SHORT COMMUNICATION

Protective effect of medicinal plants and *Bacillus licheniformis* BCR 4-3 on white shrimp (*Litopenaeus vannamei*) challenged with *Vibrio parahaemolyticus*

Efecto protector de plantas medicinales y Bacillus licheniformis BCR 4-3 en el camarón blanco (Litopenaeus vannamei) retado con Vibrio parahaemolyticus

Efeito protetor de plantas medicinais e Bacillus licheniformis BCR 4-3 em camarão branco (Litopenaeus vannamei) desafiado com Vibrio parahaemolyticus

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Abstract

Background: Up to 100% mortality can occur in shrimp farming due to viral and bacterial diseases. Prophylactic methods to decrease mortality, such as natural additives that do not generate bacterial resistance and environmental problems, are currently under research. **Objective:** To evaluate the effect of medicinal plants (aloe, basil, ginger, and garlic) and *Bacillus licheniformis* BCR 4-3 added to the water on the survival of *Litopenaeus vannamei* shrimp challenged with *Vibrio parahaemolyticus* IPNGS16. **Methods:** Two bioassays assessed a mixture of the four powdered plants (4 g/kg of feed) every two days, and the bacillus (1×10^6, 2×10^6 and 3×10^6 CFU/L) added to the water was evaluated every three and seven days. Before each bioassay, the mean lethal concentration of *Vibrio* was determined. **Results:** The mixture of medicinal plants in the feed and *B. licheniformis* BCR 4-3 in the water increased up to 70% the survival rate of *Litopenaeus vannamei* challenged with *V. parahaemolyticus* IPNGS16, representing a potential tool for preventing acute hepatopancreatic necrosis disease (AHPND) infections in commercial shrimp farms.

Keywords: *acute hepatopancreatic necrosis disease; aloe; basil; Bacillus licheniformis; garlic; ginger; Litopenaeus vannamei; shrimp; Vibrio parahaemolyticus*.

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Resumen

Antecedentes: Las enfermedades virales y bacterianas ocasionan pérdidas por mortalidad de hasta un 100% en camaronicultura. Actualmente se investiga el uso de métodos profilácticos tales como aditivos naturales que no generen resistencia bacteriana ni problemas ambientales. **Objetivo:** Evaluar el efecto de plantas medicinales (sábila, albahaca, jengibre y ajo) y *Bacillus licheniformis* BCR 4-3, adicionado en el agua, sobre la supervivencia del camaron *Litopenaeus vannamei* desafiado con *Vibrio parahaemolyticus* IPNGS16. **Métodos:** En dos bioensayos se evaluó cada dos días una mezcla de las cuatro plantas en polvo (4 g/kg de alimento) y el bacilo se adicionó al agua (1×10^6, 2×10^6 y 3×10^6 UFC/L) y se evaluó cada tres y siete días. Antes de cada bioensayo, se determinó la concentración letal media de *Vibrio*. **Resultados:** La mezcla de plantas medicinales en el alimento y *B. licheniformis* BCR 4-3 en el agua mejoró hasta un 70% la tasa de supervivencia de *Litopenaeus vannamei* retado con *V. parahaemolyticus* IPNGS16, representando una herramienta viable para prevenir infecciones por necrosis hepatopancreática aguda (AHPND) en granjas camaroneras comerciales.

Palabras clave: *ajo; albahaca; Bacillus licheniformis; camarón; enfermedad de necrosis hepatopancreática aguda; jengibre; Litopenaeus vannamei; sábila; Vibrio parahaemolyticus.*

Resumo

Antecedentes: Na carcinicultura, doenças virais e bacterianas causam perdas por mortalidades de até 100% da produção. Atualmente está sendo pesquisado o uso de métodos profiláticos como aditivos naturais que não gerem resistência bacteriana e problemas ambientais. **Objetivo:** Este estudo avaliou o efeito de plantas medicinais (babosa, manjericão, gengibre e alho) e *Bacillus licheniformis* BCR 4-3, adicionado na água, na sobrevivência do camarão *Litopenaeus vannamei* desafiado com *Vibrio parahaemolyticus* IPNGS16. **Métodos:** Em dois bioensaios, uma mistura das quatro plantas em pó (4 g/kg de ração) foi avaliada a cada 2 dias, e o bacilo (1×10^6, 2×10^6 e 3×10^6 UFC/L) adicionado à água foi avaliada a cada 3 e 7 dias. Antes de cada bioensaio, foi determinada a concentração letal média de *Vibrio*. **Resultados:** A mistura de plantas medicinais na ração e *B. licheniformis* BCR 4-3 na água melhorou a taxa de sobrevivência de até 70% de *Litopenaeus vannamei* desafiado com *V. parahaemolyticus* IPNGS16 adicionado na água, tornando esta mistura um tratamento viável para a prevenção de infecções agudas da doença da necrose hepatopancreática (AHPND) em fazendas comerciais de camarão.

