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Biocontrol of Acute Hepatopancreatic Necrosis Disease (AHPND)

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BIOCONTROL OF ACUTE HEPATOPANCREATIC
NECROSIS DISEASE (AHPND)

BY

JASON LAPORTE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

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MASTER OF SCIENCE THESIS

OF

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2017

ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) causes mass mortalities in farmed penaeid shrimp and has proven difficult to control using typical disease control measures. The causative agent of AHPND has been identified as *Vibrio parahaemolyticus* strains possessing the 69 kbp plasmid pVPA3-1 containing genes homologous with *Photobacterium* insect-related (Pir) toxin-like genes (*pirA*- and *pirB*-like). Probiotics have been used successfully in shrimp aquaculture to control disease outbreaks caused by pathogenic *Vibrio*, but there are currently no probiotics available that have been proven to control AHPND. The goal of this study was to screen and characterize marine bacterial isolates as potential agents to prevent *Artemia* nauplii and *Litopenaeus vannamei* post-larvae (PL) mortality by the pathogen *Vibrio parahaemolyticus*. Twelve candidate probiotic organisms were tested in an *Artemia* sp. model. *Phaeobacter inhibens* was the only candidate probiont that significantly increased the survival of *Artemia* nauplii challenged with AHPND *V. parahaemolyticus* ($p < 0.001$). Candidate probionts *Pseudoalteromonas piscicida*, *Pseudoalteromonas flavipulchra*, and *Pseudoalteromonas arabiensis* were lethal to *Artemia* nauplii ($p < 0.001$). Six species of candidate probiotic organisms were tested in *L. vannamei*. *P. inhibens* was the only candidate probiont tested which was not harmful to *L. vannamei* PLs and significantly increased the survival of PLs challenged with AHPND *V. parahaemolyticus* ($p < 0.001$). Genome analysis of *V. parahaemolyticus* PSU5579 revealed the presence of the multiple putative virulence genes including nine hemolysins, six secreted proteases, and six secretion systems including one T3SS and two T6SS. The genome also contains the 69 kbp pVPA3-1

plasmid encoding the *pirA*- and *pirB*-like toxin genes. Genome analysis of *Bowmanella denitrificans* JL63 revealed several gene clusters potentially involved in the production of the following antibacterial compounds: colicin V (or bacteriocin), lanthionine, the broad-spectrum antibacterial protein marinocine encoded by the *lodAB* operon, a secreted hemolysin-type calcium-binding bacteriocin, lantipeptide, bacteriocin, and a nonribosomal peptide.

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DEDICATION

I dedicate this thesis to my parents Carol and James Ionata. Thank you for always believing in me and encouraging me to further my education and professional career. I am unendingly grateful for your support and continuous confidence in me.

I would also like to dedicate this thesis to Linda LaPorte. Thank you for your continuous love and support. I am incredibly grateful that we share a passion for microbiology and that I was able to undertake this journey through graduate school together with you.

PREFACE

The following thesis has been prepared in manuscript format according to the guidelines of the Graduate School of the University of Rhode Island. This thesis contains a literature review and three manuscripts.

The first manuscript “Biocontrol of acute hepatopancreatic necrosis disease (AHPND)” will be submitted to BMC Microbiology.

The second manuscript “Draft genome sequence of *Vibrio parahaemolyticus* PSU5579, isolated during an outbreak of acute hepatopancreatic necrosis disease (AHPND) in Thailand.” will be submitted to Genome Announcements.

The third manuscript “Draft genome sequence of *Bowmanella denitrificans* JL63, a bacterium isolated from whiteleg shrimp (*Litopenaeus vannamei*) that can inhibit the growth of *Vibrio parahaemolyticus*” will be submitted to Genome Announcements.

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Literature Review

Introduction

In 2009 an emerging disease now known as acute hepatopancreatic necrosis disease (AHPND) began to affect penaeid shrimp farms in southern China [1, 2]. The disease has spread to Vietnam, Malaysia, Thailand, and Mexico and global losses from AHPND are estimated to amount to more than one billion US dollars annually [2-5]. AHPND causes serious production losses in affected areas which negatively impacts local employment, social welfare, and international markets [6]. The causative agent of AHPND has been identified as *Vibrio parahaemolyticus* strains possessing the 69 kbp plasmid pVPA3-1 containing genes homologous with *Photorhabdus* insect-related (Pir) toxin-like genes (*pirA*- and *pirB*-like) [2, 7, 8]. AHPND has proven difficult to control using typical disease control measures such as water disinfection and antibiotic treatment [9, 10].

Beneficial microbes known as probiotics have been used to improve the health and disease tolerance of terrestrial farm animals since the 1940s, and research on probiotics in aquaculture has continued to increase since the late 1980s [11-14]. Studies have shown that probiotics can be used in aquaculture to prevent diseases in a variety of farmed species while also improving harvest yields [14-21]. Probiotics can provide various benefits in aquaculture including improvement of water quality, enhancement of nutrition of host species, reduced incidence of diseases, higher survival rates, and improved host immune response [15, 16, 22]. Probiotics have been used successfully in shrimp aquaculture to control disease outbreaks caused by pathogenic *Vibrio* spp. [14-18] and may have the potential to control AHPND. Probiotics provide an alternative to the use of antibiotics in aquaculture, which have

become increasingly controversial and ineffective due to the emergence of antibiotic resistance in bacteria [15, 23-25]. Members of the genus *Phaeobacter* have been shown to be effective probiotic organisms by protecting cod and turbot larvae from the pathogen *Vibrio anguillarum* [26, 27], as well as eastern oyster (*Crassostrea virginica*) larvae from the pathogens *Aliiroseovarius crassostreae* CV919-312^T and *Vibrio coralliilyticus* RE22 [19, 28]. The marine bacterium *Phaeobacter inhibens* S4Sm is an excellent biofilm former [28], produces the broad-spectrum antibiotic tropodithietic acid (TDA) [28], can quench/inhibit the quorum sensing-dependent production of the virulence factor protease in *V. coralliilyticus* RE22 [29], and is non-toxic to eukaryotic organisms [30], which makes it an ideal candidate for the control of bacterial diseases in aquaculture such as AHPND.

Main Body

Acute Hepatopancreatic Necrosis Disease (AHPND)

Aquaculture is the world's fastest growing food production sector with cultured shrimp increasing at an annual rate of 16.8 % [31]. In 2007, shrimp harvested from aquaculture surpassed wild-caught shrimp, and in 2013, aquaculture produced 4.45 million metric tons of shrimp [32]. As of 2012, the shrimp farming industry was worth an estimated \$19.4 billion [32]. Southeast Asia and China have the largest and most productive shrimp farming regions in the world with 77% of globally produced shrimp coming from Asia [32]. In 2009 an emerging disease first called early mortality syndrome (EMS) began to affect shrimp farms in southern China [1]. The disease has recently been given a more descriptive name, acute hepatopancreatic necrosis disease (AHPND) [2]. Since its emergence, the disease has spread to Vietnam, Malaysia, Thailand, and Mexico [2-4]. AHPND affects both whiteleg shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*) and can lead to 100% mortality in affected populations [3]. The causative agent of AHPND has been identified as *V. parahaemolyticus* strains possessing the 69 kbp plasmid pVPA3-1 containing genes homologous with *Photorhabdus* insect-related (Pir) toxin-like genes (*pirA*- and *pirB*-like) [2, 7].

Initial studies determined that the pathology of AHPND is limited to the hepatopancreas (HP) which suggests that the disease may have a toxin-mediated etiology [19, 33]. It has also been shown that cell-free supernatant from *V. parahaemolyticus* strains possessing pVPA3-1 can cause AHPND, supporting the conclusion that a toxin is associated with the disease [19]. AHPND develops

approximately eight days after ponds are stocked with shrimp post-larvae (PLs) and severe mortalities occur within the first 20-30 days [19, 33]. Early signs of AHPND include a pale to white HP, reduced HP size, empty stomach, and empty midgut (Figure 1) [19]. Histological analysis of the HP has revealed three stages of AHPND: initial, acute, and terminal. In the initial stage, the epithelial cells of the HP are elongated into tubular lumen and there is a reduction of the vacuole size in R (resorptive) and B (blister like) cells [33]. In the acute stage, the tubular epithelium is necrotic with severe desquamation of the cells showing hemocytic infiltration as a response to the necrotic epithelium [33]. In the terminal stage of the disease, the HP tubules show a severe inflammatory response and the tubular epithelium becomes entirely necrotic with massive sloughing of epithelial cells (Figure 2) [33-35]. At this stage, low levels of *Vibrio* can be found in the necrotic tissue in the HP and higher loads of *Vibrio* can be found in the stomach [33]. Additionally, there is increased hemocyte infiltration and black streaks or spots develop in the HP due to melanin deposition from hemocyte activity [19-33]. The absence of an inflammatory response that is usually elicited by a pathogen during the early stages of AHPND strongly supports the conclusion that this disease has a toxin-mediated etiology [2].

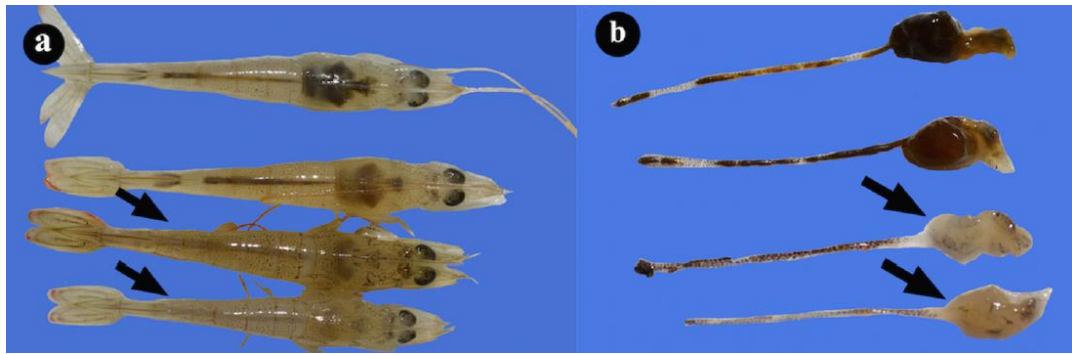


Figure 1. (a) Photographs of hepatopancreases from healthy *L. vannamei* shrimp (upper two) and shrimp naturally infected with AHPND (arrows). (b) The hepatopancreas without external membrane shows atrophy and white color [33].

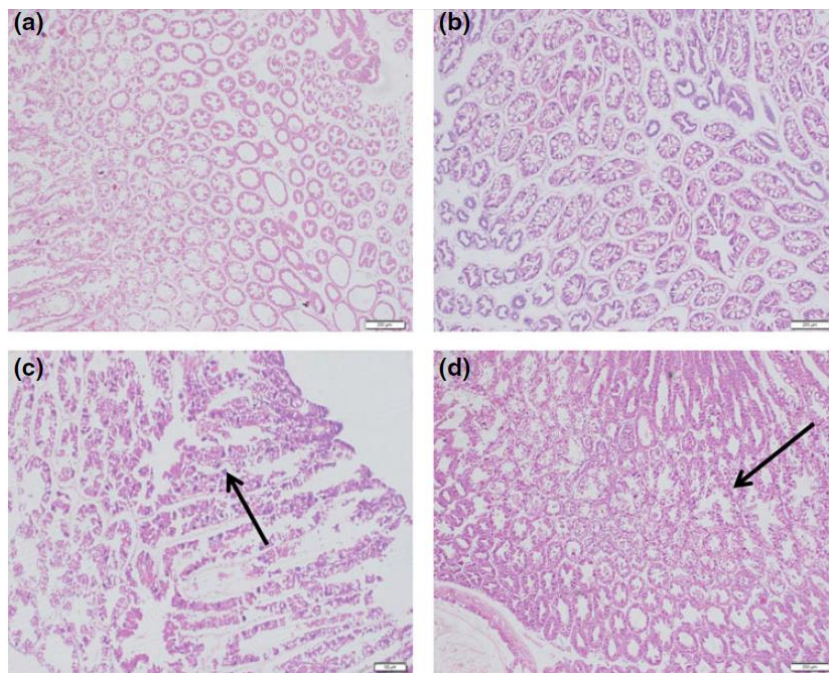


Figure 2. Histopathological analysis of hepatopancreas of shrimp challenged by immersion with AHPND *V. parahaemolyticus*. Necrosis and sloughing (arrows) of hepatopancreas were observed when challenged at 10^5 CFU/ml (c), and these signs were more severe at 10^6 CFU/ml (d); however, non-AHPND pathology was found at 10^3 CFU/ml (a) and 10^4 CFU/ml (b) [35]. Sloughing can be observed as cells round up and detach into the tubule lumens.

AHPND causes serious production losses in affected areas which negatively impacts local employment, social welfare, and international markets [6]. Global losses from shrimp disease are estimated to amount to around three billion US dollars annually [31] with losses from AHPND amounting to more than one billion US dollars annually [5]. Disease prevention can be challenging for shrimp farmers because most farmers do not have the resources to treat seawater before it is used to fill their ponds [36] and by the time shrimp are showing signs of AHPND, it is difficult to treat as antibiotic treatment has proven unsuccessful in most cases [10]. Additionally, treating water sources with chlorine, ozone, or UV before stocking does not provide total sterility [9]. Further, disinfection of water perturbs the natural microbial balance and leaves the environment open to opportunistic bacteria which survived disinfection. This can actually favor the growth of *Vibrio* as *Vibrio* grow rapidly after their competitors are removed [37]. *V. parahaemolyticus* has been reported to have a generation time as short as 12 minutes [38].

Current recommendations to prevent AHPND outbreaks in shrimp farms include the use of greenwater systems [39] or the application of biocontrol strategies such as probiotics [5], phage [40], or *Bdellovibrio*-and-like organisms (BALOs) [41]. It has been observed that AHPND is less prevalent in ponds colonized by copepods [39]. Copepods require a constant supply of phytoplankton and bacteria as feed, so their presence in an indicator of a mature ecosystem [42]. The use of greenwater systems has also been observed to reduce the incidence of AHPND [39]. Greenwater systems are characterized by a mature micro-algal and bacterial community. These systems have been shown to maintain decreased *Vibrio* levels and decreased animal

mortality [43, 44]. The beneficial effect of greenwater systems can be attributed to the algal and bacterial production of antibacterial substances [45, 46] and compounds which quench/inhibit quorum sensing-dependent production of virulence factors in pathogens [47]. Additionally, the bacteria in greenwater systems compete with pathogens for available nutrients and occupy niches which would otherwise be left open for invading pathogens [46]. Occurrences of overgrowth of pathogenic bacteria such as *Vibrio* spp. in shrimp grow-out ponds can be reduced by minimizing disturbances such as water disinfection which lead to sudden variations in nutrient levels, and by colonizing pond water with nonpathogenic bacteria and/or algae [48].

Probiotic bacteria have been used successfully in shrimp aquaculture to control disease outbreaks caused by pathogenic *Vibrio* spp. [14-16]. A recent study determined that the probiotics which are currently commercially available to shrimp farmers in Malaysia are not effective at controlling AHPND [49]. More research needs to be conducted to develop and test new probiotic formulations which may have the potential to control AHPND. The use of phage has also been proposed as a potential strategy to control AHPND, and a virulent *Siphoviridae* phage, pVp-1, has been shown to have effective bacteriolytic activity against 74% of AHPND strains of *V. parahaemolyticus* tested, but has yet to be tested in an aquaculture setting [40]. Another promising biocontrol strategy to prevent AHPND involves the use of BALOs. A recent study isolated a BALO, identified as *Bacteriovorax* sp. BV-A, from a sediment samples in a shrimp farm in Thailand, which could kill all AHPND strains of *V. parahaemolyticus* tested as well as *Vibrio vulnificus*, *Vibrio cholerae*, and *Vibrio alginolyticus* [41]. *Bacteriovorax* sp. BV-A was also shown to increase the survival of

L. vannamei PLs challenged with AHPND *V. parahaemolyticus* by 50% [41]. In field studies, BALOs in combination with photosynthetic bacteria have been shown to provide increased survival rates of Chinese mitten crab (*Eriocheir sinensis*) and decreased *Vibrio* concentrations in cultured pufferfish (*Fugu obscurus*) [50]. The use of BALOs is a promising prospect for the control of diseases caused by bacterial pathogens in aquaculture.

Vibrio parahaemolyticus

Vibrio is a genus of Gram-negative motile marine bacteria of the family Vibrionaceae within the Gammaproteobacteria [51]. Members of this genus are facultative anaerobes with a curved-rod shape [52]. *Vibrio* species can be found in a wide range of aquatic environments, including the water column, in association with hosts (both pathogenic and symbiotic), and even in extreme habitats (hydrothermal vents) [53]. Pathogenicity in *Vibrio* is not species dependent, but rather strain specific as different strains of the same species can cause diseases in different hosts, or can be nonpathogenic [54, 55]. Many species of *Vibrio* are pathogenic and can cause disease in humans (e.g. *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*) [53, 56, 57], fish (*V. anguillarum*, *V. alginolyticus*, *V. harveyi*) [53, 58], bivalves (*V. coralliilyticus*, *V. tubiashii*, *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus*) [59], coral (*V. coralliilyticus*) [60], or shrimp (*V. parahaemolyticus*, *V. harveyi*, *V. campbellii*, *V. alginolyticus*) [53]. The aquaculture industry suffers multibillion-dollar losses due to these pathogens annually [5, 53, 61].

