DOI: https://doi.org/10.52973/rcfcv-e32148 Revista Científica, FCV-LUZ / Vol. XXXII, rcfcv-e32148, 1 - 9

Efficacy of BACTI-NIL®AQUA in Experimental infection with *Vibrio parahaemolyticus* in juvenile of *Litopenaeus vannamei*

Eficacia de BACTI-NIL®AQUA en infección experimental con *Vibrio parahaemolyticus* en juveniles de *Litopenaeus vannam*ei

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ABSTRACT

This work aims to evaluate looks at the antibacterial efficacy of BACTI-NIL®AQUA when added to the feed of *Penaeus vannamei* in experimental infection with *Vibrio parahaemolyticus* (M0904AHPND+strain). Results show that adition of BACTI-NIL®AQUA at 3,000 and 5,000 part per million (ppm), causes inhibition growth zones of 15.00 ± 0.50 milimeters (mm) and 17.00 ± 0.30 mm, respectively. The challenge with *V. parahaemolyticus* resulted in 60% survival for organisms fed doses 3,000 ppm of BACTI-NIL®AQUA and 60% for those fed 5,000 ppm of BACTI-NIL®AQUA, resulting in twice the amount of survival as opposed to 13.33% (4 organism) in the positive control at 24 hours post-infection. Histopathological alterations in the hepatopancreas with hemocytic infiltration within the intertubular connective tissue were observed. Also, tubules with severe cell detachment and tubular atrophy were detected in the positive control organisms, and organisms treated with of BACTI-NIL®AQUA only had vermiform structures in the tubular lumen, cell detachment and infiltration of hemolymph in intertubular connective tissue. According to the analysis of the studied variables, it can be concluded that of BACTI-NIL®AQUA is a promising alternative for *V. parahaemolyticus* control in shrimp culture.

Key words: BACTI-NIL®AQUA; *Vibrio parahaemolyticus*; *Penaeus vannamei*; acute hepatopancreatic necrosis disease; early mortality syndrome

RESUMEN

El objetivo de esta investigación fue evaluar la eficacia de dos dosis de BACTI-NIL®AQUA adicionada al alimento en una infección experimental de 24 horas (h) en juveniles de *Penaeus vannamei*. Los resultados de este estudio mostraron que BACTI-NIL®AQUA, a concentraciones de 3.000 y 5.000 partes por millón (ppm) inhibe el crecimiento de *Vibrio parahaemolyticus* (cepa M0904AHPND+), causante de la enfermedad de necrosis hepatopancreática aguda en camarones. En estas concentraciones, se observaron zonas de crecimiento de 15,00 ± 0,50 milímetros (mm) para 3.000 ppm y 17,00 ± 0,30 mm para 5.000 ppm, respectivamente. Se obtuvo una sobrevivencia del 60% para ambas dosis, el doble de sobrevivencia que el control positivo 13,33% (4 organismos) a las 24 h post-infección. En los organismos control positivo se observaron alteraciones histopatológicas en los túbulos del hepatopáncreas con desprendimiento celular severo e infiltración hemocítica dentro del tejido conectivo intertubular. En los organismos tratados con BACTI-NIL®AQUA solo se observaron estructuras vermiformes en el lúmen de los túbulos del hepatopáncreas. Con los resultados del presente estudio se puede concluir que BACTI-NIL®AQUA adicionado al alimento es una alternativa prometedora para el control de *V. parahaem*olyticus en cultivo de camarón.

Palabras clave: BACTI-NIL®AQUA; *Vibrio parahaemolyticus*; *Penaeus vannamei*; enfermedad de la necrosis hepatopancreática aguda; síndrome de la mortalidad temprana

Received: 11/05/2022 Accepted: 15/06/2022 Published: 31/07/2022

INTRODUCTION

Marine shrimp farming has increased dramatically, from around 100 tons in the 1980s to about 4.5 million tons in 2018; the top five producers being China, Thailand, Vietnam, Indonesia, and Ecuador. The Pacific white shrimp (*Litopenaeus vannamei*) is the main cultivated aquatic species in the world, with a value of USD 18.46 billion in 2018 [10].

Despite this success, shrimp farming continues to suffer important economic losses due to the impact of mainly viral diseases. More recently, more concerns arised when bacterial diseases such as Acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome (EMS) started to showed. This is a bacterial shrimp disease due to the action of Pir A and B toxin secreted by *Vibrio parahaemolyticus* [31] capable of destroying the cells (E, R, F, and B) of the hepatopancreas, causing detachment of the tubular epithelial cells, hemocytic infiltration, and very marked necrosis of the hepatopancreas [36]. In the terminal phase, in addition to the shedding of epithelial cells, a massive secondary bacterial infection occurs [26, 36]. *Vibrio parahaemolyticus, V. campbellii, V. owensii*, and *V. punensis* have been proved to cause AHPND.

However, the mechanisms underlying the burgeoning number of *Vibrio* species that cause AHPND is not complete known. All of AHPND-causing *Vibrio* bacteria (V_{AHPND}) harbor a highly homologous plasmid (designated as pVA1-type) carrying *pirAB* ^{vp} toxin genes [12]. AHPND is characterized by sudden and massive mortalities (100%) in post larvae or juveniles with 30 to 35 days (d) of culture [12, 25].