Palavras-chave: *alho; babosa; Bacillus licheniformis; camarão; doença necrose hepatopancreática aguda; jengibre; Litopenaeus vannamei; manjericão; Vibrio parahaemolyticus.*

Introduction

The Pacific white shrimp (*Penaeus vannamei*) is one of the most economically important shrimp species. According to the Food and Agriculture Organization (FAO), 5.8 million tons were produced in 2022, contributing to 51.7% of the global crustacean production (FAO, 2022). However, the intensification of aquaculture has promoted conditions favoring the spread of infectious diseases by diverse pathogens, including viruses, bacteria, fungi, and parasites (Doyle, 2016).

The early mortality syndrome, also known as acute hepatopancreatic necrosis disease (AHPND), was first reported in China in 2009 and subsequently spread throughout the world causing economic losses of more than one billion USD (Zorriehzahra and Banaederakhshan, 2015; Shinn *et al.*, 2018). AHPND is caused by a bacterial agent of the *Vibrio parahaemolyticus* species that is transmitted orally and colonizes the gastrointestinal tract of shrimp producing a binary toxin that induces tissue destruction and hepatopancreatic dysfunction. Signs and symptoms of infection include lethargy, empty stomach and intestine, a pale or whitish appearance of the hepatopancreas, and shedding of epithelial cells from the hepatopancreas tubules into the tubule lumen (Zorriehzahra and Banaederakhshan, 2015; Lin *et al.*, 2017).

The use of natural additives in feed or water to reduce diseases in cultivated shrimp has consistently increased over the last decade. These additives include plants, beta glucans, prebiotics, and probiotics (Mudagandur and Yuanan, 2009; Huynh *et al.*, 2011; Medina-Beltrán *et al.*, 2012). Diverse plant species containing bioactive compounds that modulate inflammatory and immunological processes *in vivo* and *in vitro* have been tested (Yin *et al.*, 2016; Cardoso *et al.*, 2017; Ho *et al.*, 2017). Other organisms, such as yeasts and bacteria, have been used as probiotics to improve water quality, increase the immune response, and provide nutrients, secondary metabolites, and enzymes to shrimp cultures (Escamilla-Montes *et al.*, 2015; Alamillo, *et al.*, 2017; Gao *et al.*, 2017; Luna-González *et al.*, 2017). This study aimed to determine the effect of medicinal plants (garlic, aloe, ginger, and basil) and *Bacillus licheniformis* BCR 4-3 (Escamilla-Montes *et al.*, 2015) on survival of *Litopenaeus vannamei* infected with *V. parahaemolyticus* IPNGS16.

Materials and Methods

The study complied with the Mexican Official Standard NOM-062-ZOO-1999 technical specifications for the production, care, and use of laboratory animals.

Shrimp were obtained from Acuícola Cuate Machado, a shrimp farm located in Guasave, Sinaloa, Mexico. The larval stock was delivered to the farm certifying that they were free of WSSV, IHHNV, and *V. parahamenolyticus*. Shrimp were acclimated in 1000-L plastic tanks with seawater at 30 PSU and constant aeration at the aquaculture laboratory of Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional.

The diet consisted of a powdered plant mixture (PPM) containing *Allium sativum* bulbs (garlic, 1.5 g/kg of feed), *Aloe vera* leaves (aloe 0.5 g/kg of feed), *Ocimum sanctum* leaves and stems (basil, 1.0 g/kg of feed), and *Zingiber officinale* underground stems (ginger, 1.0 g/kg of feed). The diet was prepared with a commercial feed (35% protein, Purina®, MX) and pulverized in a coffee grinder. Distilled water (410 ml/kg of feed) was added to form a paste with the feed, the plant mixture, and gelatin (40 g/kg feed). The diet was pelleted in a meat mill. The additive of interest was replaced with cellulose in the control treatment.