Vibrio spp. are known to possess a number of virulence factors including enterotoxins, cytotoxins such as the multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins which use a type I secretion system (T1SS), siderophores, adhesion factors/biofilm formation (type I pili), extracellular polysaccharides, hemagglutinins, type III secretion systems (T3SS), type VI secretion systems (T6SS), and lytic enzymes including hemolysins, proteases, lipases, and chitinases, most of which use type II secretion systems (T2SS) [53, 62-64]. Virulence gene expression in *Vibrio* is regulated by quorum sensing and has been studied extensively in *V. harveyi* [53, 64]. *V. harveyi* uses a three-channel quorum-sensing system (Figure 3), secreting chemical signal molecules that include HAI-1 (Harveyi autoinducer 1), AI-2 (Autoinducer 2), and CAI-1 (Cholera autoinducer 1) [53]. The concentration of these molecules in the extracellular environment is proportional to cell density. These autoinducers are detected at the cell surface by membrane bound histidine sensor kinase proteins that feed a phosphorylation/dephosphorylation signal transduction pathway which controls the production of the quorum-sensing master regulator protein LuxR (*V. harveyi*)/OpaR (*V. parahaemolyticus*) [53]. LuxR/OpaR directly activates the Lux operon, whereas most of the other genes regulated by quorum sensing are controlled indirectly [53]. Several species of *Vibrio*, including *V. cholerae* and *V. parahaemolyticus*, have virulence factors which are controlled by the ToxR regulon [53]. In *V. cholerae*, the ToxR regulon controls the expression of the *ctx* gene encoding the cholera toxin [53]. In *V. parahaemolyticus* the *toxR* operon controls the expression of the thermostable direct hemolysin gene (*tdh*) as well as the T3SS [65,

66]. The *toxR* operon is found in both clinical and environmental isolates of *V. cholerae* and *V. parahaemolyticus* [67].

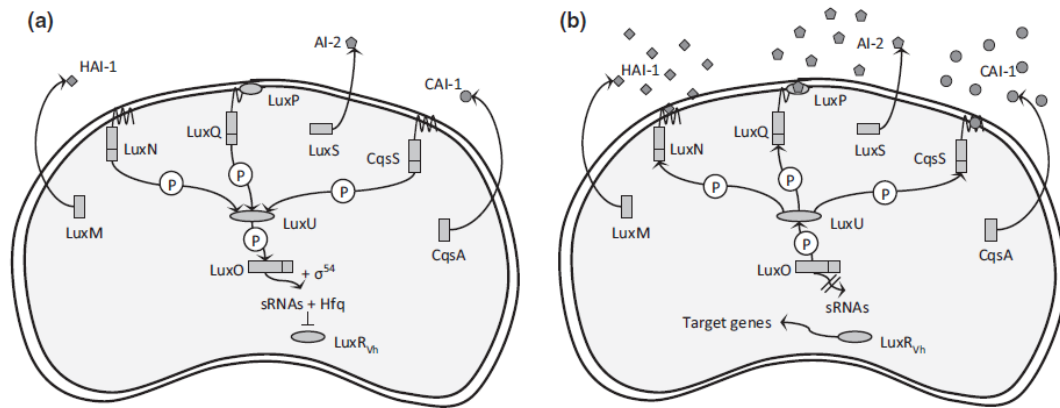


Figure 3. Quorum sensing in *Vibrio harveyi*. The LuxM, LuxS and CqsA enzymes synthesise the autoinducers HAI-1, AI-2 and CAI-1, respectively. These autoinducers are detected at the cell surface by the LuxN, LuxQ and CqsS two-component receptor proteins, respectively. Detection of AI-2 by LuxQ requires the periplasmic protein LuxP. (a) In the absence of autoinducers, the receptors autophosphorylate and transfer phosphate to LuxO via LuxU. Phosphorylation activates LuxO, which together with σ^{54} activates the production of five small regulatory RNAs (sRNAs). These sRNAs, together with the chaperone Hfq, destabilise the mRNA encoding the transcriptional regulator LuxR_{Vh}. Therefore, in the absence of autoinducers, the LuxR_{Vh} protein is not produced. (b) In the presence of high concentrations of the autoinducers, the receptor proteins switch from kinases to phosphatases, which results in dephosphorylation of LuxO. Dephosphorylated LuxO is inactive and, therefore, the sRNAs are not formed and the transcriptional regulator LuxR_{Vh} is produced. P, phosphotransfer [53].

Vibrio parahaemolyticus is commonly found in marine coastal waters and estuarine environments including water, sediment, suspended particles, plankton, fish

and shellfish [53]. Strains of this species are a leading cause of seafood-associated bacterial gastroenteritis globally and can also cause eye, ear, and wound infections [68]. While most environmental strains of *V. parahaemolyticus* are not pathogenic to humans, strains possessing the *tdh* and *trh* genes and the T3SS2 gene cluster are pathogenic [69, 70]. Strains of *V. parahaemolyticus* are also an important shrimp pathogen and have been identified as the causative agent of AHPND. One of the challenges of preventing and treating AHPND is the high frequency of antibiotic resistance found in *V. parahaemolyticus* isolates. Jiang et al. [71] found that 100% of the *V. parahaemolyticus* strains they isolated in China were resistant to ampicillin and cephalosporin and 43.7% were resistant to streptomycin. Shaw et al. [72] found that 68% of *V. parahaemolyticus* strains isolated in Maryland, USA were resistant to penicillin and Al-Othrubai et al. [73] found that 21.5% of *V. parahaemolyticus* strains isolated in Malaysia were resistant to ciprofloxacin. Additionally, Kongrueng et al. [74] tested AHPND strains of *V. parahaemolyticus* isolated in Thailand and found that all of these isolates were resistant to ampicillin and erythromycin.

AHPND strains of *V. parahaemolyticus* have been identified as those possessing the 69 kbp plasmid pVPA3-1 containing genes homologous with *Photobacterium* insect-related (Pir) toxin-like genes (*pirA*- and *pirB*-like) [2, 7, 8]. These genes are located within a 3.5 kbp fragment flanked by inverted repeats of a transposase-coding sequence (1 kbp) which is a mobile genetic element that can induce horizontal gene transfer [2]. The GC content of the *pirA*- and *pirB*-like genes is only 38.2%, which is considerably lower than that of the rest of the plasmid (45.9%), suggesting that these genes were recently acquired [2]. Similar to the Pir

toxins that affect insects, the Pir-like toxins act as binary proteins, which form a heterodimer and both *pirA*- and *pirB*-like genes are required for pathogenesis [2, 8, 75]. The crystal structure of the PirAB-like heterodimer has similar structural topology to that of the *Bacillus* Cry insecticidal toxin-like proteins, despite the low sequence identity (<10%), which suggests that the putative PirAB-like toxin might emulate the functional domains of the Cry protein and its pore-forming activity [75]. While the PirA- and PirB-like toxins affect the hepatopancreas in shrimp, the Pir toxins primarily affect the midgut of insects, which may suggest different mechanisms of action [2].

While AHPND strains of *V. parahaemolyticus* have been shown to possess between 1 and 121 copies of the pVPA3-1 plasmid per cell [2, 8], the copy number of this plasmid does not correlate with virulence [8]. Instead, the amount of secreted PirA- and PirB-like proteins determines virulence to shrimp [8]. AHPND strains of *V. parahaemolyticus* have been shown to possess other virulence factors as well, including T3SS1, T6SS1, and T6SS2 genes [74]. Additionally, a unique sequence encoding a type IV pilus has been found in the genomes of AHPND strains of *V. parahaemolyticus* isolated in Thailand and Mexico [7, 76], but was not detected in strains isolated in India [34]. It has also been shown that AHPND strains of *V. parahaemolyticus* lack the *tdh* and *trh* genes [7, 8, 19, 33, 34] as well as the T3SS2 gene [7, 34, 74] required for pathogenesis in humans, indicating that these strains are not human pathogens. The role of virulence factors other than the PirA- and PirB-like toxins in the pathogenesis of AHPND strains of *V. parahaemolyticus* to shrimp has yet to be determined.

Probiotics in aquaculture

For more than 70 years, beneficial microbes known as probiotics have been used to improve the health and disease tolerance of terrestrial farm animals such as swine and chickens [11-13]. Probiotics are defined as “live microorganisms, conferring a healthy benefit to the host when being consumed in adequate amounts” [77]. Probiotics are now widely used for enhancing production of land animals due to the fact that they are better, cheaper, and more effective in promoting animal health than antibiotics or chemical substances [21]. Research on the use of probiotics in aquaculture dates back to the late 1980s and has continued to increase since then [14]. Studies have shown that probiotics can be used in aquaculture to prevent diseases in bivalves (oysters, scallops), fish (salmon, cod, trout, halibut, turbot, catfish), and crustaceans (shrimp, *Artemia* spp.) [14-18, 20, 21]. Although probiotics can prevent disease when applied prophylactically, they are not meant to be used therapeutically and are unlikely to cure animals which are already infected with a pathogen [78, 79].

Currently the main rate limiting factor in the shrimp aquaculture industry is disease control. Intensive (high-density) shrimp culture systems have become common practice because they produce substantially higher shrimp yields than do semi-intensive systems [80]. This intensification comes at a cost however, and results in stressful environmental changes which can cause problems for shrimp [81] and also increase their susceptibility to disease [82]. Although vaccines have been developed against several bacterial diseases in fish, such vaccines are not successful in shrimp or any other invertebrates due to the lack on an adaptive immune system [20]. In aquaculture, bacterial disease is generally controlled through water disinfection and

the application of antibiotics both prophylactically and therapeutically. The use of antibiotics in aquaculture has become increasingly controversial and ineffective due to the emergence of antibiotic resistance in bacteria [15, 23-25]. Water disinfection also has limited success and in some cases may actually increase the likelihood of an outbreak, most notably in controlling diseases caused by *Vibrios* spp. such as AHPND [9, 10, 39]. Additionally, disinfecting water with chlorine has been shown to increase the proportion of multiple antibiotic resistance bacteria [83].

The overuse of antibiotics in aquaculture has become a major concern due to the emergence of antibiotic resistance in bacteria and the potential for residual contamination in harvested fish and shellfish. The aquaculture industry uses massive quantities of antibiotics which are released into the environment [24, 37]. For example, antibiotic usage in shrimp farms in Thailand in 1994 was estimated to be as much as 500 – 600 tonnes [37]. The leaching of these antibiotics into the environment contributes to the development of antibiotic resistance determinants in bacteria which can be spread to other species by horizontal gene transfer [24, 84]. These determinants can spread by horizontal gene transfer to bacteria of the terrestrial environment as well, including human and animal pathogens [24]. Studies have shown that antibiotic resistance determinants of *Salmonella enterica* serotype Typhimurium DT104, which caused several outbreaks of salmonellosis in humans and animals in Europe and the USA, likely originated in aquaculture settings of the Far East [24]. One study found that in the presence of tetracycline concentrations below the minimum inhibitory concentration (MIC), the rate of gene transfer between *V. cholerae* and *Aeromonas salmonicida* increased 100-fold [84]. Celli et al. [85]

proposed a molecular mechanism which may explain this increased rate of gene transfer in the presence of tetracycline. Transfer of the conjugative transposon Tn916, possessing the tetracycline resistance determinant *tetM*, requires excision of the element and is dramatically increased in the presence of tetracycline [85].

Tetracycline-based transcriptional attenuation of *pal_{orf12}* allows for transcription of *orf7* and *orf8* from the *tetM* promoter [85]. ORF7 and ORF8 then activate the promoter P_{*orf7*} which directs the expression of the transfer functions in the transposon allowing for transfer of the element [85].

There is also public health concern over potential exposure of human consumers to antibiotic residues or other chemical contaminants in shrimp harvested from aquaculture [86]. Undetected consumption of antibiotics in food can cause allergy and toxicity problems, alter normal flora and increases susceptibility to infections, and select for antibiotic-resistant bacteria [24]. In 2006, there was an antibiotic residue crisis in the flatfish industry in China where 25,000 tonnes of turbot could not be sold, costing the industry an estimated 200 million Euro [50]. For these reasons, the use of probiotics in aquaculture is becoming an increasingly popular alternative to the use of antibiotics [15].

Probiotics provide several benefits in aquaculture including improvement of water quality, enhancement of nutrition of host species, reduced incidence of diseases, higher survival rates, and improved host immune response [15, 16, 22]. Although there have been many studies on probiotics in aquaculture, they are still not widely used. Greenwater systems are a new water management strategy which use mature microalgal and bacterial communities and have been shown to have reduced *Vibrio*

levels and increased animal survival rates [43, 44]. Probiotics can be used not only as a biocontrol strategy, but can also be used in conjunction with algae treatment to make greenwater systems. In aquaculture, probiotics are typically added to the feed or directly into the culture water [15]. Probiotics have been used successfully in shrimp aquaculture to control disease outbreaks caused by pathogenic *Vibrio* spp. while also improving harvest yields [14-18]. The most common probiotics used in aquaculture are photosynthetic bacteria (purple non-sulfur bacteria), antagonistic bacteria (*Pseudoalteromonas* spp., *Flavobacterium* spp., *Alteromonas* spp., *Phaeobacter* spp., *Bacillus* spp.), microorganisms for improving digestion (lactic acid bacteria and yeast), bacteria for improving water quality (nitrifying bacteria, denitrifiers), and predatory bacteria that kill other bacteria (e.g. BALOs) [50].

Candidate probiotic organisms are typically selected based on their ability to produce antibacterial and/or antivirulence compounds. These compounds give an ecological advantage to the producing bacteria against other microorganisms, and may also provide an advantage against bacteriovorous eukaryotic predators [30]. Some organisms that have been shown to inhibit the growth of bacterial pathogens also produce compounds that are toxic to eukaryotic organisms [30, 87, 88]. These organisms should be tested thoroughly before being applied in aquaculture as they might cause adverse effects on the farmed animals, their prey species (algae, rotifers, or *Artemia* spp.), or the humans who consume them. Therefore, the toxicity of the live bacterial cultures on the target organisms should be tested for any adverse effects before being applied commercially. The candidate probiotics should be used to

challenge the target species under conditions which mimic the aquaculture setting before being tested in large scale.

Artemia spp. have been used as a model organism not only for toxicology studies but also to test the effectiveness of probiotic bacteria and the role of quorum sensing in pathogenesis [30, 89-93]. *Artemia* spp. are useful model organisms because they adapt easily to changes in nutrients, salinity, temperature, and oxygen, are easy to culture, are resistant to manipulation, and have a short life cycle [94]. Toxicology studies using *Artemia* sp. to evaluate potential probiotic organisms have shown that both *P. inhibens* and *Ruegeria mobilis* are innocuous to these organisms while *Pseudoalteromonas piscicida*, *Pseudoalteromonas rubra*, *Photobacterium halotolerans*, and *V. coralliilyticus* are lethal and therefore should not be used as probiotics in aquaculture [30].

The success of probiotic organisms can be attributed to several specific properties: 1) Improvement of water quality through the reduction of ammonia, nitrate, nitrite, phosphate, and carbon [95]. 2) Antagonistic activity through the production of compounds which inhibit the growth of pathogenic bacteria. Antagonistic bacteria have been shown to significantly decrease the concentration of *Vibrio* in shrimp ponds [96]. The production of inhibitory compounds against pathogens *in vitro* does not guarantee that potential probiotic organisms will be effective *in vivo* [97], but is still an important property of probiotic organisms which are effective [28, 95]. 3) Competition for attachment sites which is likely to serve as the first barrier of defense against invading pathogenic bacteria [28, 95]. 4) Competition for nutrients or available energy. Verschuere et al. [89] showed that pre-colonization of *Artemia* sp.

culture water with non-antagonistic probiotic bacteria protected *Artemia* sp. from the pathogenic effects of a *Vibrio proteolyticus* and hypothesized that this protection may be due to competition with the pathogen for available nutrients. 5) Enhancement of host digestion through the production of enzymes which can break down chitin, starch, protein, cellulose, and lipids [16]. 6) Stimulation of host immune response [15]. Although shrimp lack an adaptive immunity, they still possess an innate immune system that effectively protects them from harmful microorganisms and probiotic treatment has been shown to modulate the cellular and humoral immune responses in shrimp [95]. 7) Production of siderophores which compete with pathogens for ferric iron in the iron-limited environment of the host [15]. 8) Production of acyl-homoserine lactones (AHLs) which quench/inhibit the quorum sensing-dependent production of virulence factors in pathogens [29, 95]. Quorum sensing has been shown to be one of the virulence mechanisms of many pathogenic bacteria, including *V. harveyi* and *V. parahaemolyticus* [53, 98]. Organisms that are not harmful to host species and possess some, if not all, of these properties make ideal candidates for use as probiotics in aquaculture.

***Phaeobacter inhibens* S4Sm**

The Roseobacter clade consists of organisms that occupy diverse marine niches and colonize both biotic and abiotic surfaces including sediments, phytoplankton, invertebrates, and vertebrates [28, 99-102]. This clade is an important member of the marine microbiota, accounting for ~4 % to as much as ~40 % of bacterial DNA from the ocean depending on location, and plays an important role in

the organic sulfur cycle of the ocean by degrading dimethylsulfoniopropionate (DMSP) [100, 103-106]. Roseobacter clade members are also dominant primary surface colonizers [102, 107, 108] and are known to produce biologically active secondary metabolites [28, 109-112]. These secondary metabolites play an important role in the symbiotic relationship between *Phaeobacter gallaeciensis* and some marine algae species such as *Emiliania huxleyi* [109-111]. Algae provide a carbon and sulfur source for *P. gallaeciensis* in the form of DMSP and in return, the bacteria produce an algal growth promoter, phenylacetic acid, as well as a broad-spectrum antibiotic, tropodithietic acid (TDA), which suppresses the growth of parasitic bacteria [28, 109-112]. The bacteria switch from a mutualist to an opportunistic pathogen however, when the algae begin to senesce [109, 110]. Under these conditions the algae release p-coumaric acid, which triggers the bacteria to produce potent algaecides, roseobacticide A and B [109, 110].

