Research in Veterinary Science 171 (2024) 105204

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

In vitro and *in vivo* evaluation of the efficacies of commercial probiotics and disinfectant against acute hepatopancreatic necrosis disease and luminescent vibriosis in *Litopenaeus vannamei*

Leobert D. de la Peña [a,*], Germin L. Moquera [a], Edgar C. Amar [a], Jose Louis A. Castellano [a], Nikko Alvin R. Cabillon [b], Joey I. Arboleda [e], Joseph Keith Paulo T. Nava [a], Rodolfo V. Zamora Jr [c], Peter De Schryver [d]

[a] Southeast Asian Fisheries Development Center, Aquaculture Department (SEAFDEC/AQD), Tigbauan, Iloilo 5021, Philippines
[b] Department of Animal Sciences, Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel
[c] Biosolutions International Corporation, #15 Clipper Ave., Bayview Village, Tambo, Parañaque City, Manila 1701, Philippines
[d] INVE Technologies, NV Hoogveld 93, 9200 Dendermonde, Belgium
[e] Department of Biology, University of Crete Heraklion, Crete, Greece

ARTICLE INFO

Keywords:
Shrimp
Commercial probiotics
Disinfection
Diseases
Bacillus
Aquaculture

ABSTRACT

The bioactivities of two commercially available probiotics and one chemical disinfectant were tested against strains of *Vibrio parahaemolyticus* (VP_{AHPND}) and *V. harveyi*. This study aimed to determine shrimp pathogenic Vibrios' *in vitro* and *in vivo* sensitivities to commercial probiotics and a chemical disinfectant. The probiotics and disinfectant were tested first *in vitro,* followed by the *in vivo* trials. Results showed that upon administration of probiotics either through diet or adding into the tank water, the survivability of shrimp was increased during challenge with VP_{AHPND} and *V. harveyi*. Also, the disinfectant was tested against the same pathogens and showed positive bactericidal effects at 2500 ppm and 5000 ppm. The present findings suggest that adding probiotics to the rearing water or the shrimp feeds effectively prevents infection by lowering the load of pathogenic bacteria. In comparison, the effectiveness of the disinfectant (PUR) depends on its appropriate concentration and timing of application. It is not only limited to rearing water but is also applicable for decontaminating pond liners, tanks, and other paraphernalia.

1. Introduction

Diseases caused by bacteria of genus *Vibrio* constitute some of the significant threats to the shrimp aquaculture industry worldwide. For instance, luminescent vibriosis caused by *V. harveyi* has led to a significant decline in the shrimp aquaculture industry (Austin and Zhang, 2006). Currently, the emerging pathology of acute hepatopancreatic necrosis disease (AHPND) is caused by several *Vibrio* species, such as *V. parahaemolyticus* (VP_{AHPND}), *V. owensii*, *V. harveyi*, and *V. campbellii* (Lee et al., 2015; Liu et al., 2015; Kondo et al., 2015; Dong et al., 2017), that were heavily affecting shrimp production. It started in Southeast Asia (Lightner et al., 2012; Flegel and Lo, 2014; de la Peña et al., 2015; Kondo et al., 2015) and then rapidly spread to Mexico (Nunan et al., 2014; Soto-Rodriguez et al., 2015) and, more recently, to the USA (Dhar et al., 2019).

Infectious diseases, including those caused by *Vibrio* species, are severe problems affecting aquaculture worldwide, and several different approaches have been used to control them. Traditionally, bacterial contaminations have been dealt with using chemical compounds (Gomez-Gil et al., 2000). Many hatcheries have resorted to antibiotics, but their indiscriminate use has led to the emergence of antibiotic-resistant bacterial strains, which may transfer their resistance to other bacteria *via* horizontal gene transfer (Gilbert and McBain, 2003; Holmström et al., 2003). Consequently, the use of antibiotics, particularly as a prophylactic measure, has been highly discouraged.

Several alternative strategies for disease control that enhance the animals' immune function through alterations in the gut microbiota have been proposed and applied in aquaculture. A blend of organic acids and essential oils has been shown to benefit the gut microbiota and improve the immune response and disease resistance of Pacific white-leg

* Corresponding author at: Buyu-an, Tigbauan, Iloilo 5021, Philippines.
E-mail address: leobertd@seafdec.org.ph (L.D. de la Peña).

https://doi.org/10.1016/j.rvsc.2024.105204
Received 21 December 2023; Received in revised form 25 February 2024; Accepted 27 February 2024
Available online 1 March 2024
0034-5288/© 2024 Elsevier Ltd. All rights reserved.

shrimp (*Litopenaeus vannamei*) (He et al., 2017). Biofloc rearing systems are another sustainable alternative that has been used to control vibriosis in L. *vannamei* (Aguilera-Rivera et al., 2019). However, one of the most noteworthy advances in terms of sustainable disease control in aquaculture is the use of probiotics. According to the modern broad definition applicable in the aquaculture context, a probiotic is a live microbial adjuvant that has a beneficial effect on the host by modifying the host-associated or ambient microbial community, ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment (Verschuere et al., 2000). Probiotics have been widely used to control vibriosis in shrimp aquaculture (Aribah and Wahyudi, 2022; Rajyalakshmi et al., 2021).

Nonetheless, doubts about the efficacy of probiotics have emerged (Decamp et al., 2008), which may be due to ineffective products sold as probiotics (Browdy et al., 2001). According to the latter study, different types of bacteria present in the product, their mode of delivery, and their abundances should be analyzed before using the probiotics. Therefore, it is essential to perform a thorough analysis of any commercial product, considering all these parameters into account. Additionally, most probiotic studies have been performed *in vivo* in hatchery experiments (Gomez-Gil et al., 2000; Werasan and Prapansak, 2019). Thus, rigorous laboratory studies in which variables are tightly controlled need to be performed.

Another strategy used to control the diseases caused by pathogens, including *Vibrio* species, is chemical disinfectants (Puente et al., 1992; Park et al., 2004; Mainous et al., 2010; Collymore et al., 2014; Wang et al., 2015). However, their efficacy appears to be highly dependent on the organic load of the water (De Bodt and Defoirdt, 2018). Moreover, some disinfectants adversely affect fish and water quality (Leal et al., 2018). Hydrogen peroxide, for instance, was not toxic to turbot (*Scophthalmus mixumus*) but increased its stress levels when provided at high doses, which accelerated the tenacibaculosis outbreak (Avendaño-Herrera et al., 2006). Formalin, another common disinfectant in aquaculture, damages fish gills, alters mucous cells, and adversely affects the biological filters (Leal et al., 2018). Calcium and sodium hypochlorite are also the most commonly used disinfectants in Southeast Asian shrimp farming but are highly toxic to aquatic organisms and can negatively affect the environment (Primavera et al., 1993). Thus, proper use of disinfectants requires knowledge of the farmed species, the disease to be treated, water quality, and the chemical itself. For instance, potassium peroxymonosulfate combined with sulfamic acid, malic acid, sodium polymetaphosphate, and sodium chloride are oxidizing agents. The latter are the active substances of a commercially available disinfectant effective against major aquatic bacterial and viral pathogens. It is advantageous for being biodegradable, potent, and with a strong oxidizing property.

