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Research Article

Combination of Organic Acids and Essential Oil (Aquavibra™) in the Diet Improves Survival of *Penaeus vannamei* Infected with *Vibrio parahaemolyticus* Causing Acute Hepatopancreatic Necrosis Disease

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ABSTRACT

Supplementation of a combination of organic acids and essential oils (Aquavibra™) was evaluated for its effect during experimental infection of *Vibrio Parahaemolyticus* Isolate-XN89 that caused Acute Hepatopancreatic Necrosis Disease (VPAHPND) in white shrimp, *Penaeus vannamei* as compared to that of an antibiotic. Initially, the *in vitro* Minimum Bactericidal Concentration (MBC) and Minimum Biofilm Inhibitory Concentration (MBIC) of Aquavibra™ against VPAHPND isolate XN89 were determined to be 3.12 mg/ml -1.56 mg/ml and 2 mg/ml, respectively. Aquavibra™ exhibited efficient biofilm reduction (85%), which was comparable to that of chloramphenicol (88%). An experimental challenge was conducted in shrimp juveniles with XN89 at 10^5 CFU/mL (LC50) following a 42-day feeding period using a diet supplemented with 0.5% of Aquavibra™. The growth and FCR between the Aquavibra™ supplemented group, and the control were not significantly different (P>0.05) after the feeding period. The experimental challenge resulted in 83% mortality in the infected control group. Antibiotic supplementation in feed using Trimethoprim-Sulfamethoxazole (Bactrim®) resulted in significant improvement in survival (61.54%), while the Aquavibra™ group showed comparable survival rate (60%) at 120 h post-challenge. Histopathological analysis revealed hepatopancreatic tissues with normal tubule epithelia in the uninfected group and severe collapsed hepatopancreatic tubule epithelia in the infected control group. However, the histopathology of moribund shrimp from Aquavibra™ and antibiotic supplemented groups revealed a mix of normal and thin collapsed hepatopancreatic epithelia, indicating less bacterial colonization of pathogens in hepatopancreas. Results from the present study indicate that dietary supplementation of Aquavibra™ at 0.5% is promising against AHPND in white shrimp.

Keywords: Shrimp disease, Bacterial infection, Biofilm, Sustainable aquaculture

INTRODUCTION

Bacteria belonging to the genus Vibrio are the most common pathogens in shrimp aquaculture worldwide associated with diseases such as tail necrosis, loose shell syndrome, running mortality syndrome, and red disease in shrimp. Among the pathogenic vibrio's, a strain of *V. parahaemolyticus* that harbours the pVA1 plasmid encoding binary toxins, Parp and PirVpB is reported to cause Acute Hepatopancreatic Necrosis Disease (AHPND) affecting both the black tiger shrimp, Penaeus monodon and white shrimp. Prominent symptoms seen in

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affected shrimp include an empty gut and an atrophied, pale hepatopancreas. Histopathology shows the sloughing of the hepatopancreatic tubule epithelial cells and haemolytic infiltration. Besides, non-AHPND strains of *V. parahaemolyticus* are implicated in the white gut syndrome that affects *P. vannamei*. Isolation and characterization of several Vibrio species from the hemolymph of *P. vannamei* showed the presence of antibiotic-resistant genes [1].

Essential oils are lipophilic compounds and reported to inhibit the growth of various pathogenic bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Staphylococcus aurous*. The antibacterial activity of essential oils and organic acids countering several pathogenic organisms, including Vibrio sp., has been reported. The effect of a specific combination of organic acids and essential oils on the growth performance and survival of *P. vannamei* following experimental challenge with VPAHPND isolate XN89 was evaluated in this study [2].

MATERIALS AND METHODS

Bacterial Strain

The VPAHPND isolate XN89 strain used in the study was initially isolated from AHPND affected shrimp in 2014. The isolated strain was characterized at National Centre for Genetic Engineering and Biotechnology, Thailand and was maintained at the Department of Aquaculture and Aquatic Resources Management, Asian Institute of Technology, Thailand [3]. The bacterial suspension to be used in the challenge study was prepared by sub-culturing the XN89 strain in Tryptic Soy Broth (TSB) containing 2% NaCl, followed by incubation at 30°C for 24 h.

