymes on biofilm formation of *V. parahaemolyticus* were later investigated (Panel D). The results demonstrated a slight decrease but no significant difference under the treatment of respectively QQ enzyme of AiiA and YtnP (*P*-value was 0.19 and 0.41). Besides, the addition of different types of exogenous AHLs presented no difference on biofilm formation of 17SZ (*P*-value all above 0.05), which indicated that AHL mediated QS might not be the only pathway on the combined intermediation of 17SZ on biofilm formation.

Considering the unsatisfactory inhibition on biofilm formation by QQ enzymes, the DH82 pellets and the functional products in different fractions, intracellular QQ enzymes in the lysate fraction, extracellular antibacterial peptides in the supernatant of bacterial culture, and the salting-out crude antimicrobial proteins, were investigated for their effects on the growth of *V. parahaemolyticus.* As shown in **Table 2**, the results of antagonistic activity demonstrated positive antibacterial ability by bacterial supernatant (with inhibitory zone at a diameter of 19.4 $\pm$ 0.2 mm) and cell pellets of DH82 (with inhibitory zone at a diameter of 16.5 $\pm$ 0.3 mm), and negative inhibition by the cell lysate; whilst the crude proteins extracted from the supernatant (approximately 100 times concentrated) presented a larger inhibitory zone at a diameter of 24.3 $\pm$ 0.2 mm. The results indicated that the antibacterial property of DH82 was contributed by the secreted extracellular peptides, but not the intracellular products containing QQ enzymes in cell lysate.

In vitro Assessment of *Vibrio* Control by Different Fractions of DH82

As the status transition between biofilm and planktonic cells of *V. parahaemolyticus*, an *in vitro* assessment was designed to investigate the synergistic effects of functional products from DH82 on the *Vibrio* control. The cell pellets, cell lysate, supernatant of overnight culture, and an enzyme mixture of AiiA and YtnP were used to assess the function of different fractions from DH82 on microbial antagonism against *V. parahaemolyticus*. As shown in **Figure 2**, the DH82 lysate significantly decreased the biofilm formation of 17SZ (*P*-value was 0.0045) and showed no difference to the planktonic bacteria; whilst the supernatant of DH82 containing secreted anti-microbial peptides also limited the biofilm formation (*P*-value was 0.0026) and reduced approximately half of the planktonic 17SZ. The whole pellets of DH82 demonstrated a dose dependent magnitude reduction on planktonic 17SZ and slight down-regulation of the biofilm formation, including the few biofilms

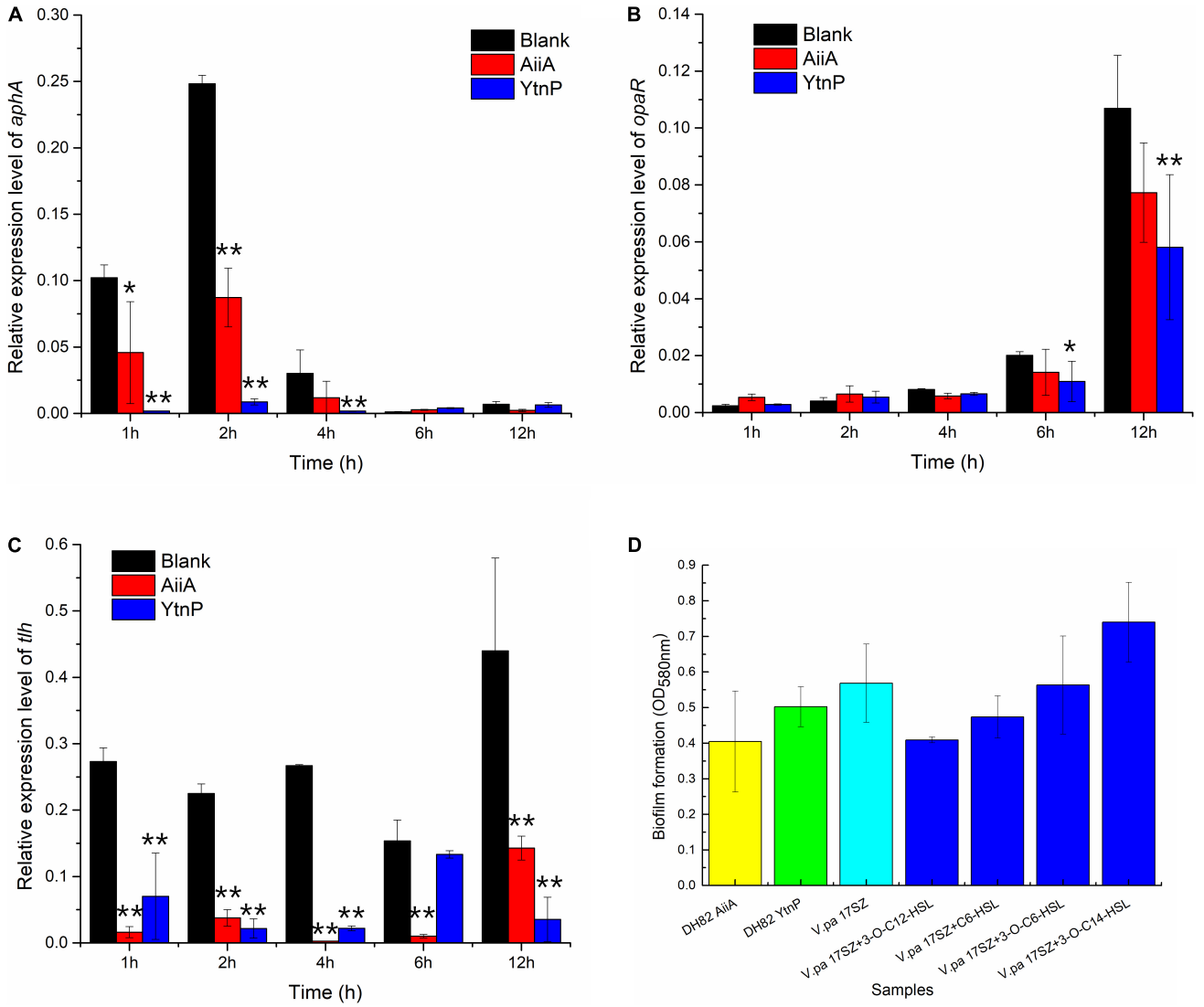

FIGURE 1 | Effect of quorum quenching (QQ) enzymes on the regulation of QS against *Vibrio parahaemolyticus.* Fifty microgram/milliliter QQ enzyme was used to treat the 17SZ culture [OD_{600} 0.1, 1:100 (v/v) inoculation] in LB at 30°C. RNA from bacterial culture sampled at 1, 2, 4, 6, and 12 h was used as temperate for RT-qPCR, using 16S rRNA as internal reference. Panel **(A)** is the effect on the expression of *aphA*; Panel **(B)** is the effect on the expression of *opaR;* Panel **(C)** is the effect on the expression of *tlh*; Panel **(D)** is the effect of QQ enzyme on biofilm formation of 17SZ. The error bars are presented as the standard deviation. Statistical analysis results are presented by a significant difference indicated by ** where $p < 0.01$ and * where $p < 0.05$.

