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Evaluation of the Efficacy of Probiotics *in vitro* Against *Vibrio parahaemolyticus*, Causative Agent of Acute Hepatopancreatic Necrosis Disease in Shrimp

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Abstract

Acute hepatopancreatic necrosis disease (AHPND) is a devastating disease affecting aquaculture shrimp industry around the world. Probiotics may provide an effective approach to reduce the detrimental impact of this disease in shrimp ponds. The present study was conducted to determine the inhibitory effects of probiotics against the *Vibrio parahaemolyticus* strain that causes AHPND. Probiotic solutions (whole microbial cultures and supernatants) consisting of three microbial cultures including *Lactobacillus casei*, *Saccharomyces cerevisiae*, and *Rhodopseudomonas palustris* individually and in various combinations were tested against *V. parahaemolyticus*. Disk diffusion tests and challenge tests in liquid media were conducted. Findings revealed inhibition zones with greater diameters in disks treated with whole microbial cultures (min: 7.83 mm, max: 11.33 mm) in comparison to those treated with only supernatants (min: 7.00 mm, max: 8.50 mm). Results from the challenge test showed greater inactivation of the pathogen after 48 h (6.56 ± 0.07 to 5.43 ± 0.03 $\log_{10}$ reduction) when treated with *L. casei* and *L. casei* in combination with other two probiotics. In conclusion, the probiotic solutions that included *L. casei*, the combination of *L. casei* and *R. palustris*, and the combination of *L. casei*, *S. cerevisiae* and *R. palustris* have the potential for inhibiting AHPND in shrimp aquaculture.

Keywords: Probiotics; Lactic acid bacteria; Yeast; Photosynthetic bacteria; AHPND; Shrimp aquaculture

Introduction

Acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome, is a devastating disease that affects aquaculture shrimp populations in South Asia and the Americas. The disease has had overarching impacts not only on shrimp farms, but also on communities who depend on shrimp farming for their livelihood [1]. The pathogen responsible for acute hepatopancreatic necrosis disease is a pathogenic strain of *V. parahaemolyticus* that produces a binary toxin. To our knowledge, there are no treatments that can adequately control this disease and that have been scientifically proven to cause no adverse side effects on the production system or the environment. Moreover, the use of antibiotics to treat this disease may have detrimental effects on public health since this practice can result in pathogens acquiring antibiotic resistance [2].

The Fisheries and Aquaculture report published by the Food and Agriculture Organization [3], emphasized probiotics as a promising alternative to mitigate the effects of AHPND, along with an adequate water management program, consistent monitoring, and stocking of ponds at lower population densities. The multiple mechanisms of action of probiotics previously studied in humans and commercial animals have led to an increased interest in testing their potential for aquaculture use [4]. Among the mechanisms of actions postulated in aquaculture systems is the competitive exclusion phenomenon by which probiotic microbes colonize the epithelial cells of the host, preventing the adhesion of pathogenic bacteria external to the GI tract [5]. Additionally, probiotic microbes have the potential to secrete inhibitory substances such as enzymes, bacteriocins, hydrogen peroxide, and short chain organic acids such as lactic, acetic, butyric, and propionic acids [6].

While some probiotics have shown beneficial effects in improving the water quality of aquaculture systems by degrading organic matter and converting it into CO_2 [7], others have shown increases in survival against pathogenic bacterial infections [8,9]. For instance, tilapia (*Oreochronis niloticus*) fed with commercial probiotics consisting of species of the genus *Bacillus*, *Lactobacillus*, *Clostridium*, and *Saccharomyces* showed a 30% increase in survival after being infected with *Edwardsiella tarda* [10]. In addition, Pacific white shrimp (*Litopenaeus vannamei*) treated with *Bacillus subtilis* strain L10 and G1 showed up to 13.5% increase in survival after a *V. harveyi* infection [11]. The available literature regarding the effects of these probiotics against the pathogenic *V. parahaemolyticus* strain that causes AHPND is limited. Accordingly, the objective of the present study was to determine the inhibitory effects of probiotics in whole cultures as well as their metabolites (in supernatant) against the AHPND pathogenic strain of *V. parahaemolyticus in vitro*.