The potential of *Vibrio spp.* that can act as a probiotics were conducted by Thompson et al. (2010). In addition, Aguilllahi-Arpanahi et al. (2018) tested the efficacies of *Bacillus subtilis* and *B. licheniformis* against *Vibrio* infection in shrimp. Also, *Bacillus pumilus,* isolated from penaeid shrimp, was tested *in vitro* against marine pathogens (Hill et al., 2009). However, to date, there are no published articles to validate the combined effect of *in vitro and in vivo* trials of *B. pumilus, B. licheniformes, and B. subtilis* in L. *vannamei* against economically significant shrimp bacterial diseases. Thus, the present study aimed to determine the *in vitro* and *in vivo* sensitivities of VP_{AHPND} and *V. harveyi* to commercial probiotics with *Bacillus spp.* as active components and a commercial disinfectant against AHPND and Luminescent vibriosis in L. *vannamei.*

2. Materials and methods

2.1. Sources and isolation of pathogens

Three isolates of VP_{AHPND} (VP_{AHPND} 1212, VP_{AHPND} 1213, and VP_{AHPND} 1217) were obtained from diseased shrimp during a natural AHPND outbreak in a farm in Negros Occidental, Philippines in 2015. They have the same source and draft genome sequence as VP strain PH1339 (Penir et al., 2019). *V. harveyi* isolate (PN-9801) was obtained from diseased shrimp during an outbreak of luminescent vibriosis in a farm in Negros Occidental, Philippines, in 1998 (de la Peña et al., 2001). These bacterial isolates were stocked in Nutrient Broth (Merck, Germany) supplemented with 1.5% NaCl (Merck, Germany) and 20% glycerol (Merck, Germany) and then stored at the bio-freezer (−80 °C). Before the experiments, bacterial isolates were revived on Nutrient Agar (Pronadisa, Spain) plates supplemented with 1.5% NaCl and incubated for 18–24 h at room temperature (28–30 °C).

2.2. In vitro assays

2.2.1. Probiotics

2.2.1.1. Isolation and identification of probiotic components. Three bacterial strains were present in the commercial probiotic PRO-2 (Sanolife® INVE Aquaculture, Belgium), a highly concentrated microbial mixture for feed. *B. pumilus, B. licheniformes, and B. subtilis* were reisolated by inoculation on Tryptic Soy Agar (Oxoid, USA) supplemented with 1.5% NaCl and were incubated at 28 °C overnight. Purified colonies were identified by gram and spore stainings and were further confirmed using API 50CH (Biomerieux, France) and conventional Bergey's Scheme of Identification for *Bacillus* species (Breed et al., 1944).

2.2.1.2. Co-culture assay. The modified method of Lavilla-Pitogo et al. (1998) was followed in the co-culture assay. The test pathogens (VP_{AHPND} 1212 and *V. harveyi*) and commercial probiotics (PRO W, Sanolife® INVE Aquaculture, Belgium) were separately grown overnight on Nutrient agar supplemented with 1.5% NaCl (NA^{+}). Bacterial colonies were harvested after 18–24 h and suspended in sterile seawater. The test pathogens were first added to a flask containing 100 mL of the sterile seawater, followed by the PRO W. The initial concentration of PRO W and pathogenic bacteria were 1×10^3 CFU mL^{-1} and 1×10^2 CFU mL^{-1}, respectively. The PRO-W is a probiotic mixture specifically formulated to stabilize phytoplankton blooms in marine and freshwater aquaculture ponds. PRO W is composed of two *Bacillus* strains: *B. subtilis* and *B. licheniformis* which are both spore-forming. The minimum concentration of these two *Bacillus* strains in PRO-W is 5×10^{10} CFU g^{-1}. According to the manufacturer's instruction, when applied in the pond, the required amount of 2×10^2 g PRO-W ha^{-1} m^{-1} gives a final concentration of 1×10^3 CFU mL^{-1} of *Bacillus*. Mixed cultures were maintained in a shaker at 115 rpm throughout the tests. Bacterial counts were carried out upon inoculating test containers daily for three to five days on NA^{+} and vibrio chromogenic agar (VCA) using standard spread plate methods. All cultures were maintained at room temperature (28 °C).

2.2.1.3. Disinfectant. PUR (Sanocare® INVE Aquaculture, Belgium) is a highly effective disinfectant specifically formulated for use in aquaculture against *Vibrio spp.* The manufacturer's recommended concentration is 0.5 g ton^{-1} of rearing water for routine bacteria control in the grow-out pond. Sanocare PUR contains a surfactant, organic acids oxidizing agent (potassium peroxymonosulfate), inorganic buffer (sodium polymetaphosphate), and hypochlorous acid (which is produced by the chemical reaction of sodium chloride, potassium monopersulfate, and sulphamic acid. It is used for grow-out pond water for routine control of bacteria and disinfection of tools and equipment that function by denaturing the proteins and lipids of microorganisms (Dvorak, 2008).

2.2.1.4. Disc diffusion method. The disc diffusion method was used to evaluate the antibacterial activity of PUR. Five to ten colonies of the four *Vibrio spp.* (VP_{AHPND} 1212, VP_{AHPND} 1213, and VP_{AHPND} 1217, and PN-9801) isolates were inoculated on Tryptic Soy Broth with 1.5% NaCl (TSB^{+}), incubated for two to three hours, then adjusted to the 0.5

McFarland's turbidity standard (Cockerill, 2012), *i.e.*, to 1.5 × 10^8 CFU mL $^{-1}$. A sterile cotton-tipped swab was dipped into each pathogenic strain suspension and swabbed thoroughly on a Mueller Hinton Agar with a 1.5% NaCl (MHA^+) plate from edge to edge. Sterilized filter paper discs (6 mm) were impregnated with 20 µl of PUR (250 mg mL $^{-1}$) and then introduced on the MHA^+ plates 30–60 min after pathogen inoculation. For the positive and negative controls, these were impregnated with 0129 *Vibrio*-static and sterile standard saline solution, respectively. The zone of inhibition was measured after 18–24 h of incubation at room temperature (28–30 °C).

2.2.1.5. Agar well diffusion method. *Vibrio spp.* isolates were inoculated on TSB^+, and the turbidity was adjusted based on 0.5 McFarland's turbidity standard. A cork borer was used to make a hole with a 6 to 8-mm diameter punched aseptically to the MHA^+ plates previously inoculated with the pathogenic *Vibrio spp.* (VP_{AHPND} 1212, VP_{AHPND} 1213, and VP_{AHPND} 1217, and PN-9801). Then, 100 µl volume of the different concentrations of the PUR was introduced into the well. For the positive and negative controls, these were impregnated with 0129 *Vibrio*-static and sterile standard saline solution, respectively. The plates were incubated at room temperature (28–30 °C), and the zone of inhibition was measured after 18–24 h.

2.2.1.6. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Tests for MICs and MBCs were conducted according to (Baticados et al., 1990) with some modifications. Firstly, PUR was dissolved in distilled water to obtain a 100 µg mL $^{-1}$ working solution. *Vibrio spp.* isolates were emulsified in TSB^+ to turbidity equal to 0.5 McFarland's turbidity standard and then diluted to 10^4 CFU mL^{-1} final concentrations. The PUR working solution was added to the *Vibrio spp.* solutions at final concentrations of 50, 25, 12.5, 6.25, 3.125, 1.5, and 0 (control) µg mL^{-1}. Two replicates per concentration were used for two trials per isolate. The tubes containing each isolate and PUR mixture were incubated for 18–24 h at ambient temperature and then examined visually to determine bacterial growth. The lowest concentration of PUR at which no bacterial growth was observed was considered the MIC. Those isolates showed no turbidity was spread on NA^+ plates (0.1 mL of each isolate suspension; two replicates per concentration) to determine the MBC. Plates were incubated for 18–24 h at ambient temperature and then examined for the presence of bacterial colonies. Colonies were enumerated by direct counting, and the lowest concentration of PUR that resulted in no bacterial growth was considered the MBC.