formed by DH82, compared with the control groups of 17SZ and different dose injections of DH82. The treatment of mixed enzymes of AiiA and YtnP also presented a similar tendency on biofilm control to that of bacterial pellets and lysate, but no significant reduction on planktonic 17SZ.

In vivo Assessment of *Vibrio* Control Under Bath Challenge

Compared with the *in vitro* experiment, the *in vivo* experiment of bacterial challenge in shrimp trial experiment is designed to assess the synergistic effects of DH82 strain with the multiple functional products on *Vibrio* control in aquaria with pump-in seawater, which contained the inoculated *Bacillus* and *Vibrio* and other microorganisms from marine environments. We monitored the total amount of planktonic microorganisms in the culture system, as shown in **Figure 3**, compared with the blank control, the total number of *Bacillus* and *Vibrio* in the

TABLE 2 | Antagonistic activity of DH82 against *Vibrio parahaemolyticus* 17SZ.

Fractions of DH82	Inhibitory zone (diameter/mm)
Supernatant	19.4 ± 0.2
Cell pellets	16.5 ± 0.3
Cell lysate	NA
Crude proteins from supernatant	24.3 ± 0.2

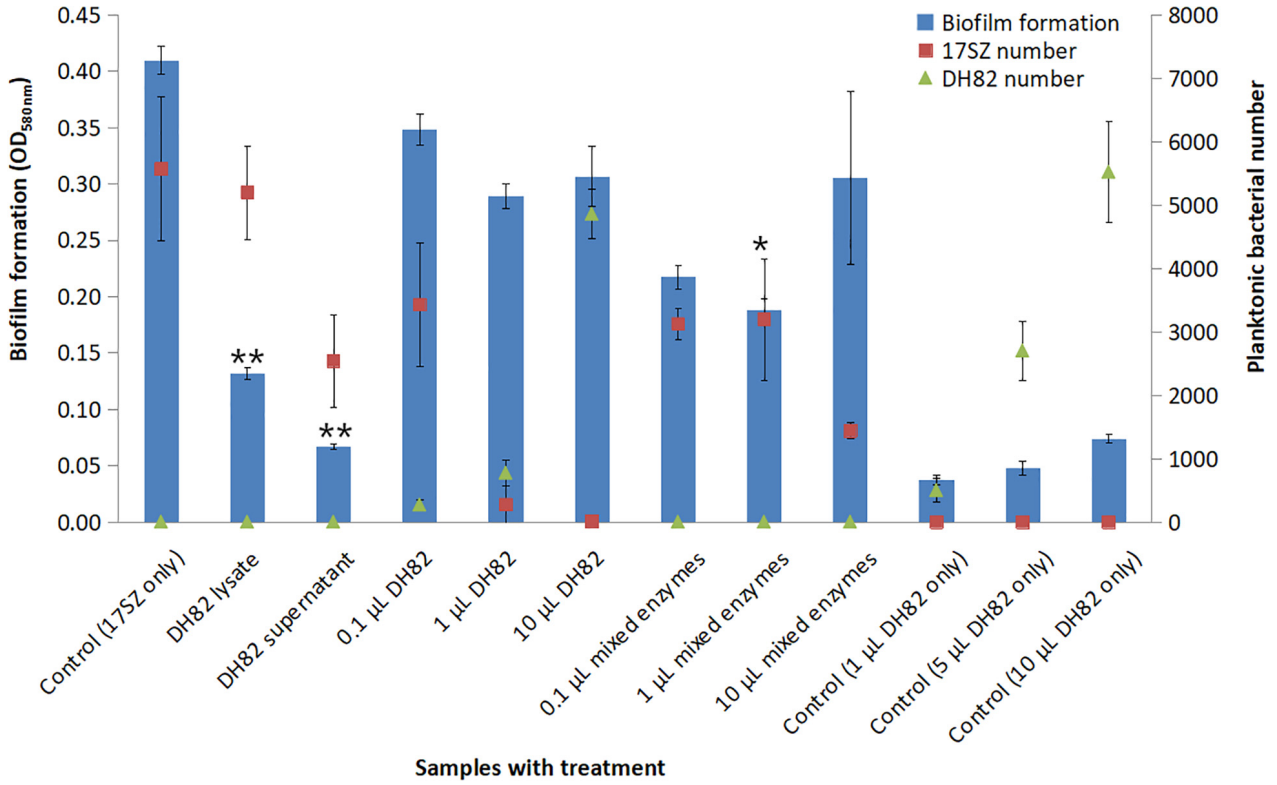

FIGURE 2 | Effect of fractions of DH82 on the biofilm and planktonic bacterial amount of *Vibrio parahaemolyticus*. Each 100 μL of DH82 supernatant and lysate, 0.1, 1, and 10 μL DH82 overnight culture (10^7 CFU/mL), 0.1, 1, and 10 μL QQ enzyme (AiiA and YtnP mixture) were respectively mixed with 1 μL of 17SZ overnight culture ($OD_{600\ nm}$ 0.1, 10^8 CFU/mL) in 200 BM medium, compared with 17SZ with PBS as a negative control, the mixtures were incubated in 30°C for 3 days. The biofilms were analyzed by crystal violet assay, and the supernatants of biofilm cultures were spread on Luria–Bertani (LB) agar plates to calculate the planktonic bacterial number. The biofilms are presented by blue bars, the number of planktonic bacteria of 17SZ is presented ■ in red, the DH82 by ▲ in green. The error bars are presented as the standard deviation. The error bars are presented as the standard deviation. Statistical analysis results are presented by the significant difference indicated by * where $p < 0.05$ and ** where $p < 0.01$.

treatment group with DH82 and 17SZ alone in the system showed quantitative advantages respectively 1 day after the bacterial solution was added for bath challenge. In the treatment group with a mixture of DH82 and 17SZ, although *Vibrio* was still countable, its number was significantly lower than that in the single challenge group with 17SZ alone, which indicated the bacterial inhibition of DH82 against *Vibrio*.