Antibiotics are used in aquaculture to control the development of bacteria during the production process. However, misuse has led to antibiotic resistance in both humans and animals. At present, the use of these compounds is being restricted by the resistance they can induce in different groups of microorganisms, either by mismanagement of the effective doses or by new mechanisms that allow them to generate resistance [1, 7]. The shrimp industry requires other alternatives to inhibit microbial flora in production systems. Among the sustainable strategies applied to modulate the intestinal microflora of shrimp, wide varieties of natural compounds are used, such as organic acids (OA).

Specific OA alone or in combination are one of the alternatives for nutritionally sustainable and environmentally friendly production. OA are oxygenated compounds derived from hydrocarbons, and have been widely used in formulations for animal nutrition. OA included in balanced food function as preservatives, lowering the pH and reducing microbial growth; although the main application is as an antimicrobial with action in the digestive tract [1, 27].

The main effect of OA in shrimp is due to the fact that undissociated molecules penetrate the cell walls of Gram-negative pathogenic bacteria and acidifies their cytoplasmic pH, for which the bacterial cell needs to neutralize its pH through the proton pump H^+-ATPase, causing excessive energy expenditure. As undissociated OA molecules continue to penetrate the bacterial cell walls, they rapidly deplete their energy reserves, ultimately leading to cell death [27]. However, there are very few studies on the antibacterial efficacy of OA *in vivo* and *in vitro*, in shrimp with experimental infections. The primary antimicrobial action of BACTI-NIL®AQUA (a synergistic blend of OA mixture) is by altering the cell cytoplasm pH of bacteria and those that are sensitive to such changes are inhibited, thus reducing harmful bacteria within the gastrointestinal tract of the host animal.

The aim of the study was to determine the effect of dietary supplementation of BACTI-NIL®AQUA (a synergistic blend of OA mixture) on survival to *V. parahaemolyticus* (M0904AHPND+strain) infection in *L. vannamei* and alterations in hepatopancreas using wet analysis and histopathological analysis.

MATERIALS AND METHODS

Bacterial suspension preparation (inoculum)

A sample of *V. parahaemolyticus* used in this experiment was isolated from shrimp farms affected by AHPND in north-western Mexico and cryopreserved (Panasonic-U53VA-PA. USA) at –80°C [33]. The strain was recovered from cryovials, inoculated in 10 mililiters (mL) of tryptic soy broth (TSB) + 2.0% NaCl (TSB+ Bioxon), and incubated in a rotary shaker (nb-205L N-BIOTEK. México) at 30°C for 24 hours (h). Bacterial cells were washed by centrifugation (Refrigerated centrifuge FELISA-TE-CR12-México) (2330 x Gears (*G*) for 20 minutes (min) at 20°C) and the optical density (OD600 nanomer (-nm-) was adjusted to 1.0 [21]. One hundred microliters (µL) aliquot were inoculated in 40 mL of TSB in triplicate; these samples were incubated in a rotary shaker (VWR-Scientific-1516, USA) at 30 ± 1°C for 24 h, bacterial growth was estimated by total viable count (TVC) on TCBS agar plates (BD Difco).

Minimum inhibitory concentration (MIC) of BACTI-NIL®AQUA (a synergistic blend of organic acids) against *V. parahaemolyticus.*

The MIC was determined in triplicate at concentrations of 500; 1,000; 1,500; 2,000; 3,000; 4,000; 5,000 (provided by the supplier), 8,000; and 9,000 parts per million (ppm) with pH 7.0, 7.5, and 8.0, adding 100 µL of the bacterial inoculum $1x10^8$ Colonies Former Units (CFU)·mL^{-1} and incubated (Shaking incubator-NB205L-Biotek-USA) at 30 ± 1°C for 24 h with constant stirring at a 125 revolution per minutes (rpm). The samples were visually evaluated and those that did not show turbidity were established as MIC [18, 20].

Minimum bactericidal concentration (MBC) of BACTI-NIL®AQUA (a synergistic blend of organic acids) against *V. parahaemolyticus.*

MBC was determined in triplicate with the concentrations that did not produce turbidity (MIC), by adding 100 µL of the bacterial inoculum $1x10^8$ CFU·mL^{-1} in glass tube with tryptic soy broth (Bioxon© TSB, Mexico) and 2.0% NaCl, for 24 h, at 30 ± 1°C. The lowest concentration, in which colonies former units (CFU) did not occur, was considered MBC [20]. A positive (bacterial inoculum $1x10^8$ CFU·mL^{-1}) and a negative control (only tryptic soy broth) were used to ensure adequate bacterial growth during the incubation period and sterility of the mediums [15, 39].

Sensitivity of BACTI-NIL®AQUA against *V. parahaemolyticus*

The bactericidal capacity of the BACTI-NIL®AQUA was determined by Bauer *et al.* [3] with each experiment done in triplicate. A colony was selected and placed in a sterile saline solution until its turbidity matched a Mac Farland standard 0.5 solution [21]. The test was performed as follows:100 µL of bacterial suspension was added at a concentration of $1×10^8$ CFU·mL^{-1} and swabbed on the surface of Mueller-Hinton agar (MHA) plates, supplemented with 2.5% NaCl, pH 8.4. Sterile OXOID discs (OXOID antimicrobial susceptibility test discs) measuring 8 milimeters (mm) diameter were impregnated with three concentrations of with the BACTI-NIL®AQUA (10, 20 and 50 µL) in triplicate including a negative control (only sterile OXOID discs) and incubated at 30 ± 1°C for 24 h. According to the test for bacterial

sensitivity to antibiotics, effectiveness was classified according to the inhibition diameter proposed by Celikel and Kavas [8], and it was achieved.