Materials and Methods

Probiotics used and cultural conditions

Three probiotic microorganisms were used in this study, a lactic acid bacterium (L) (*Lactobacillus casei* NBRC 15883), a yeast (Y) (*Saccharomyces cerevisiae* NBRC 0333), and a *photosynthetic bacterium* (P) (*Rhodopseudomonas palustris* NBRC 100419). Certified pure cultures of the three probiotic microorganisms were purchased from Nite Biological Resource Center (NBRC, Chiva, Japan). *L. casei*

was revived by preparing three consecutive overnight cultures on Criterion™ *Lactobacilli* deMan, Rogosa and Sharpe (MRS) broth (Hardy Diagnostics, Springboro, OH), and incubating at 37°C for 24 h. Subsequently, the third culture was plated on MRS agar and incubated under the same conditions. Colonies were randomly selected from the plates for identification and further culturing. The permanent stock culture was prepared following the protocol of Li et al. [12] with modifications. The bacterial culture was stored at -80°C (TSX Ultra-Low Freezer, Thermo Fisher Scientific, Inc, Asheville, NC) in 2 ml tubes containing MRS broth supplemented with 40% (v/v) glycerol. *S. cerevisiae* was revived by preparing three consecutive overnight cultures on Difco™ yeast mold (YM) broth (Becton, Dickinson and Company, Sparks, MD) and incubating at 30°C for 24 h, followed by plating on YM broth with 1.5% agar [13]. Colonies were then randomly selected for species confirmation and further culturing. The permanent stock culture was stored at -80°C in 2 ml tubes containing YM broth supplemented with 40% (v/v) glycerol. *R. palustris* was revived and cultured on Van Neil's medium (VN broth) supplemented with 0.20% of Bacto™ peptone (Becton, Dickinson and Company, Sparks, MD) in transparent transport tubes and placed under full spectrum light at an intensity of approximately 4,000 lux for 48 h at room temperature [14]. Subsequently, the culture was plated on VN broth with 1.5% agar and incubated for 48 to 72 h under the same conditions. The permanent stock culture was stored at -80°C in 2 ml tubes containing VN broth supplemented with 40% (v/v) glycerol.

Confirmation of probiotics

Overnight cultures of the probiotic microbes were obtained following the protocol described earlier. DNA was extracted from the overnight cultures for species identification by using the Mo Bio PowerWater® DNA isolation kit (Mo Bio Laboratories, Inc, Carlsbad, CA) following the manufacturer's protocol for water samples. For bacterial DNA, the 515F and 806R primers were used [15,16], and for fungal DNA, the ITS1 and ITS4 were used [17]. PCR products were sequenced by conventional Sanger sequencing at the University of Arizona Genetics Core where a 3730 Automated DNA Analyzer (Applied Biosystems, Foster City, CA) was used to generate sequences. Identification was based on best matches obtained in the Basic Local Alignment Search Tool (BLAST) [18] with an e-value cutoff of 0.0 and maximum identity of 95% or greater.