The probiotic used was *B. licheniformis* BCR 4-3 grown in trypticase soy broth (TSB) medium at 32 °C for 48 h. Subsequently, the medium was centrifuged at 3900 *g* for 20 min, the supernatant was decanted, and the bacterial pellet was washed twice with 50 ml saline solution (2.5% NaCl), centrifuging at 3,900 g×20 min in each wash. The obtained pellet was resuspended in saline solution before

being inoculated in the water of the experimental cultures, according to Escamilla-Montes *et al.* (2015). The pathogenic *V. parahaemolyticus* IPNGS16 strain was grown in a TSB medium with 2.5% NaCl at 30 °C for 18 h. The pathogenic*V. parahaemolyticus* IPNGS16 strain was grown in TSB medium containing 2.5% NaCl at 30 °C for 18 h. The bacterial culture was centrifuged at 3,900 g×20 min and the pellet was resuspended in 2.5% NaCl saline. The bacterial culture was centrifuged at 3,900 g×20 min and the pellet was resuspended in 2.5% NaCl saline. The bacterial suspension was brought to an Optical Density of 1.0 (595 nm) in a Thermo Spectronic Genesys 2® spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA). Shrimp were infected with the *Vibrio* strain, inoculating the bacteria in water at 10, 100, 1,000, 1,000, 10,000, and 100,000 CFU/ml in a parallel bioassay where mortalities were recorded for 92 h, obtaining the mean lethal concentrations (LC_{50}) (López-León *et al.*, 2016) 1.8×10^5 CFU/ml (Bioassay 1) and 1.05×10^5 CFU/ml (Bioassay 2) by probit analysis (Finney *et al.*, 1952) using package PASW Statistics 18.

Bioassay 1

In the first bioassay, shrimp post-larvae (12.5 ± 2.5 mg) were cultured for 34 d. Before the experiments, a shrimp batch from the stock was analyzed by PCR to rule out the presence of *V. parahaemolyticus* IPNGS16, which causes AHPND. The experiment was carried out in glass tanks (5 L) with 4 L of filtered seawater (20 µm) at 30 PSU and constant aeration. Shrimp were fed twice a day (09:00 and 17:00 h) with commercial feed (30% protein, Purina®, MX) adjusting the feed amount according to shrimp biomass. Ten post-larvae were placed per tank. The bioassay consisted of five treatments in triplicate as follows: I) negative control, commercial feed (CF); II) positive control, CF + *V. parahaemolyticus* IPNGS16 (LC_{50}); III) CF + *B. licheniformis* BCR 4-3 (1×10^6 CFU/L) in water each 7 d + *V. parahaemolyticus* IPNGS16 (LC_{50}); IV) CF with PPM (4 g/kg of feed) each 2 d + *V. parahaemolyticus* IPNGS16 (LC_{50}); V) *B. licheniformis* BCR 4-3 (1×10^6 CFU/L) in water each 7 d + CF with PPM (4 g/kg of feed) each 2 d + *V. parahaemolyticus* IPNGS16 (LC_{50}). The calculated LC_{50} was 1.8×10^5 CFU/ml. Dissolved oxygen, pH, and salinity were determined daily. Tanks were siphoned every four days, and 50% of the water was replaced until day 31. On day 31, shrimp were exposed to *V. parahaemolyticus* IPNGS16 strain added to the water (1.8×10^5 CFU/ml). During the *Vibrio* challenge, water was neither cleaned nor replaced. Dead organisms were quantified and survival was determined every 6 h for three days.

Bioassay 2

In the second bioassay, shrimp post-larvae (17.5 ± 2.5 mg) were cultured for 34 d. The experiment was carried out in glass tanks (5 L) with 4 L of filtered seawater (20 µm) at 30 PSU and constant aeration. Shrimp were fed at 09:00 and 17:00 h with CF (30% protein, Purina®, MX). Twelve shrimp were placed per tank. Four treatments in triplicate were used: I) negative control, commercial feed (CF); II) positive control, CF + *V. parahaemolyticus* IPNGS16 (LC_{50}); III) CF with PPM (4 g/kg of feed) each 2 d + *B. licheniformis* (2×10^6 CFU/L) in water each 3 d + *V. parahaemolyticus* IPNGS16 (LC_{50}); IV) AC + PPM (4 g/kg of feed) each 2 d + *B. licheniformis* (3×10^6 CFU/L) in water each 3 d + *V. parahaemolyticus* IPNGS16 (LC_{50}). Dissolved oxygen, pH, and salinity were recorded daily. Tanks were siphoned every four days and 50% water was replaced until day 31. On day 31, shrimp were exposed to 1.05×10^5 CFU/ml *V. parahaemolyticus* IPNGS16 strain added to the water. Water was neither cleaned nor replaced during the *Vibrio* challenge. Dead organisms were quantified, and survival was determined every 6 h for three days. Survival at the end of the study was calculated as follows: Survival (%) = (N_f/N_i) * 100. Where N_i= Initial number of shrimps, and N_i= Final number of shrimp.