The activity of PUR against VP_{AHPND} 1212 and *V. harveyi* was tested using different concentrations from 0.5 ppm to 4.5 ppm. The VP_{AHPND} 1212 and *V. harveyi* cultures were grown overnight on NA^+, harvested, and suspended in sterile seawater. Initial densities were ≤ 10^4 CFU mL^{-1}. PUR was directly added to the flasks containing bacterial cultures. Bacterial counts were carried out upon inoculation of test containers every hour for three days on NA^+ and vibrio chromogenic agar (VCA) using standard spread plate methods. All cultures were maintained at room temperature (28 °C).

2.3. In vivo assays

2.3.1. Animals

AHPND and luminescent vibrio-free *L. vannamei* with an average body weight (ABW) of 2.67 g were obtained from a shrimp farm in Zarraga, Iloilo, Philippines (stocked post-larvae produced from SPF breeders). Representative samples were taken for AHPND and luminous bacteria detection using PCR and microbiological tests, respectively. The shrimps were maintained in a constantly aerated flow-through seawater system with 32 ppt salinity and 30–32 °C temperature at the Infection Building of the Southeast Asian Fisheries Development Center/ Aquaculture Department (SEAFDEC/AQD) before the start of the experiment. They were given a commercial shrimp starter feed at a 3% body weight feeding rate (per the manufacturer's feed rate guideline). These shrimp were used in all of the *in vivo* experiments.

2.3.1.1. Tolerance test. The tolerance of L. *vannamei* was tested using concentrations double the MICs and MBCs of the PUR disinfectant and determined in a 24-h static bioassay procedure according to Baticados et al. (1990). Shrimp (ABW 2.67 g) were stocked in 8-L glass aquaria at a density of 15 individuals/aquarium^{-1}. Water temperature was maintained at 30–32 °C, and the aquaria were covered to minimize chemical degradation. The shrimp were not fed until the end of the tolerance test. The effect of the different concentrations of PUR on shrimp morphology (*e.g.*, color change), survival, and behavior were determined.

2.3.1.2. Effect of PRO-W and PUR disinfectant on L. vannamei. Fifteen 100-L capacity tanks were used for five treatments, with three replicates in each treatment and 30 shrimps (ABW 2.67 g) per tank. PRO-W treatment was administered through the addition of probiotic cell suspension of 2 × 10^2 g PRO-W ha^{-1} (1 x 10^3CFU mL^{-1}) or 2 × 10^6 g of PRO-W ha^{-1} (1 x 10^7CFU mL^{-1}) twice a day (9:00 AM and 3:00 PM) for seven days. PUR disinfectant was added directly to the tanks at a concentration based on the tolerance test (0.5 ppm and 10 ppm). The control group was in a separate tank without PRO-W and PUR. Shrimps were fed twice daily with a commercial shrimp starter feed at 3% body weight. Water temperature was maintained at 30–32 °C, continuous aeration was provided in the tanks, and about 50 % (50%) of the rearing water was changed daily. Before PRO-W addition, feces, and uneaten feeds were removed from the water early in the morning.

2.3.1.3. Preparation of probiotic feed. Feed was prepared by adding 0 (control), 1, and 5 g of PRO-2 kg^{-1} commercial shrimp starter feed. Mixing was done manually based on the manufacturer's protocol. Briefly mix the required quantity with clean freshwater or good-quality fish oil. Then, the pellets were top-dressed with the prepared mixture. Finally, the pellets were allowed to absorb the mixture between 30 min to 2 h. Feed for control treatment was treated with sterile freshwater only. Aliquoted feeds were stored at 4 °C before use.

2.3.1.4. Effect of feed containing PRO-2 on L. vannamei. Twelve 100-L tanks were used for the feeding trial (0 (control), 1, and 5 g of PRO-2 kg^{-1} feeds). Four tanks were assigned per group with 30 shrimp (ABW 7.46 g) $tank^{-1}$. Shrimp in experimental tanks were fed with the commercial shrimp pelleted diet containing PRO-2 (1 and 5 g kg^{-1} of feed at 3% of its body weight day^{-1}), while control tanks were given commercial feed only without PRO-2. Water temperature was maintained at 30–32 °C with continuous aeration, and 50% of the rearing water was changed daily. The bacterial load of the gut and rearing water was determined and monitored. Initial measurements of body length and weight were taken, and weekly monitoring of size increments was conducted up to one month of the feeding trial.

2.3.1.5. Vibrio spp. challenge assay. After one month of feeding with PRO-2 or seven days of PUR or PRO-W addition in the rearing water, shrimp were subjected to bacterial challenges. The ABW of shrimp used per treatment were 13.22 g, 4.58 g, and 4.61 g, respectively. VP_{AHPND} 1212 (the only strain used in the co-culture experiment) and *V. harveyi* PN9801 were administered through immersion and injection, respectively. Sixty shrimp were used for each treatment (20 shrimp per replicate). The desired concentration of VP_{AHPND} was 5 × 10^4 CFU mL^{-1} (Amar et al. unpublished data), and *V. harveyi* was l × 10^6 CFU mL^{-1} (de la Peña et al., 2001).

The challenge test procedure for the *V. harveyi* isolate followed the method of de la Peña et al. (2001) with modifications. The 24-h culture of *V. harveyi* was inoculated into apparently healthy shrimp by intramuscular injection at a concentration of 1 × 10^6 CFU mL^{-1}. Control

shrimp received 100 uL sterile seawater. Rearing water was replaced at 80% daily to maintain water quality, and the shrimp were not fed during the challenge period. The mortality was observed for one week.

A modified method of Tran et al. (2013) was used in the challenge test for VP_{AHPND}. Five pure single colonies of VP_{AHPND} were inoculated in 10 mL of TSB^{+}, incubated at 30 °C for two hours, and then inoculated to 250 mL of TSB^{+} with vigorous shaking at approximately four hours or until $OD_{600} = 0.8$ abs (1×10^8 CFU mL^{-1}). Twenty shrimp per replicate were directly immersed in 50-L tanks containing a bacterial concentration of 5×10^4 CFU mL^{-1} for five days or until the mortality rate reached 100%. Cumulative mortality was recorded from day one up to the 7th-day post-immersion.

2.3.1.6. Statistical analyses. Data are presented as means ± standard deviations. One-way ANOVA was used to compare the effect of PRO—W, PRO-2, and PUR in the growth and survival of L. *vannamei* using the statistical package SPSS (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.). Significance was set at $p < 0.05$.