Preparation of probiotic solutions for treatment

Probiotic solutions were prepared from overnight cultures of the three microorganisms (i.e. *L. casei*, *S. cerevisiae*, and *R. palustris*) using methods described previously. However, the nutrient media were enriched with 2% NaCl in the case of *L. casei* and *S. cerevisiae*, and 1% NaCl in the case of *R. palustris*. This was done to account for the salinity of the media used in the challenge test since *V. parahaemolyticus* is a slightly halophilic bacterium [19]. Seven probiotic solutions made of pure cultures were evaluated in this study, either individually or in combination. Three probiotic solutions consisted of one microbial type each; *L. casei* (L), *S. cerevisiae* (Y), and *R. palustris* (P). The next batch of three probiotic solutions consisted of combinations of two microbial types; *L. casei* and *S. cerevisiae* (L+Y), *L. casei* and *R. palustris* (L+P), and *S. cerevisiae* and *R. palustris* (Y+P). The last probiotic solution consisted of a combination of all three microorganisms: *L. casei*, *S. cerevisiae*, and *R. palustris* (L+Y+P). For those probiotic solutions made of two or more microbial types, overnight cultures of individual microbes were briefly vortexed in an analog vortex mixer (VWR, Troemner, LLC, Thorofare, NJ) and mixed in equal volumes. In addition to the probiotic solutions prepared for this study, a commercial probiotic (CP) treatment (EM®, EMRO Inc., Tucson, AZ) was included and prepared following the manufacturer's recommendation. EM®, commercially available as EM1® or EM Camarón®, is a proprietary probiotic formulation owned and managed by EM Research Organization in Okinawa, Japan. This microbial consortium contains multiple species of lactic acid bacteria, yeast and photosynthetic bacteria.

Preparation of pathogen culture

Pure culture of *V. parahaemolyticus* strain 13-028/A3 (VP_{AHPND}), originated in Vietnam [20], was obtained from Dr. Donald Ligthner's collection at the Aquaculture Pathology Laboratory at the University of Arizona, Tucson, AZ. The bacterial inoculum was prepared following the method described by Tang et al. [21] with modifications. In brief, a 100-µl aliquot of a pure bacterial culture was poured into a 250 ml flask containing 50 ml of Criterion™ tryptic soy broth (Hardy Diagnostics, Springboro, OH) with 2% NaCl (TSB+). The flask with a magnetic stir bar was placed on a stir plate in an incubator at 29°C ± 0.5°C with a slight rotation of 120 rpm for 3 h. Consequently, the contents of the flask were poured into a 500 ml flask containing 450 ml of TSB^+ and placed into the incubator on a stir plate for 20 h under the same conditions. The final bacterial concentration of the overnight culture (9 $\log_{10}$ CFU ml^{-1}) was confirmed via direct plate count on Criterion™ thiosulfate citrate bile salts sucrose (TCBS) agar (Hardy Diagnostics, Springboro, OH) and by measuring the optical density (600 nm) using a spectrophotometer.

Disk diffusion test

To test the inhibitory effects of probiotics against *V. parahaemolyticus* on solid media, the disk diffusion test described by Hendrikson [22] was performed with modifications. Agar plates containing 20 ml of Criterion™ tryptic soy agar (TSA) (Hardy Diagnostics, Springboro, OH) with 2% NaCl (TSA+) were prepared beforehand. After probiotic solutions and overnight culture of the pathogen were obtained, a sterile cotton swab was dipped into the pathogen culture. The pathogen was cultured onto the TSA+ plates by streaking these plates thoroughly with the cotton swab over the entire surface, so that a thick mat can be formed. Subsequently, previously sterilized paper disks were soaked in the probiotic solutions, both individual ones and combinations, for 15 min to obtain an inoculum level of approximately 8 log CFU per disk of *L. casei* and *R. palustris*, and 7 log CFU per disk in the case of *S. cerevisiae*. In addition, paper disks were prepared similarly using the commercial probiotic. Then, treated disks were aseptically placed using forceps onto the agar surface of the TSA+ plates inoculated with the pathogen. No more than 4 treated disks were arranged per plate to avoid overlapping of the zones of inhibition. Two different controls were included in this study. The first was a positive control (+Ctrl) in which paper disks were soaked into the overnight culture of the pathogenic strain. The second was a negative control (-Ctrl) in which paper disks were soaked in TSB +. The plates were incubated at 29°C ± 0.5°C for 24 h [21]. The diameters of the zones of inhibition were measured in millimetres. Additionally, the same experimental setting was used with only the supernatant of probiotic solutions, which was obtained by centrifugation of the overnight cultures of the three microbial types at 8000 rpm for 2 min. The disks were soaked in 100 µl of the supernatant solutions for 15 min. All treatments, for both the bacterial cultures and

the supernatants, were conducted in duplicates and the experiment was carried out three times on different days.