In the subsequent weekly sampling, the total amount of planktonic bacteria in the aquaria fluctuated greatly affected by the environment. The addition of DH82 in the first week did not show the quantitative advantage of *Bacillus* in the following 3 weeks, whilst *Bacillus* was continuously detected and dominant in the treatment group of continuous addition of DH82, and *Vibrio* was inhibited at a low level. In addition, it could be seen that a high number of *Vibrios* were detected in the treatment group of 17SZ alone during the whole bacterial bath challenge period. Since there was no artificial addition of *Vibrios* after the first day, considering the good biofilm-forming capacity of 17SZ observed in the *in vitro* experiment, and compared with the *Vibrio* number in other treatment groups, it could be inferred that the planktonic *Vibrios* might transform from the biofilm statues and cause continuous influence to the shrimps.

High throughput sequencing on 16s rRNA was also used to analyze the bacterial community of gut microbiota on genus level, between the 17SZ infected shrimps (int_20) and those treated with continuous dietary DH82 after *Vibrio* infection (int_15), compared with the blank groups (int_12) and those treated with DH82 in the first week (int_13) as control.

The richness of microbial community from each sample was analyzed by the Alpha index, as shown in **Table 3**. The results of OTU coverage in each group were all above 99.84%, which indicated the good recovery and reliable reflection of the tested samples. The results of a comprehensive assessment on the four indexes of Ace, Chao, Shannon, and Simpson demonstrated adverse impact on the richness of bacterial community caused by *Vibrio* infection, and recovery under the treatment of continuous DH82.

The microbial community of each group is shown in **Figure 4**. The DH82 treated shrimps were observed with significantly more abundant *Bacillus* sp. existed (respectively took 7.266% out of 7.457% in the gut of DH82 feed shrimps, and only 0.03143% out of 0.03404% in 17SZ infected ones), and significant lower richness and abundance of *Vibrio* sp. in the intestine (respectively 0.3842% in DH82 treated groups and 5.57% in 17SZ infected

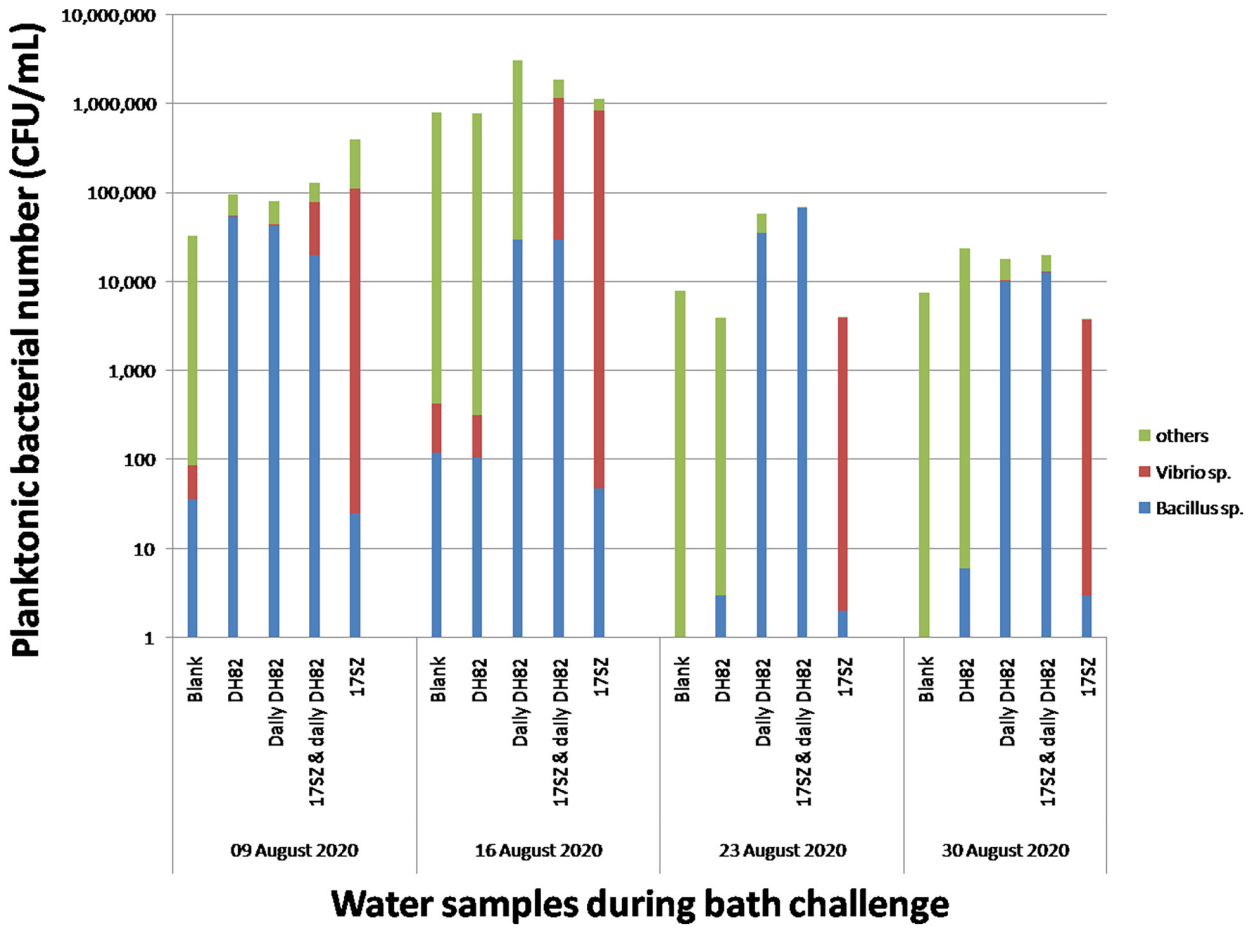

FIGURE 3 | Effects of DH82 on *Vibrio* control under bath challenge. Each 100 mL bacterial culture (OD_{600nm} 0.1) was individually poured into aquaria for bacterial bath challenge to white shrimps with the final concentration of 10^5 CFU/mL. The water samples in each group were collected before water exchange and spread on Luria–Bertani (LB) agar plates to calculate the bacterial number of *Bacillus* sp. and total amount, and on SCPB agar plates to calculate the number of *Vibrio* sp. The number of *Bacillus* sp. is presented by column in blue, *Vibrio* sp. in red and other bacteria in green. The vertical axis in the stacked column graph is marked as a logarithmic scale.