Phaeobacter inhibens S4Sm, formerly classified as *P. gallaeciensis* S4Sm [107], is a member of the α -Proteobacteria from the Roseobacter clade. *P. inhibens* S4Sm is a pleomorphic rod with 1-2 flagella on one or both poles and can elongate and form rosettes in stationary phase [107]. It is a heterotrophic strict aerobe and grows optimally at temperatures between 18 and 30 °C [107]. *P. inhibens* S4Sm is a probiotic organism that can protect eastern oyster (*C. virginica*) larvae from bacterial pathogens [28, 107]. Pretreating oyster larvae with *P. inhibens* S4Sm significantly increases their survival after challenge with either *A. crassostreae* CV919-312^T or *V. coralliilyticus* RE22 (Figure 4) [107]. *P. gallaeciensis* can also protect cod and turbot larvae from *V. anguillarum*, the causative agent of vibriosis [26, 27].

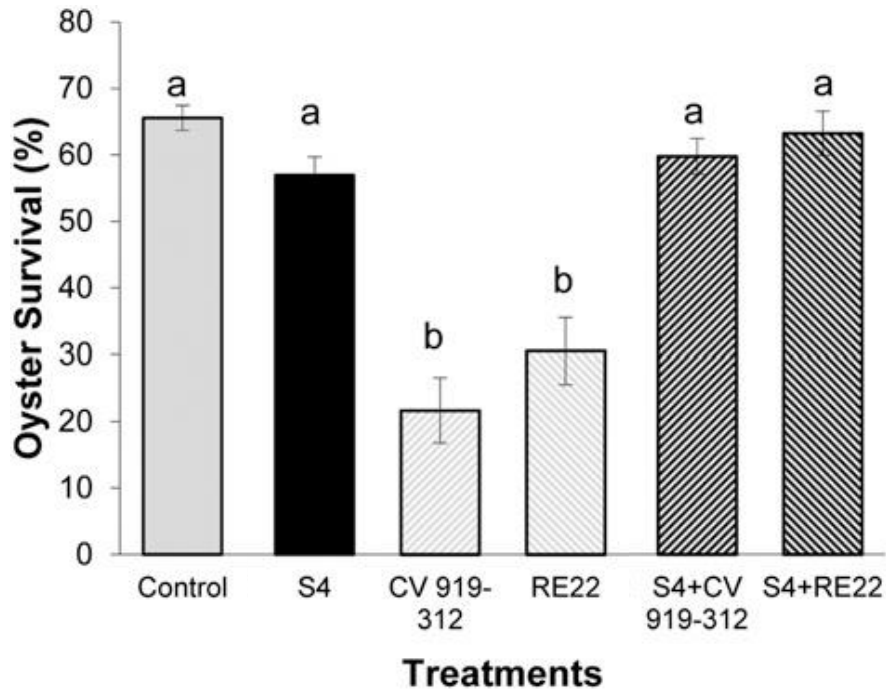


Figure 4. Effect of preincubation of larval oysters with candidate probiont *P. inhibens* S4Sm at 10^4 CFU/ml on survival (% \pm SD) 24 h after challenge with bacterial pathogens *A. crassostreae* CV919-312^T and *V. coralliilyticus* RE22 at 10^5 CFU/ml. The candidate probionts were introduced 24 h before larvae were challenged. Different letters indicate statistical significance among groups (1-way ANOVA, $p < 0.05$) [107].

The probiotic activity of *P. inhibens* S4Sm can be attributed to at least three factors: 1) excellent biofilm forming ability [28]; 2) production of TDA [28]; and 3) ability to quench/inhibit the quorum sensing-dependent production of the virulence factor protease in *V. coralliilyticus* RE22 [29]. Toxicology studies using *Artemia* sp. and *Caenorhabditis elegans* have shown that both *P. inhibens* as well as purified TDA are innocuous for these organisms [30]. *P. inhibens* S4Sm produces a more robust biofilm than the fish pathogen *V. anguillarum* or the oyster pathogens *A. crassostreae* or *V. coralliilyticus* (Table 1) [28]. Knockout mutants of *P. inhibens* S4Sm which

were deficient in biofilm formation (*exoP*) or antibiotic production (*clpX*) were shown to provide significantly less protection to oyster larvae after challenge with *V. coralliilyticus* RE22 compared to wild-type *P. inhibens* S4Sm, demonstrating the importance of these activities for probiotic function (Figure 5) [28]. Additionally, TDA knockout mutants of *P. gallaeciensis* do not protect cod larvae challenged with *V. anguillarum* as well as wild type *P. gallaeciensis* [26] and do not reduce cell densities of *V. anguillarum* as well as wild type *P. gallaeciensis* [113]. *P. inhibens* S4Sm has also been shown to produce acyl-homoserine lactones (AHLs) which down-regulate the virulence factor protease activity in *V. coralliilyticus* by disrupting the quorum-sensing pathway that activates protease transcription of *V. coralliilyticus* [29].

Table 1. Quantification of biofilm formation by measuring optical density at 580 nm (OD₅₈₀) of crystal violet dye attached to the cells forming biofilms on glass tubes at 27 °C under static conditions at 60 h [28].

	OD₅₈₀
<i>P. inhibens</i> S4Sm	3.89±0.06
<i>A. crassostreae</i> CV919	0.52±0.08 ^b
<i>V. anguillarum</i> NB10	0.58±0.02 ^b
<i>V. coralliilyticus</i> RE22	0.54±0.02 ^b

^bStatistically significant difference ($p < 0.05$) compared to S4Sm.

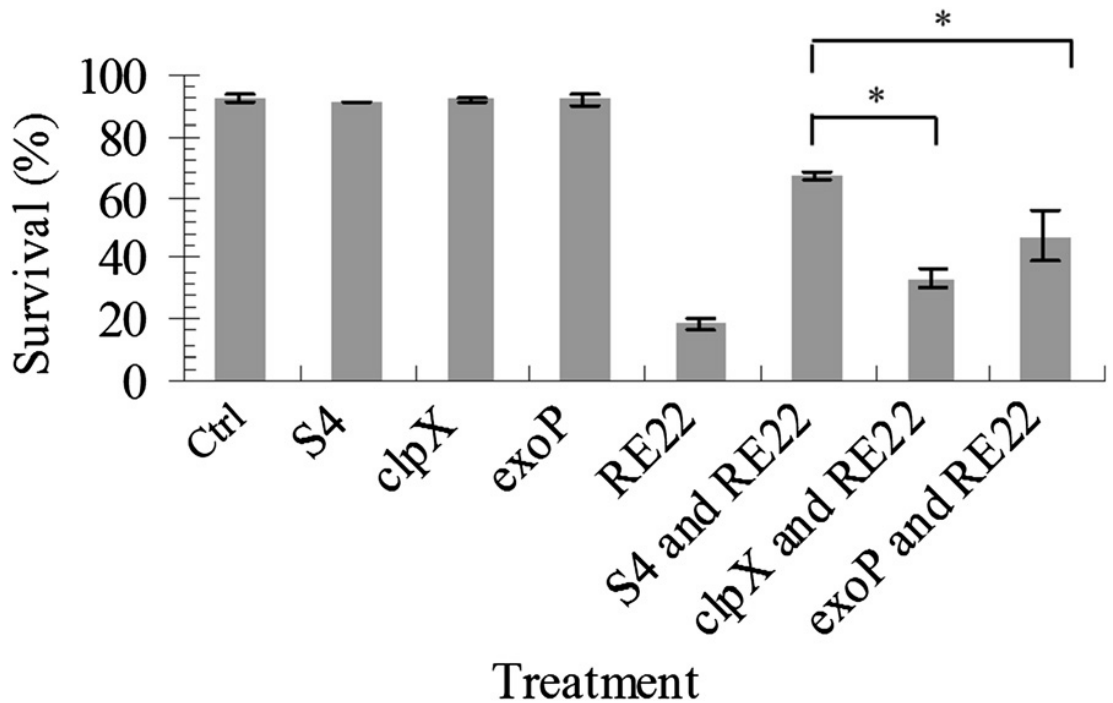


Figure 5. Oyster larvae survival in the presence of *P. inhibens* strains after challenge with *V. coralliilyticus* RE22. The *P. inhibens* S4Sm strains (10^4 CFU/ml) were introduced 24 h before larvae were challenged with *V. coralliilyticus* RE22 (10^5 CFU/ml). Oyster larvae survival (% \pm SD) was determined 24 h after challenge with RE22. Bars marked with an asterisk (*) show significant differences ($p < 0.05$). Error bars represent one standard deviation [28].

TDA is effective against a range of both Gram-positive and Gram-negative bacteria [114] and acts as a protonophore, which collapses the proton motive force in target cells [115]. It has also been shown that resistance to TDA is hard to select [114] and the *tdaR3* gene, which is predicted to encode for a γ -glutamyl-cyclotransferase, is required for TDA resistance [115]. In the TDA resistance model proposed by Wilson et al. [115], TdaR3 facilitates the glutamate-dependent acid-response system by converting glutathione to 5-oxo-proline, which is then hydrolyzed to glutamate via a

5-oxoprolinase. This glutamate is then decarboxylated to form γ -aminobutyric acid (GABA), which is exchanged by an antiporter for glutamate, resulting in the export of 1 H⁺ per glutamate [115]. The strong biofilm forming ability combined with production of antivirulence compounds (AHLs) and a broad-spectrum antibiotic (TDA) which pathogens are unlikely to become resistant to, make *P. inhibens* S4Sm a promising candidate for use as a probiotic to control bacterial diseases in aquaculture such as AHPND.

Goals of this study

The overall goal of this study was to isolate and characterize bacteria inhibitory towards the growth of *V. parahaemolyticus* and determine if they can be used to prevent or reduce losses due to the AHPND strains of *V. parahaemolyticus* in aquaculture systems. AHPND causes significant losses in the shrimp aquaculture industry and current strategies to control the disease are not effective [9, 10]. The use of probiotics has the potential to control AHPND, but new formulations are needed as it has been shown that probiotics which are currently available to shrimp farmers in Malaysia are not effective at controlling AHPND [49].

The first aim of this investigation was to isolate potential probiotic bacteria from the environment which can inhibit the growth of *V. parahaemolyticus*, quantify their biofilm formation, and identify their species. More than 300 bacterial isolates were cultured from a variety of sources and used in a zone of inhibition assay to determine if they could inhibit the growth of *V. parahaemolyticus* on an agar surface.

Biofilm formation was then quantified using the crystal violet method. The 16S rRNA gene for each isolate was sequenced for species-level identification.

The second aim of this study was to determine if any of the candidate probiotic organisms can increase the survival of *Artemia* nauplii challenged with AHPND *V. parahaemolyticus*. A model system using *Artemia* nauplii challenged with AHPND *V. parahaemolyticus* PSU5579 was developed and used to test candidate organisms for probiotic activity.

The third aim of this study was to determine if any of the candidate probiotic organisms can increase the survival of *L. vannamei* PLs challenged with AHPND *V. parahaemolyticus*. An assay using *L. vannamei* PLs challenged with AHPND *V. parahaemolyticus* PSU5579 was developed and used to test candidate organisms for probiotic activity.

The fourth aim was to obtain the genomic sequence of *V. parahaemolyticus* PSU5579 and *Bowmanella denitrificans* JL63. Prior to this research, no whole genomic sequences were available for any organisms in the *Bowmanella* genus and no bioactive secondary metabolites produced by members of this genus had been identified. The genomes of these organisms were sequenced and subsequently annotated by various software programs. The genome of *V. parahaemolyticus* PSU5579 was analyzed for potential virulence factors and the genome of *B. denitrificans* JL63 was analyzed for genes responsible for the production of compounds which can inhibit the growth of *V. parahaemolyticus*.

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Manuscript I

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Title: Biocontrol of acute hepatopancreatic necrosis disease (AHPND)

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Abstract

Acute hepatopancreatic necrosis disease (AHPND) causes mass mortalities in farmed penaeid shrimp and has proven difficult to control using typical disease control measures. The causative agent of AHPND has been identified as *Vibrio parahaemolyticus* strains possessing the 69 kbp plasmid pVPA3-1 containing genes homologous with *Photobacterium* insect-related (Pir) toxin-like genes (*pirA*- and *pirB*-like). Probiotics have been used successfully in shrimp aquaculture to control disease outbreaks caused by pathogenic *Vibrio* spp., but there are currently no probiotics available that have been proven to control AHPND. The goal of this study was to screen and characterize marine bacterial isolates as potential agents to prevent *Artemia* nauplii and *Litopenaeus vannamei* post-larvae (PL) mortality by the pathogen *V. parahaemolyticus*. Twelve candidate probionts were tested in an *Artemia* sp. model. *Phaeobacter inhibens* was the only candidate probiont tested that could significantly increase the survival of *Artemia* nauplii challenged with AHPND *V. parahaemolyticus* ($p < 0.001$). Candidate probionts *Pseudoalteromonas piscicida*, *Pseudoalteromonas flavipulchra*, and *Pseudoalteromonas arabiensis* caused mortality in *Artemia* nauplii ($p < 0.001$). Six species of candidate probionts were tested in *L. vannamei*. *P. inhibens* was the only candidate probiont tested which was not harmful to *L. vannamei* PLs and significantly increased the survival of PLs challenged with AHPND *V. parahaemolyticus* by 41% ($p < 0.001$).

Introduction

In 2009 an emerging disease now known as acute hepatopancreatic necrosis disease (AHPND) began to affect penaeid shrimp farms in southern China [1, 2]. The disease has spread to Vietnam, Malaysia, Thailand, and Mexico with global losses from AHPND estimated to be more than one billion US dollars annually [2-5]. AHPND affects both whiteleg shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*) and can lead to 100 % mortality in affected populations [3]. The disease causes serious production losses in affected areas, which negatively impacts local employment, social welfare, and international markets [6]. The causative agent of AHPND has been identified as *Vibrio parahaemolyticus* strains possessing the 69 kbp plasmid pVPA3-1 containing genes homologous with *Photobacterium* insect-related (Pir) toxin-like genes (*pirA*- and *pirB*-like) [2, 7]. AHPND has proven difficult to control using typical disease control measures such as water disinfection and antibiotic treatment [8, 9].

Disease prevention can be challenging for shrimp farmers because most farmers do not have resources necessary to treat seawater before it is used to fill their ponds [10]. Further, by the time shrimp show signs of AHPND, it is difficult to treat as antibiotic treatment has proven unsuccessful in most cases [9] and antibiotic treatment will select for antibiotic resistant bacteria. Additionally, treating water sources with chlorine, ozone, or UV before stocking does not provide total sterility [8]. Further, disinfection of water perturbs the natural microbial balance, leaving the environment open to opportunistic bacteria that survive disinfection, and can actually favor the growth of *Vibrio* species, which grow rapidly after their competitors are

removed [11]. Occurrences of overgrowth of pathogenic bacteria such as *Vibrio* in shrimp grow-out ponds can be reduced by minimizing disturbances such as water disinfection that can lead to sudden variations in nutrient levels, and by colonizing pond water with nonpathogenic bacteria and/or algae [12].

Current recommendations to prevent AHPND outbreaks in shrimp farms include the use greenwater systems [13] or the application of biocontrol strategies such as probiotics [5], phage [14], or *Bdellovibrio*-and-like organisms (BALOs) [15]. Studies have shown that probiotics can be used in aquaculture to prevent diseases in a variety of farmed species while also improving harvest yields [16-23]. Probiotics provide several benefits in aquaculture including improvement of water quality, enhancement of nutrition of host species, reduced incidence of diseases, higher survival rates, and improved host immune response [16, 18, 24]. Probiotics have been used successfully in shrimp aquaculture to control disease outbreaks caused by pathogenic *Vibrio* spp. [16-18, 20, 21] and may have the potential to control AHPND. Probiotics provide an alternative to the use of antibiotics in aquaculture, which have become increasingly controversial and ineffective due to the emergence of antibiotic resistance in bacteria [16, 25-27]. Currently there are no probiotics commercially available to shrimp farmers that have proven to be effective at preventing AHPND. A recent study determined that the probiotics which are available to shrimp farmers in Malaysia are not effective at controlling AHPND [28].

Before potential probiotic organisms can be used in aquaculture, they must be tested to confirm that no pathogenic effects can occur in the host. *Artemia* spp. have been used as a model organism not only for toxicology studies but also to test the

effectiveness of probiotics and the role of quorum sensing in pathogenesis [29-36]. *Artemia* spp. are useful model organisms because they adapt easily to changes in nutrients, salinity, temperature, and oxygen, are easy to culture, are resistant to manipulation, have a short life cycle, and are inexpensive [35].

In this study, ten newly isolated potential probionts, as well as two oyster probionts, *Phaeobacter inhibens* S4Sm and *Bacillus pumilus* RI06-95, were identified as having *in vitro* antibiotic activity against an AHPND strain of *V. parahaemolyticus*. These 12 candidate probionts were tested *in vivo* for their ability to protect *Artemia* nauplii or *L. vannamei* post-larvae (PL) from AHPND *V. parahaemolyticus* challenge. It was found that *P. inhibens* S4Sm was the only candidate probiont tested which significantly increased the survival of *Artemia* nauplii challenged with AHPND *V. parahaemolyticus*. All species of *Pseudoalteromonas* tested were found to be pathogenic to *Artemia* sp. *P. inhibens* S4Sm was also the only candidate probiont which was not harmful to *L. vannamei* PLs and significantly increased the survival of PLs challenged with AHPND *V. parahaemolyticus*.

Materials and Methods

Bacterial strains and growth conditions

Candidate probionts used in this study are listed in Table 1. All bacteria were grown for 24 h at 27 °C with shaking. All *Bacillus* strains were grown in 2×LB30IOS (20 g/L tryptone, 10 g/L yeast extract, 30 g/L Instant Ocean[®], pH 7). *P. inhibens* S4Sm, *Bowmanella denitrificans* JL63, and all *Pseudoalteromonas* strains were grown

in LB30IOS (10 g/L tryptone, 5 g/L yeast extract, 30 g/L Instant Ocean[®], pH 7). *V. parahaemolyticus* strains were grown in LB20 (10 g/L tryptone, 5 g/L yeast extract, 20 g/L NaCl, pH 8). Spontaneous streptomycin-resistant mutants were selected by passing on increasing concentrations of streptomycin, up to 200 µg/ml. These strains are indicated by “Sm” at the end of their strain name. All bacterial strains were maintained and stored in 25% glycerol stocks at -80 °C.