3. Results

3.1. In vitro assay

3.1.1. Probiotics

Twelve bacterial isolates were randomly obtained from PRO-2. All isolates were Gram and spore stain-positive. These twelve isolates were characterized and identified according to the conventional Bergey's Scheme for Identification of *Bacillus spp.* (Breed et al., 1944) and API 50CH (Biomerieux, France). From these, seven were identified as *B. pumilus,* three as *B. licheniformes, and* two as *B. subtilis* with prevalence of 11.8%, 44.1%, and 44.1%, respectively. The API50CH assay returned a 99.9% match with *B. pumilus*, *B. licheniformes*, and *B. subtilis* with no mismatched substrates (Table 1). *B. pumilus* has a small semi-moist and opaque colony with a matte texture. *B. licheniformes* is semi-dry with irregular colony size and with serrated margin. *B. subtilis* has a mucoid colony with a round and entire margin. *B. pumilus* gave negative results for both nitrate and starch hydrolyses tests, while *B. licheniformes* and *B subtilis* were both positive for nitrate and starch hydrolyses tests.

In the co-culture assay, mixed culture of PRO W with VP_{AHPND} 1212 and with *V. harveyi* (PN-9801) isolates, the pathogen population decreased at days 1 and 2 for treatment PRO-W plus VP_{AHPND} 1212 and with recovery starting at day 3. PRO-W mixed with *V. harveyi* (PN-9801) showed a decreased pathogen population on day 1 and recovered on day 2 (Fig. 1).

3.1.2. Disinfectant

The sensitivity of the three VP_{AHPND} (VP_{AHPND} 1212, VP_{AHPND} 1213, and VP_{AHPND} 1217) and one *V. harveyi* (PN-9801) isolates were tested against a commercial disinfectant PUR using the disc diffusion assay technique. Isolate VP_{AHPND} 1217 showed sensitivity to PUR at 2000 ppm and above, whereas the other two, VP_{AHPND} 1212 and 1213 and *V. harveyi* (PN-9801), showed sensitivities at 10,000 ppm PUR and above. All strains showed increasing sensitivity (measured by the diameter of the zone of inhibition) with increasing concentrations of PUR (Table 2). Also, the sensitivity of the same *Vibrio spp.* isolates to PUR were tested using the well-agar diffusion assay technique. VP_{AHPND} 1212 and 1213 showed sensitivity to PUR starting at 10,000 ppm, whereas VP_{AHPND} 1217 and *V. harveyi* (PN-9801) showed sensitivity at 20,000 ppm (Table 2).

The MIC and MBC of PUR were determined for VP_{AHPND} 1212, VP_{AHPND} 1213, VP_{AHPND} 1217, and *V. harveyi* (PN-9801). In all cases, MIC was 2500 ppm, and MBC was 5000 ppm, resulting in a MIC/MBC ratio of 2. Specifically, 0.5 ppm of PUR was effective against *V. harveyi* (PN-9801). The bacterial population was completely inhibited three hours after application (Fig. 2). Although PUR concentrations between 0.5 ppm and 4.5 ppm were tested against VP $_{AHPND}$ 1212, those up to 4.0 ppm were insufficient to inhibit this bacterial strain (data not shown) completely. PUR at 4.5 ppm started to inhibit VP_{AHPND} 1212 upon application up to 18 h. Eventually, after 24 h onwards, VP_{AHPND} 1212 started to recover, and the bacterial population was comparable to that of the control treatment at 168 h (Fig. 3).

3.2. In vivo assays

3.2.1. Effect of PRO-2 on the growth and survival of L. vannamei

Litopenaeus vannamei were fed for four weeks with 0 (control), 1, and 5 g of PRO-2 kg^{-1} of feed, which showed no significant differences in final body weight and body length (Table 3). The survival rate of L. *vannamei* was also similar across treatments. Initial, middle, and final samplings for total plate count of bacterial load in the rearing water in all treatments ranges from 3.8×10^2 to 2.3×10^5 CFU mL^{-1} while total vibrio count ranges from 0 to 4.3×10^3 cfu mL^{-1}. No luminous bacteria were detected in the rearing water in all treatments. Furthermore, the total plate count of bacterial load in shrimp gut ranges from 9.3×10^4 to 3.3×10^5 cfu gut^{-1} in 0 (control) and 1 g of PRO-2 kg^{-1} of feed while 1.0×10^6 to 1.0×10^7 cfu gut^{-1} in 5 g of PRO-2 kg^{-1} of feed. Total vibrio count ranges from 1.5×10^1 to 3.2×10^4 cfu gut^{-1} in all treatments. Luminous bacteria was detected in control shrimp at 5.0×10^2 cfu gut^{-1}.

After four weeks of feeding with PRO-2, *L. vannamei* was challenged with VP_{AHPND} or *V. harveyi*. In the VP_{AHPND} challenge, shrimp fed with either 1 or 5 g of PRO-2 kg^{-1} of feed showed a significantly higher survival (76.67% and 90.00%, respectively) than control shrimp (6.67%) (Table 3). Similarly, in the *V. harveyi* challenge, shrimp fed with 1 g of PRO-2 kg^{-1} of feed had significantly higher survival (96.67%) than shrimp fed with either 5 g (73.33%) of PRO-2 or no PRO-2 added to the feed (70.00%) (Table 3).

3.2.2. Effect of PRO-W in L. vannamei

There were no significant differences in final body weight after seven

Table 1
Isolation and identification of *Bacillus spp.* components of PRO-2*.

Isolate #	Catalase	Oxidase	Nitrate	Starch hydrolysis	Bergey's ID	API 50CH ID
1	+	−	+	+	*B. subtilis*	*B. subtilis*
2	+	−	+	+	*B. licheniformes*	*B. licheniformes*
3	+	−	−	−	*B. pumilus*	*B. pumilus*
4	+	−	−	−	*B. pumilus*	*B. pumilus*
5	+	−	+	+	*B. subtilis*	*
6	+	−	+	+	*B. licheniformes*	*
7	+	+	−	−	*B. pumilus*	*B. pumilus*
8	+	−	−	−	*B. pumilus*	*B. pumilus*
9	+	−	−	−	*B. pumilus*	*B. pumilus*
10	+	−	+	+	*B. licheniformes*	*
11	+	+	−	−	*B. pumilus*	*B. pumilus*
12	+	−	−	−	*B. pumilus*	*B. pumilus*

* – test not conducted.

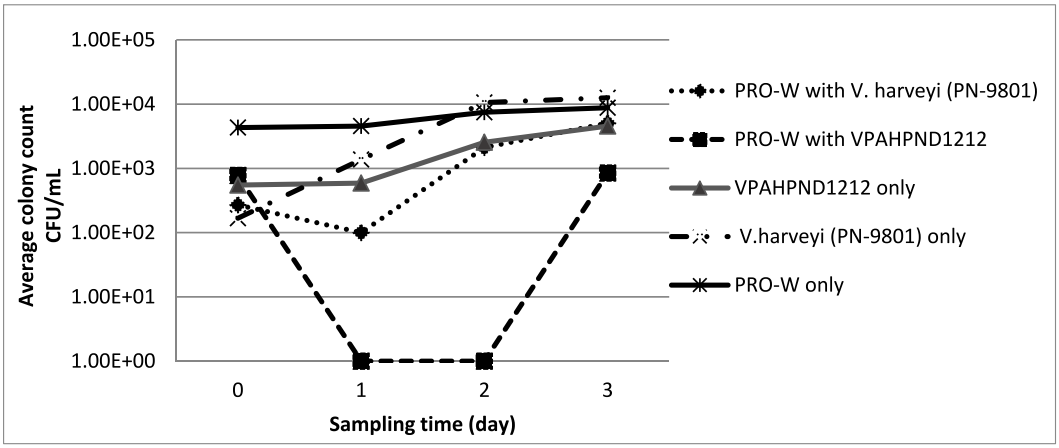


Fig. 1. The activity of PRO-W against *V. harveyi* and VP_{AHPND}.