TABLE 3 | Richness and diversity of intestinal bacterial community.

Sample	Treatment	Coverage	Ace	Chao	Shannon	Simpson
int_12	Blank	99.88%	357.4140	380.1818	3.6718	0.0514
int_13	DH82 in the first week	99.84%	354.4368	383.5000	3.7506	0.0430
int_15	17SZ and continuous DH82	99.84%	353.2687	371.3704	3.4722	0.0597
int_20	17SZ	99.91%	242.6522	229.0303	2.5436	0.2268

groups), where both *P*-values were lower than 1E-15. Among these, the *Bacillus* community mostly contributed *B. velezensis* on a species level, whose corresponding OTU sequence was 100% matched to that of 16s rDNA of *B. velezensis*, according to the analysis of further alignment. The results verified the successful colonization of DH82 and the effective inhibition against pathogenic *Vibrio* in shrimp guts.

The enzyme activities of ACP, AKP, SOD, and LZM in shrimp serum were assessed to demonstrate the non-specific immune response of shrimp to the challenge by bacterial bath, as shown in **Figure 5**.

The results demonstrated that ACP activity (Panel A) significantly decreased under the challenge of *V. parahaemolyticus* (*P*-value was 0.013), even with the control of DH82 (*P*-value was 0.030), which indicated the irreversible adverse effects of the pathogen to shrimp on the growth factor of ACP. Moreover, the additional DH82 in the first week improved the ACP activities, however, there was no significant difference to the blank group, and somehow the continuous treatment of DH82 also presented adverse effects to ACP activity.

A similar tendency was observed in the data of AKP activities (Panel B), that the initial delivery of DH82 showed no difference

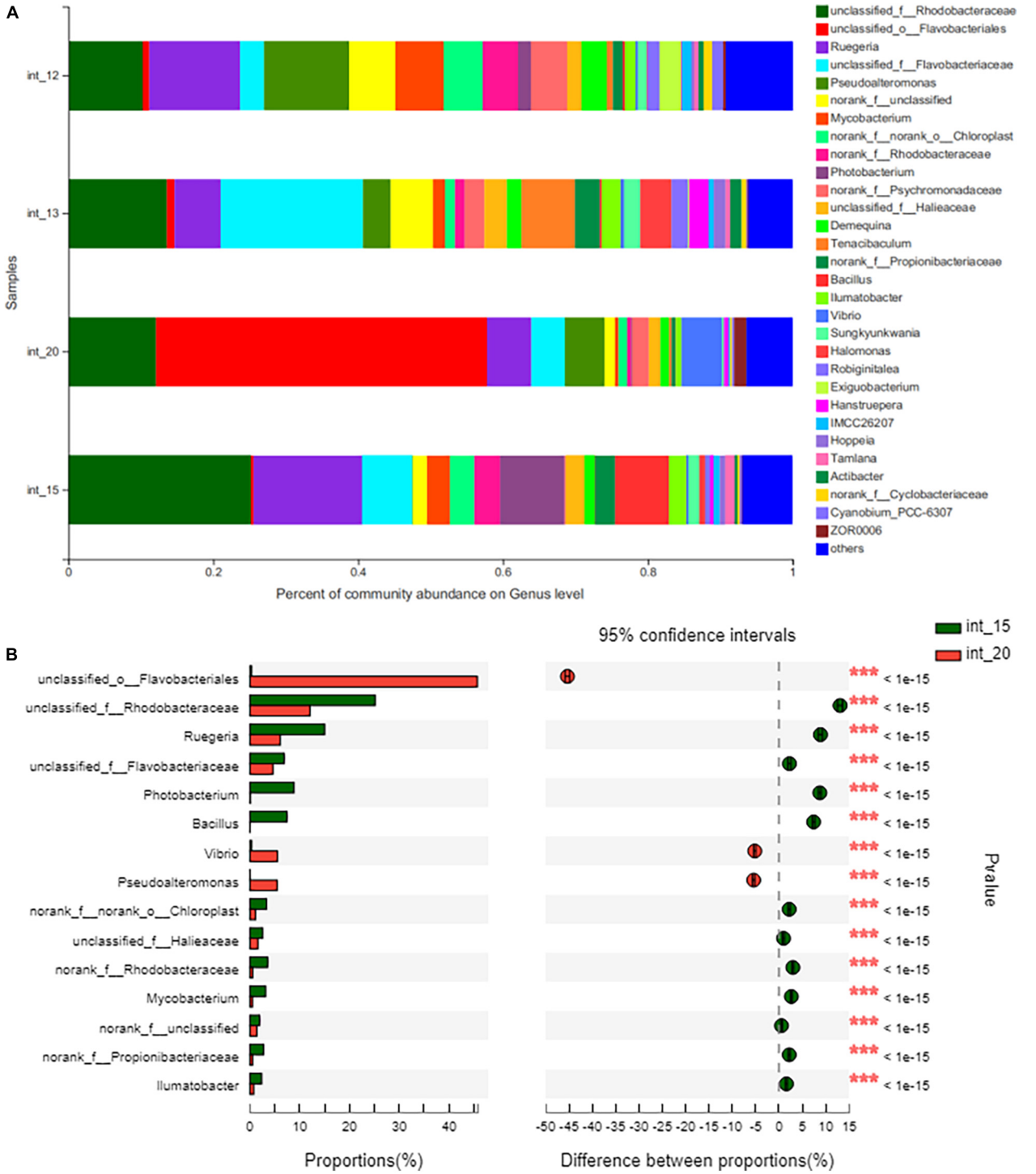

FIGURE 4 | Intestinal bacterial community of shrimps under bath challenge. The total genomic DNA from the intestine sample of the shrimps under the treatment of blank (int_12), DH82 in the first week (int_13), 17SZ then continuous with DH82 (int_15), and the 17SZ only (int_20) were respectively performed high throughput sequencing on the 16s rRNA. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 and classified on genus level. The OTUs were analyzed by Fisher's exact test, to compare the difference of abundance between treatments. Panel **(A)** is the microbial structure presented by relative community abundance, the bar plot of *Bacillus* is arrowed in red and that of *Vibrio* in blue; Panel **(B)** is the difference of abundance with DH82 treatment after 17SZ infection, the top 15 genera with the highest abundance were listed, including the *Bacillus* sp. and *Vibrio* sp. Statistical analysis results are presented by a significant difference indicated by *** where $p \leq 0.001$.