Food preparation

Commercial feed (Purina 35) was pulverized using a domestic mill. Subsequently, BACTI-NIL®AQUA was added to make a pre-mix and, then, distilled water was added at 40°C (Fisherbrand Termo Fisher Scien – 37200t-China). Pellets were restored using a domestic meat grinder (3 mm in diameter), dried in an oven (LBI, 30CH220V, MEXICO) at 40°C for 12 h, and stored (Samsung, RT45VNSW5, MEXICO) at 4°C, according to Morales-Covarrubias *et al.* [22].

Sensitivity of BACTI-NIL®AQUA in food

The test was performed as follows: 100 µL of bacterial suspension was added at a concentration of 1×10^8 CFU·mL^{-1} and swabbed on the surface of Mueller-Hinton agar (MHA) plates, supplemented with 2.5% NaCl, pH 8.4. The feed pellets with BACTI-NIL®AQUA including a negative control (feed pellets without BACTI-NIL®AQUA) and incubated at 30 ± 1°C for 24 h.

Food consumption and palatability were assessed using the method proposed by Morales-Covarrubias *et al.* [22]. Acrylic aquariums (25x30x28-CIAD-México) with five liters (L) of water each were used in the present bioassay. One organism was placed in each aquarium, using five repetitions with each concentration of 3,000 and 5,000 ppm of BACTI-NIL®AQUA, and control (food without OA).

The organisms were fed the diets, providing 30% of their body weight for five consecutive d. After a period of 4 h, the uneaten food (without feces) was recovered from each aquarium, rinsed with distilled water to remove the salt, and, subsequently, dried in the oven (40°C). They were cooled (LG GR 282SVF-USA) and weighed to estimate food consumption with the following formula:

$$Weight\ of\ food\ consumed = A - B$$

Where A is the total weight of the initial food, and B the weight of recovered food.

Experimental animals

A total of 200 juvenile *L. vannamei* were purchased from local commercial hatchery with a certificate specifying that were not detected of white spot syndrome vírus (WSSV), infectious hypodermal and hematopoietic necrosis vírus (HHNV) and *V. parahamenolyticus*. The organisms were acclimated in Center for Research in Food and Development (CIAD) for 1 week in 600 L tanks with filtered (10 micro mol -µM-) seawater (33 salinity) disinfected by ultraviolet (UV) radiation. Each tank had individual aeration, constant temperature (30 ± 1°C), and a photoperiod of 12 h light: 12 h dark. Shrimp were fed Camaronina™ daily, which contains 35% protein and 9% lipids at 3% total biomass. Shrimp were fed 3 times at d.

Before the assay, 25 shrimp (10% prevalence [16]) were removed from the batch (200 juvenile) to determine their health status by bacteriological analysis, wet mount analysis [16, 24]. Polymerase Chain Reaction (PCR) using commercial kits (IQ2000™ GeneReach Biotechnology Corp., Taiwan) and histological analysis for acute hepatopancreatic necrosis Disease (AHPND), white spot syndrome vírus (WSSV), infectious hypodermal and hematopoietic necrosis vírus (IHHNV) and Necrotizing hepatopancreatitis bacterium (NHPB) [16, 36].

Bioassay (for effectiveness and survival record)

A bioassay was conducted for 24 h in 10 L glass tanks with 10 shrimps (3-4 grammes (gr)), not detected pathogens and intermolt stage) with three replicates per treatment and constant aeration. In total, 2 treatments of the organic acids mix were used: 3,000 ppm; 4,000 ppm and two controls (positive and negative). Before infection, an acclimatizing period of 24 h was allowed. The established control conditions during the test were: 30 ± 1°C, seawater 30% salinity, pH 7.5 – 8.0, ammonium < 0.1 miligrams (mg) L^{-1} and oxygen 6 – 8 mg L^{-1}.

Infection (bacterial inoculum)

Fifty mL of bacterial inoculum concentration of 1×10^8 UFC·mL^{-1} was added directly to an experimental aquarium final concentration water 1×10^6 UFC·mL^{-1} containing 10 shrimps, for all treatments and controls. For the negative controls the same concentration of autoclave heat inactivated bacteria was added (120°C for 15 min by autoclave (FE-405U-México)).

First feeding was administered after 15 min of inoculation and then every 4 h until the end of the experiment (24 h) [22].

To evaluate survival rate, 3 replicates from each treatment were used with 10 shrimps replicate, for a total of 30 shrimps in each treatment and 120 shrimps overall. The survival rate was calculated as the survival probability at any particular time (S_t) [11]. A total of 30 shrimps were used to evaluate the AHPND disease by wet mount and histological analysis. The surviving shrimps were also fixed in Davidson´s solutions at the end of the experiment.