Inhibition assay in liquid media

After preparation of the probiotic solutions and overnight culture of the pathogen, 50 ml transport tubes were filled with 46 ml of TSB+. Then, 2 ml aliquots of VP_{AHPND} were added into the transport tubes. For each probiotic treatment, 2 ml aliquot of the probiotic solution was dispensed into the same transport tube making up the total volume to 50 ml. The tubes were incubated at 30°C. Two different controls were used in this assay: a positive control (+Ctrl) where the pathogen was not challenged with any probiotic solution and a negative control (-Ctrl) where TSB+ was not inoculated with either probiotics or the pathogen. Simultaneously, acidity of the media was measured, and samples were taken at the beginning of the incubation period and every 6 h thereafter, for up to 48 h. Samples were serially diluted using peptone water with 2% NaCl and plated on selective media. For enumeration of VP_{AHPND}, TCBS with ampicillin was used. Ampicillin was added to TCBS to prevent the growth of probiotic bacteria. For the enumeration of probiotics, the broth media previously described for each probiotic microbe were used to which 1.5% bacteriological grade Criterion™ agar (Hardy Diagnostics, Springboro, OH) and streptomycin were added. Preliminary studies showed that VP_{AHPND} was highly susceptible to streptomycin, which prevented the growth of the pathogen in the nutrient media used for each probiotic. Plates were incubated under the conditions described earlier. All treatments were conducted in duplicates during each repeat. A total of three repeats of the experiment were conducted on different days for statistical significance.

Statistical analysis

Data processing was performed using R statistical software [23] with the PMCMR [24] and STATS [23] packages. The Shapiro-Wilk normality test was used to determine the normality of the data [25]. For data following normal distribution, the Analysis of Variance (ANOVA) at a 1% significance level was performed followed by post-hoc analysis Tukey's Honest Significance Test. If data were not normally distributed, the nonparametric analysis of variance Kruskal-Wallis test was utilized with pairwise comparisons using Tukey's and Kramer's Nemenyi test with Tukey-Distribution approximation for independent samples [16,26].

Results

Disk diffusion tests

The disk diffusion test on solid media (Figure 1) showed greater diameters of inhibition zones for the treatments in which disks were treated with microbial cultures when compared to those of their respective supernatants. The treatments that included *L. casei* (L=11.33 ± 0.76 mm, L+Y=10.33 ± 0.31 mm, L+PB=11.00 ± 1.32 mm, and L+Y +PB=10.50 ± 0.50 mm) as well as the commercial probiotic treatment (11.17±1.36 mm), demonstrated larger zones of inhibition than those that did not include *L. casei* in the formulation (Y=8.17 ± 0.29 mm and PB=7.83 ± 0.29 mm). Although the combination of *S. cerevisiae* and *R. palustris* (9.33±1.04 mm) showed no significant difference (P>0.05) when compared to the treatments including *L. casei*, the average diameters of the zones of inhibition caused by this combination treatment were lower. Furthermore, the results from the disk diffusion test using only supernatants showed that there was no significant difference among treatments including *L. casei* in their formulation (7.95 ± 0.44 mm), in comparison to those treatments without this lactic acid bacterium: the combination of *S. cerevisiae* and *R. palustris* (7.00 ± 0.20 mm), and the treatments with *S. cerevisiae* (7.00 ± 0.20 mm) and *R. palustris* (7.00 ± 0.30 mm) used individually.