Table 2
Sensitivities of VP_{AHPND} and *V. harveyi* to PUR.

PUR (ppm)	Disc Diffusion Assay				Well-Agar Diffusion Assay			
	Average diameter of inhibition zone (mm)				Average diameter of inhibition zone (mm)			
	V. harveyi (PN-9801)	VP_{AHPND} 1212	VP_{AHPND} 1213	VP_{AHPND} 1217	*V. harveyi* (PN-9801)	VP_{AHPND} 1212	VP_{AHPND} 1213	VP_{AHPND} 1217
0.5	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0
1000	0	0	0	0	0	0	0	0
2000	0	0	0	6.9	0	0	0	0
5000	0	0	0	7.2	0	0	0	0
10,000	6.7	7.7	6.9	7.3	0	7.7	7.5	0
20,000	8.1	8.4	9.4	8.2	11.75	8.4	13.8	9.6
250,000	9.5	9.5	9.9	9.8	–	–	–	–
Positive Control	16.3	11.6	12.7	12.9	18.5	16.9	20.7	19.9
Negative Control	0	0	0	0	0	0	0	0

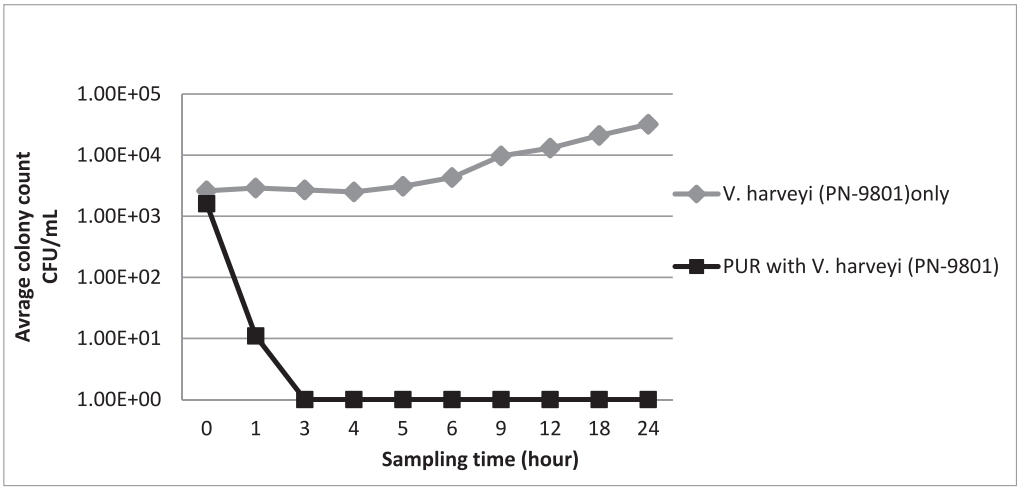


Fig. 2. Antibacterial activity of PUR (4.5 ppm) against *V. harveyi.*

days of applying either 2×10^2 g PRO-W ha^{-1} (4.91 g) or 2×10^6 of PRO-W ha^{-1} (4.37 g) in the rearing water. However, survival after seven days was relatively lower in the negative control (91.70%) (no PRO-W added) compared to the 2×10^2 (93.30%) or 2×10^6 g of PRO-W ha^{-1} (95.00%) treatments (Table 4).

After seven days of water treatment with PRO—W, *L. vannamei* was challenged with VP_{AHPND} and *V. harveyi*. There was no significant difference in percent survival during the VP_{AHPND} and *V. harveyi* challenge. But shrimp treated with 2×10^2 g and 2×10^6 g of PRO-W ha^{-1} showed higher survival (30.0% and 3.3%, respectively) than control shrimp (0%) during the *V. harveyi* challenge. During the VP_{AHPND} challenge, the water treatment with 2×10^6 g of PRO-W ha^{-1} produced relatively higher shrimp survival (40.0%) than the control (0%) or 2×10^2 of PRO-W ha^{-1} treatments (3.3%) (Table 4).

3.2.3. *Effect of PUR in L. vannamei*

The tolerance of L. *vannamei* to PUR was tested using concentrations ranging from 0 to 10,000 ppm (*i.e.*, double the MIC determined above).

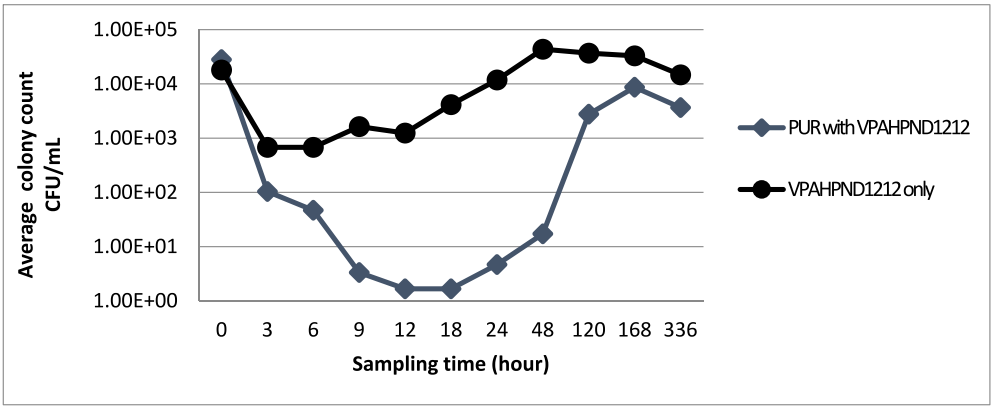


Fig. 3. Antibacterial activity of PUR (4.5 ppm) against VP_{AHPND}.

Table 3
Average body weight (ABW) and survival of *L. vannamei* after 4 weeks of feeding with PRO-2 and when challenged with *V. harveyi* and VP_{AHPND}.

Treatment	Feeding Trial		*V. harveyi* (PN-9801)	VP_{AHPND} (VP1212)
PRO-2	ABW	Survival	Survival	Survival
(g kg^{-1} of feed)	(g)	(%)	(%)	(%)
0	13.62 ± 0.7[a]	69.15 ± 6.3[a]	70.00 ± 3.3[a]	6.67 ± 6.5[a]
1	12.80 ± 0.3[a]	72.52 ± 11.1[a]	96.67 ± 3.3[b]	76.67 ± 10.0[b]
5	13.23 ± 0.3[a]	71.65 ± 12.5[a]	73.33 ± 6.7[a]	90 ± 10.0[b]

Letters denote significant difference at $p < 0.05$.

Table 4
Average body weight (ABW) and survival of *L. vannamei* after the addition of PRO-W or PUR to the rearing water for 1 week and when challenged with *V. harveyi* and VP_{AHPND}.