Isolation of candidate probiotic bacteria

Environmental samples, such as seawater or small marine invertebrates, such as shrimp, were collected for the isolation of bacteria (Table 1.). Seawater collected from Narragansett Bay, Rhode Island, USA was serially diluted in sterile artificial seawater (ASW) (30 g/L Instant Ocean[®], pH 8, autoclaved) and 100 µl of each dilution was spread on YP30IOS agar (5 g/L peptone, 1 g/L yeast extract, 30 g/L Instant Ocean[®], 15 g/L agar, pH 7.5) and incubated at 27 °C for 24-48 h. Small marine invertebrates were blended in sterile ASW in a sterile blender, serially diluted in sterile ASW, and 100 µl of each dilution was spread on YP30IOS agar and incubated at 27 °C for 24-48 h. Isolated bacterial colonies were picked from the YP30IOS agar and inoculated into LB30IOS and incubated at 27 °C with shaking for 24-48 h before being used in zone of inhibition assays.

16S rRNA gene sequencing

Genomic DNA was isolated using the Bio Basic EZ-10 Spin Column Bacterial Genomic DNA Miniprep Kit. Primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') were used to amplify the 16S rRNA gene [37]. Extracted gDNA (1 µl at a concentration of 10-60 ng/µl) was combined with 1 µl of each primer (stock solution, 10 µmol), 9.5 µl nuclease-free water, and 12.5 µl of QIAGEN *Taq* PCR master mix. Reaction conditions were 95 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 53 °C for 30 s, and 72 °C for 1.5 min; and a final extension at 72 °C for 5 min. PCR products were sequenced at the University of Rhode Island Genomics and Sequencing Center.

Zone of inhibition assay

Zones of inhibition were quantified using a modification of a method described previously [38]. *V. parahaemolyticus* PSU5429 was grown for 24 h in LB20, diluted 10³-fold, and 100 µl of this diluted culture was spread on YP30IOS agar. The candidate probionts (10 µl of a 24 h culture) were then spotted on the same plate. Plates were incubated at 27 °C for 24-48 h. Inhibition zones were measured between growth of the candidate probiont (edge of spot) and the *V. parahaemolyticus* lawn (edge of lawn). Each candidate probiont was tested three times.

Biofilm assay

Biofilm formation was quantified using a modification of the crystal violet staining method [39]. Bacteria were grown for 24 h before being diluted 10³-fold into 200 µl of YP30IOS in a polystyrene 96-well plate which was then incubated at 27 °C for 24 h under static conditions. Biofilms in the wells were washed with ASW twice, stained with 0.2% crystal violet for 20 min, washed twice with ASW, and biofilm-bound crystal violet was eluted with 95% ethanol for 30 min before being measured at OD₅₈₀. Each candidate probiont was tested in three wells per experiment and each experiment was repeated twice.

Characterization of *V. parahaemolyticus* strains using duplex PCR for the detection of *pirA*- and *pirB*-like genes

Ten strains of *V. parahaemolyticus* isolated from shrimp farms located in Pattani and Songkla provinces, southern Thailand during an AHPND outbreak were screened for *pirA*- and *pirB*-like genes. These *V. parahaemolyticus* strains were gifted to us from Wenjing Zhao at the Mekalanos Lab, Harvard Medical School. Genomic DNA was isolated using the Bio Basic EZ-10 Spin Column Bacterial Genomic DNA Miniprep Kit. Primers VpPirA-284F (5'-TGACTATTCTCACGATTGGACTGR-3'), VpPirA-284R (5'-CACGACTAGCGCCATTGTTA-3'), VpPirB-392F (5'-TGATGAAGTGATGGGTGCTC-3'), and VpPirB-392R (5'-TGTAAGCGCCGTTTAACTCA-3') were used to amplify the *pirA*- and *pirB*-like genes [2]. 1 µl of extracted gDNA (10-60 ng/µl) was combined with 1 µl of each

primer (10 μ mol), 7.5 μ l nuclease-free water, and 12.5 μ l of QIAGEN *Taq* PCR master mix. Reaction conditions were 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. PCR products were separated on a 2% agarose gel containing ethidium bromide (0.4 μ g/ml) and visualized on a UV transilluminator. Presence of *pirA*-like is indicated by a band at 284 bp, while presence of *pirB*-like is indicated by a band at 392 bp.

***Artemia* challenge**

Artemia cysts (0.075 g) were hatched in an inverted 60 ml syringe, covered with perforated plastic wrap, containing 60 ml HEPPS-buffered (10 mM, pH 8.2) sterile ASW at 28 °C for 24 h with constant aeration and fluorescent light. To maintain pH and maximize *Artemia* hatching rate, an increased buffer capacity is required to avoid a drop in the pH due to the acid produced by cysts during hatching [40]. Hatched nauplii (2 ml, containing approximately 400 nauplii) were transferred to 50 ml centrifuge tubes containing 26.3 ml HEPPS-buffered (10 mM, pH 8.2) ASW. Nauplii were fed autoclaved *Escherichia coli* K-12 cells at a final concentration of 10^7 cells/ml during hatching and every 24 h. Candidate probionts were washed twice in sterile ASW by centrifugation at $6,000 \times g$ for ten minutes at 4 °C. Nauplii were treated with candidate probionts after hatching and every 24 h. The centrifuge tubes were placed on a rotator set to 4 rpm at 30 °C. After incubation for 24 h, 1.6 ml YP30IOS was added to each tube and nauplii were challenged with 1×10^5 CFU/ml washed *V. parahaemolyticus* cells. *V. parahaemolyticus* cells were washed twice in

sterile ASW by centrifugation at $5,000 \times g$ for five minutes at room temperature. To count nauplii, the tubes were inverted five times to mix the nauplii suspension, 1 ml of the nauplii suspension was transferred into each well of a tri-well petri dish containing 5 ml of ASW with 0.05% agar (to slow nauplii movement during counting), and nauplii were viewed under a dissecting microscope. Nauplii were counted at 24 h and 72 h to quantify survival. Nauplii which showed any signs of movement were counted as alive. Each treatment was tested in three tubes and was repeated twice. Water in each tube was not changed during the experiment.

Whiteleg shrimp (*Litopenaeus vannamei*) post-larvae challenge

Litopenaeus vannamei PLs were purchased from Miami-Aquaculture (Boynton Beach, Florida, USA) and maintained at room temperature in ASW in a 20 L aquarium tank with filtration (Hagen[®] AquaClear[®] 50 power filter, Mansfield, MA) and weekly water changes. PLs (approximately 10 mm – 20 mm in length) were fed Hagen[®] Fluval[®] (Mansfield, MA) Shrimp Granules daily. Six PLs were transferred to 250 ml bottles with 200 ml ASW containing 200 µg/ml streptomycin and provided aeration through a sterile air stone. Streptomycin-resistant candidate probionts were washed twice in sterile ASW by centrifugation at $6,000 \times g$ for ten minutes at 4 °C. PLs were treated with probiotics at 1×10^6 CFU/ml at the beginning of the experiment and every 24 h. After a 24 h pretreatment period, PLs were challenged with 1×10^6 CFU/ml washed streptomycin-resistant *V. parahaemolyticus* PSU5579Sm cells. *V. parahaemolyticus* PSU5579Sm was washed twice in sterile ASW by centrifugation at $5,000 \times g$ for five minutes at room temperature. PLs were incubated at 30 °C and

survival was quantified 48 h post-challenge. Each treatment was tested in three bottles and the experiment was repeated twice. Water in each bottle was not changed during the experiment.

Statistical analysis

Statistical data analysis was performed using the Student's t-test. Data with $p < 0.05$ was considered to be statistically significant.

Results

Isolation of candidate probiotic bacteria and 16S sequencing

More than 300 bacterial isolates were screened for antibiotic activity against *V. parahaemolyticus* PSU5429 by zone of inhibition (ZOI) assay. A total of 30 isolates were found to inhibit the growth of *V. parahaemolyticus* PSU5429. The 16S rRNA genes of these isolates were sequenced to identify their species, and these data combined with the ZOI and biofilm data were analyzed to rule out strains that were isolated more than once. This analysis revealed that of the 30 original isolates, 10 were unique strains (Table 1), while the remaining 20 isolates were duplicates. Two oyster probiotic organisms, *P. inhibens* S4Sm and *B. pumilus* RI06-95, previously identified by Karim et al. [41], were also tested by ZOI and found to inhibit the growth of *V. parahaemolyticus* PSU5429. *B. pumilus* HR1 was gifted to us by Hilary Ranson at the Rowley lab, University of Rhode Island.

Table 1. Candidate probiotic organisms that produce a zone of inhibition against *V. parahaemolyticus* PSU5429.

Candidate Probiot	Source	Location of collection	Reference
<i>Phaeobacter inhibens</i> S4Sm	Oyster shell	Rhode Island	Karim et al. [41]
<i>Bacillus pumilus</i> RI06-95	Marine sponge	Narrow River, RI	Karim et al. [41]
<i>Pseudoalteromonas piscicida</i> GR1	Seawater	Galilee, Narragansett, RI	This study
<i>Pseudoalteromonas flavipulchra</i> GR4	Seawater	Galilee, Narragansett, RI	This study
<i>Pseudoalteromonas flavipulchra</i> JL1	Seawater	Upper Pond, South Kingstown, RI	This study
<i>Pseudoalteromonas piscicida</i> JL12	Seawater	Upper Pond, South Kingstown, RI	This study
<i>Pseudoalteromonas piscicida</i> JL15	Brine shrimp	Critter Hut, Wakefield, RI	This study
<i>Pseudoalteromonas flavipulchra</i> JL18	Brine shrimp	Critter Hut, Wakefield, RI	This study
<i>Pseudoalteromonas arabiensis</i> JL29	Brine shrimp	Critter Hut, Wakefield, RI	This study
<i>Bowmanella denitrificans</i> JL63	Whiteleg shrimp	SKy8 Shrimp Farm, Stoughton, MA	This study
<i>Bacillus pumilus</i> JL70	Gulf shrimp hindgut	Gulf of Mexico	This study
<i>Bacillus pumilus</i> HR1	Lobster shell	Narragansett Bay, RI	This study

Zone of inhibition assay

Zones of inhibition (ZOI) produced by candidate probionts against *V. parahaemolyticus* PSU5429 were quantified to evaluate each organism's ability to inhibit *V. parahaemolyticus* growth on an agar surface. ZOIs are areas around the candidate probiont spot where *V. parahaemolyticus* was plated, but was not able to grow due to the presence of growth-inhibiting compound(s) secreted by the candidate probiont. Of the 12 candidate probionts, *Pseudoalteromonas flavipulchra* JL1 and

GR4 and *Pseudoalteromonas piscicida* JL15 produced the largest ZOIs (6.4 mm, 4.6 mm, and 5.9 mm, respectively) (Figure 1). *P. inhibens* S4Sm, *Ps. piscicida* GR1 and JL12, and *Ps. flavipulchra* JL18 produced moderate ZOIs (1.5 mm, 1.7 mm, 2.2 mm, and 1.2 mm, respectively) (Figure 1). *Pseudoalteromonas arabiensis* JL29, *B. denitrificans* JL63, and *B. pumilus* RI06-95, JL70, and HR1 produced the smallest ZOIs (0.1 mm, 0.3 mm, 0.4 mm, 0.7 mm, and 0.6 mm, respectively) (Figure 1).

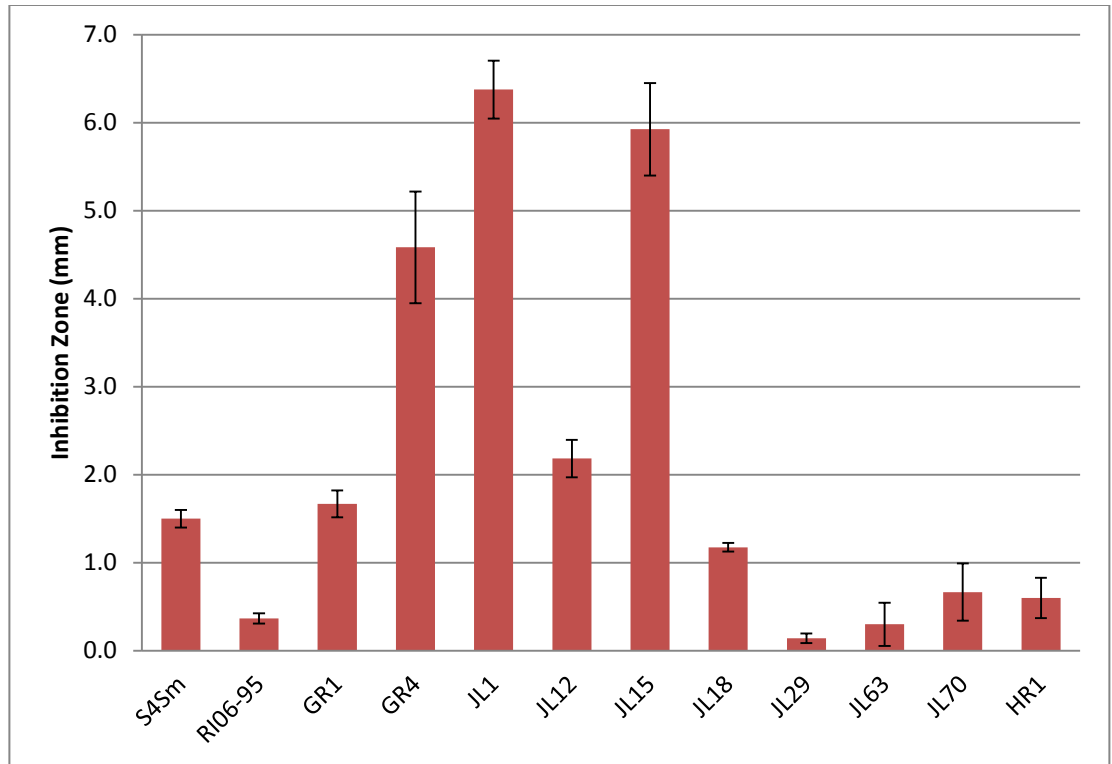


Figure 1. Zones of inhibition produced by candidate probionts against *V. parahaemolyticus* PSU5429. 100 μ l of a 10^3 -fold diluted stationary phase *V. parahaemolyticus* culture was spread on YP30 agar and 10 μ l of a stationary phase culture of each candidate probiont was spotted over the *V. parahaemolyticus* lawn. Plates were incubated at 27 °C for 24-48 h. Inhibition zones were measured between growth of the candidate probiont and the *V. parahaemolyticus* lawn. Representative of three independent experiments. Error bars equal one standard deviation.

Biofilm assay

The biofilm forming ability of each candidate probiont, as well as *V. parahaemolyticus* PSU5579, was quantified to determine if any of the candidate probionts can form stronger biofilms than *V. parahaemolyticus*. Of the 12 candidate probionts, *P. inhibens* S4Sm, *B. pumilus* RI06-95, *Ps. piscicida* GR1, *Ps. flavipulchra*

JL1, *Ps. piscicida* JL12, and *Ps. flavipulchra* JL18 produced the strongest biofilms ($OD_{580} = 3.7, 3.3, 3.2, 3.2, 3.0,$ and 3.4 respectively) (Figure 2). *Ps. flavipulchra* GR4, *Ps. piscicida* JL15, *B. denitrificans* JL63, and *B. pumilus* JL70 produced moderate biofilms ($OD_{580} = 2.3, 2.1, 2.4,$ and 2.6 respectively) while *Ps. arabiensis* JL29 and *B. pumilus* HR1 were the weakest biofilm formers ($OD_{580} = 1.3$ and 0.8 respectively) (Figure 2). *V. parahaemolyticus* PSU5579 produced a weak biofilm ($OD_{580} = 0.7$) (Figure 2). The only candidate probiont that did not produce a significantly stronger biofilm than *V. parahaemolyticus* PSU5579 was *B. pumilus* HR1 ($p=0.565$) (Figure 2). The eleven other candidate probionts all produced significantly stronger biofilms than *V. parahaemolyticus* PSU5579 ($p<0.006$) (Figure 2).

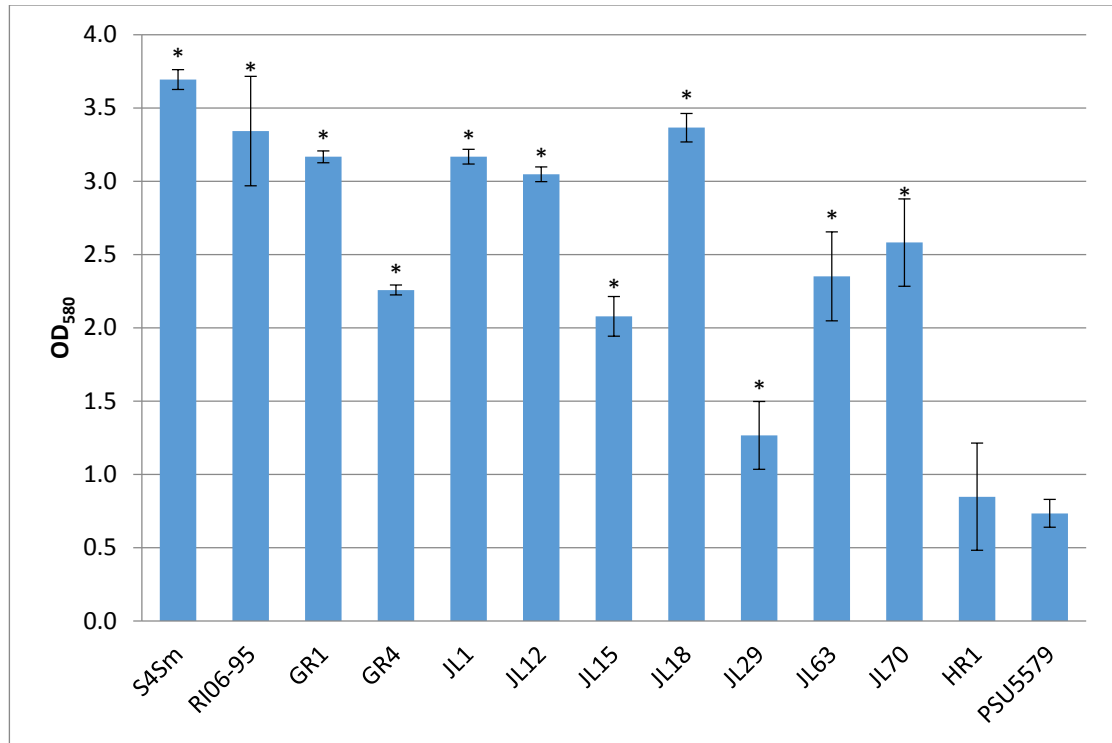


Figure 2. Biofilm formation quantified by the crystal violet method. Cultures were grown, as described in the Methods, in 96-well plates for 24 hours at 27 °C. Biofilms in the wells were washed with ASW, stained with 0.2% crystal violet, washed again, and biofilm-bound crystal violet was eluted with 95% ethanol before the optical density was measured at 580 nm (OD₅₈₀). Representative of three independent experiments with three technical replicates per experiment. Error bars equal one standard deviation. * indicates statistically significant difference from *V. parahaemolyticus* PSU5579 (t-test, $p < 0.05$).