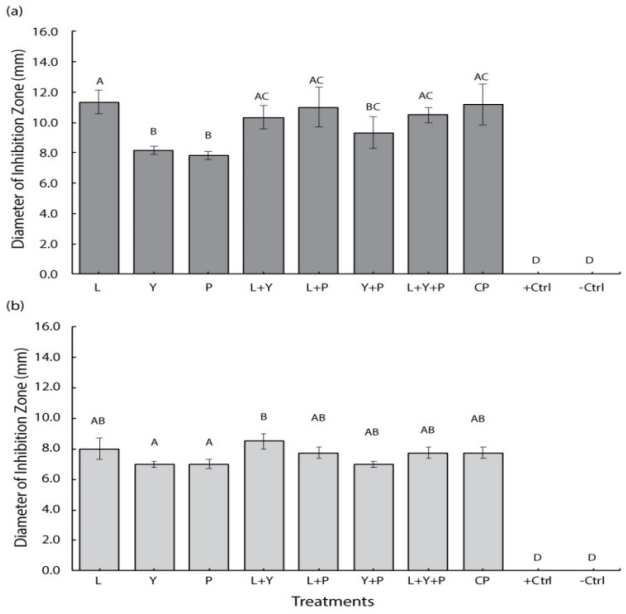

Figure 1: Diameters of inhibition zones (mm) of probiotic treatments using the complete microbial culture (a) and their respective supernatants (b). Data represent mean ± SD. Treatments: (L) *L. casei*, (Y) *S. cerevisiae*, (P) *R. palustris*, (L+Y) *L. casei*+*S. cerevisiae*, (L+P) *L. casei*+*R. palustris*, (Y+P) *S. cerevisiae*+*R. palustris*, (L+Y+P) *L. casei*+*S. cerevisiae*+*R. palustris*, (CP) Commercial Probiotic EM1®, (+Ctrl) positive control, and (–Ctrl) negative control. Bars with similar letters represent no significant difference between those treatments (P>0.05).

Inhibition assay in liquid media

The surviving population of VP_{AHPND} after challenging it with various probiotic solutions over a 48-h period is depicted in Figure 2. The results indicated that after 12 h, some probiotics treatments (L, L +Y, L+P, L+Y+P) showed a bactericidal effect, as evidenced by an average difference of 0.97 $\log_{10}$ CFU ml^{-1} in comparison to the population of the positive control, while others (Y, P, and Y+P) did not show any effect. The treatment with the commercial probiotic caused a decrease of 2.37 $\log_{10}$ CFU ml^{-1} after 12 h. From 24 to 36 h, all treatments showed different degrees of pathogen inactivation. The four treatments showing the greatest difference in comparison to the positive control (8.09 ± 0.09 $\log_{10}$ CFU ml^{-1}) after 24 h were the commercial probiotic (3.30 ± 0.10 $\log_{10}$ CFU ml^{-1}), the combination of *L. casei* and *R. palustris* (5.22 ± 0.20 $\log_{10}$ CFU ml^{-1}), the combination of *L. casei*, *S. cerevisiae* and *R. palustris* (5.48 ± 0.12 $\log_{10}$ CFU ml^{-1}), and *L. casei* (6.00 ± 0.22 $\log_{10}$ CFU ml^{-1}) alone. After 36 h, the commercial probiotic (1.87 ± 0.04 $\log_{10}$ CFU ml^{-1}), the combination of *L. casei*, *S. cerevisiae* and *R. palustris* (2.03 ± 0.06 $\log_{10}$ CFU ml^{-1}), the combination of *L. casei* and *R. palustris* (2.18 ± 0.06 $\log_{10}$ CFU ml^{-1}),

and the combination of *L. casei* and *S. cerevisiae* (3.35 ± 0.17 $\log_{10}$ CFU ml^{-1}) showed the greatest bactericidal effect against *V. parahaemolyticus*. After 48 h, four treatments including *L. casei*, the combination of *L. casei* and *S. cerevisiae*, the combination of *L. casei*, *S. cerevisiae* and *R. palustris*, and the commercial probiotic treatments showed no VP_{AHPND} survivors.