Physicochemical parameters (mean ± standard deviation) were maintained within the optimal range according to Brock and Main (1994). In bioassay one, salinity was between 29.7 ± 2.3 and 30 ± 3.0 PSU, temperature between 26.8 ± 5.4 and 27.3 ± 6.1 °C, oxygen between 5.1 ± 0.1 and 5.3 ± 0.1 mg/L, and pH between 8.4 ± 0.2 and 8.7 ± 0.2. In the second bioassay, salinity was between 30 ±

3.0 and 31 ± 2.0 PSU, temperature between 26.5 ± 0.5 and 27.5 ± 0.5 °C, oxygen between 5.0 ± 0.1 and 5.2 ± 0.2 mg/L, and pH between 8.2 ± 0.6 and 8.6 ± 0.3.

Data analysis

Percent survival data were arcsine transformed and then analyzed by one-way ANOVA (Statistica, version 7.0). If significant differences were found among negative control and treatments, Tukey's honestly significant difference (HSD) test was used to identify the source differences ($p < 0.05$) (Orellana-Suarez and Cañarte-Vélez, 2020).

Results

Survival in Bioassay 1

Final survival was 100% in the negative control, 30 ± 15.27% in the positive control, 80 ± 10% in treatment III, 96.67 ± 3.33% in treatment IV, and 100% in treatment V. Survival in the negative control and treatments III, IV, and V were significantly higher than positive control II with the *Vibrio* strain ($p<0.05$; Figure 1).

Survival in Bioassay 2

Shrimp survival in treatments III and IV (71.51 ± 10.26 and 100%, respectively) was significantly different from the positive control II (34.09 ± 4.17%; $p<0.05$). Survival in treatment IV (100%) was significantly higher compared to treatment III (71.51 ± 10.26%; $p<0.05$). In addition, a significantly higher survival ($p<0.05$) was observed in the negative control (100%) as compared to the positive control II (34.09 ± 4.17%; Figure 2).

Discussion

Significantly higher survival was recorded in the treatments with plants and bacilli when shrimp were challenged with *V. parahaemolyticus* IPNGS16. Plants such as aloe, ginger, garlic, and basil have been tested to prevent bacterial

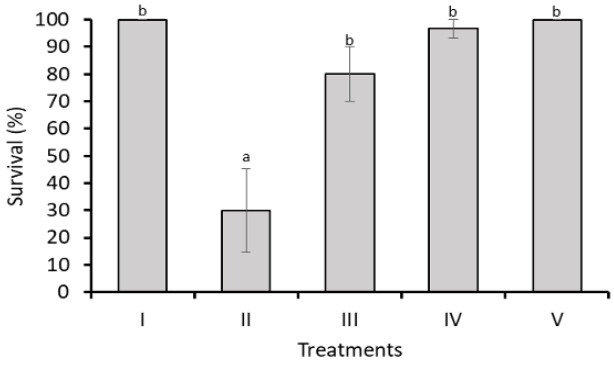

Figure 1. Survival of *Litopenaeus vannamei* treated with plants and *B. licheniformis* BCR 4-3 and challenged with *V. parahaemolyticus* IPNGS16. Treatments: I) negative control, commercial feed (CF); II) positive control, CF + *V. parahaemolyticus* IPNGS16 (LC_{50}); III) CF + *B. licheniformis* BCR 4-3 (1×10^6 CFU/L) in water each 7 d + *V. parahaemolyticus* IPNGS16 (LC_{50}); IV) CF with PPM (4 g/kg of feed) each 2 d + *V. parahaemolyticus* IPNGS16 (LC_{50}); V) *B. licheniformis* BCR 4-3 (1×10^6 CFU/L) in water each 7 d + CF with PPM (4 g/kg of feed) each 2 d + *V. parahaemolyticus* IPNGS16 (LC_{50}). Means ± standard errors are indicated. Different letters indicate significant differences.