Treatment		Treatment Trial		*V. harveyi*	VP_{AHPND}
				(PN-9801)	(VP1212)
PRO-W (g ha^{-1})	PUR (ppm)	ABW (g)	Survival (%)	Survival (%)	Survival (%)
0	0	4.45 ± 0.9[a]	91.7 ± 6.0	0 ± 0.0[a]	0 ± 0.0[a]
2.00E+02	0	4.91 ± 0.9[a]	93.3 ± 3.3	30 ± 10.0[a]	3.3 ± 3.3[a]
2.00E+06	0	4.37 ± 0[a]	95 ± 0	33.3 ± 10.0[a]	40 ± 0[a]
0	0.5	4.58 ± 0.7[a]	88.3 ± 6.0	0 ± 0.0[a]	0 ± 0.0[a]
0	10	4.80 ± 0.9[a]	96.7 ± 3.3	0 ± 0.0[a]	0 ± 0.0[a]

Letters denote significant difference at $p < 0.05$.

The shrimp died within five minutes of exposure to PUR from 2500 ppm (equal to the MIC) and above. Exposure was tolerable at 0.5 and 10 ppm of PUR, with 97% and 95% survival, respectively, after 24 h. No color and behavioral changes were noted on the recovered dead shrimp. The tolerable doses of PUR (0.5 and 10 ppm) were then used to treat the rearing water for seven days, which did not affect the final body weight or survival of L. *vannamei* (Table 4).

Shrimp reared in water with PUR for seven days and then challenged with either VP_{AHPND} or *V. harveyi* showed no significant differences in survival with either 0.5 ppm or 10 ppm PUR treatments.

4. Discussion

The results obtained *in vitro* showed the inhibitory action of commercial probiotics (PRO 2 and PRO W) against the growth of VP_{AHPND} and *V. harveyi*. The bactericidal action of disinfectant (PUR) at high concentrations was also noted. The *in vivo* results showed that PRO 2 and PRO W can improve the survivability rate of the shrimp challenged by VP_{AHPND} and *V. harveyi*.

Some studies have shown that probiotic *Bacillus* strains improved growth performance, feed utilization, immune parameters, and improved resistance against VP_{AHPND} in whiteleg shrimp, *L. vannamei* (Madani et al., 2018; Kewcharoen and Srisapoome, 2019).

Although Silva et al. (2012) found that adding *Bacillus* species to the rearing water or feeds of L. *vannamei* improved the growth and survival of their zoea and mysis, a similar trend was not found for post-larval stages. This conforms with the present study that the addition of PRO-2 or PRO-W showed no effects on the growth and survival of adult shrimp throughout the feeding trial.

Bacillus species can outcompete other bacteria from the gut flora of shrimp by resisting colonization of pathogens through competition, releasing metabolites and producing substances with bactericidal or bacteriostatic effects such as bacteriocins, proteases and many others. This could prevent the colonization of pathogens thus increasing the shrimp resistance to diseases (Jamal et al., 2019). In addition, these probiotic bacteria can enhance shrimp immunity due to their phenol oxidative and phagocytic activities (Li et al., 2007; Tseng et al., 2009) and due to the secretion of quorum-quenching enzymes that can act as quorum-sensing blockers, thus protecting against infection (Zhao et al., 2015). The *in vitro* results obtained in the present study agree with these documented activities of *Bacillus* species, as the commercial probiotics PRO-W showed antibacterial activity against *V. harveyi* and VP_{AHPND}. A recent study showed that a *B. pumilus* strain supplied with the feed could colonize the L. *vannamei* gastrointestinal tract, resulting in improved growth and immunity and increased resistance to VP_{AHPND} (Kewcharoen and Srisapoome, 2019). PRO W is a composite component of two *Bacillus spp.* Based on the result, when added to *V. harveyi* and VP_{AHPND}, it effectively lowers the growth of the pathogen population after 24 and 48 h, respectively (Fig. 3). Furthermore, the study of Vaseeharan and Ramasamy (2003) supported the positive effect of probiotics as it showed that the growth of pathogenic *V. harveyi* was controlled by non-pathogenic *B. subtilis* BT23 also under *in vivo* and *in vitro* conditions.

In treatment *V. harveyi* with PRO W, the increase of the pathogen population after 24 h was probably due to the decrease of *Bacillus spp.* components wherein it could not reach the optimum density to inhibit the growth of *V. harveyi* effectively. Therefore, a potential probiotic co-culture must either be supplied regularly or be able to colonize and multiply in the host (Vaseeharan and Ramasamy, 2003).

Many researchers have continuously investigated the effects of commercial probiotics on aquaculture. Some of these studies have not shown any positive effects on the growth parameters or survival rate of the shrimp. For instance, the research of Far et al. (2009) on using commercial probiotic *B. subtilis* applied as positive control showed no significant effects on the growth and survival rates of L. *vannamei*.

Shariff et al. (2001) also reported that treatment of *P. monodon* with a commercial *Bacillus* probiotic did not significantly increase survival. As Gomez-Gil et al. (2000) said, the efficacy of a probiotic application depends on many factors, such as species composition, application level, frequency of application, and environmental conditions. The above-mentioned studies evidently support the results of our present study on the addition of PRO-2 to the shrimp feed that showed no significant effect on the growth and survival among treatments compared to the control group. However, it gave a higher survival rate when the animals were challenged with *Vibrio spp.* isolates. At 1 g kg^{-1} of feed, PRO-2 was effective against both *V. harveyi* and $VP_{AHPND,}$ and it can be safely administered prophylactically. This dosage may potentially be increased to 5 g kg^{-1} of feed during VP_{AHPND} outbreaks based on its significantly higher survival rate compared to the control group. In the case of *V. harveyi* outbreak, the dosage can be maintained at 1 g kg^{-1} of feed.

With regard to PRO—W, it can be safely added to the water at 2.0×10^2 g $ha^{-1,}$ according to the present study. Still, this amount is insufficient to improve the shrimp's survivability during the *V. harveyi* challenge. The preliminary results with 2.0×10^6 g of PRO-W ha-1 indicated that this dosage might be more effective during outbreaks, although not indicated for prophylactic purposes, but further investigation is needed to validate this result. Based on the results, determining the exact dose and the duration of application is recommended to confirm its efficacy further.

The present data also showed that the commercial disinfectant PUR is bactericidal against *Vibrio spp. in vitro* with a MIC/MBC ratio of 2, which is considered bactericidal (MBC/MIC $\leq$4) according to the criteria of Marmonier (1990). This effect, however, was only observed at doses that were lethal for the shrimp. Tolerable doses of PUR had no beneficial effect when the shrimp were challenged with either VP_{AHPND} or *V. harveyi*, and therefore, based on the current results, we can recommend its addition to the rearing water only for disinfection purposes but not directly on the shrimp itself. PUR should be used at 5000 ppm or higher to clean and disinfect tanks and paraphernalia. Potassium peroxymonosulfate, the main active ingredient of PUR, has indeed been recommended to disinfect nets when rearing zebrafish (*Danio rerio*) (Collymore et al., 2014). Similarly, Avendaño-Herrera et al. (2006) recommended using hydrogen peroxide at 240 ppm to disinfect the tanks and treat the water before the introduction of turbot.

Our challenge tests were performed in adult shrimp, but the results would likely have been more striking in larval shrimp, as probiotics are more effective at these developmental stages (Silva et al., 2012). Also, challenge trials by pathogen injection on shrimp likely produce a much more acute effect than natural infection during an outbreak. Therefore, testing the probiotics in naturally occurring outbreaks is vital, as their effects might be more substantial in such conditions. Furthermore, it will be essential to test the potentially additive or synergistic effects of the combined use of probiotics in the water and the feed.