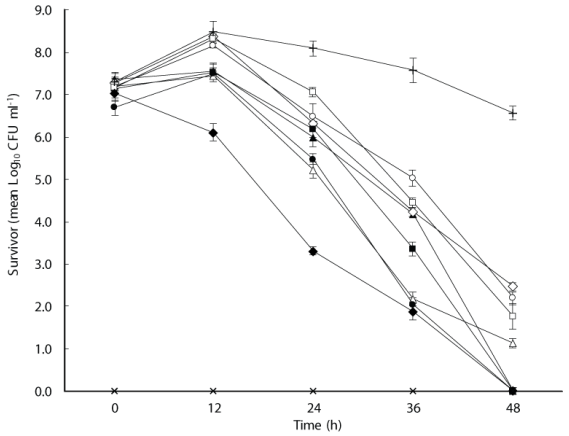

Figure 2: Bacterial population ($\log_{10}$ CFU ml^{-1}) of VP_{AHPND} at different time intervals after being challenged with probiotic solutions. Data represent mean ± SE. Treatments: (x) negative control, (+) positive control, (▲) *L. casei*, (○) *S. cerevisiae*, (◇) *R. palustris*, (▪) *L. casei*+*S. cerevisiae*, (Δ) *L. casei*+*R. palustris*, (□) *S. cerevisiae*+*R. palustris*, (●) *L. casei*+*S. cerevisiae*+*R. palustris*, (♦) Commercial Probiotic EM•1®.

Furthermore, statistical analysis of variance revealed that the commercial probiotic treatment, the combination of *L. casei* and *R. palustris*, the combination of *L. casei* and *S. cerevisiae*, the treatment with *L. casei* alone, and the treatment including all three microbes were significantly different ($P<0.05$) in comparison to the positive control.

Changes in acidity levels in the liquid media as a result of microbial growth were observed (Table 1). Statistical analyses revealed no significant difference in media pH values between probiotic treatments and the positive control ($P<0.05$). Initial pH values started at an average of 6.22 and decreased over 48 h in comparison to the negative control. The treatment including the combination of *L. casei*, *S. cerevisiae* and *R. palustris* reached the highest acidity with a pH of 4.83 followed by the treatment with the combination of *L. casei* and *S. cerevisiae*, and the combination of *L. casei* and *R. palustris*, with a pH of 4.88 in both cases. In the case of the commercial probiotic, the average pH value (5.58) was slightly higher than the pH value obtained in the positive control (5.4).

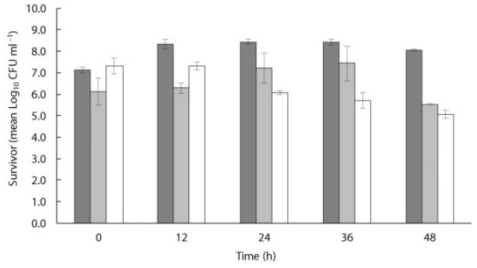

Figure 3: Population of probiotic microorganisms at different time intervals in TSB+ inoculated with VP_{AHPND}. Data represent mean ± SD. Probiotic microbes: (■)*L. casei*, (▩)*S. cerevisiae*, (□)*R. palustris*