Experimental Animals

Penaeus vannamei juveniles with an initial body weight of 3.4 g ± 0.3 g were maintained in 2000 L capacity tanks. Each tank containing aerated brackish water was equipped with a biological filter in a closed recirculating culture system. The temperature, salinity, pH and dissolved oxygen of rearing water were maintained at 28°C-30°C, 15 ppt, 7.5-8.0 and ≥ 5.0 mg/l, respectively. Juveniles were fed a commercial crumble feed daily at 5% of their body weight [4].

Antimicrobial Agent

A supplement containing organic acids and essential oil (Aquavibra™) was obtained from Kevin Aqua Science™, Chennai, and used for the evaluation of their efficacy against the pathogenic strain of *V. parahaemolyticus* XN89.

Determination of Minimum Bactericidal Concentration (MBC)

The MBC of Aquavibra™ against *V. parahaemolyticus* XN89 was determined by the broth dilution method followed by dot assay. Five percentage test solution was prepared by dissolving 2.5 g of product in 50 ml of 50% Dimethyl Sulfoxide (DMSO). Sterile Zobell marine broth (100 µl) was then added to all the wells of a 96-well microliter plate, followed by 100 µl of test solution added to the first well, and subsequently, two-fold serial dilutions were made in a concentration range between 25 mg/ml and 0.012 mg/ml. Similarly, in the antibiotic group, chloramphenicol (Sigma-C0378-25G) was prepared in sterile saline and added in a concentration range between 0.5 mg/ml to 0.001 mg/ml [5]. The cell density of V parahaemolyticus XN89 was adjusted (OD600 nm=0.5) using sterile Zobell marine broth. One hundred microliters of *V. parahaemolyticus* was added to all the wells. Media control (sterile broth alone), Vibrio control (broth+*V. parahaemolyticus* XN89), and DMSO control (50% DMSO+broth) were maintained in different wells followed by incubation of the plates at 37°C for 16 h. After incubation, 3 µl of culture from each well was dotted on the HI chrome vibrio agar plate to confirm the inhibition of Vibrio growth. The complete inhibition of Vibrio growth in Hichrome vibrio agar plate indicated the MBC. The experiments were carried out in triplicates [6].

Determination of Minimal Biofilm Inhibitory Concentration (MBIC)

An improved crystal violet method was used to estimate the MBIC of Aquavibra™. A five-percentage stock solution was prepared, as mentioned above. Test solutions were prepared from the stock by diluting with pre-autoclaved marine broth. A narrow range of concentrations were prepared, from 20 mg/ml to 2 mg/ml. In a 96-well microliter plate, 100 µL of test solutions were added into the respective wells, and *V. Parahaemolyticus* XN89 at the appropriate cell density (OD600 nm=0.5) was added into all the wells. Vibrio control (broth+*V. parahaemolyticus*), media control (broth alone), and antibiotic control (chloramphenicol at 1 mg/ml+*V. parahaemolyticus*) were maintained in separate wells. After the incubation of plates at 37°C for 24 h, sterile deionized water was used to wash the planktonic cells three times gently. The plates held in an inverted position were patted dry with a piece of paper towel. After drying, 100 µl of 0.2% crystal violet stain was added to each well and incubated at 37°C for 15 min [7-10]. Any non-adherent stain was removed by washing with sterile deionized water followed by air-drying of the plates. The plates were incubated for 30 min at room temperature, and OD590 was measured after solubilizing the adherent dye using glacial acetic acid and ethanol (80:20) (v/v). The BIC value with a maximum inhibition of biofilm was then quantified according to the following formula.

$$MBIC = \left[\frac{OD\ Vibrio\ control - OD\ Test\ products}{OD\ Test\ products}\right] \times 100$$

The experiments were carried out in triplicates.