Wet mount analysis

Immediately after the survival challenge test, diagnosis through wet mount analysis was done to asses if the surviving or moribund shrimps had organ and tissue alterations. Their organs and tissue were removed, dissected and squash mounted with sterile seawater then examined under the light microscope Olympus BX 60-USA and photo-documented using an Olympus Infinity 2 camera-USA [16, 23, 34].

Histopathological analysis

Bioassay organisms displaying behaviors such as positionment in aquarium bottom, decubitus and movement of the scaphognathite (moribund) were extracted and fixed with Davidson solution for conventional histological processes [4, 24, 36]. Specimens were paraffin-embedded, cut into 4 µM sections, stained with hematoxylin and eosin, and reviewed under the light microscope to detect AHPND and alterations in hepatopancreas [4, 14, 33, 36].

Lesion severity was graded accordingly to the G-grading system [16] with G0 being negative and G4 as the highest severity of AHPND. Briefly, tissues graded as G0 are without lesions associated with AHPND; G1 are mild focal lesions; G2 and G3 are moderate, locally extensive to multifocal lesions; and G4 are severe, multifocal to diffuse lesions. Slides were observed under Olympus-USA (BX60) microscopy and photo-documented using an Olympus (Infinity 2) camera.

Statistical analysis

Statistical analysis was conducted with R 3.3.1 (R Windows®) software. The experimental infection was analyzed by two-way ANOVA (2x2) ($\alpha<0.05$). Factor A levels were (P) organic acid group, and (C) positive control group. Factor B levels were (V) tested with *V. harveyi*,

and (S) negative control with the same amount, though the bacteria were inactivated by heat. When the analysis of variance indicated the difference between the factors, the Holm-Sidak test was used with a significance level of 0.05 [19, 40].

RESULTS AND DISCUSSION

Determination of the MIC and CMB

The results of observations of the MIC test showed that the BACTI-NIL®AQUA had the activity of inhibiting the growth of *V. parahaemolyticus* (M0904AHPND+strain) bacteria with a minimum concentration of 3,000 ppm (FIG. I).

Results of observations of the MIC test visually in tubes to 3,000 ppm (appear clear), while those that appear cloudy indicate bacterial growth is found in positive controls and tubes with concentrations of 500 ppm to 1,500 ppm. The observations showed control (-) there was no growth of of *V. parahaemolyticus* bacterial colonies and no growth of other bacterial colonies, meaning that there was no contamination during the dilution of BACTI-NIL®AQUA. The observations showed that control (+) there was a growth of bacterial colonies, meaning that the suspension of *V. parahaemolyticus* 1.0 x 10^8 CFU·mL^{-1} that was used for the living conditions was not contaminated by other bacteria, as evidenced by the formation of bacterial colonies by bacterial colonies of *V. parahaemolyticus* for which the MIC values also corresponded to MBC.

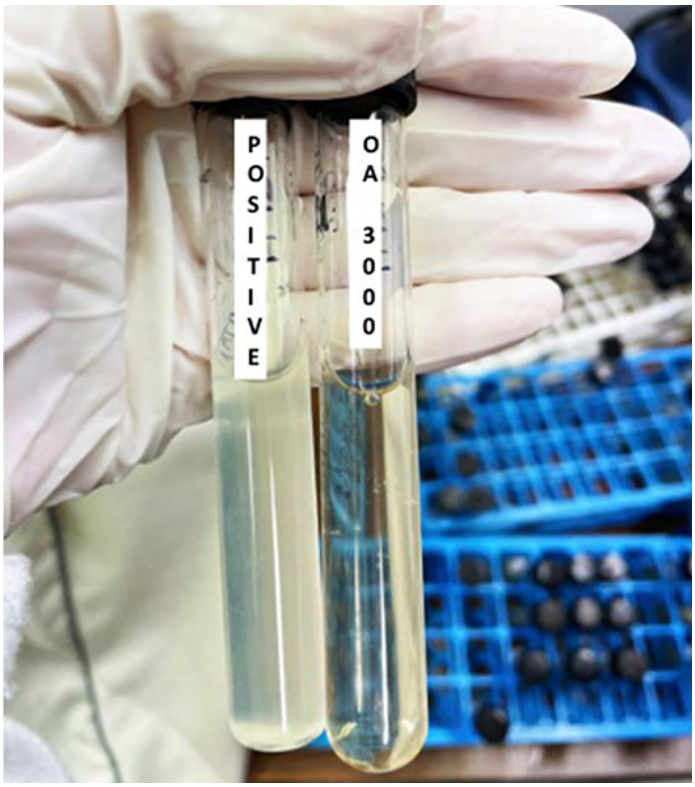

FIGURE 1. MIC test showed that the BACTI-NIL®AQUA had the activity of inhibiting the growth of *V. parahaemolyticus* (M0904AHPND+strain) bacteria with a minimum concentration of 3,000 ppm (OA3000) appear clear, while those that appear cloudy indicate bacterial growth is found in positive control (POSITIVE)

Antibacterial sensitivity in Petri dish and food

TABLE I illustrates the results of antibacterial sensitivity (mm) with respect to the concentration of the treatment, with an inhibition zone greater than 20.0 ± 0.50 mm and 9,000 ppm of BACTI-NIL®AQUA, i.e., 'extremely sensitive'. The doses of 5,000 and 3,000 ppm, with inhibition zone of 17.0 ± 0.03 mm and 15.0 ± 0.50 mm, were within the 'very sensitive' range (FIG. 2). The 'sensitive' doses were 1,500 ppm, with an inhibition zone of 11.0 ±0.10 mm, and 500 ppm, with a diameter of 10.0 ± 0.03 mm. In food, the dose of 5,000 ppm had a diameter of 14.0 ± 0.06 mm (very sensitive), and the dose of 3,000 ppm a diameter of 13 ± 0.1 mm (sensitive)(FIG. 3). Based on these results, it was decided to use the concentrations of 3,000 and 5,000 ppm to perform the efficacy test.