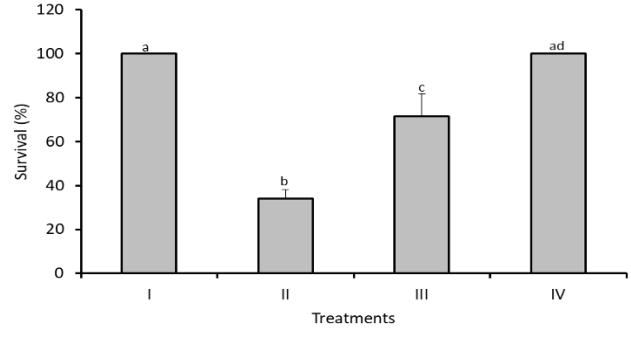

Figure 2. Survival of L. vannamei treated with plants and *B. licheniformis* BCR 4-3 and challenged with *V. parahaemolyticus* IPNGS16. Treatments: I) negative control, commercial feed (CF); II) positive control, CF + *V. parahaemolyticus* IPNGS16 (LC_{50}); III) CF with PPM (4 g/kg of feed) each 2 d + *B. licheniformis* (2×10^6 CFU/L) in water each 3 d + *Vibrio* (LC_{50}); IV) AC + PPM (4 g/kg of feed) each 2 d + *B. licheniformis* (3×10^6 CFU/L) in water each 3 d + *Vibrio* (LC_{50}). Means ± standard errors are indicated. Different letters indicate significant differences.

and viral diseases in white shrimp culture (Chang *et al.*, 2012; Nurtjahyani and Hadra, 2016; Abdel-Tawwab *et al.*, 2021; Amoah *et al.*, 2021). Plants are a source of secondary metabolites (Guerriero *et al.*, 2018) of interest for aquaculture to prevent and treat diseases and it is known that combinations of medicinal plants can have better effect than individual plants since bioactive compounds can act in synergy (Más-Toro *et al.*, 2017). It is worth mentioning that plants -in addition to antimicrobial and immunostimulant molecules- contain molecules, such as phytic acid and tannins, that can affect cultured organisms (Bairagi *et al.*, 2002; El-Adawy, 2002; Flores-Miranda *et al.*, 2014); thus, the amount and frequency of application in feed should be determined.

These substances play a role in modulating inflammatory (inflammatory inhibitors) or immunological (cytokines and IgE production) processes *in vivo* and *in vitro* (Cardoso *et al.*, 2017). These substances also participate in the first line of defense against infections and affect the innate and adaptive immune system (Cardoso *et al.*, 2017). The concentration of bioactive compounds depends on specific factors of each plant such as climatic conditions, soil characteristics, plant age, harvesting season, and extraction method (Orzechowski *et al.*, 2005; Çirak *et al.*, 2007).

The frequency and dose of feed additive supplementation determine their protective effect. Sajevan *et al.* (2009) mention that overdose and continuous application cause fatigue in the immune system. Regarding the high survival rates of treatments with bacilli in the water added at different concentrations and frequencies, the bacterium *B. licheniformis* BCR-4-3 shows a great ability to adhere to abiotic surfaces (Escamilla-Montes *et al.*, 2015). Although it does not show antagonistic activity against *V. parahaemolyticus* (Escamilla-Montes *et al.*, 2015), it shows competitive exclusion due to production of hydrogen peroxide or adhesion inhibitors as well as nutrient competition (Bjorn *et al.*, 2003; Farzanfar, 2006). García-Medel *et al.* (2020) found significantly higher survival in shrimp fed the same *Bacillus* strain and challenged with *V. parahaemolyticus* IPNGS16. Previous reports found resistance of shrimp fed strains of *Bacillus subtilis* (L10 and G1) against *Vibrio* strains (Zokaeifar *et al.*, 2012; Liu *et al.*, 2014). Furthermore, *Bacillus* sp. added for 30 days to the feed of other shrimp species, such as *Penaeus monodon*, reduced infection caused by *V. parahaemolyticus* (Sekar *et al.*, 2016). In contrast, Vidal *et al.* (2018) found no significant difference in survival of L. vannamei post-larvae fed with *Bacillus cereus* (1.0×10^8 CFU/g) and challenged with *V. alginolyticus* and *V. parahaemolyticus*.

In conclusion, a mixture of medicinal plants in the feed and *B. licheniformis* BCR 4-3 in the water improved up to 70% survival of L. vannamei challenged with *V. parahaemolyticus* IPNGS16 added to the water, representing a potential tool for preventing acute hepatopancreatic necrosis disease (AHPND) infections in commercial shrimp farms.

Declarations

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Conflicts of interest

The authors declare they have no conflict of interest regarding financial support or personal relationships.

Author contributions

Karime A. Valdez-Chávez: shrimp culture, feeding trial, data analysis, manuscript drafting. Ruth Escamilla-Montes: microbiology, manuscript drafting. Antonio Luna-González: experiment design, manuscript revision. Jesús A. Fierro-Coronado: data analysis, manuscript revision. Héctor A. González-Ocampo: manuscript drafting. Cesar Orozco-Medina: manuscript revision.

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