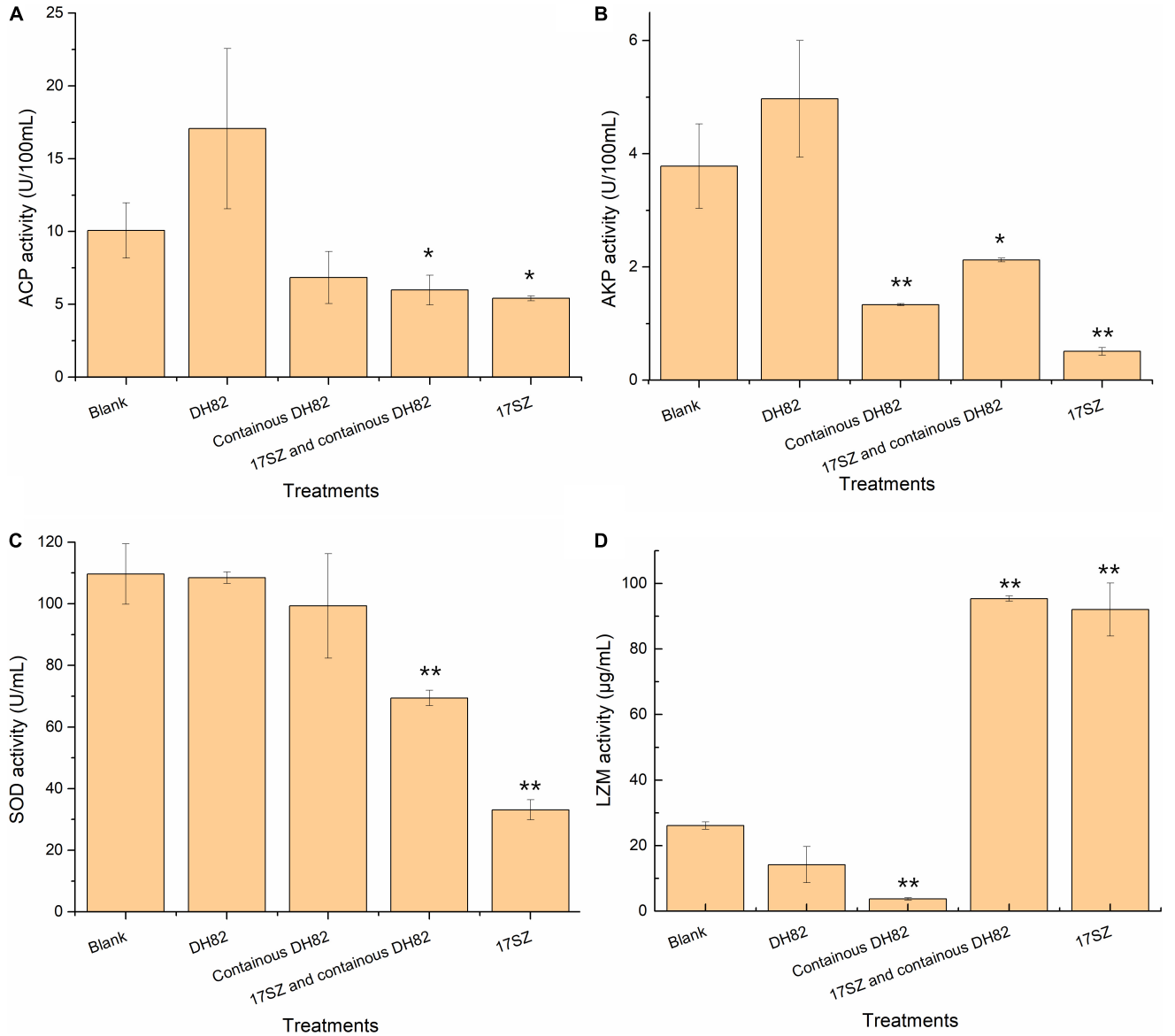

FIGURE 5 | Effect of bacterial treatments to acid phosphatase (ACP), alkaline phosphatase (AKP), superoxide dismutase (SOD), and lysozyme (LZM) activities in the serum of shrimps. Each 100 mL bacterial culture (OD_{600nm} 0.1) was individually poured into aquaria for bacterial bath challenge to shrimps with final concentration of 10^5 CFU/mL. The enzyme activities of serum under the treatment of blank (negative control), DH82 in the first week, continuous DH82 in each week, 17SZ then continuous with DH82, and the 17SZ only as infection control are presented, panel **(A)** is ACP, panel **(B)** is AKP, panel **(C)** is SOD, and panel **(D)** is LZM. The error bars are presented as the standard deviation. Statistical analysis results are presented by significant difference that indicated by ** where $p < 0.01$ and * where $p < 0.05$.

on the effect of the host. However, the long-term giving of DH82 led to a significant reduction in AKP activity, the continuous dietary DH82 significantly relieved the adverse effects caused by *V. parahaemolyticus* (*P*-value was 3.2E-06) and showed no difference to that of the blank (*P*-value was 0.018).

The result of SOD activity (Panel C) also presented a consistent tendency under the challenge of *V. parahaemolyticus* to that of phosphatases, where both the initial treatment and continuous treatment of DH82 showed no difference in SOD activity to that of the control. In addition, even though the infection of 17SZ generated a decrease in SOD activities, the emerging treatment of DH82 significantly relieved the influence (*P*-value was 0.00011), which indicated that the treatment of DH82 at the presence of *V. parahaemolyticus* did contribute to the non-response immune response scavenging for free radicals.