TABLE I
Bacti-nil®aqua concentrations and measurements of inhibition halos. All concentrations were impregnated on oxid antimicrobial susceptibility test discs

BACTI-NIL®AQUA	
Concentration (ppm)	**Halo (mm)**
500	10.0 ± 0.03
1,500	11.0 ± 0.10
3,000	15.0 ± 0.50
5,000	17.0 ±0.03
9,000	20.0 ± 0.50

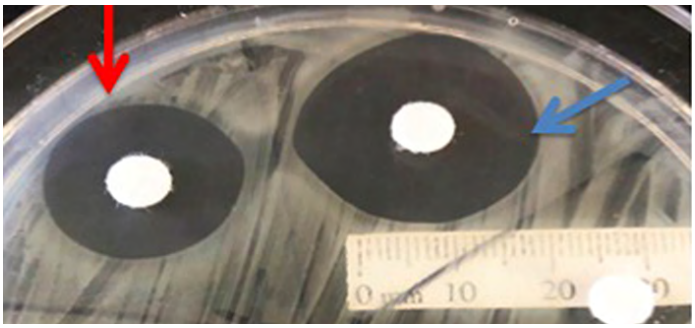

FIGURE 2. Agar diffusion method with filter paper discs on MHA with BACTI-NIL®AQUA at the doses of 5,000 and 3,000 ppm, with inhibition zone of 17.0 ± 0.03 mm (blue arrow) and 15.0 ± 0.50 mm (red arrow)

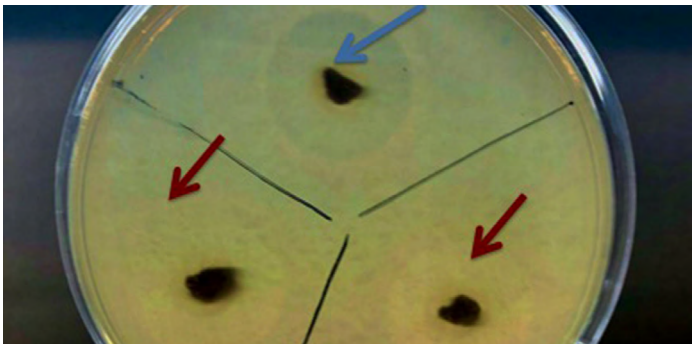

FIGURE. 3. Agar diffusion method with food MHA with BACTIL-NIL®AQUA at the doses of 5,000 (blue arrow) and 3,000 ppm (red arrow), with inhibition zone of 14.0 ± 0.06 mm (very sensitive), and the dose of 3,000 ppm a diameter of 13.0 ± 0.10 mm (sensitive)

Food consumption assessment

A favorable intake was observed thirty min after feeding, since the organisms immediately captured the food. The intestines were filled with continuous strand of feces when evacuated, for which it was considered a positive acceptance for the consumption of the food with the two concentrations of the mixtures OA. The shrimp consumed approximately 30 miligrams (mg) of food per d with a dose of 3,000 ppm, and 25 $mg \cdot d^{-1}$ with a dose of 5,000 ppm (TABLE II).

TABLE II
Food consumption per day

Days	Food consumption	
	BACTIL-NIL®AQUA	
	3,000 ppm (mg)	5,000 ppm (mg)
1	30	25
2	31	27
3	32	23
4	29	26
5	30	24

Antibacterial capacity of BACTIL-NIL®AQUA assessed *in vivo*

The average consumption of food with or without acid was not significantly different between the treatments and the controls ($P<0.05$). All the shrimps of the positive control exhibited red antennae and uropods ten min after inoculation (FIG.4A). After 30 min the shrimps exhibited muscle opacity, cramping, and erratic swimming with permanence at the bottom of the tank (FIG. 4B). After two h, the shrimps had empty intestines with pale hepatopancreas (FIG. 4C), and swam in the decubitus position with static lapses (FIG.4D).

Mortality occurred after 3 h of bacterial inoculation, ending after 19 h with 86.66% (26 shrimps) of accumulated mortality. At the end of the test period (24 h), the negative control showed normal swimming behavior, without color change, and survival of 100% (30 organisms) (FIG. 5).

Organisms treated with BACTI-NIL®AQUA at a dose of 3,000 ppm exhibited clinical signs 3 h after infection, with reddish coloration of antennae and uropods, muscular opacity, cramping, discoloration of the hepatopancreas, and swam in the decubitus position with permanence at the bottom of the aquarium. Seven h after infection, mortality rates were recorded (12 organisms). These rates decreased and there was a survival rate of 60% at the end of the 24 h bioassay (FIG.5).