Duplex PCR for the detection of *pirA*- and *pirB*-like genes

In order to confirm that the putative AHPND strains of *V. parahaemolyticus* contained the genes necessary to cause this shrimp disease, ten strains of *V. parahaemolyticus* isolated from shrimp farms located in Pattani and Songkla provinces, southern Thailand during an AHPND disease outbreak were screened for

pirA- and *pirB*-like genes by duplex PCR. Six of these strains (PSU5429, PSU5495, PSU5501, PSU5507, PSU5520, and PSU5579) possess both *pirA*- and *pirB*-like genes (Figure 3). Three of these strains (PSU5580, PSU5585, and PSU5599) possess only *pirA*-like and one strain (PSU5587) lacks both *pirA*- and *pirB*-like genes (Figure 3). Strains which possess both *pirA*- and *pirB*-like genes have the potential to cause AHPND. All six strains possessing both *pirA*- and *pirB*-like genes were used in a preliminary experiment to challenge *Artemia* nauplii. Of the six strains tested, *V. parahaemolyticus* PSU5579 induced the highest mortality rate in *Artemia* nauplii (Figure S1) and was chosen as the strain to be used in future experiments. For this preliminary experiment, *Artemia* were not fed during hatching and were challenged with *V. parahaemolyticus* strains immediately after hatching without the 24 h pretreatment period used in other experiments. This protocol was later modified (as described in the Materials and Methods) to allow for a 24 h pretreatment period.

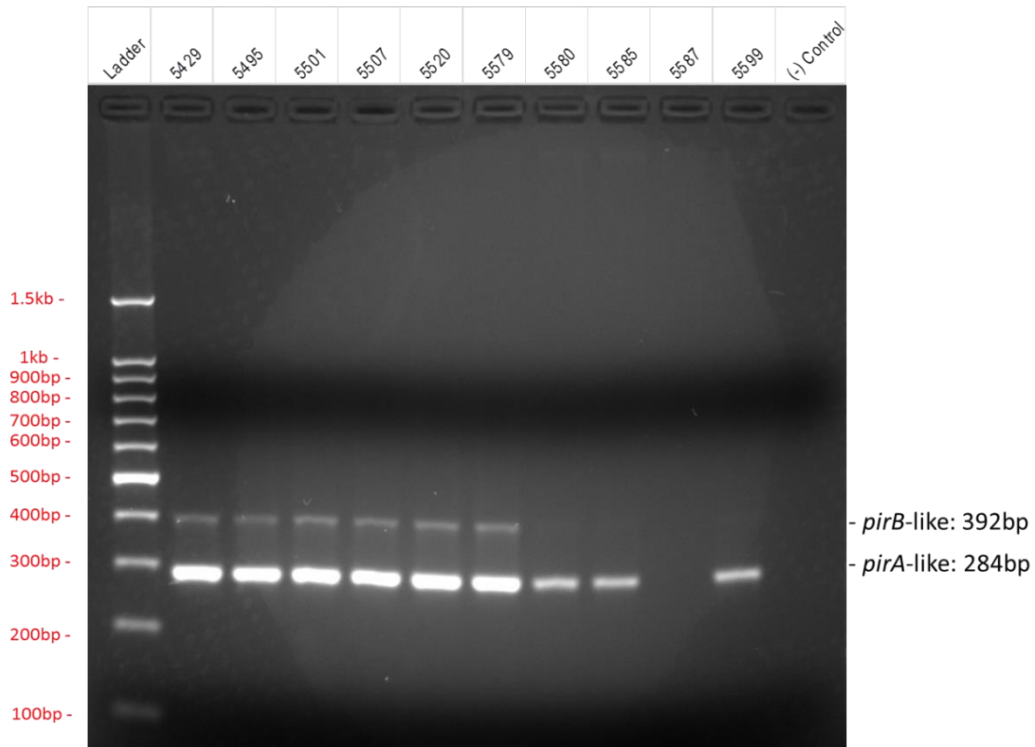


Figure 3. Duplex PCR detection of *pirA*- and *pirB*-like genes found on the pVPA3-1 plasmid. PCR amplification was performed using primers VpPirA-284F/R and VpPirB-392F/R and was viewed on a 2% agarose gel containing ethidium bromide (0.4 µg/ml) as described by Han et al. [2]. Presence of a *pirA*-like amplicon is indicated by a band at 284 bp, while presence of a *pirB*-like amplicon is indicated by a band at 392 bp.

Artemia challenge studies

To determine if any of the candidate probiotic organisms have the potential to prevent AHPND, an assay was developed to test if candidate probionts could protect *Artemia* nauplii from AHPND *V. parahaemolyticus* challenge. This assay also served as a test to determine if any of the candidate probionts can be harmful to crustaceans, such as *Artemia* sp., under certain conditions. For this assay, the addition of 1.6 ml

YP30IOS allowed for *V. parahaemolyticus* PSU5579 to consistently induce a 53% - 71% mortality rate in *Artemia* nauplii when applied at 10^5 CFU/ml 24 h after hatching (Figure S2). When *V. parahaemolyticus* PSU5579 was applied at 10^4 CFU/ml 24 h after hatching, the mortality rate was lower (39% - 49%) (Figure S2). The higher mortality rate induced by *V. parahaemolyticus* PSU5579 when applied at 10^5 CFU/ml provides a range of survival between the challenged and unchallenged controls where a level of protection provided by probiotic organisms can be detected, which is why this concentration was used for these experiments. Without the addition of YP30IOS, challenging *Artemia* nauplii 24 h after hatching with *V. parahaemolyticus* PSU5579 at 10^6 or 10^7 CFU/ml only induced 0% or 28% mortality, respectively (Figure S3).

Twelve candidate probiotic organisms were tested for their potential ability to kill *Artemia* nauplii, as well as their ability to protect nauplii from challenge with *V. parahaemolyticus* PSU5579. *Artemia* nauplii pretreated with 10^6 CFU/ml *P. inhibens* S4Sm for 24 h exhibited a statistically significant 1.8-fold increase in survival (70% survival) when challenged with *V. parahaemolyticus* PSU5579 ($p < 0.001$) compared to the *Artemia* challenge control (38% survival) (Figure 4). *Artemia* nauplii pretreated with *P. inhibens* S4Sm at 10^6 CFU/ml had 32% higher survival after challenge with *V. parahaemolyticus* PSU5579 than those pretreated with *P. inhibens* S4Sm at 10^5 CFU/ml (Figure S4). None of the other eleven probiont candidate isolates were able to protect *Artemia* nauplii from infection and death when challenged with *V. parahaemolyticus* PSU5579 (Figures 5 and 6). *Artemia* nauplii treated with *P. inhibens* S4Sm at 10^6 CFU/ml daily for 72 h (probiotic control) exhibited a 17.6%

decline in survival (to 75%) compared to the untreated control (91%) ($p=0.001$) (Figure 4).

Candidate probionts *B. pumilus* strains RI06-95, JL70, and HR1 tested at 10^4 , 10^5 , and 10^6 CFU/ml did not have any significant effect on the survival of *Artemia* nauplii challenged with *V. parahaemolyticus* PSU5579 ($p>0.094$) (Figure 5). Survival of *Artemia* challenged with *V. parahaemolyticus* PSU5579 averaged 33%, while survival of *Artemia* treated with the various *B. pumilus* strains and then challenged ranged from 32-42%. In contrast, *Ps. piscicida* strains GR1, JL12, and JL15, *Ps. flavipulchra* strains GR4, JL1, and JL18, *Ps. arabiensis* JL29, and *B. denitrificans* JL63 tested at 10^4 CFU/ml all significantly decreased (0% vs. 37%) the survival of *Artemia* nauplii challenged with *V. parahaemolyticus* PSU5579 ($p<0.001$) (Figure 6). In all cases, the *Artemia* treated with these candidate probionts and then challenged with PSU5579 showed 100% mortality. Further, *Artemia* treated only with the candidate probionts *Ps. flavipulchra* JL1, *Ps. piscicida* JL15, and *Ps. arabiensis* JL29 at 10^5 CFU/ml daily (with no *V. parahaemolyticus* challenge) exhibited significantly decreased survival ($p<0.001$) (with only 0%, 5%, 4% survival, respectively) (Figure 7). The untreated control *Artemia* exhibited 85% survival. Treating *Artemia* nauplii daily with *B. denitrificans* JL63 at 10^5 CFU/ml for 72 h reduced survival to 62%, but this decline (compared to the control) was not significant ($p=0.063$) (Figure 7).

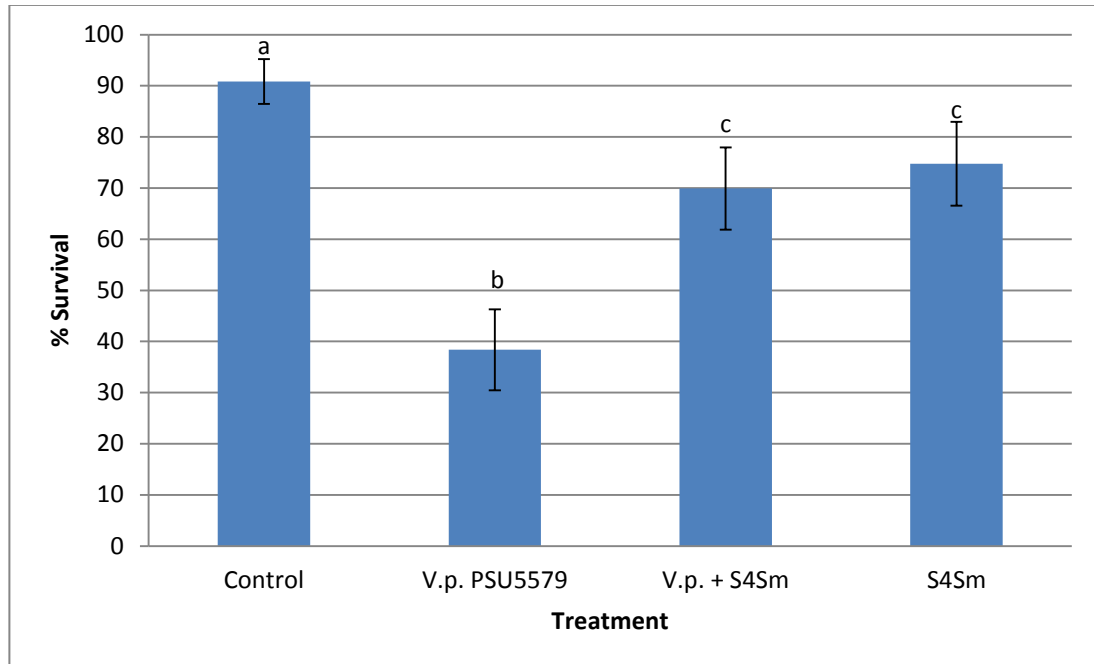


Figure 4. Effect of preincubation of *Artemia* nauplii with *P. inhibens* S4Sm at 10^6 CFU/ml for 24 h on survival 48 h after challenge with *V. parahaemolyticus* PSU5579 at 10^5 CFU/ml. *P. inhibens* S4Sm was added at the start of the experiment and every 24 h. Representative of three independent experiments with three technical replicates per experiment. Error bars equal one standard deviation. Different letters indicate statistical significance among groups (t-test, $p < 0.05$).

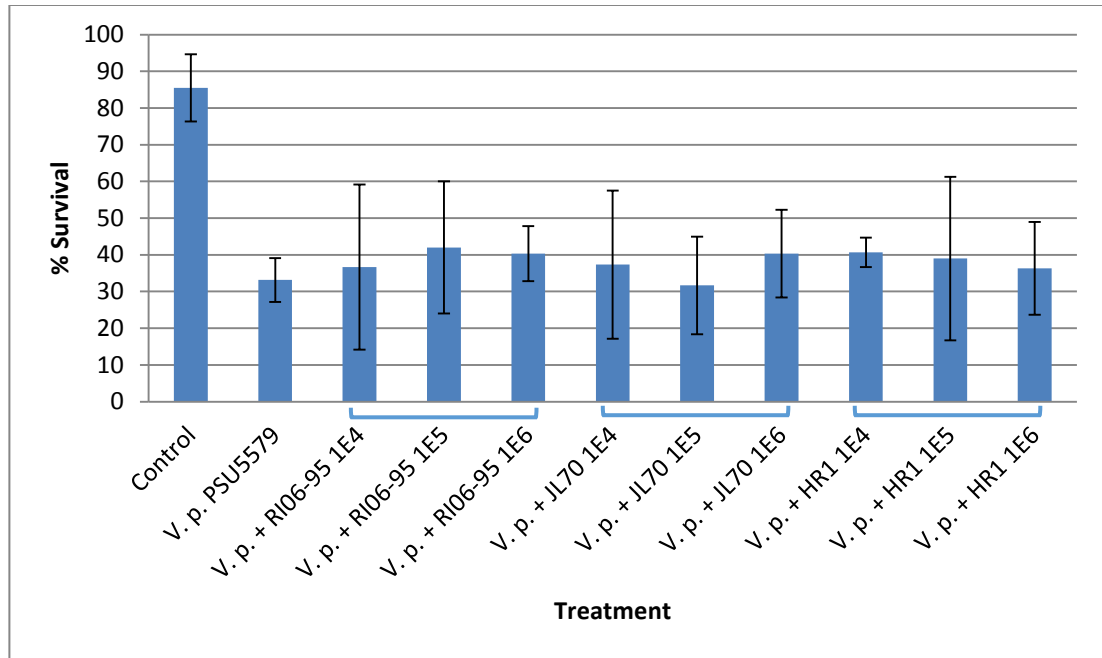


Figure 5. Effect of preincubation of *Artemia* nauplii with *B. pumilus* RI06-95, JL70, and HR1 at 10^4 , 10^5 , and 10^6 CFU/ml for 24 h on survival 48 h after challenge with *V. parahaemolyticus* PSU5579 at 10^5 CFU/ml. Candidate probionts were added at the start of the experiment and every 24 h. Representative of three independent experiments with three technical replicates per experiment. Error bars equal one standard deviation. * indicates statistically significant difference from the *V. parahaemolyticus* PSU5579 treatment (t-test, $p < 0.05$).

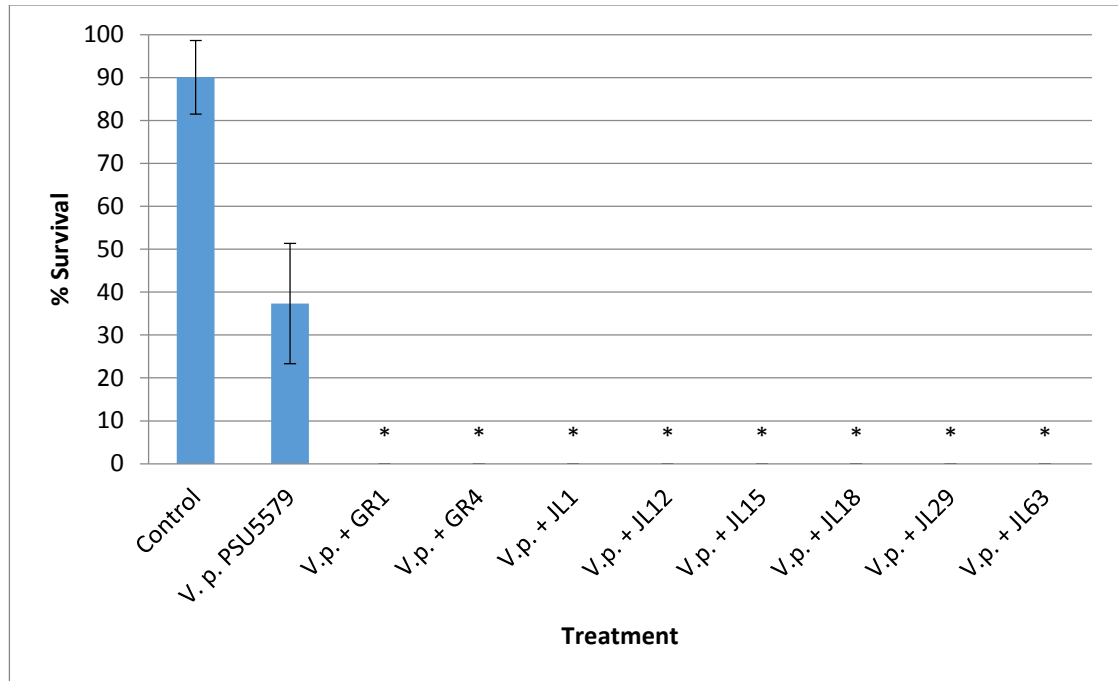


Figure 6. Effect of preincubation of *Artemia* nauplii with *Ps. piscicida* GR1, JL12, and JL15, *Ps. flavipulchra* GR4, JL1, and JL18, *Ps. arabiensis* JL29, and *B. denitrificans* JL63 at 10^4 CFU/ml for 24 h on survival 48 h after challenge with *V. parahaemolyticus* PSU5579 at 10^5 CFU/ml. Candidate probionts were added at the start of the experiment and every 24 h. Representative of three independent experiments with three technical replicates per experiment. Error bars equal one standard deviation. * indicates statistically significant difference from the *V. parahaemolyticus* PSU5579 treatment (t-test, $p < 0.05$).