In conclusion, probiotic interventions improved shrimp's survivability rate against pathogenic *Vibrios*. Application of PRO 2 at 1 g kg^{-1} of feed can help improve the survival of L. *vannamei* against VP_{AHPND} and *V. harveyi*. However, during challenging conditions such as VP_{AHPND} outbreaks, the dosage can be increased to 5 g kg^{-1} of feed. The application of PRO W is recommended to be extended for more than seven days to effectively improve the survival *of L. vannamei* against VP $_{AHPND}$ and *V. harveyi*. Chemical disinfectant (PUR) at high concentrations (2500 ppm & 5000 ppm) is recommended to disinfect pond liners, tanks, and other paraphernalia. Moreover, if added to the rearing water, appropriate concentration and timing of application are advised.

Ethics approval

No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

Funding

This work was supported by the Southeast Asian Fisheries Development Center/Aquaculture Department for the support (FH-01-C2016T).

CRediT authorship contribution statement

Leobert D. de la Peña: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Germin L. Moquera:** Writing – original draft, Methodology, Formal analysis, Data curation. **Edgar C. Amar:** Writing – review & editing, Validation, Resources, Methodology. **Jose Louis A. Castellano:** Writing – review & editing, Data curation. **Nikko Alvin R. Cabillon:** Writing – review & editing, Data curation. **Joey I. Arboleda:** Writing – review & editing, Data curation. **Joseph Keith Paulo T. Nava:** Writing – original draft, Writing – review & editing. **Rodolfo V. Zamora:** Resources, Funding acquisition. **Peter De Schryver:** Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

Acknowledgements

Special thanks to the Southeast Asian Fisheries Development Center/Aquaculture Department for the support (FH-01-C2016T), the shrimp farm for providing experimental animals, and the Fish Health Section staff for assistance during this study. The authors acknowledge Miguel Leal for their comments to improve the quality of the manuscript.

References

Aguilera-Rivera, D., Prieto-Davó, A., Rodríguez-Fuentes, G., Escalante-Herrera, K.S., Gaxiola, G., 2019. A vibriosis outbreak in the Pacific white shrimp, *Litopenaeus vannamei,* reared in bio floc and clear seawater. J. Invertebr. Pathol. 167, 107246.

Aguilllahi-Arpanahi, D., Soltani, E., Jafaryan, H., Soltani, M., Naderi-Samani, M., Campa-Córdova, A.I., 2018. Efficacy of two commercial and indigenous probiotics, *Bacillus subtilis* and *Bacillus licheniformis,* on growth performance, immuno-physiology and resistance response of juvenile white shrimp (*Litopenaeus vannamei*). Aquaculture 496 (1), 43–49.

Aribah, D., Wahyudi, A.T., 2022. The effectiveness of marine bacterial microcapsules in controlling vibriosis disease caused by the infection of *Vibrio parahaemolyticus* in white shrimp *Litopenaeus vannamei*. Aquaculture 549, 737795.

Austin, B., Zhang, X.H., 2006. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Lett. Appl. Microbiol. 43, 119–124.

Avendaño-Herrera, R., Magariños, B., Irgang, R., Toranzo, A.E., 2006. Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophthalmus maximus*). Aquaculture 257, 104–110.

Baticados, M.C.L., Lavilla-Pitogo, C.R., Cruz-Lacierda, E.R., de la Peña, L.D., Sunaz, N.A., 1990. Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. Dis. Aquat. Org. 9, 133–139.

Breed, R.S., Murray, E.G., Hitchens, A.P., 1944. The outline classification used in the Bergey manual of determinative bacteriology. Bacteriol. Rev. 8, 255–260.

Browdy, C.L., Bratvold, D., Stokes, A.D., McIntosh, R.P., 2001. Perspectives on the application of closed shrimp culture systems. In: The Rising Tide, Proceedings of the Special Session on Sustainable Shrimp Farming. The World Aquaculture Society, Baton Rouge, LA, USA, pp. 20–34.

Cockerill, F., 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard. CLSI.

Collymore, C., Porelli, G., Lieggi, A., Lipman, N.S., 2014. Evaluation of 5 cleaning and disinfection methods for nets used to collect zebrafish (*Danio rerio*). J. Am. Assoc. Lab. Anim. Sci. 53, 657–660.

De Bodt, J., Defoirdt, T., 2018. Impact of the organic load on the efficacy of chlorine disinfection against acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus*. J. Fish Dis. 41, 1609–1612.

de la Peña, L.D., Lavilla-Pitogo, C.R., Paner, M.G., 2001. Luminescent vibriosis associated with mortality in pond cultured shrimp *Penaeus vannamei*: species composition. Fish Pathol. 36 (3), 133–138.

de la Peña, L.D., Cabillon, N.A.R., Catedral, D.D., Amar, E.C., Usero, R.C., Monotilla, W. D., Saloma, C.P., 2015. Acute hepatopancreatic necrosis disease (AHPND) outbreaks