The shrimps treated with 5,000 ppm exhibited the same changes in behavior and coloration as the organisms treated with the 3,000 ppm. Mortality (12 shrimp) occurred 6 h after infection, and there was a survival rate of 60% at the end of the test (FIG.5).

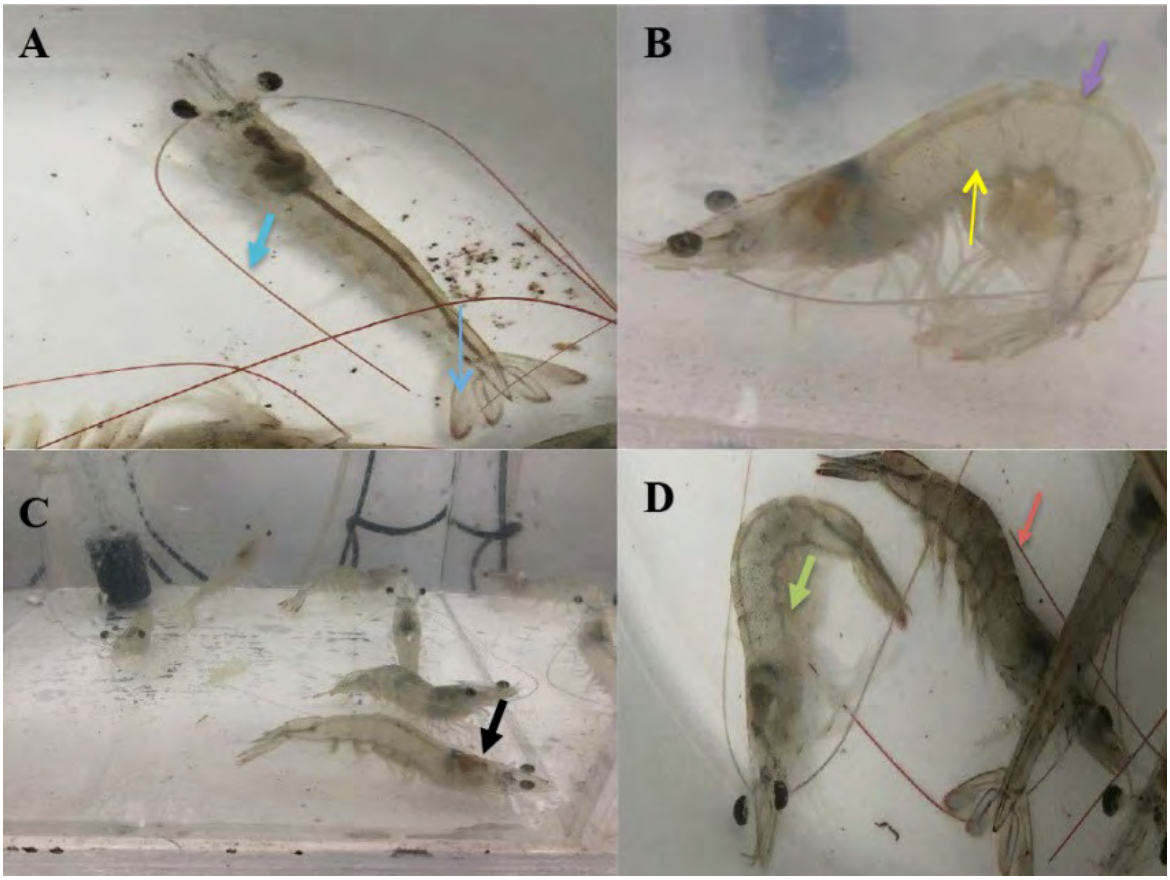

FIGURE 4. Positive control shrimps with red antennae and uropods (FIG. 4A), muscular opacity, cramping, erratic swimming with permanence at the bottom of the tank (FIG. 4B), pale hepatopancreas (FIG. 4C), and swimming in the decubitus position with static lapses (FIG. 4D)

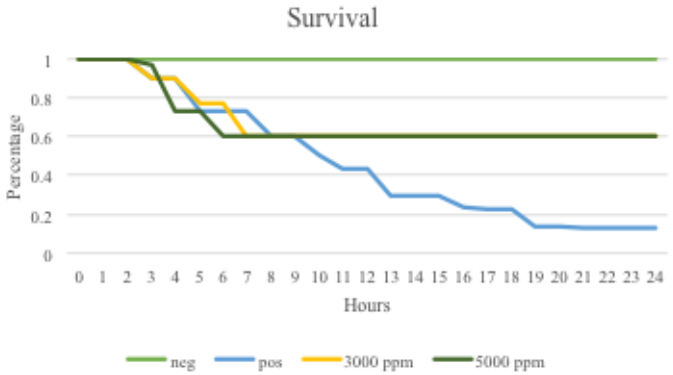

FIGURE 5. Rates of survival at the end of the 24 h bioassay

Statistically, no significant differences were observed in the organisms treated with 3,000 and 5,000 ppm, since the survival rate was the same 24 h after infection (P>0.05). Significant differences were observed between controls and treatments when performing the Holm-Sidak multiple comparison test (P<0.05).