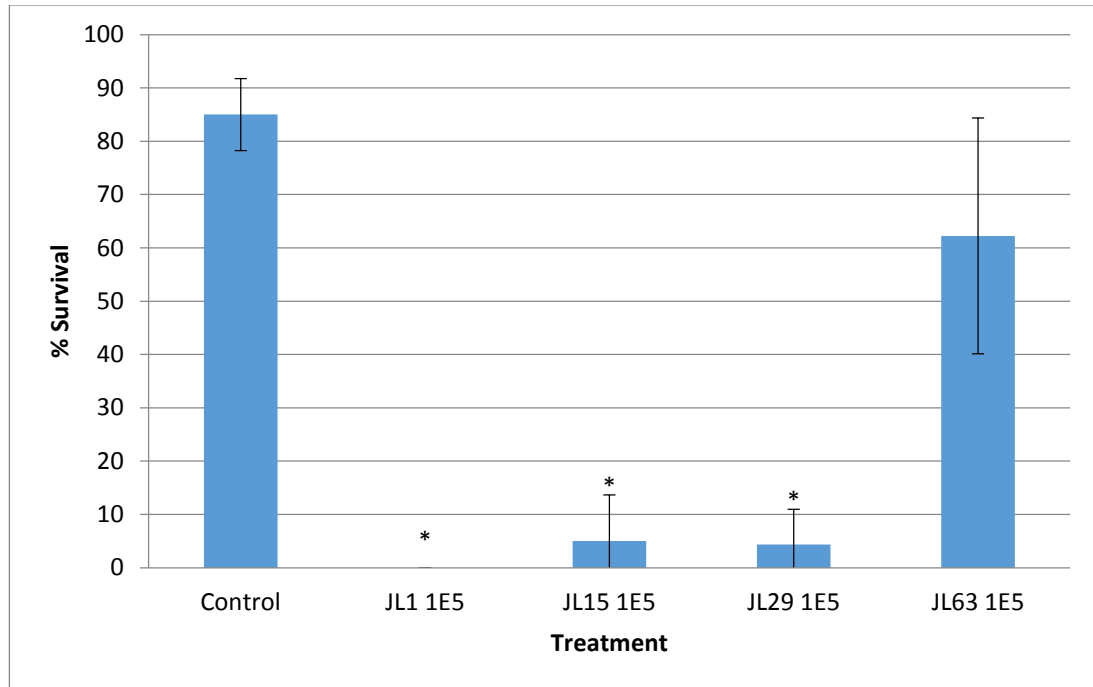


Figure 7. Survival of *Artemia* nauplii treated with *Ps. flavipulchra* JL1, *Ps. piscicida* JL15, *Ps. arabiensis* JL29, and *B. denitrificans* JL63 at 10^5 CFU/ml daily for 72 h. Representative of three independent experiments with three technical replicates per experiment. Error bars equal one standard deviation. * indicates statistically significant difference from the Control (t-test, $p < 0.05$).

Whiteleg shrimp (*Litopenaeus vannamei*) post-larvae challenge

An assay was developed to test if any of the candidate probionts could protect *L. vannamei* PLs from AHPND *V. parahaemolyticus* challenge. An initial experiment determined that *V. parahaemolyticus* PSU5579Sm induces a high mortality rate (67%) in *L. vannamei* PLs when applied at 10^6 CFU/ml at the start of the experiment; however, when PLs were incubated for 24 h prior to the addition of *V. parahaemolyticus*, the mortality rate was reduced to 33% (Figure S5). This may be due to the growth of commensal bacteria from the shrimp during the 24 h

preincubation period, which then compete with *V. parahaemolyticus* for available nutrients. This 24 h preincubation period is important because probiotics usually require a pretreatment period to effectively protect animals from pathogen challenge [42, 43]. This issue was resolved through the addition of 200 µg/ml streptomycin to the PL water at the start of the experiment which allowed for a 24 h preincubation period without a reduced *V. parahaemolyticus*-induced mortality rate when applied at 10^6 CFU/ml (67%) (Figure S5). Challenging *L. vannamei* PLs with 10^5 CFU/ml *V. parahaemolyticus* PSU5579Sm after a 24 h preincubation period with streptomycin only induced a 5% mortality rate (Figure S5).

In order to determine if any of the candidate probionts are harmful to shrimp, *L. vannamei* PLs were treated with each species of candidate probiont at 10^6 CFU/ml daily for 72 h. PLs treated with *P. inhibens* S4Sm, *Ps. piscicida* JL15Sm, *Ps. arabiensis* JL29Sm, *B. denitrificans* JL63Sm, or *B. pumilus* RI06-95Sm daily for 72 h did not exhibit any significantly decline in survival that ranged from 92-98% when compared to untreated control PLs with 97% survival ($p>0.187$) (Figure 8). The only species that significantly decreased the survival of *L. vannamei* PLs was *Ps. flavipulchra* JL1Sm with 83% survival compared to 97% in the untreated control ($p=0.008$) (Figure 8).

In an effort to determine if any of the candidate probionts can protect *L. vannamei* PLs from *V. parahaemolyticus* challenge, PLs were pretreated with candidate probionts at 10^6 CFU/ml for 24 h before *V. parahaemolyticus* PSU5579Sm challenge and, as described previously, the candidate probionts were also added every 24 h. Both *P. inhibens* S4Sm and *Ps. flavipulchra* JL1Sm significantly increased the

survival of *L. vannamei* PLs challenged with *V. parahaemolyticus* PSU5579Sm to 87%, 80%, respectively ($p < 0.001$). Infection control PLs exhibited only 46% survival (Figure 9). As seen with the *Artemia* challenge experiments, treatments with *P. inhibens* S4Sm increased survival by nearly 2-fold. *Ps. piscicida* JL15Sm, *Ps. arabiensis* JL29Sm, *B. denitrificans* JL63Sm, and *B. pumilus* RI06-95Sm did not significantly affect the survival of *L. vannamei* PLs challenged with *V. parahaemolyticus* PSU5579Sm with survival of PLs ranging from 35-54% ($p > 0.051$) (Figure 9). These results indicate that *P. inhibens* S4Sm is the only candidate probiotic organism tested which is not harmful to *L. vannamei* PLs and can significantly increase the survival of PLs challenged with AHPND *V. parahaemolyticus*.

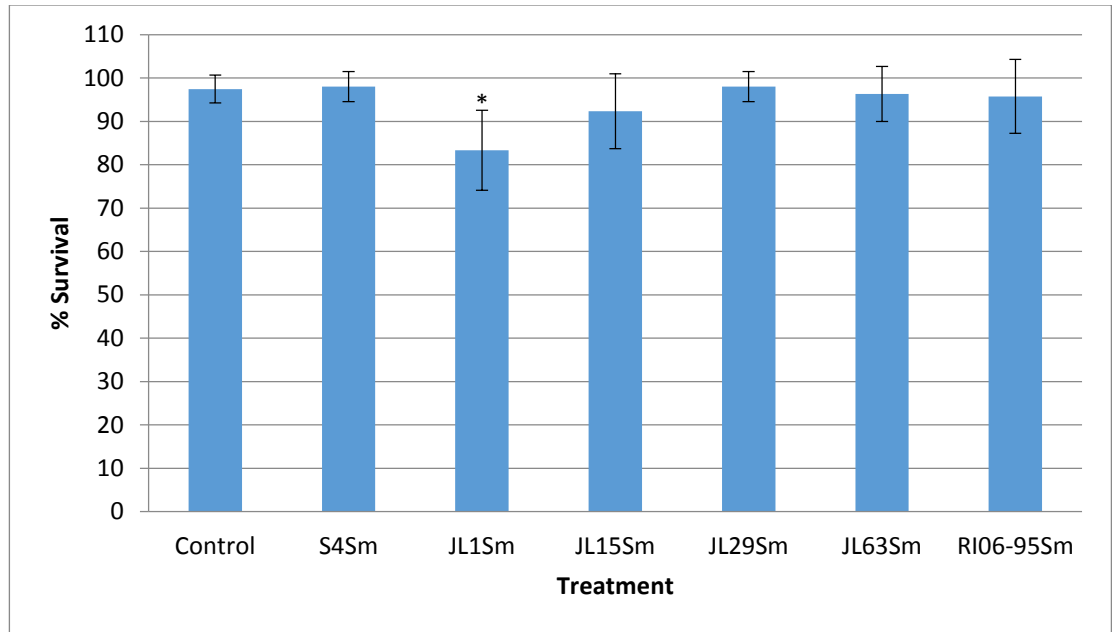


Figure 8. Survival of *L. vannamei* PLs treated with *P. inhibens* S4Sm, *Ps. flavipulchra* JL1Sm, *Ps. piscicida* JL15Sm, *Ps. arabiensis* JL29Sm, *B. denitrificans* JL63Sm, and *B. pumilus* RI06-95Sm at 10^6 CFU/ml daily for 72 h. Representative of three independent experiments with three technical replicates per experiment. Error bars equal one standard deviation. * indicates statistically significant difference from the Control (t-test, $p < 0.05$).

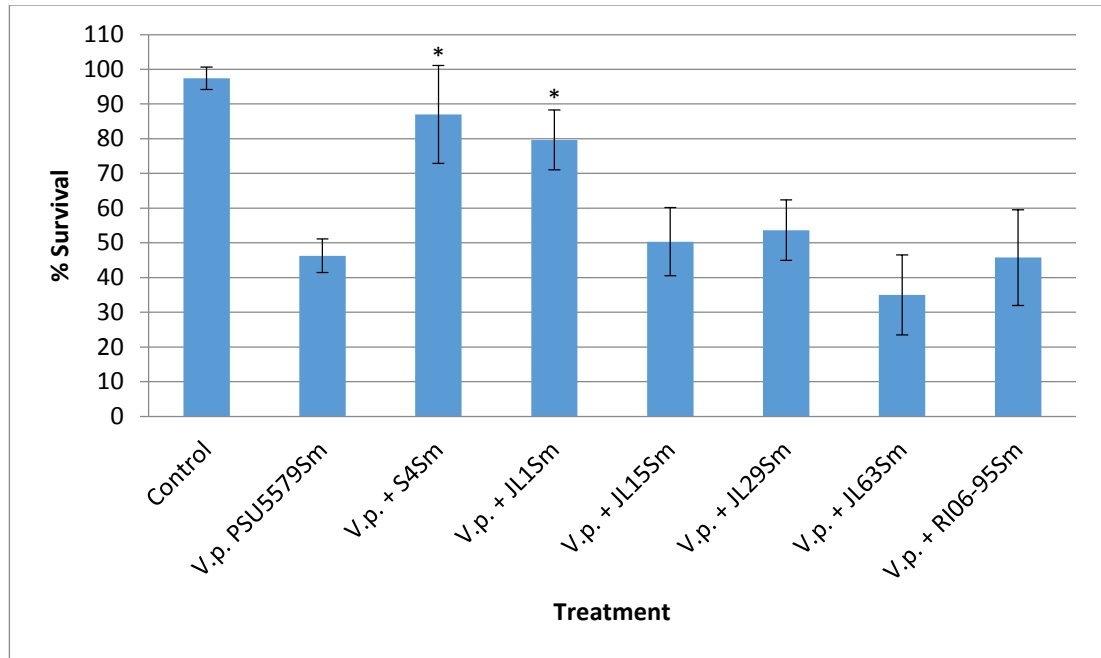


Figure 9. Effect of preincubation of *L. vannamei* PLs with *P. inhibens* S4Sm, *Ps. flavipulchra* JL1Sm, *Ps. piscicida* JL15Sm, *Ps. arabiensis* JL29Sm, *B. denitrificans* JL63Sm, and *B. pumilus* RI06-95Sm at 10^6 CFU/ml for 24 h on survival 48 h after challenge with *V. parahaemolyticus* PSU5579Sm at 10^6 CFU/ml. Candidate probionts were added at the start of the experiment and every 24 h. Representative of three independent experiments with three technical replicates per experiment. Error bars equal one standard deviation. * indicates statistically significant difference from the *V. parahaemolyticus* PSU5579Sm treatment (t-test, $p < 0.05$).

Discussion

The present study aimed to determine if probiotic bacteria can decrease the mortality rate of *L. vannamei* challenged with AHPND *V. parahaemolyticus* and if *Artemia* spp. can be used as a model organism to evaluate the potential of candidate probionts to control AHPND. Candidate probionts were selected by screening for production of compounds inhibitory toward the growth of *V. parahaemolyticus*. This

characteristic is an important property for probiotics used for disease control [38, 44], and is commonly used as a primary test in selecting candidate probionts, but does not necessarily guarantee that candidate probionts will be effective at protecting live host organisms such as shrimp [45]. Candidate probionts were characterized and tested for their ability to protect both *L. vannamei* and *Artemia* sp. from AHPND *V. parahaemolyticus* challenge. This study identified *P. inhibens* S4Sm as a bacterial candidate, which has the potential to be used as a probiotic for control of AHPND in penaeid shrimp aquaculture. This study also showed that *Artemia* sp. can be used to identify probionts that protect *L. vannamei* from AHPND *V. parahaemolyticus* challenge. Under the conditions used in this study, *Artemia* sp. were also shown to have a higher sensitivity than *L. vannamei* to organisms which are harmful to crustaceans and, therefore, can be used to identify organisms that should not be used in shrimp aquaculture.

Twelve bacterial strains were selected as candidate probionts for control of AHPND because of their antagonistic properties against *V. parahaemolyticus*. Two of these strains, *P. inhibens* S4Sm and *B. pumilus* RI06-95, were oyster probionts previously identified by Karim et al. [41]. The other ten strains were isolated during this study based on their ability to inhibit the growth of *V. parahaemolyticus* and were identified by 16S rRNA genes sequencing. The production of antimicrobial compounds by these organisms, as determined by ZOI assay, suppresses the growth of *V. parahaemolyticus*, allowing them to outcompete *V. parahaemolyticus* for nutrients and energy sources. Probiotics with known antagonistic activity have been shown to decrease the concentration of *Vibrio* spp. in black tiger shrimp (*P. monodon*) rearing

water [46, 47]. Antagonistic probionts have also been shown to inhibit the colonization of *P. monodon* by *V. harveyi* through competitive exclusion [48].

The biofilm forming ability of the twelve candidate probionts as well as *V. parahaemolyticus* PSU5579 was quantified by the crystal violet method using polystyrene 96-well plates. Biofilm formation is an important characteristic for probiotic activity because competition for attachment sites within the host is likely to serve as the first barrier of defense against invading pathogenic bacteria [16, 38, 44]. Eleven of the twelve candidate probionts produced significantly stronger biofilms than *V. parahaemolyticus* PSU5579 under the conditions tested ($p < 0.006$). The only candidate probiont that did not produce a significantly stronger biofilm than *V. parahaemolyticus* PSU5579 was *B. pumilus* HR1 ($p = 0.565$). The biofilm assay used in this study provides insight into the biofilm forming ability of the organisms tested, but is not comprehensive and may not be predictive of how well organisms will be able to colonize a host. Some of the organisms tested may form stronger biofilms on a biotic surface than on an abiotic surface, and the biofilm forming ability of these organisms may be underestimated using this assay. However, other organisms may form strong biofilms on a variety of surfaces. For example, *P. inhibens* S4Sm, which formed the strongest biofilm on polystyrene of any of the organisms tested in this study, has also been shown to form a strong biofilm on borosilicate glass [38]. Zhao et al. [38] made an *exoP*-knockout mutant of *P. inhibens* S4Sm to study the contribution of biofilm forming ability to the probiotic activity of this organism. The *P. inhibens* S4Sm *exoP* mutant had 60% reduced biofilm forming ability and oyster larvae pretreated with this mutant before *Vibrio coralliilyticus* challenge had 30%

lower survival than larvae pretreated with wild-type *P. inhibens* S4Sm, indicating that biofilm formation is important for the probiotic activity of *P. inhibens* S4Sm [38].

All twelve candidate probionts demonstrated antagonistic activity against *V. parahaemolyticus* on an agar surface and eleven of the twelve candidate probionts also form stronger biofilms than *V. parahaemolyticus* on a polystyrene surface. Organisms with both of these characteristics may be able to competitively exclude the pathogen from colonizing the host and the surrounding environment, thereby limiting the proliferation of the pathogen and reducing the likelihood of disease. Attachment to the host and production of antimicrobial compounds are critical factors for the ability of lactic acid bacteria to exclude pathogens in both humans [49, 50] and fish [51]. Verschuere et al. [31] quantified the colonization of *Artemia* nauplii by nine candidate probionts as well as the ability of these organisms to protect *Artemia* nauplii from *Vibrio proteolyticus* challenge and observed a correlation between colonization potential and the protective ability of the candidate probionts [31]. All twelve candidate probionts used in this study showed promising results *in vitro*; however, these results were not predictive of their effectiveness *in vivo*, possibly due to toxicity to the host or other undetermined factors.