in *Penaeus vannamei* and *Penaeus monodon* cultured in the Philippines. Dis. Aquat. Org. 116, 251–254.
Decamp, O.D., Moriarty, J.W., Lavens, P., 2008. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquac. Res. 39, 334–338.
Dhar, A.K., Piamsomboon, P., Caro, L.F.A., Kanrar, S., Adami Jr., R., Juan, Y.S., 2019. First report of acute hepatopancreatic necrosis disease (AHPND) occurring in the USA. Dis. Aquat. Org. 132, 241–247.
Dong, X., Wang, H., Zou, P., Chen, J., Liu, Z., Wang, X., Huang, J., 2017. Complete genome sequence of *Vibrio campbellii* strain 20130629003S01 isolated from shrimp with acute hepatopancreatic necrosis disease. Gut Pathogens 9, 1–5.
Dvorak, G., 2008. Disinfection 101. In: Center Food Security and Public Health, pp. 1–20.
Far, H.Z., Saad, C.R.B., Daud, H.M., Harmin, S.A., Shakibazadeh, S., 2009. Effect of *Bacillus subtilis* on the growth and survival rate of shrimp (*Litopenaeus vannamei*). Afr. J. Biotechnol. 8 (14).
Flegel, T., Lo, C., 2014. Announcement regarding free release of primers for specific detection of bacterial isolates that cause acute hepatopancreatic necrosis disease (AHPND). Aquac. Asia 19, 30.
Gilbert, P., McBain, A.J., 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clin. Microbiol. Rev. 16, 189–208.
Gomez-Gil, B., Roque, A., Turnbull, J.F., 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191, 259–270.
He, W., Rahimnejad, S., Wang, L., Song, K., Lu, K., Zhang, C., 2017. Effects of organic acids and essential oils blend on growth, gut microbiota, immune response and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) against *Vibrio parahaemolyticus*. Fish Shellfish Immunol. 70, 164–173.
Hill, J., Baiano, J., Barnes, A., 2009. Isolation of a novel strain of *Bacillus pumilus* from penaeid shrimp that is inhibitory against marine pathogens. J. Fish Dis. 32, 1007–1016.
Holmström, K., Gräslund, S., Wahlström, A., Poungshompoo, S., Bengtsson, B.E., Kautsky, N., 2003. Antibiotic use in shrimp farming and implications for environmental impacts and human health. Int. J. Food Sci. Technol. 38, 255–266.
Jamal, M.T., Abdulrahman, I.A., Al Harbi, M., Chithambaran, S., 2019. Probiotics as alternative control measures in shrimp aquaculture: a review. J. Appl. Biol. Biotechnol. 7 (3), 69–77.
Kewcharoen, W., Srisapoome, P., 2019. Probiotic effects of *Bacillus* spp. from Pacific white shrimp (*Litopenaeus vannamei*) on water quality and shrimp growth, immune responses, and resistance to *Vibrio parahaemolyticus* (AHPND strains). Fish Shellfish Immunol. 94, 175–189.
Kondo, H., Van, P.T., Dang, L.T., 2015. Draft genome sequence of non-*Vibrio parahaemolyticus* acute diseased shrimp in Vietnam. Genome Announc. 3, 2014–2015.
Lavilla-Pitogo, C.R., Leaño, E.M., Paner, M.G., 1998. Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent vibrios in the rearing environment. Aquaculture 164, 337–349.
Leal, J.F., Neves, M.G.P., Santos, E.B., Esteves, V.I., 2018. Use of formalin in intensive aquaculture: properties, application and effects on fish and water quality. Rev. Aquac. 10, 281–295.
Lee, C.T., Chen, I.T., Yang, Y.T., Ko, T.P., Huang, Y.T., Huang, J.Y., Lo, C.F., 2015. The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proc. Natl. Acad. Sci. U.S.A. 112, 10798–10803.
Li, K., Zheng, T., Tian, Y., Xi, F., Yuan, J., Zhang, G., Hong, H., 2007. Beneficial effects of *Bacillus licheniformis* on the intestinal microflora and immunity of the white shrimp, *Litopenaeus vannamei*. Biotechnol. Lett. 29, 525–530.
Lightner, D.V., Redman, R.M., Pantoja, C.R., Noble, B.L., Tran, L., 2012. Early mortality syndrome affects shrimp in Asia. Glob. Aquac. Advoc. 15, 40.
Liu, L., Xiao, J., Xia, X., Pan, Y., Yan, S., Wang, Y., 2015. Draft genome sequence of *Vibrio owensii* strain SH-14, which causes shrimp acute hepatopancreatic necrosis disease. Genome Announc. 3, 3354.
Madani, N.S.H., Adorian, T.J., Farsani, H.G., Hoseinifar, S.H., 2018. The effects of dietary probiotic Bacilli (*Bacillus subtilis* and *Bacillus licheniformis*) on growth performance, feed efficiency, body composition and immune parameters of whiteleg shrimp (*Litopenaeus vannamei*) postlarvae. Aquac. Res. 49 (5), 1926–1933.wc.
Mainous, M.E., Smith, S.A., Kuhn, D.D., 2010. Effect of common aquaculture chemicals against *Edwardsiella ictaluri* and *E. tarda*. J. Aquat. Anim. Health 22, 224–228.
Marmonier, A.A., 1990. Introduction Aux Techniques D'étude des Antibiotiques. In: Bactériologie Médicale. Techniques Usuelles, Doin, Paris, pp. 227–236.
Nunan, L., Lightner, D., Pantoja, C., Gomez-Jimenez, S., 2014. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Dis. Aquat. Org. 111, 81–86.
Park, J.H., Seok, S.H., Cho, S.A., Baek, M.W., Lee, H.Y., Kim, D.J., Park, J.H., 2004. Safety and protective effect of a disinfectant (STEL water) for white spot syndrome viral infection in shrimp. Dis. Aquat. Org. 60, 253–257.
Penir, S.M.U., de la Pena, L.D., Cabillon, N.A.R., Bilbao, A.D.P., Amar, E.C., Saloma, C.P., 2019. Draft genome sequence of *Vibrio parahaemolyticus* strain PH 1339, which causes acute hepatopancreatic necrosis disease in shrimp in the Philippines. Microbiol. Resour. Announc. 8 e01020–19.
Primavera, J.H., Lavilla-Pitogo, C.R., Ladja, J.M., Pena, M.D., 1993. A survey of chemical and biological products used in intensive shrimp farms in the Philippines. Mar. Pollut. Bull. 26, 35–40.
Puente, M.E., Vega-Villasante, F., Holguin, G., Bashan, Y., 1992. Susceptibility of the brine shrimp Artemia and its pathogen *Vibrio parahaemolyticus* to chlorine dioxide in contaminated seawater. J. Appl. Bacteriol. 73, 465–471.
Rajyalakshmi, K., Babu, M.K., Shabana, S., Satya, A.K., 2021. Identification and screening of probiotics as a biocontrol agent against pathogenic vibriosis in shrimp aquaculture. Ann. Roman. Soc. Cell Biol. 25 (6), 12292–12305.
Shariff, M., Yusoff, F.M., Devaraja, T.N., Rao, P.S., 2001. The effectiveness of a commercial microbial product in poorly prepared tiger shrimp, *Penaeus monodon* (Fabricius), ponds. Aquac. Res. 32 (3), 181–187.
Silva, E.F., Soares, M.A., Calazans, N.F., Vogeley, J.L., do Valle, B.C., Soares, R., Peixoto, S., 2012. Effect of probiotic (*Bacillus* spp.) addition during larvae and postlarvae culture of the white shrimp *Litopenaeus vannamei*. Aquac. Res. 44, 13–21.
Soto-Rodriguez, S.A., Gomez-Gil, B., Lozano-Olvera, R., Betancourt-Lozano, M., Morales-Covarrubias, M.S., 2015. Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. Appl. Environ. Microbiol. 81, 1689–1699.
Thompson, J., Gregory, S., Plummer, S., Shields, R.J., Rowley, A.F., 2010. An *in vitro* and *in vivo* assessment of the potential of *Vibrio* spp. as probiotics for the Pacific white shrimp, *Litopenaeus vannamei*. J. Appl. Microbiol. 109, 1177–1187.
Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons, K., Lightner, D.V., 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis. Aquat. Org. 105, 45–55.
Tseng, D.Y., Ho, P.L., Huang, S.Y., Cheng, S.C., Shiu, Y.L., Chiu, C.S., Liu, C.H., 2009. Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20. Fish Shellfish Immunol. 26, 339–344.
Vaseeharan, B., Ramasamy, P., 2003. Control of pathogenic Vibrio spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett. Appl. Microbiol. 36 (2), 83–87.
Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. 64, 655–671.
Wang, W., Li, M., Li, Y., 2015. Intervention strategies for reducing *Vibrio parahaemolyticus* in seafood: a review. J. Food Sci. 80, R10–R19.
Werasan, K., Prapansak, S., 2019. Probiotic effects of *Bacillus* spp. from Pacific white shrimp (*Litopenaeus vannamei*) on water quality and shrimp growth, immune responses, and resistance to *Vibrio parahaemolyticus* (AHPND strains). Fish Shellfish Immunol. 94, 175–189.
Zhao, J., Chen, M., Quan, C.S., Fan, S.D., 2015. Mechanisms of quorum sensing and strategies for quorum sensing disruption in aquaculture pathogens. J. Fish Dis. 38, 771–786.