Experimental Design for LC50

The effective lethal concentration (LC50) of *V. parahaemolyticus* XN89 against *P. vannamei* was identified by studying the survival of shrimp in different Vibrio concentrations. Thirty shrimp were maintained in each experimental challenge tanks in duplicates and acclimatized for 30 h before the challenge. A commercial feed without any supplements was fed to the shrimp during this period 11. The different final concentrations (10^2 CFU/ml, 10^3 CFU/ml, 10^4 CFU/ml, 10^5 CFU/ml and 10^6 CFU/ml) of *V. parahaemolyticus* XN89 were prepared based on the OD at 600 nm and total plate count. Mortality pattern was observed for five days of post-immersion challenge, and the LC50 value was calculated [11].

Growth Study

The feeding trial consisted of a control group, with no test prod-

uct and a treatment group, supplemented with Aquavibra™ at recommended dose (0.5% inclusion in the feed). Commercial crumble feed was used to prepare experimental diets with the aid of binders. Five grams of Aquavibra™ was added to 140 ml of 0.5% carboxymethylcellulose for the preparation of a binder solution. Subsequently, the binder solution was added into a mixer containing 1 kg of feed and mixed for 5 min at 100 rpm [12]. After completion of mixing, the prepared feed was dried in an oven at 50°C for 15 min and stored in sealed plastic bags under refrigeration at 4°C.

The shrimp were transferred from SPF stock to glass tanks (150 L capacity) equipped with biological filter and aerated brackish water in a closed recirculating culture system. After acclimatization, a total of 30 shrimp were stocked in each tank. All the experimental groups were in triplicates, and shrimp were fed at 7% of their body weight in all the groups. The feeding times were maintained at 6:00 a.m., 10:00 a.m., 2:00 p.m. and 6:00 p.m. with commercial crumble feed [13-15]. The body weight and feed intake were measured, and Feed Conversion Ratio (FCR) was calculated for 42 days.

Experimental Design for the Immersion Challenge

The trial comprised of four groups, including an uninfected control, an infected control, positive control, and Aquavibra™ supplemented group as treatment. The first three groups had shrimp from the control group in the growth study Bactrim® (Trimethoprim/sulfamethoxazole, Roche) was used in the positive control group at a dose of 30 mg/kg of feed. The antibiotic was supplemented through the feed for five days before the challenge. For the preparation of antibiotic feed, 480 mg of Bactrim® was dissolved in 40 ml of DMSO. From this stock solution, 2.5 ml was mixed in 140 ml of 0.5% carboxymethylcellulose for the preparation of a binder solution [Lightner DV, and subsequently, the binder solution was mixed with the feed and stored in sealed plastic bags at 4°C. The shrimp were transferred from the tanks for growth study to the challenge tanks, each with a capacity of 20 L, in duplicates [16]. In each tank, 15 shrimp were stocked and acclimatized for 40 h. After completion of the acclimatization period, shrimp were immersed at the LC50 dose (10^5 CFU/ml) of *V. parahaemolyticus* XN89 suspension, while sterilized seawater replaced the bacterial suspension in the uninfected group. The mortality rate of shrimp was recorded every 3 h up to 120 h of post-immersion. The immersion challenge study was repeated with the same groups of shrimp to confirm the results; however, after a shorter duration of supplementation. The shrimp were fed with respective supplement feed for seven days and then they were transferred to challenge tanks each of 20 L capacity, at a stocking density of 15 shrimp per tank and three replicates per group. The shrimp mortality rate was recorded at every 3 h up to 96 h post-immersion. The moribund and survived shrimp were used for histopathology analysis at 96 h post-immersion challenge [17-20].

Statistical Analysis

Data were analysed by Analysis of Variance (ANOVA) using Graph Pad Prism version 6.01. Statements of statistical significance are declared when P<0.05. The lethal dose (LC50) was determined by probit analysis at 72 h post-immersion [21-25].