Artemia spp. are an advantageous model organism to test the effectiveness of probiotics at reducing pathogen-induced mortality [31, 34, 36]. Verschuere et al. [31] found several probionts that provide total protection to *Artemia* nauplii from *V. proteolyticus*. Pretreatment of *Artemia* nauplii with yeast (*Saccharomyces boulardii*) also provides total protection from *Vibrio harveyi* challenge [36]. *Bacillus licheniformis* and *Pseudomonas aeruginosa* have also been shown to provide nearly

maximum survival (78%) to *Artemia* nauplii from non-AHPND *V. parahaemolyticus* [34]. This study is the first to test candidate probionts in an AHPND *V. parahaemolyticus*-challenged *Artemia* sp. model.

An AHPND strain of *V. parahaemolyticus* was identified which caused rapid mortalities in both *Artemia* nauplii and *L. vannamei* PLs. Animal models using *Artemia* nauplii and *L. vannamei* PLs challenged with *V. parahaemolyticus* PSU5579 were optimized to produce high survival rates (83% – 97%) in unchallenged controls and significantly decreased survival rates (33% – 46%) in AHPND *V. parahaemolyticus*-challenged controls ($p < 0.001$). It is important for experiments testing the effect of probiotic treatment on pathogen-challenged animals to have healthy animals and a significant pathogen-induced mortality rate in order for a level of probiotic protection to be detectable [52].

Pretreatment of *Artemia* nauplii with the twelve candidate probionts before *V. parahaemolyticus* PSU5579 challenge revealed that *P. inhibens* S4Sm was the only candidate probiont that significantly increased *Artemia* nauplii survival. None of the other eleven probiont candidates were able to protect *Artemia* from *V. parahaemolyticus* PSU5579 under the conditions tested. It was observed that *Artemia* nauplii treated with *P. inhibens* S4Sm at 10^6 CFU/ml daily for 72 h had reduced survival (75%) compared to the untreated control (91%). It should be noted however, that of the candidate probionts tested in this model as probiotic controls, *P. inhibens* S4Sm produced the smallest decline in *Artemia* nauplii survival. *P. inhibens* S4Sm is a strict aerobe [53] and may have depleted the oxygen level in the sealed tubes when applied daily at 10^6 CFU/ml. Neu et al. [29] found that *P. inhibens* is innocuous to

Artemia sp. however. Neu et al. [29] treated *Artemia* nauplii with 10^7 CFU/ml *P. inhibens* only once and 100 ml of the nauplii solution was incubated in 250 ml bottles with shaking at 90-100 rpm at 25 °C for 48 h. The decline in survival of *Artemia* nauplii treated with *P. inhibens* observed in this study but not observed by Neu et al. [29] may be due to any one or combination of the following factors: the addition of YP30IOS to the nauplii solution, the additional 24 h of incubation, or the sealed tubes used in this study.

Candidate probionts *B. pumilus* strains RI06-95, JL70, and HR1 were tested at $10^4 - 10^6$ CFU/ml, but did not significantly affect the survival of *Artemia* nauplii challenged with *V. parahaemolyticus* PSU5579 at 10^5 CFU/ml ($p > 0.094$). It was not determined if *B. pumilus* strains could protect *Artemia* nauplii from lower concentrations of *V. parahaemolyticus* PSU5579. Although *B. pumilus* did not have a significant effect on the survival *Artemia* nauplii challenged with AHPND *V. parahaemolyticus* in this study, there still remains the possibility that *B. pumilus* may have an effect if applied to *L. vannamei* PLs in an aquaculture setting where the concentration of AHPND *V. parahaemolyticus* may be lower than the concentration used in this study.

The remaining candidate probionts, *Ps. piscicida* strains GR1, JL12, and JL15, *Ps. flavipulchra* strains GR4, JL1, and JL18, *Ps. arabiensis* JL29, and *B. denitrificans* JL63 when applied at 10^4 CFU/ml all significantly decreased the survival of *Artemia* nauplii challenged with *V. parahaemolyticus* PSU5579 ($p < 0.001$). In all cases, the *Artemia* nauplii treated with these candidate probionts and then challenged with *V. parahaemolyticus* PSU5579 showed 100% mortality. *Artemia* treated only with *B.*

denitrificans JL63 daily at 10^5 CFU/ml reduced survival to 62%, but not significantly ($p=0.063$). Additionally, *Artemia* treated only with candidate probionts *Ps. flavipulchra* JL1, *Ps. piscicida* JL15, and *Ps. arabiensis* JL29 at 10^5 CFU/ml daily (with no *V. parahaemolyticus* challenge) exhibited 95% mortality. All three species of *Pseudoalteromonas* when applied at 10^5 CFU/ml daily for 72 h induced a higher mortality rate in *Artemia* nauplii than *V. parahaemolyticus* PSU5579 when applied once at 10^5 CFU/ml. Results similar to these were shown by Neu et al. [29] who determined that *Ps. piscicida* and *Pseudoalteromonas rubra* are lethal to *Artemia* nauplii when applied at 10^7 CFU/ml and induced 95-99% mortality in 48 h.

A *L. vannamei* challenge assay was developed to test if any of the candidate probionts could protect *L. vannamei* PLs from AHPND *V. parahaemolyticus*. This assay involved a 24 h probiotic pretreatment period, which is usually required for probiotics to effectively protect animals from pathogen challenge [42, 43]. This 24 h pretreatment period allows the probionts to colonize the host and begin producing antimicrobial compounds before the pathogen can take hold. During this period, the probiont cells may begin to divide and reach a higher density, allowing them to compete with fast growing pathogens such as *V. parahaemolyticus*, which has been shown to have a generation time as short as 12 minutes [54]. For example, the oyster probiont *P. inhibens* S4Sm (generation time = 1.9 h [41]) can inhibit the oyster pathogen *V. coralliilyticus* RE22 from colonizing glass coverslips, but only when *P. inhibens* S4Sm is allowed to precolonize the coverslip for 24 h prior to the introduction of the pathogen [38].

For the *L. vannamei* challenge assay an initial experiment determined that a 24 h preincubation period prior to 10^6 CFU/ml *V. parahaemolyticus* challenge reduced *V. parahaemolyticus*-induced *L. vannamei* mortality from 67%, when *L. vannamei* were challenged at 0 h, to 33% even without the addition of candidate probionts. This assay uses a much higher PL density than that of even super-intensive shrimp farming practices which use a maximum density of 7 PLs per 10 L [55]. Due to this high PL density, commensal bacteria from the shrimp likely grew to a high density during the 24 h preincubation period. These commensal bacteria then compete with *V. parahaemolyticus* for available nutrients, which may explain why *V. parahaemolyticus*-induced *L. vannamei* mortality was reduced. To inhibit the growth of commensal bacteria from the PLs, streptomycin (200 µg/ml) was added to the shrimp culture water. The addition of streptomycin restored *V. parahaemolyticus*-induced *L. vannamei* mortality to 67% when applied at 10^6 CFU/ml (Figure S5). A similar approach using streptomycin treatment is used in mouse models to allow both pathogenic and nonpathogenic bacteria to colonize the gastrointestinal tract [56-59]. Streptomycin treatment renders mice highly susceptible to enteric pathogens due to the elimination of commensal facultative intestinal bacteria [59]. This study showed that streptomycin treatment has the same effect on *L. vannamei* by increasing their susceptibility to AHPND *V. parahaemolyticus*.

Representatives of each species of candidate probiont were tested to determine if they were harmful to *L. vannamei* PLs. *Ps. flavipulchra* JL1Sm was the only species tested that significantly decreased the survival of *L. vannamei* PLs ($p=0.008$), but survival was only reduced from 97% to 83% during the 72 h treatment period.

Extending this treatment period further or applying *Ps. flavipulchra* to *L. vannamei* in an aquaculture setting could potentially reduce *L. vannamei* survival beyond the level found in this study. *Ps. flavipulchra* was also found to induce 100% mortality in *Artemia* nauplii under the conditions used in this study. *Ps. flavipulchra* should not be used in crustacean aquaculture due to the harmful effect this organism has on both *Artemia* sp. and *L. vannamei* identified in this study. *Ps. piscicida* and *Ps. arabiensis* also induced 95% mortality in *Artemia* nauplii. The harmful effect these organisms had on *Artemia* was not detected in *L. vannamei*, but is an indication that these bacteria could be harmful under different conditions or during an extended treatment period.

Pretreatment of *L. vannamei* PLs with the six candidate probiont species before *V. parahaemolyticus* PSU5579Sm challenge revealed that both *P. inhibens* S4Sm and *Ps. flavipulchra* JL1Sm significantly increased PL survival compared to the challenged control ($p < 0.001$). Similar to the *Artemia* challenge experiments, pretreatment of PLs with *P. inhibens* S4Sm increased survival by 1.9-fold. Interestingly, while *Ps. flavipulchra* JL1Sm exhibited a small increase in mortality to *L. vannamei* PLs, this bacterium also increased the survival of PLs challenged with *V. parahaemolyticus* PSU5579Sm. Of the candidate probionts tested, *Ps. flavipulchra* JL1 produced the largest ZOI against *V. parahaemolyticus*. Although *Ps. flavipulchra* JL1Sm may negatively affect *L. vannamei*, it also appears to prevent *V. parahaemolyticus* from causing AHPND. *Ps. flavipulchra* was far more harmful to *Artemia* nauplii than to *L. vannamei* PLs, which explains why a level of protection from *V. parahaemolyticus* was not detected in the *Artemia* sp. model. As in the

Artemia sp. model, *Ps. piscicida* JL15Sm, *Ps. arabiensis* JL29Sm, *B. denitrificans* JL63Sm, and *B. pumilus* RI06-95Sm did not significantly increase the survival of *L. vannamei* PLs challenged with *V. parahaemolyticus* PSU5579Sm ($p>0.051$).

These results indicate that the *Artemia* sp. model used in this study makes a good substitute for *L. vannamei* to study the effects of probiotics on AHPND. The *Artemia* sp. model successfully identified *P. inhibens* S4Sm as being the only candidate probiont tested that is not harmful to crustaceans and can prevent AHPND. The *Artemia* sp. model also identified *B. pumilus* as having no effect on AHPND. *Artemia* were also more sensitive to the harmful effects of *Pseudoalteromonas* spp. and *B. denitrificans* than *L. vannamei*, demonstrating that these organisms may be harmful to shrimp if used under different conditions and/or long-term.

Phaeobacter inhibens S4Sm is the only candidate probiont tested that is not harmful to *L. vannamei* PLs and can significantly increase the survival of both *L. vannamei* PLs and *Artemia* nauplii challenged with AHPND *V. parahaemolyticus*. Although twelve strains of candidate probionts produced promising results *in vitro*, our study showed that biofilm formation and growth-inhibiting activity toward a particular pathogen *in vitro* are not necessarily predictive of how a candidate probiont would perform *in vivo*. This study found that under the conditions used, *Ps. flavipulchra*, *Ps. piscicida*, and *Ps. arabiensis* were lethal to *Artemia* sp. and *Ps. flavipulchra* was also harmful to *L. vannamei*. It has been shown that some organisms, such as *Pseudoalteromonas* spp., that produce compounds inhibitory toward the growth of bacterial pathogens are also toxic to eukaryotic organisms [29, 60, 61]. Neu et al. [29] determined that *Pseudoalteromonas luteoviolacea* strains

S2607 and S4060 produce the antibacterial compound pentabromopseudilin which is lethal to *Artemia* nauplii. *Ps. piscicida* S2049 has also been shown to produce several bromoalterochromides [62] which are inhibitory toward *Bacillus subtilis* [63] and toxic to sea urchins [62]. *Ps. rubra* produces prodigiosin [64] which is antagonistic toward bacteria [65] and toxic to algae [66] and eukaryotic parasites [67].

Bowmanella denitrificans did not significantly decrease the survival of *Artemia* sp. or *L. vannamei* in probiotic controls, but did decrease the survival of these animals when challenged with *V. parahaemolyticus*, indicating that this organism potentiates *V. parahaemolyticus* infection. *V. parahaemolyticus* possess two type VI secretion systems (T6SS) [68] which may allow *V. parahaemolyticus* to kill other bacteria and potentially mediate host colonization. This effect has been shown for *Vibrio cholerae* which uses a T6SS to perturb the host's natural microbiota and enhance the pathogen's colonization of the host [69]. By killing bacteria colonizing the host's gastrointestinal tract, *V. cholerae* opens up space which it can then colonize. Gildberg et al. [70] demonstrated potentiation of the fish pathogen *Aeromonas salmonicida* by lactic acid bacteria. The lactic acid bacteria were able to colonize the intestine of Atlantic salmon (*Salmo salar*) fry, but surprisingly increased the mortality of fry challenged with *A. salmonicida* [70].

Bacteria belonging to the genus *Bacillus* are some of the most common organisms used as probiotics in aquaculture, have been shown to be effective probiotics for penaeid shrimp, and can reduce incidence of disease caused by *Vibrio* spp. [16, 22, 71, 72]. However, this study found that in the conditions used in these experiments, *B. pumilus* was not able to reduce the mortality rate of *Artemia* sp. or *L.*

vannamei challenged with AHPND *V. parahaemolyticus*. This lack of *in vivo* protection by a candidate probiont with promising *in vitro* activity has been shown before. For example, *Pseudomonas fluorescens* can protect rainbow trout (*Oncorhynchus mykiss*) from *Vibrio anguillarum* [73] but does not protect salmon (*S. salar*) from *A. salmonicida*, even though *P. fluorescens* can inhibit the growth of *A. salmonicida in vitro* [45]. This emphasizes the need to test candidate probionts for each unique host-pathogen combination *in vivo* before application in aquaculture.

Members of the genus *Phaeobacter* have been shown to be effective probiotic organisms for the protection of cod and turbot larvae from the pathogen *V. anguillarum* [74, 75], as well as eastern oyster (*Crassostrea virginica*) larvae from the pathogens *Aliiroseovarius crassostreae* CV919-312^T and *V. coralliilyticus* RE22 [19, 38]. This study demonstrated that *P. inhibens* S4Sm can also protect *Artemia* sp. and *L. vannamei* from AHPND *V. parahaemolyticus*. The probiotic activity of *P. inhibens* S4Sm has been studied and can be attributed to at least three factors: 1) excellent biofilm forming ability [38]; 2) production of the broad-spectrum antibiotic tropodithietic acid (TDA) [38]; and 3) ability to inhibit the quorum sensing-dependent production of the virulence factor protease in *V. coralliilyticus* [76]. It has also been shown that resistance to TDA is hard to select [77], making it unlikely that pathogens will develop resistance to this probiotic over time.

In conclusion, *P. inhibens* S4Sm has great potential for application in whiteleg shrimp (*L. vannamei*) aquaculture for prevention of AHPND. *P. inhibens* S4Sm is a strong biofilm former, showed antibiotic activity against *V. parahaemolyticus in vitro*, and provided protection to both *Artemia* sp. and *L. vannamei in vivo*. Application of

P. inhibens S4Sm as a probiotic in shrimp aquaculture provides an advantageous alternative to the use of antibiotics for disease control.

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Supplemental Data

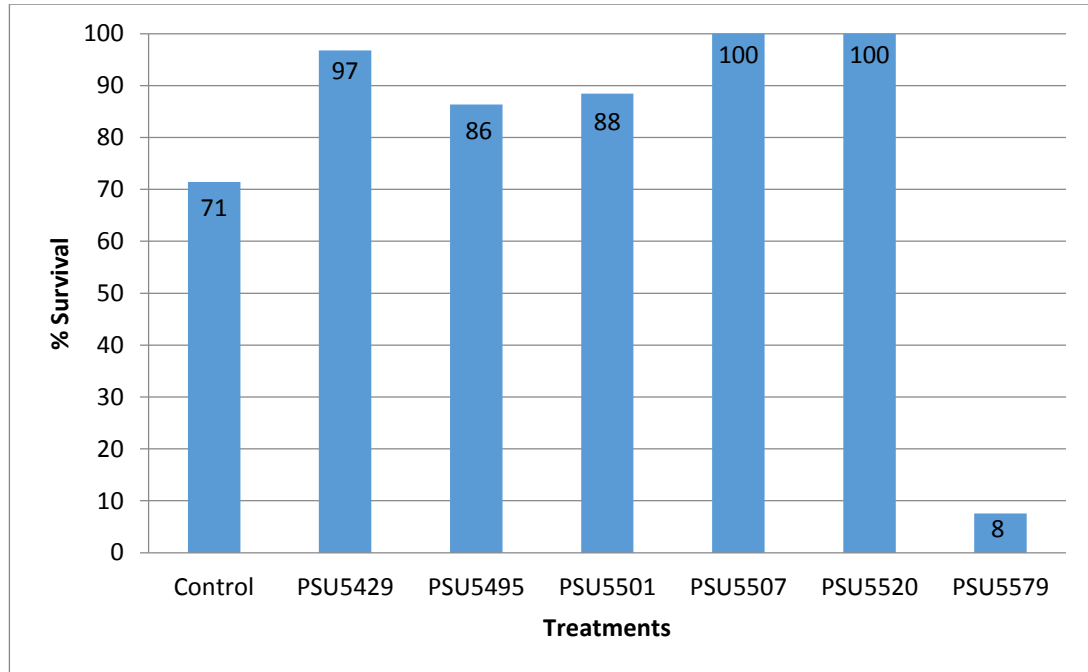


Figure S1. Survival of *Artemia* nauplii challenged immediately after hatching with suspected AHPND *V. parahaemolyticus* strains at 10^6 CFU/ml. Representative of one independent experiment with three technical replicates.