Treatments	pH values at Time Intervals				
	0 h (mean ± S.D.)	12 h (mean ± S.D.)	24 h (mean ± S.D.)	36 h (mean ± S.D.)	48 h (mean ± S.D.)
L	6.22 ± 0.01	5.64 ± 0.03	5.2 ± 0.03	4.96 ± 0.04	4.9 ± 0.02
Y	6.24 ± 0.01	5.87 ± 0.01	5.51 ± 0.02	5.36 ± 0.03	5.04 ± 0.03
P	6.22 ± 0.00	5.75 ± 0.03	5.46 ± 0.01	5.19 ± 0.06	5.13 ± 0.01
L+Y	6.24 ± 0.00	5.67 ± 0.02	5.17 ± 0.02	4.91 ± 0.08	4.88 ± 0.03
L+P	6.24 ± 0.01	5.64 ± 0.03	5.15 ± 0.02	5.01 ± 0.02	4.88 ± 0.02
Y+P	6.23 ± 0.01	5.67 ± 0.02	5.2 ± 0.02	5.03 ± 0.02	5.09 ± 0.03
L+Y+P	6.23 ± 0.01	5.66 ± 0.01	5.08 ± 0.01	4.95 ± 0.02	4.83 ± 0.01
CP	6.2 ± 0.00	6.19 ± 0.11	5.86 ± 0.05	5.82 ± 0.02	5.58 ± 0.05
+Ctrl	6.14 ± 0.03	5.73 ± 0.03	5.59 ± 0.03	5.47 ± 0.05	5.4 ± 0.03
- Ctrl	7.05 ± 0.03	7.05 ± 0.01	7.12 ± 0.03	7.11 ± 0.03	7.14 ± 0.03

Table 1: pH of the liquid media at different time intervals during challenge of VP_{AHPND} with various probiotic solutions. (L) *L. casei*, (Y) *S. cerevisiae*, (P) *R. palustris*, (L+Y) *L. casei*+*S. cerevisiae*, (L+P) *L. casei*+*R. palustris*, (L+Y+P) *L. casei*+*S. cerevisiae*+*R. palustris*, (CP) Commercial Probiotic EM1®, (+Ctrl) positive control, and (–Ctrl) negative control.

Changes in the populations of the probiotic microbes over the 48 h challenge test were observed (Figure 3). The population of *L. casei* increased by 1.23 $\log_{10}$ CFU ml^{-1} from 0 to 12 h; however, there were no appreciable changes from 12 to 36 h. From 36 to 48 h, the population of *L. casei* had a slight decrease of 0.36 $\log_{10}$ CFU ml^{-1}. *S. cerevisiae* showed no significant changes in population from 0 to 36 h. However, there was a slight decrease at 48 h. The average population of *R. palustris* declined after 24 h, but no significant changes were evident thereafter.

Discussion

In aquaculture, probiotics have been applied to improve water quality and to control bacteria associated with emerging diseases. Some types of probiotics found to be effective in inhibiting the growth of certain bacteria include the commercial product INVE Sanolife® (INVE, Belgium) containing Gram-positive *Bacillus* sp. [27] as well as Procreatin 7 (Safmex, Mexico) containing the yeasts *S. cerevisiae* [28] and *Debaryomyces hansenii* [29]. Probiotics were initially used in land dwelling vertebrates such as in calves to reduce the incidences and duration of diarrhea [30], in chickens to increase resistance to coccidiosis [31], and in sows to improve fertility [32]. In many cases, probiotics were found to improve animal health through many different mechanisms of action, such as competitive exclusion, secretion of antimicrobial substances, disruption of quorum sensing, and immune-modulatory effects on the host [33]. Beyond their effective application in vertebrates, probiotics have also been successfully evaluated in invertebrates. A study found that probiotic solutions containing *Streptomyces* in combination with bacteria of the genus *Bacillus* improved growth parameters and immune response in Pacific white shrimp (*Litopenaeus vannamei*) [34]. Similar findings were demonstrated in freshwater prawns (*Macrobrachium rosenbergii*) when their diet was supplemented with *Bacillus licheniformis* [35]. Although the efficacy of commercial probiotics to inhibit the growth of deleterious bacteria has been evaluated in shrimp *L. vannamei* [27], their effects on the causative agent of AHPND, *V. parahaemolyticus* strain 13-028/A3, are currently unknown.