RESULTS

MBC and MBIC

The MBC and MBIC values of Aquavibra™ are given in Table 1. MBC of 3.12 mg/ml-1.56 mg/ml was observed against *V. parahaemolyticus* XN89 at the concentration of 1 × 104.5. MBIC concentration of 2 mg/ml and biofilm reduction of 85% observed for Aquavibra™ was comparable to those of the antibiotic group, which were 1 mg/ml and 88%, respectively **(Table 1)**.

Table 1: The *in vitro* MBC and MBIC of tested compounds. Note: MBC=Minimum Bactericidal Concentration; MBIC=Minimum Biofilm Inhibitory Concentration

	MBC (mg/ml)	MBIC (mg/ml)	Reduction of biofilm % (at respective MBIC)
Aquavibra™	3.12-1.56	2	85
Chloramphenicol	0.003-0.002	1	88

LC 50 of *V. parahaemolyticus* XN89

The mortality of shrimps began 6 h post-immersion, and the higher dose of *V. parahaemolyticus* XN89 (1×10^6 CFU/ml) resulted in 100% mortality at 48 h [26,27]. The mortality gradually increased in mid dosage groups, and the LC50 value for the experimental animals against the *V. parahaemolyticus* XN89 isolate was determined to be 1×10^5 CFU/ ml based on probit analysis **(Figure 1)**.

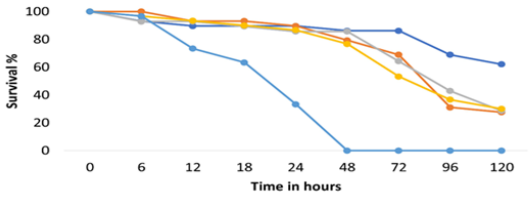

Figure 1: Survival of *P. vannamei* (n=30) exposed to five concentrations of *V. parahaemolyticus* XN89 in the LC50 study. Note: (—●—) 1.00E+02; (—●—) 1.00E+03; (—●—) 1.00E+04; (—●—) 1.00E+05; (—●—) 1.00E+06.

Growth Study

Feeding the juvenile shrimp with Aquavibra™ at 0.5% level had no significant (P>0.05) influence on their growth and FCR compared to the control group. The total body weight gain of the Aquavibra™ treatment and control in 42 days of the trial were 7.42 g ± 0.99 g and 7.39 g ± 1.58 g, while the FCR obtained were 0.87 and 0.96, respectively. However, the survival of the experimental shrimp was low, both in control (43%), and Aquavibra™ treatment (44%), possibly due to the cannibalism particularly associated with shrimp moulting since no substrates were used in the tanks **(Figure 2)**.

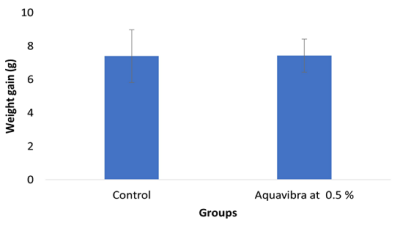

Figure 2: Total body weight gain of experimental *P. vannamei* after 42 days feeding period. Data represented as mean ± SD; n=3 (30 shrimp in each replicate): (P >0.05)

Experimental challenge with *V. parahaemolyticus* XN89: The experimental challenge resulted in 83% mortality in the infected group at 120 h post-challenge. Histological examination of moribund shrimp from infected control showed severe collapsed hepatopancreatic tubule epithelia, one of the characteristics of AHPND (Figure 3-B). The uninfected group immersed with sterile seawater showed normal hepatopancreatic tissues with normal tubule epithelia. The supplementation of Aquavibra™ at 0.5% inclusion, resulted in a better survival rate (60%) than the infected control (16.67%), and was comparable to that of the positive control group (61.54%). The repetition of the same experiment was performed, and a similar trend was observed [28-30]. A significantly higher ($P<0.05$) survival rate of 93% was observed in the Aquavibra™ supplemented group after 96 h, post-challenge while 100% survival was observed in the positive control group. The histology of moribund shrimp from both groups revealed a mix of normal and thin collapsed hepatopancreatic epithelia **(Figures 3 to 5)**.