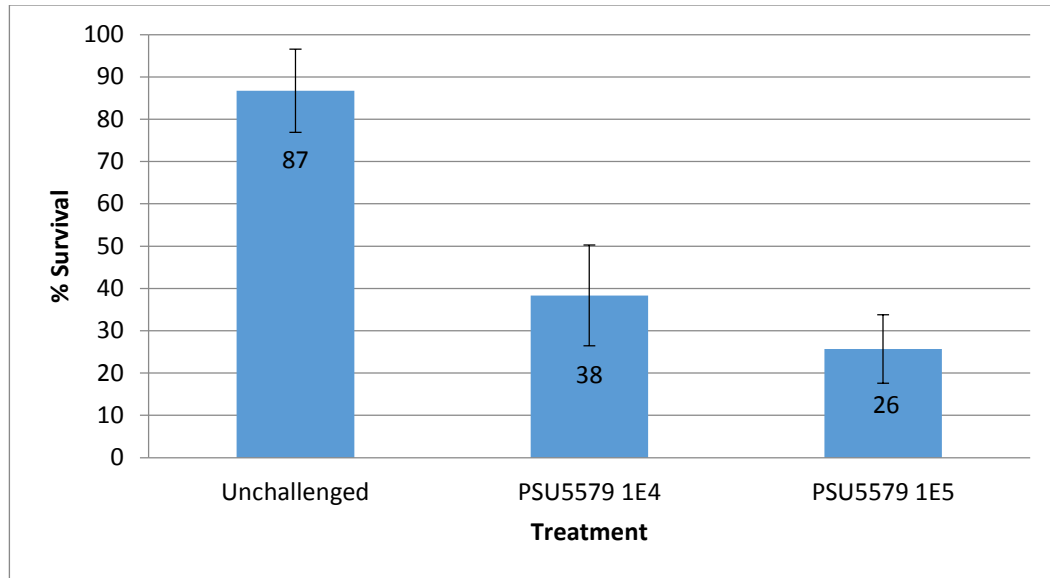


Figure S2. Survival of *Artemia* nauplii challenged 24 h after hatching with *V. parahaemolyticus* PSU5579 at 10^4 or 10^5 CFU/ml. Representative of three independent experiments with three technical replicates per experiment. Error bars equal one standard deviation.

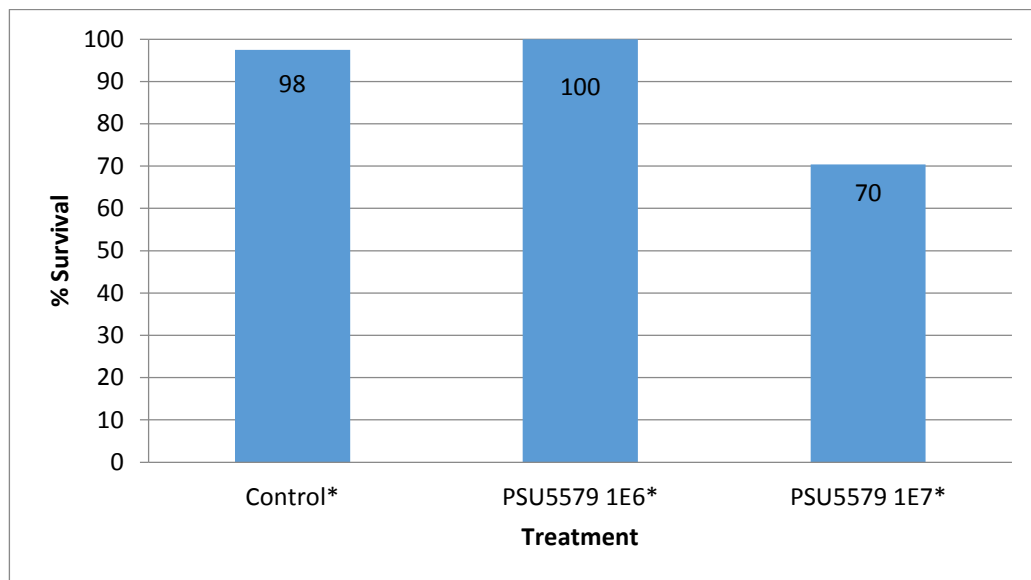


Figure S3. Survival of *Artemia* nauplii challenged 24 h after hatching with *V. parahaemolyticus* PSU5579 at 10^6 or 10^7 CFU/ml. *, without the addition of YP30IOS. Representative of one independent experiment with three technical replicates.

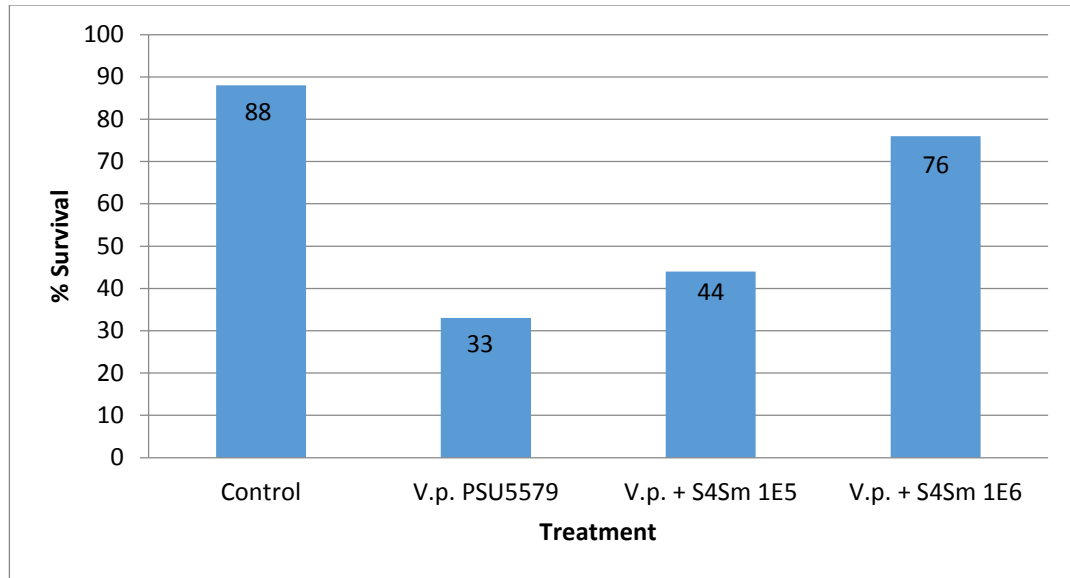


Figure S4. Effect of preincubation of *Artemia* nauplii with *P. inhibens* S4Sm at 10^5 or 10^6 CFU/ml for 24 h on survival 48 h after challenge with *V. parahaemolyticus* PSU5579 at 10^5 CFU/ml. *P. inhibens* S4 was added at the start of the experiment and every 24 h. Representative of one independent experiment with three technical replicates.

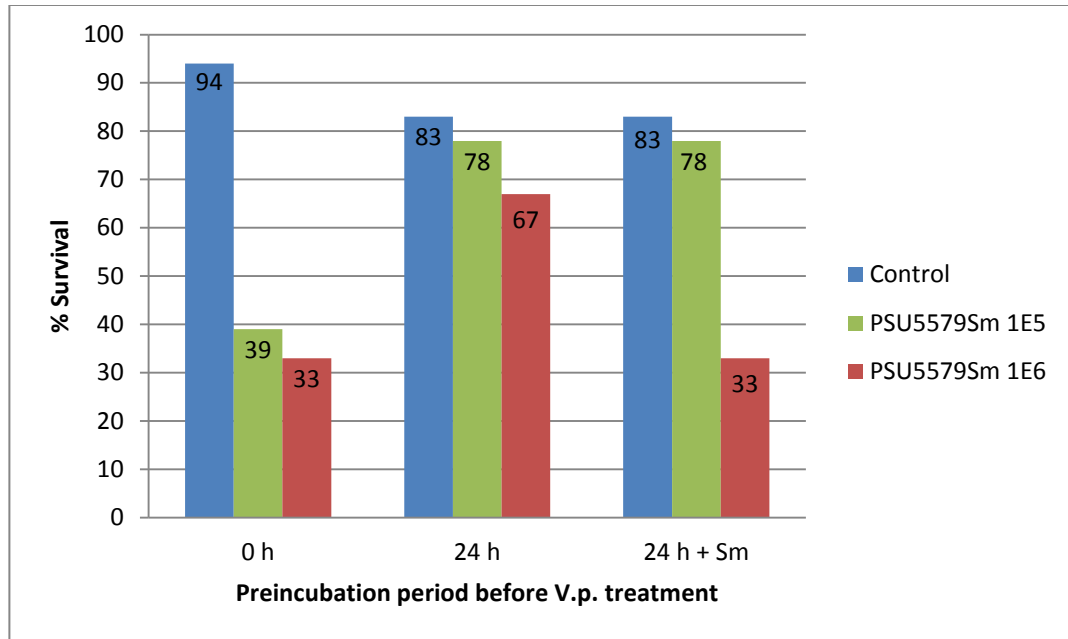


Figure S5. Survival of *L. vannamei* PLs 48 h post-treatment with *V. parahaemolyticus* PSU5579Sm applied at 10^5 or 10^6 CFU/ml at the start of the experiment (0 h), 24 h after the start of the experiment (24 h), or 24 h after the start of the experiment with the addition of 200 μ g/ml streptomycin (24 h + Sm). Representative of one independent experiment with three technical replicates.

Manuscript II

Publication status: Preparing to submit to Genome Announcements

Title: Draft Genome Sequence of *Vibrio parahaemolyticus* PSU5579, Isolated During an Outbreak of Acute Hepatopancreatic Necrosis Disease (AHPND) in Thailand.

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Key Words: Acute hepatopancreatic necrosis disease (AHPND), *Vibrio parahaemolyticus*, *Litopenaeus vannamei*

Abstract

Acute hepatopancreatic necrosis disease (AHPND) causes high mortalities in shrimp farms around the world (1-6). We announce here the draft genome sequence of one AHPND strain of *V. parahaemolyticus* and describe virulence factors that may play a role in its pathogenicity.

Body

In 2009 an emerging disease now known as acute hepatopancreatic necrosis disease (AHPND) began to affect shrimp farms in southern China and has since spread to Vietnam, Malaysia, Thailand, and Mexico (1-6). AHPND causes serious production losses in affected areas which negatively impacts local employment, social welfare, and international markets (5). The causative agent of AHPND has been identified as *Vibrio parahaemolyticus* strains possessing the 69-kbp plasmid pVPA3-1 encoding genes homologous to the *Photobacterium* insect-related (Pir) toxin-like genes (*pirA*- and *pirB*-like) (6-8). *V. parahaemolyticus* PSU5579 was isolated from shrimp farms located in southern Thailand during a disease outbreak and has been shown to induce high mortality rates via bath immersion at 10^5 CFU/ml against whiteleg shrimp (*Litopenaeus vannamei*).

V. parahaemolyticus PSU5579 was grown overnight in yeast-tryptone broth supplemented with 2% NaCl (LB20) at 27°C. Genomic DNA was isolated using the Promega Wizard genomic DNA purification kit, and DNA was resuspended in 2 mM Tris-HCl buffer (Bio Basic). Sequencing was performed at the Rhode Island

Genomics and Sequencing Center using an Illumina MiSeq Sequencer. Sequence trimming was performed using CLC Genomics Workbench (version 9.5.3) resulting in 2,743,364 paired-end reads averaging 192 bp in size. Contigs with a coverage of ≥ 34 were assembled using SPAdes genomic assembler (version 3.1.1) (9). The resulting contigs were processed using CLC Microbial Genome Finishing module using *V. parahaemolyticus* RIMD 2210633 as a reference genome. The draft genome consists of 44 contigs, with a total sequence length of 5,229,426 bp and a G+C content of 45.3%. The draft genome included the complete 69,150 bp pVPA3-1 plasmid with a G+C content of 45.9%. Gene annotation was performed using Rapid Annotations using Subsystems Technology (RAST) and resulted in 4,840 open reading frames (10-12).

The genome of *V. parahaemolyticus* PSU5579 encodes a number of lytic enzymes including two secreted collagenases, one chitinase, one extracellular lipase, phospholipases A and C, nine hemolysins including cytolysin, leukocidin, and delta-VPH, and six secreted proteases including an extracellular serine protease, three secreted trypsin-like serine proteases, and two extracellular zinc proteases including Vibriolysin. Three adherence systems were identified: a type IV pilus, a mannose-sensitive hemagglutinin type IV pilus system, and a symbiotic colonization and sigma-dependent biofilm formation gene cluster. Several iron acquisition systems were annotated including hemin, enterobactin, vibrioferrin, ferrichrome, and TonB, including the full complement of proteins responsible for the formation of the TonB-ExbB-ExbD complex. Three quorum-sensing systems are present: LuxMN, LuxSPQ, and CqsAS. Six secretion systems were identified: one type I secretion system

(T1SS), one T2SS, one T3SS, one T2/4SS, and two T6SS. The genome also encodes a capsular polysaccharide, one RTX toxin, and one beta-lactamase. The 69 kbp pVPA3-1 plasmid encodes a conjugation system as well as the *pirA*- and *pirB*-like genes located on a 3.5 kbp fragment flanked by transposases. The genome does not contain the *tdh* or *trh* genes associated with pathogenicity in humans (13).

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited in DDBJ/ENA/GenBank under the accession no. PEBT00000000. The version described in this paper is the first version, PEBT01000000.

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This work was supported by an award from CP Foods to David R. Nelson, Marta Gomez-Chiarri, David C. Rowley, and John J. Mekalanos. The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication. We thank Wenjing Zhao at the Mekalanos Lab, Harvard Medical School for providing us with the *V. parahaemolyticus* PSU5579 strain.

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Manuscript III

Publication status: Preparing to submit to Genome Announcements

Title: Draft Genome Sequence of *Bowmanella denitrificans* JL63, a Bacterium Isolated from Whiteleg Shrimp (*Litopenaeus vannamei*) that can Inhibit the Growth of *Vibrio parahaemolyticus*

Authors: Jason P. LaPorte¹, Edward J. Spinard¹, Marta Gomez-Chiari², David C. Rowley³, David R. Nelson¹

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Key Words: *Bowmanella denitrificans*, *Litopenaeus vannamei*, antibiotic

Abstract

Bowmanella denitrificans JL63 was isolated from a whiteleg shrimp (*Litopenaeus vannamei*) and was determined to have antibacterial activity against an acute hepatopancreatic necrosis disease (AHPND) strain of *Vibrio parahaemolyticus*. Here we report the draft genome sequence of this strain and identify genes potentially involved in its antibacterial activity.

Body

Bowmanella is a genus of bacteria of the family Alteromonadaceae within the Gammaproteobacteria which was first identified in 2006 (1). Currently, only three species belonging to this genus have been described: *Bowmanella denitrificans*, *B. pacifica*, and *B. dokdonensis*. *B. denitrificans* is a chemoorganotrophic bacterium capable of respiratory, but not fermentative metabolism (1). *B. denitrificans* BD1^T was the first strain of this species to be identified and is capable of anaerobic growth by carrying out denitrification while *B. denitrificans* S088 has been shown to produce a potent, heat-stable algicidal compound (1, 2). *B. denitrificans* JL63 was isolated from a whiteleg shrimp (*Litopenaeus vannamei*) and can inhibit the growth of an acute hepatopancreatic necrosis disease (AHPND) strain of *Vibrio parahaemolyticus* on an agar surface, as determined by a zone of inhibition assay (3). The *B. denitrificans* JL63 genome reported here is the first draft genome sequence of a *Bowmanella*.

B. denitrificans JL63 was grown overnight in yeast-tryptone broth supplemented with 3% artificial sea salts (LB30IOS) at 27 °C. Genomic DNA was

isolated using the Promega Wizard genomic DNA purification kit, and DNA was resuspended in 2 mM Tris-HCl buffer (Bio Basic). Sequencing was performed at the Rhode Island Genomics and Sequencing Center using an Illumina MiSeq Sequencer. Sequence trimming was performed using CLC Genomics Workbench (version 9.5.3) resulting in 2,641,396 paired-end reads averaging 180 bp in size. Contigs with a coverage of ≥ 34 were assembled using SPAdes genomic assembler (version 3.1.1) (4). The resulting contigs were processed using CLC Microbial Genome Finishing module. The draft genome consists of 39 contigs, with a total sequence length of 5,478,087 bp and G+C content of 50.4%. Gene annotation was performed using Rapid Annotations using Subsystems Technology (RAST) and resulted in 4,980 open reading frames (5-7). The 16S rRNA gene of *B. denitrificans* JL63 is 99.8% similar to *B. denitrificans* BD1^T, 99.0% similar to *B. pacifica* W3-3A, and 95.0% similar to *B. dokdonensis* UDC354. *B. denitrificans* JL63 *gyrB* and *rpoD* are 98.6% and 98.3% similar to *B. denitrificans* BD1^T and 81.2% and 80.8% similar to *B. pacifica* W3-3A, respectively.

The genome of *B. denitrificans* JL63 encodes several gene clusters potentially involved in the production of the following antibacterial compounds: colicin V (or bacteriocin), lanthionine, and the broad-spectrum antibacterial protein marinocine encoded by the *lodAB* operon. The genome also encodes a secreted hemolysin-type calcium-binding bacteriocin, an antibiotic biosynthesis monooxygenase, a type VI secretion system (T6SS), and two iron acquisition systems, hemin and TonB, including the full complement of proteins responsible for the formation of the TonB-ExbB-ExbD complex. Gene clusters for a type IV pilus as well as a mannose-sensitive hemagglutinin type IV pilus system are also present. The genome contains gene

clusters for denitrification as well as nitrate/nitrite ammonification. Three secondary metabolic gene clusters in the JL63 genome were predicted using antiSMASH (8). Gene clusters predicted to synthesize lantipeptide, bacteriocin, and a nonribosomal peptide were identified.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited in DDBJ/ENA/GenBank under the accession no. PEBU00000000. The version described in this paper is the first version, PEBU01000000.

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This work was supported by an award from CP Foods to David R. Nelson, Marta Gomez-Chiarri, David C. Rowley, and John J. Mekalanos. The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication. We thank William Robins at the Mekalanos Lab, Harvard Medical School for providing us with the whiteleg shrimp (*L. vannamei*) from which we isolated *B. denitrificans* JL63.

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