In this study, the disk diffusion test was conducted to evaluate if the probiotic microbes were effective against VP_{AHPND}. This test showed that the top five treatments with the greatest diameters of inhibition zones were the commercial probiotic, lactic acid bacteria, lactic acid bacteria combined with photosynthetic bacteria, and lactic acid bacteria combined with both yeast and photosynthetic bacteria. These treatments, including the commercial probiotic, have lactic acid bacteria of the genus *Lactobacillus* in their formulation. These results are less likely to be attributed to the efficacy of probiotics to compete for nutrients, since the solid media used is rich in nutrients. Inhibitory effects could more likely be attributed to the production of inhibitory substances [5] such as short-chain fatty acids [36] found in the overnight cultures used for the preparation of the probiotic solutions. However, short-chain fatty acids alone are not enough to cause inhibition of the pathogen as evidenced in Figure 1, where disks prepared with only supernatants achieved lower diameters of zones of inhibition in comparison to the disks prepared with whole bacterial cultures. These results may be due to further metabolic processes performed by probiotic microbes that caused the production of additional inhibitory substances. For instance, lactic acid bacteria are known for their ability to produce antibacterial peptides that interfere with essential bacterial enzymes or disrupt the permeability of the bacterial cell membrane of pathogenic bacteria [37].

The inhibition assay in liquid media showed that the treatments using lactic acid bacteria combined with yeast, lactic acid bacteria combined with photosynthetic bacteria, and lactic acid bacteria combined with yeast and photosynthetic bacteria caused inactivation of VP_{AHPND} after 12 h and reduced the populations of the pathogen from 1.13 $\log_{10}$ CFU ml^{-1} to no detection of survivors after 48 h. The commercial probiotic treatment not only had no survivors of VP_{AHPND} detected at 48 h, but also demonstrated bactericidal effects as early as 12 h after infection. Since this test was also conducted using a nutrient rich media, it is unlikely that competition for nutrients and space may be the cause of inhibition. Furthermore, lactic acid bacteria have the ability to modify their external environment by producing organic acids such as lactic, acetic, butyric, and propionic acids [38,39]. These organic acids can reduce the pH of the environment and adversely affect the normal growth and/or survival of bacteria susceptible to acidic conditions [40]. However, the results obtained in terms of the variation in media pH levels over 48 h (Table 1) suggest that acidity may not have played a major role in pathogen inactivation. Instead, it is postulated that probiotic species have the ability to produce more complex inhibitory substances such as enzymes or bacteriocins that can cause inactivation of certain pathogens [41].

Ultimately, the findings from the disk diffusion tests in solid media along with the findings from the challenge tests in liquid media indicate that probiotics, alone and in combination, are potentially viable options for inactivating or inhibiting the growth of VP_{AHPND}. Although there is limited literature discussing the effectiveness of all the probiotic microbes used in this study and other probiotics against VP_{AHPND}, *Lactobacillus* [42,43] and *Bifidobacterium* [39,44] have been shown to be the most effective probiotic bacteria for increasing disease resistance and inhibiting the effects of some pathogens that negatively impact aquaculture species. Moreover, since probiotics have the capability of consuming the nutrients that are required for pathogens to grow within living organisms [39], the present study could potentially be extended to inhibit the growth of *V. parahaemolyticus* in live shrimp. Additionally, Gram et al. [45] suggested that the mechanisms of action of probiotics should be further evaluated with live models, since inhibitory effects *in vitro* may not directly translate in targeted environments such as the gastrointestinal tract of shrimp and the water of aquaculture ponds. Probiotics have been shown to improve the innate immunity of different land and aquatic vertebrates and enhance the resistance of shrimp against common diseases, such as vibriosis, white spot syndrome and *Aeromonas hydrophila* infection [38]. The present study suggests that the combination of certain probiotics may be an effective step towards minimizing the detrimental impact of acute hepatopancreatic necrosis disease in shrimp aquaculture.

Conflict of Interests

An author on this manuscript is an employee of EM Research Organization, Inc. whose commercial probiotic was part of the treatments used in this study.

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