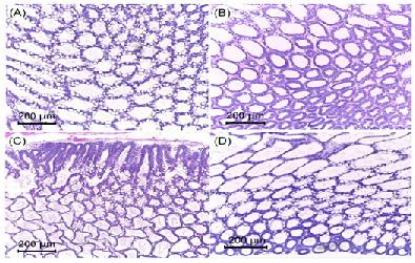

Figure 3: Photomicrographs of the shrimp hepatopancreas tissue-uninfected control a) infected control; b) antibiotic control; c) and Aquavibra™ at 0.5%; d) after 96 h post-challenge

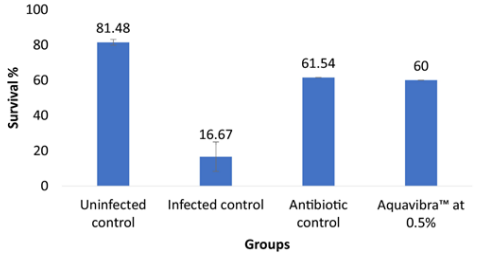

Figure 4: Survival rate (%) after the bacterial challenge of shrimp using *V. parahaemolyticus* XN89 isolate for 120 h at 10^5 CFU/ ml. (All values are represented as means where n=30)

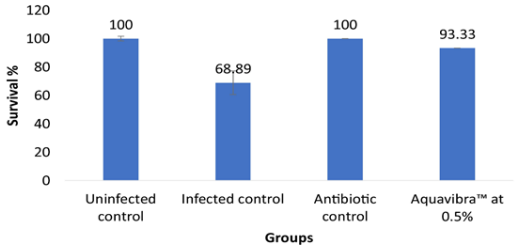

Figure 5: SSurvival rate (%) after the bacterial challenge of shrimp using *V. parahaemolyticus* XN89 isolate for 96 h at 10^5 CFU/ml. All values are represented as means where n=3 (15 shrimp in each replicate) ($P<0.05$)

DISCUSSION

Vibrios are opportunistic pathogens occupying the natural habitats of marine environments and aquaculture systems of shrimp. Infection by Vibrio, causing Acute Hepatopancreatic Necrosis Disease (AHPND) had a devastating impact on shrimp aquaculture worldwide. The initial steps of disease pathogenesis involve adsorption of pathogen and biofilm formation. Several Vibrio spp. are known to secrete the chitinase enzyme to degrade the chitin in the exoskeleton. Subsequently, the pathogen colonizes heavily in the chitin lining, including stomach resulting in biofilm formation and release of toxins. Toxins cause necrosis in the hepatopancreas leading to secondary infections and mortality [31]. Biofilm formation is highly related to bacterial intercellular communication mechanism called Quorum Sensing (QS), which plays an essential role in pathogenicity, drug resistance and gene regulations. In general antibiotics, chemotherapeutics and disinfectants are used in aquatic systems to control and treat Vibrio infections. However, the prevalence of antibiotic resistance among the bacterial strains is a significant drawback of an extensive application of antibiotics against Vibrio infections. This is evident by the resistance reported in the case of several antibiotics such as ampicillin, ciprofloxacin, cephazolin, streptomycin, cefotaxime, and cefuroxime sodium against specific isolates of *V. parahaemolyticus*. Thus, alternate strategies are needed for treatment or reduction of bacterial biofilms [32].

Antimicrobial activity of organic acids or their salts and essential oils are reported against several pathogens, including Vibrio species. In general, organic acids in the dissociated form can penetrate freely into the bacterial cell membranes and dissociate within the cytoplasm into anions and H+. A reduction in the intracellular pH and disruptions in the cytoplasmic membrane, metabolic enzymes, genetic materials, and protein synthesis, are the other impacts of organic acids. Besides, the bacterial cell consumes ATP to transport a net surplus of H+ out of cells leading to the depletion of ATP levels thus losing the ability to maintain pH homeostasis [33]. The *in vitro* anti-Vibrio activity of Aquavibra™ against *V. parahaemolyticus* XN89 revealed in the present study indicates the potential effective inhibition of pathogen by a relatively low concentration of Aquavibra™ at <3.12 mg/ml.