The data of LZM activity (Panel D) presented an interesting result. The treatment of DH82 gradually reduced the congenital immune response of shrimp on the expression of lysozyme, and the continuous addition of DH82 was observed to have a significant difference on LZM activity (*P*-value was 0.0016),

whilst the challenge of *V. parahaemolyticus* remarkably promoted the LZM activity of host (*P*-value was 0.0076) despite the addition of DH82 (*P*-value was 0.00020 compared with the blank). Considering the functional products of QQ enzymes and antibacterial peptides in DH82, the results verified the strategy of "inhibit but not kill" by DH82 against *V. parahaemolyticus* and also indicated the non-harmful property of DH82 to the shrimps.

The intestines of shrimps in each group were sampled to analyze the effect of bacteria on the intestinal structure of shrimps by bath challenge. The intestines in the blank (negative control), DH82 treated, and mixture treated groups all had thick intestinal walls and were full of feedstuffs when observed by the naked eye, compared with those of the 17SZ challenged group, which were observed to have an empty intestine, thin intestinal well, and clear inflammation.

The sections of the intestines were microscopically examined, as shown in **Figure 6**. The intestinal epithelial cells of healthy shrimp in the control group (Panel a) were closely arranged with a thick edge of intestinal epithelial cells without any swelling. The intestines of shrimps with DH82 challenge (Panel b) were observed to have a slightly thinner edge with damage to intestinal epithelial cells. However, in the group infected with *V. parahaemolyticus* (Panel c), the intestinal epithelial tissue of shrimp were nearly exfoliated and shed in the intestinal cavity, with a swollen intestinal wall. For the group treated with DH82 together with 17SZ (Panel d), the symptoms of intestinal damage were milder and they continued to have complete intestinal epithelial cells, though there were few epithelial cells separated from lamina propria and swelling on the intestinal wall was observed. The symptoms of the four groups of shrimps under bath challenge demonstrated the inhibition of DH82 on anti-inflammation and damage-reduction against *V. parahaemolyticus*.

In vivo Assessment of Bacterial Effects Under Injection Challenge

The survival rate of shrimps after injection challenge, which is shown in **Figure 7**, demonstrated a dose-dependent decreasing tendency under the bacterial treatments, and higher percent survival. Compared with the 100% survival of blank in the control group, the shrimps treated by DH82 at a dose of 10^6 CFU/mL also fully survived after 7 days, and with 80 and 40% survival respectively, when the dose of DH82 was increased to 10^7 and 10^8 CFU/mL. The challenge of 17SZ sharply reduced the number of surviving shrimp, especially at the injection dose higher than 10^7 CFU/mL, whilst the coexisting DH82 raised the survival rate at a dose of 10^6 CFU/mL and 10^7 CFU/mL and prolonged the survival time of shrimps at a dose of 10^8 CFU/mL.

As for the bacterial infection to the muscle, the shrimps in the blank and DH82 treated groups were all observed with no whitish symptoms, and recovered with a black scar at the injection spot; whilst the 17SZ infected shrimps were observed with obvious symptoms of muscle gonorrhea. The muscle

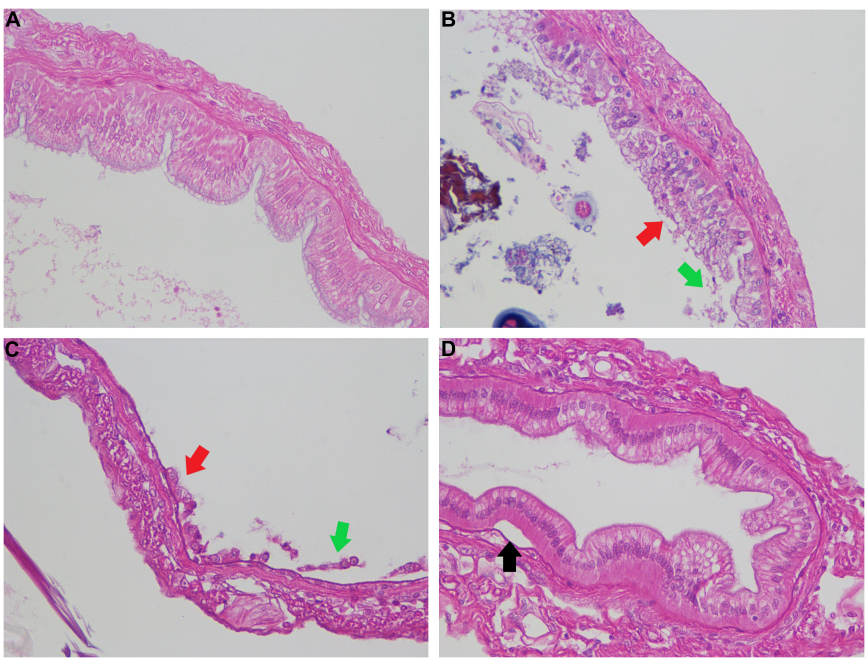

FIGURE 6 | Pathological analysis of intestinal tissue of shrimps challenged with *Vibrio parahaemolyticus*. Each 100 mL bacterial culture (OD_{600nm} 0.1) was individually poured into aquaria for bacterial bath challenge to shrimps with the final concentration of 10^5 CFU/mL. The intestine samples were performed H&E staining and imaged by microscopy. Panel **(A)** is the intestine of blank, panel **(B)** is intestine challenged with DH82, panel **(C)** is intestine challenged with 17SZ, panel **(D)** is intestine challenged with 17SZ and treated with continuous DH82. The arrows point out where the intestinal epithelial cells detach from the basement membrane (black arrows), the occurrence of basophilic hypertrophied nuclei with a reduced volume of eosinophilic cytoplasm (red arrows), and the disintegrated intestinal epithelial cells (green arrows).