Histological analysis

The organisms of the negative control did not exhibit changes in hepatopancreas. They showed normal hepatopancreatic tubules, with embryonic (E), fibrillar (F), reserve (R), and secretory or globular cells (B) (FIG. 6A).

Histopathological examinations of positive control organisms revealed separation of hepatopancreas myoepithelial layer and epithelium, infiltration of hemocytes within the interstitial sinuses, rupture and collapse of hepatopancreatic tubules, with cellular detachment of the intestine proximal region (Grade III) (FIG. 6D).

The treated organisms in antennal gland exhibited infiltration of hemocytes and pyknotic nuclei (FIG. 6B), gill with infiltration of hemocytes apical zone, minor hyperplasia and rupture and collapse of tubules (FIG. 6C) and hepatopancreas, with rupture and collapse of hepatopancreatic tubules (Grade I) (FIG. 7).

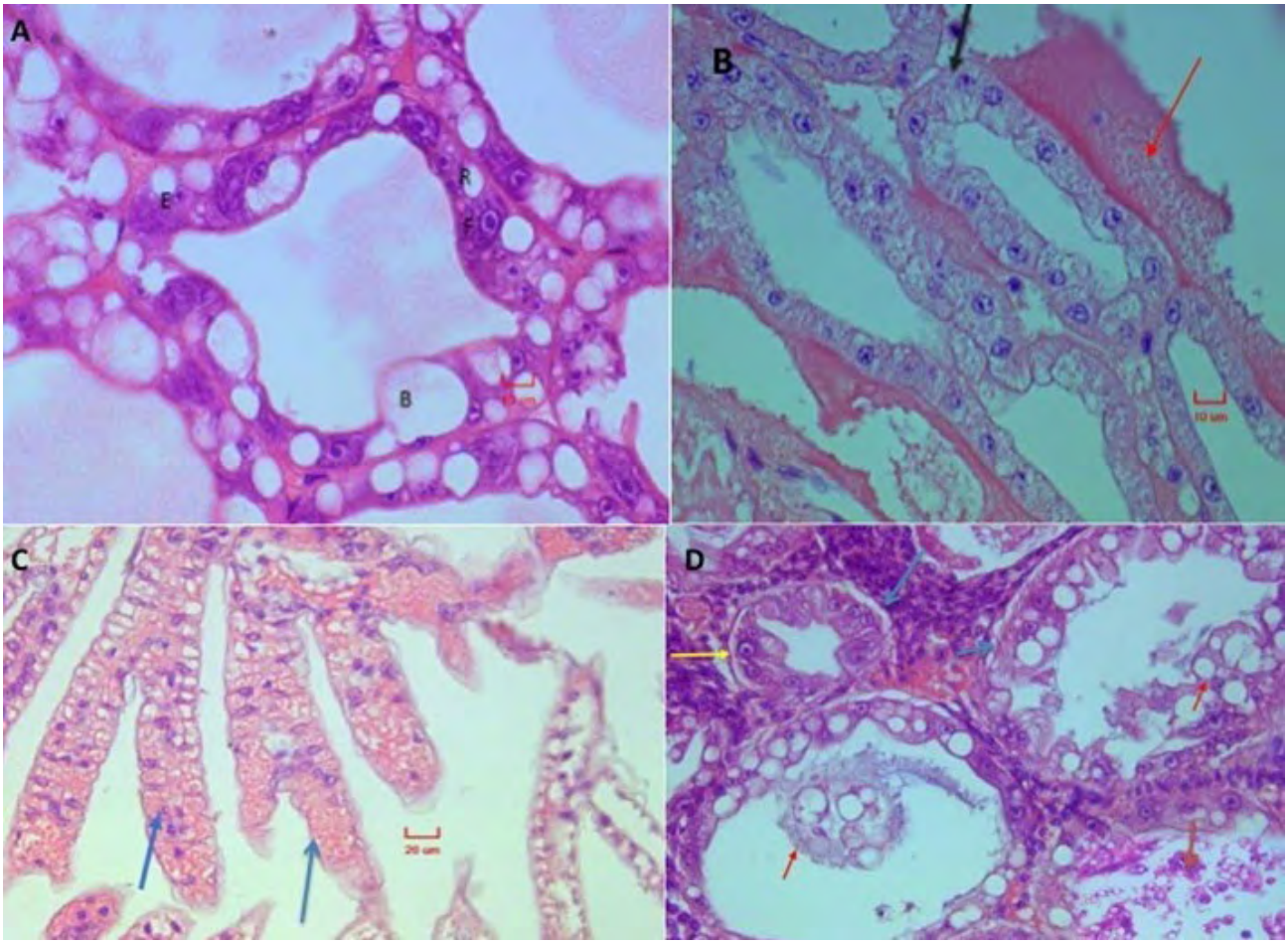

FIGURE 6. Histological sections of tissues of *Litopenaeus vannamei* showing normal, moderate to severe changes with different grades. 6A). Hepatopancreas with normal hepatopancreatic tubules, with embryonic (E), fibrillar (F), reserve (R), and secretory or globular cells (B); 6B); Antennal gland with Infiltration of hemocytes (red arrow) and pyknotic nuclei (black arrow); 6C). Hemocytes infiltration of gills apical zone (blue arrow), minor hyperplasia (blue arrow) and rupture and collapse of tubules (blue arrows); 6D). Myoepithelial layer and epithelium separation of hepatopancreas (blue arrows), infiltration of hemocytes within the interstitial sinuses (red arrows), rupture and collapse of hepatopancreatic tubules, with cellular detachment (yellow arrow; Grade III). Staining method: hematoxylin-eosin. scale bar = 20 µm