Essential oils are reported to inhibit ATPase and the enzymes involved in cytokine interactions and to disrupt the cell membrane. Extracts of several plants and their active compounds were found to effectively prevent the development of bacterial biofilm and the planktonic cells. Essential oils have emerging microbial diseases-controlling potential due to the broad spectrum of antimicrobial substances such as terpenoids and phenylpropanoids. In this study, a specific combination of organic acids and essential oils (Aquavibra™) was found to effectively inhibit the biofilm formation by *V. parahaemolyticus* XN89 with comparable efficacy with the antibiotic group. Similar results were reported in a phenolic monoterpenoid, carvacrol which showed a reduction in biofilm and the QS related gene expression of different pathogens [34].

The histology of infected shrimp followed by immersion challenge revealed massive collapsed hepatopancreatic tubule epithelia and a cumulative mortality rate of 83% by 120 h post-challenge, indicating active Vibrio infection. In general, the pathognomonic lesions of AHPND featured massive sloughing of tubule epithelial cells of the hepatopancreas of infected

shrimp. However, low bacterial challenge doses or low level of the binary toxins PirVpAB caused collapsed hepatopancreatic epithelia as observed in the present study [35-40]. The histopathology of the Aquavibra™ supplemented group revealed a mix of normal and thin collapsed hepatopancreatic epithelia, suggesting lower colonization of pathogen and consequently fewer toxins released in hepatopancreas. Also, the survival rate of the Aquavibra™ supplemented group was comparable to the antibiotic group. The results are in aligned with previous studies on photobiotic compounds against AHPND infections [41,42]. Phytochemical compounds exhibit potent antimicrobial activity against several pathogens, including Vibrio species. Dietary supplementation of a blend of natural herbs showed significant improvement in survival of shrimp against *V. parahaemolyticus* infection. Recent studies showed that Vibrio infection affects trace element homeostasis, antioxidant function and induces inflammation response in *P. vannamei*. The effectiveness of Aquavibra™ observed in the current study might also involve the antioxidant and anti-inflammatory activity of essential oils which could be further investigated.

CONCLUSION

In conclusion, the results of this study demonstrated the effectiveness of a specific combination of organic acids and essential oil (Aquavibra™) to improve survival during infection with pathogenic VPAHPND. The application of antibiotics is restricted in shrimp aquaculture as it affects the sustainability of the production and marketing process. The organic acids and essential oil blends are promising and economical alternatives to replace antibiotics. The recommended inclusion level of Aquavibra™ is 0.5% in shrimp feed. Further studies are required to evaluate Aquavibra™ for its potential to enhance both the immunity and the gut microbiome in white shrimp.

DECLARATIONS

Funding

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Conflicts of Interest

This study was funded by a research grant from Kemin Industries, South Asia to Asian Institute of Technology, aimed to test the efficacy of a product to treat bacterial infection in white shrimp. However, this funding posed no constraint in adhering to research ethics and following the Journal guidelines in the preparation of this manuscript.

Ethics Approval

The authors followed all the applicable international, national, and/or institutional guidelines for the care and use of animals in this study.

Consent to Participate

All the authors consented to participate in this research and publication of the manuscript.

Consent for Publication

All the authors consent to publish this manuscript in the Journal, Aquaculture International.

Availability of Data and Material

The supporting data are available and can be provided based on a reasonable request.

Code Availability

Not applicable

Author Contribution

Krishna R. Salin, Rajalekshmi Mukkalil, and Harikumar Sampath conceptualized this study; Amara Yakupitiyage., Saengchan Senapin, Ha Thanh Dong, and Krishna R. Salin were involved in the research design and data analysis; Harikumar Sampath wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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