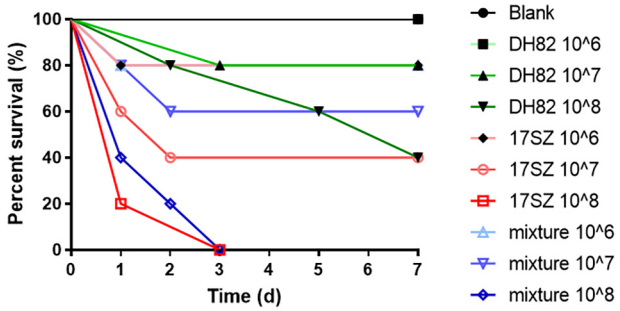

FIGURE 7 | Survival rate of shrimps after injection challenge. Fifty shrimps were randomly allocated to 10 groups, respectively injected at the muscle between 5th and 6th uromere with 1 mL of 17SZ and DH82, and the same volume of the mixture (1:1) of 17SZ and DH82 at different doses, compared with the blank of injection with PBS as control. The survival rates of each treatment are presented: the blank is presented by ● in black line; DH82 at a dose of 10^6 CFU/mL is presented by ■ in light green line, 10^7 CFU/mL by ▲ in green line, DH82 at 10^8 CFU/mL by ▼ in dark green line; 17SZ at 10^6 CFU/mL by ◆ in pink line, 10^7 CFU/mL by ○ in light red line, 10^8 CFU/mL by □ in red line; mixture at 10^6 CFU/mL by △ in light blue line, 10^7 CFU/mL by ▽ in light purple line, 10^8 CFU/mL by ◇ in blue line.

sections from survived shrimps were further performed H&E staining and examined by brightfield microscope. As shown in **Figure 8**, the muscles of shrimp infected by 17SZ (Panels c and d) were observed with serious disorder and large gaps between the muscle fibers, whilst the muscles in the group that were individually injected with DH82 (Panels a and b) were more compact and thicker for the fiber bundle. Besides, both groups were observed to have dose dependent histopathological changes on gaps between the muscle fibers, where a higher dose of bacteria challenge refers to larger gaps and more serious whitish symptoms. For the group with a mixed injection of 17SZ and DH82 (Panels e and f), the muscle fibers of challenged shrimp were tightly arranged with no histopathological changes observed, which indicated the inhibition of DH82 on the pathogenicity of 17SZ.

DISCUSSION

Using microbial antagonism to inhibit pathogen infection, by controlling the biofilm formation and virulence factor expression through QS regulation (Kalia et al., 2019), had become a novel strategy of biocontrol to replace antibiotics for the prevention and control of aquatic pathogenic bacteria such as *V. parahaemolyticus* (Defoirdt, 2013). It is currently known that the pathogenicity of *Vibrio* sp. is regulated by three parallel QS pathways, LuxM/LuxN related AHLs, CqsA/CqsS related CAI-1, and LuxS/LuxP related AI-2, all lead to the regulation of core regulatory protein LuxO (Herzog et al., 2019), then to the synergistic action of key regulatory proteins AphA (Lu et al., 2018) and OpaR (Zhang et al., 2016), which both regulate downstream exopolysaccharide synthesis genes to affect biofilm formation and virulence relative genes to release virulence factors including hemolysin.

In this article, the *in vitro* assessment of QQ enzymes demonstrated that AHL-lactonases significantly down-regulated the expression of AphA, which is mainly regulated at an early stage of low cell density, and OpaR, which plays the leading role at a later stage of high cell density, and the virulence factor tlh (**Figures 1A–C**), which verified the primary function of AHL mediated QS pathway on the pathogenicity of *V. parahaemolyticus*, and was consistent to other reports (Bzdrenga et al., 2017; Torres et al., 2018). The performance of biofilm formation under the treatment of inhibition (by AHLs degradation) or induction (by additional exogenous AHLs), both demonstrated the direct relationship between biofilm forming and the AHL mediated QS pathway (Vinoj et al., 2014; Paluch et al., 2020), which could be intervened

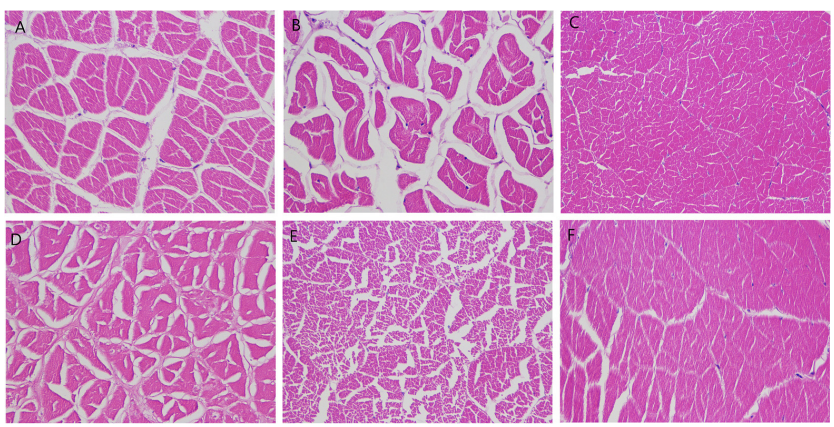

FIGURE 8 | Pathological analysis of muscle tissue of shrimps challenged with *Vibrio parahaemolyticus*. The shrimps were injected at the muscle between the 5th and 6th uromere with 1 mL of 17SZ and DH82 and the same volume of the mixture (1:1) of 17SZ and DH82. The muscle sections were performed using H&E staining and imaged by brightfield microscope with magnification of 400 times. Panel **(A)** is a medium dose of DH82 (10^7 CFU/mL), panel **(B)** is a low dose of DH82 (10^6 CFU/mL); Panel **(C)** is a medium dose of 17SZ (10^7 CFU/mL), panel **(D)** is a low dose of 17SZ (10^6 CFU/mL); Panel **(E)** is the medium dose of the mixture of DH82 and 17SZ (1:1, 10^7 CFU/mL), panel **(F)** is a low dose of the mixture of DH82 and 17SZ (1:1, 10^7 CFU/mL).

by free AHL-lactonases (**Figure 1D**) and DH82 cell pellets (**Figure 2**), and the phenomenon of non-significant difference also indicated the possibility that other two QS pathways (Henke and Bassler, 2004) might involve the regulation of biofilm formation of *V. parahaemolyticus*. Besides, the extracellular products, including anti-microbial peptides in the supernatant of the bacterial culture, also contributed to inhibition against *V. parahaemolyticus* either on the agar plate (**Table 2**) or in broth media under both planktonic and biofilm-status (**Figure 2**), which indicated the mechanism of DH82 on microbial antagonism, by using the above described dual functional products in DH82, QQ enzymes, and antibacterial peptides, to control the biomass accumulation and pathogenicity of *Vibrio* pathogens (Bai et al., 2008; Cai et al., 2019) with a synergistic effect.