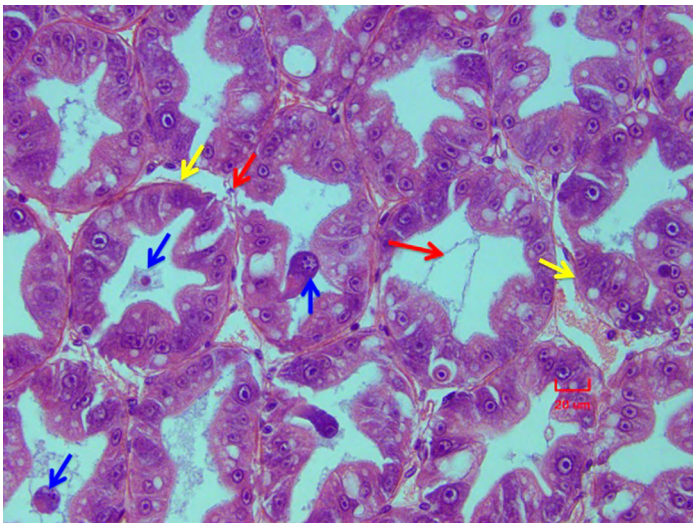

FIGURE 7. *Litopenaeus vannamei* histological section of hepatopancreas with separation of the epithelial membrane (red arrow) and epithelium (yellow arrow). Detachment of the hepatopancreatic cells (blue arrows) from proximal área to the intestine. Staining method: hematoxylin-eosin. scale bar = 20 µm

The results of the present study indicated that BACTI-NIL®AQUA, inhibites the growth of *V. parahaemolyticus in vivo* and *in vitro*, at concentrations of 3,000 and 5,000 ppm. *In vivo* was suggested that BACTI-NIL®AQUA was ingested and thus inhibited the growth of of *V. parahaemolyticus*. However, at a higher pathogens, 5,000 ppm could potentially show differences in comparison to 3,000 ppm. Adams and Boopathy [1] found that formic acid, at a concentration of 5,000 ppm, inhibited the growth of *V. harveyi*; whereas Ng *et al.* [27] observed that lactic and citric acids, at a concentration of 10,000 ppm higher than that reported in the present study, inhibited the growth of *V. harveyi*.

The survival percentage of the efficacy bioassay in the shrimps fed with the addition of 3,000 and 5,000 ppm of BACTI-NIL®AQUA was 60%. In this regard, Ng *et al.* [27] reported that prawns fed with the addition of 10,000 ppm of lactic acid had 65% survival when tested by intramuscular injection with *V. harveyi*.

The histopathological analysis indicated that the organisms fed with the doses of 3,000 and 5,000 ppm exhibited infiltration of hemolymph and hemocytes in the antennal gland, gills, intestinal epithelium, and the lymphoid organ [37, 38]. In addition, the hepatopancreas exhibited rupture and collapse of hepatopancreatic tubules (Grade I). Authors such as Anuta *et al.* [2], Ng *et al.* [29] and Romano *et al.* [30] have documented that by adding OA to shrimp (white and tiger) diets, tolerance to pathogens increased. These authors performed experimental infections with *V. harveyi* and observed minor changes such as rupture, cell detachment, and collapse of the tubules in hepatopancreas.

It has been reported that synergistic activities between OA may occur and provide a broader spectrum of antimicrobial protection than the exclusive use of one type of OA [5, 6, 9, 28, 32, 35]. Probably, this synergy favored tolerance to *V. harveyi*, since the present study showed low mortality and minor changes in organs and tissues, especially in hepatopancreas [13, 34], since only Grade I rupture and collapse of hepatopancreatic tubules was observed in organisms fed with 3,000 and 5,000 ppm of BACTI-NIL®AQUA during the efficacy test.

This result was relevant, because the hepatopancreas was the largest organ of crustaceans and fulfills different functions, including secretion of digestive enzymes, digestion and absorption, storage of mineral reserves and organic substances, metabolism of carbohydrates and lipids, distribution of reserves stored during the intermoult cycle, and catabolism of products of the ingested diets [17].

CONCLUSIONS

The results obtained in the present study allowed inferring that the doses of 3,000 and 5,000 ppm of BACTI-NIL®AQUA added to food, in experimental infection for efficacy, improved the biological response regarding bacterial infections of shrimp *L. vannamei* in comparison to those specimens who did not receive. This way, these results are certainly encouraging, given that BACTI-NIL®AQUA added to food showed beneficial properties that can help reduce the dependence on antibiotics in shrimp farming and, therefore, produce an antibiotic-free product.

ACKNOWLEDGMENT

This work was partially funded by CIAD grant 20657-MCMS.

CONFLICTS OF INTEREST

The authors of this paper deny any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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