Pathogenic *Vibrio* could exist as planktonic and biofilm status in an aquatic water system, and invade shrimps via gill to cause bacterial gill-rot disease (Shi et al., 2017) through contaminated feed and cause intestine infection (Zhou et al., 2016), or via wounds, it could cause whitish muscle disease (Sun et al., 2007). The environmental conditions such as high stocking density would motivate the agonistic behavior of shrimps (Yuchao et al., 2016), thus increasing the probability of death from wound infection, intestinal inflammation, and cross predation.

The *in vivo* study of bacterial challenge on shrimp simulated the two pathways of bacterial infection, respectively through the digestive tract and surface wounds. The findings indicate that the shrimps showed intestinal inflammation, cross contamination caused by predation, or decreased exercise ability caused by white muscle turbidity, and the vicious circle, which led to the rapid increase of mortality in the *Vibrio* infection groups and therefore make the survival rate uncountable in the experiment of bath challenge.

Since the trial experiment was completed in an open system, the newly pumped water would bring environmental microorganisms to the aquaria, and the bacteria attached sediment rolling up by typhoon attack would aggravate this problem. The results of bacterial counting from the water samples (**Figure 3**) did point out the phenomenon that the continuous addition of DH82 formed a dominant flora in the environment and effectively inhibited the *Vibrios* in aquaria (not just 17SZ but also other *Vibrios* from pump-in seawater), these findings were consistent with the data on the bacterial community of gut microbiota analyzed by high throughput sequencing (**Figure 4**).

Although Typhoon Mekkhala attacked the farming base in the first week of the experiment period after bacteria inoculation, the biochemical indexes of white shrimps under treatments all demonstrated the antibacterial and probiotic properties of DH82, in which the initial dietary DH82 promoted the growth of shrimps by increasing the ACP and AKP activities (**Figures 5A,B**), both of which were the growth factors of phosphoric acid metabolism, and further leads to weakening of shell-changing, which were regulated by ACP and AKP. However, the continuous delivery of DH82 reduced the ACP and AKP activities instead, the reason was unknown, yet based on the current experiment, the daily inoculation of DH82 with a dose of 10^5 CFU/mL consumed a lot of oxygen, which might cause additional stress to the shrimps and affect growth. Besides, DH82 enhanced the stress resistance ability of the host by increasing the SOD activity (**Figure 5C**), the additional DH82 also assists the host with antibacterial ability by increasing the LZM activity when challenged with pathogens and reducing the LZM activity when there was an absence of *V. parahaemolyticus*, which also verified the antibacterial activity of DH82 (**Figure 5D**).

Considering that the bacterial challenge might be synergistically affected by other potentially opportunistic pathogens, the subsequent injection challenge was designed to further confirm the direct effect of *Vibrio* via shrimp wound infection and the pathogenic inhibition of DH82 against 17SZ.

The physical signs of shrimps under bath challenge and injection challenge both supported evidence of the potential probiotic properties of DH82. Additional DH82 presented obvious rehabilitation on intestinal tissue against *V. parahaemolyticus*, compared with the damage caused by the pathogens in control groups (**Figure 6**), which indicated the consistent probiotic effects of DH82 on the digestion ability of the host (Vinoj et al., 2014); as for the whitish muscle disease caused by bacterial infection, the presence of DH82 remarkably raised the survival rate of shrimps (**Figure 7**), and reduced the damage of athletic ability from *V. parahaemolyticus* (**Figure 8**), which indicated the effective protection of DH82 as probiotics during shrimp growth, especially to prevent bacterial infection through wounds from fights or the shell-changing of shrimp during growth (Zhou et al., 2012).

Traditional studies on probiotic applications have investigated the immune enhancement of aquatic animals when probiotics are used as feed additives. This article was more focused on emergency treatment against *Vibrio* outbreak. The trial experiment of dietary DH82 on white shrimp farming verified the effective *in vivo* bacterial inhibition against *V. parahaemolyticus*, and also revealed the increasing survival rate of shrimp and the rehabilitation on infected intestine and muscle with additional DH82 when challenged with *V. parahaemolyticus*. The *in vitro* and *in vivo* investigation in this study both verified the QQ capacity and probiotic properties of DH82 and the functional products working along with both QQ and antibiotic against *V. parahaemolyticus*, and indicated potential application as biocontrol reagents. For example, they could be used for emergency treatment when the breaking out of pathogenic *Vibrio*, in sustainable aquaculture and green production (Interaminense et al., 2019; Lukwambe et al., 2019).

CONCLUSION

The *in vitro* and *in vivo* assessments undertaken in this study revealed that the DH82 strain could significantly reduce the biomass accumulation of *V. parahaemolyticus* 17SZ on an agar plate and in broth media, including the planktonic bacteria number and biofilm formation. It could inhibit the QS regulation of 17SZ to reduce the pathogenicity by down-regulating the expression level of primary regulator AhpA, OpaR, and the virulence factor of tlh. The delivery of dietary DH82 could

enrich their abundance, richness, and quantity in both the aquatic system and the shrimp intestine, and also inhibit the biomass of *Vibrio*, thus reducing the damage to the non-specific immune system of ACP, AKP, and SOD activities, and assisting host to undertake antibacterial activity. This can reduce the damage to the intestine, infected muscles, and survival rate caused by infection of *V. parahaemolyticus*. The findings indicated the potential application of DH82 as a biocontrol reagent to prevent and undertake biological control of *V. parahaemolyticus*. This is a potentially effective strategy for enabling immune regulation in aquatic white shrimp, especially when there is an outbreak of pathogens, which might contribute to the sustainable and green production of aquaculture.

DATA AVAILABILITY STATEMENT

The data would be available on request.

ETHICS STATEMENT

The animal study was reviewed and approved by the institutional research Ethics Committee of Huaqiao University.

AUTHOR CONTRIBUTIONS

XS contributed to the conception of the study and drafted the manuscript. JL performed the experiments of quorum sensing inhibition. JS, RL, and WL performed the experiment of bacterial challenge and enzyme activities assessment. Y-ZS contributed to design the experiment of bacterial challenge. XT and SZ helped analysis with constructive discussions. MK and KW provided the bacterial strain. XS, MK, KW, and Y-ZS contibuted to the review and correction in the revision. XS offered the funding acquisition. All authors reviewed the results and approved the final version of the manuscript.

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