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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

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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.

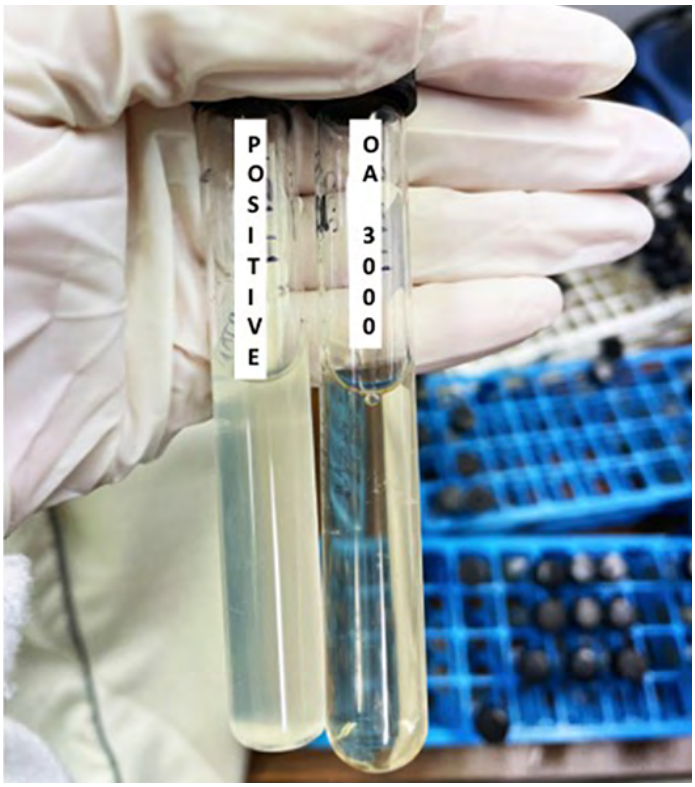

FIGURE 1. MIC test showed that the BACTI-NIL®AQUA had the activity of inhibiting the growth of *V. parahaemolyticus* (M0904AHPND+strain) bacteria with a minimum concentration of 3,000 ppm (OA3000) appear clear, while those that appear cloudy indicate bacterial growth is found in positive control (POSITIVE)

Antibacterial sensitivity in Petri dish and f d

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 f d, 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

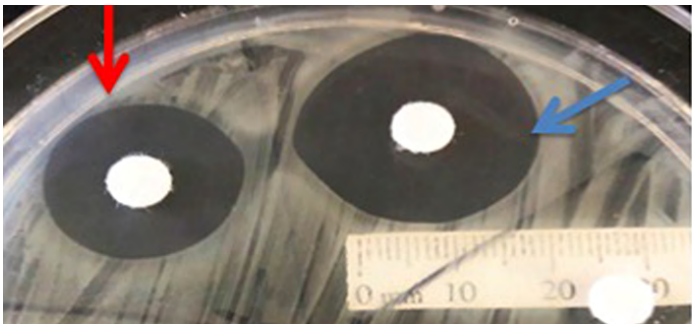

FIGURE 2. Agar diffusion method with filter paper discs on MHA with BACTI-NIL®AQUA at the doses of 5,000 and 3,000 ppm, with inhibition zone of 17.0 ± 0.03 mm (blue arrow) and 15.0 ± 0.50 mm (red arrow)

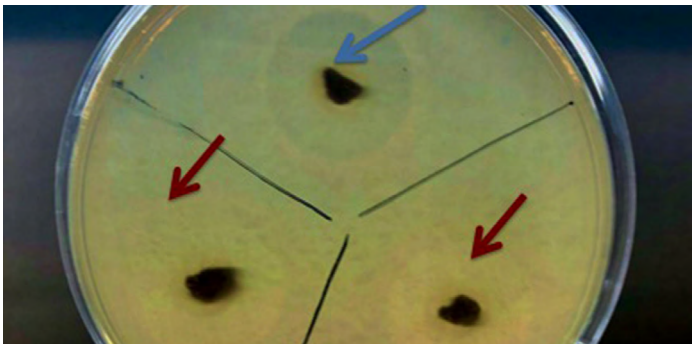

FIGURE. 3. Agar diffusion method with fMMd MA with BACTIL-NIL®AQUA at the doses of 5,000 (blue arrow) and 3,000 ppm (red arrow), with inhibition zone of 14.0 ± 0.06 mm (very sensitive), and the dose of 3,000 ppm a diameter of 13.0 ± 0.10 mm (sensitive)

Feed consumption assessment

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

TABLE II
Feed consumption per day
Feed consumption

Days	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 feed 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).

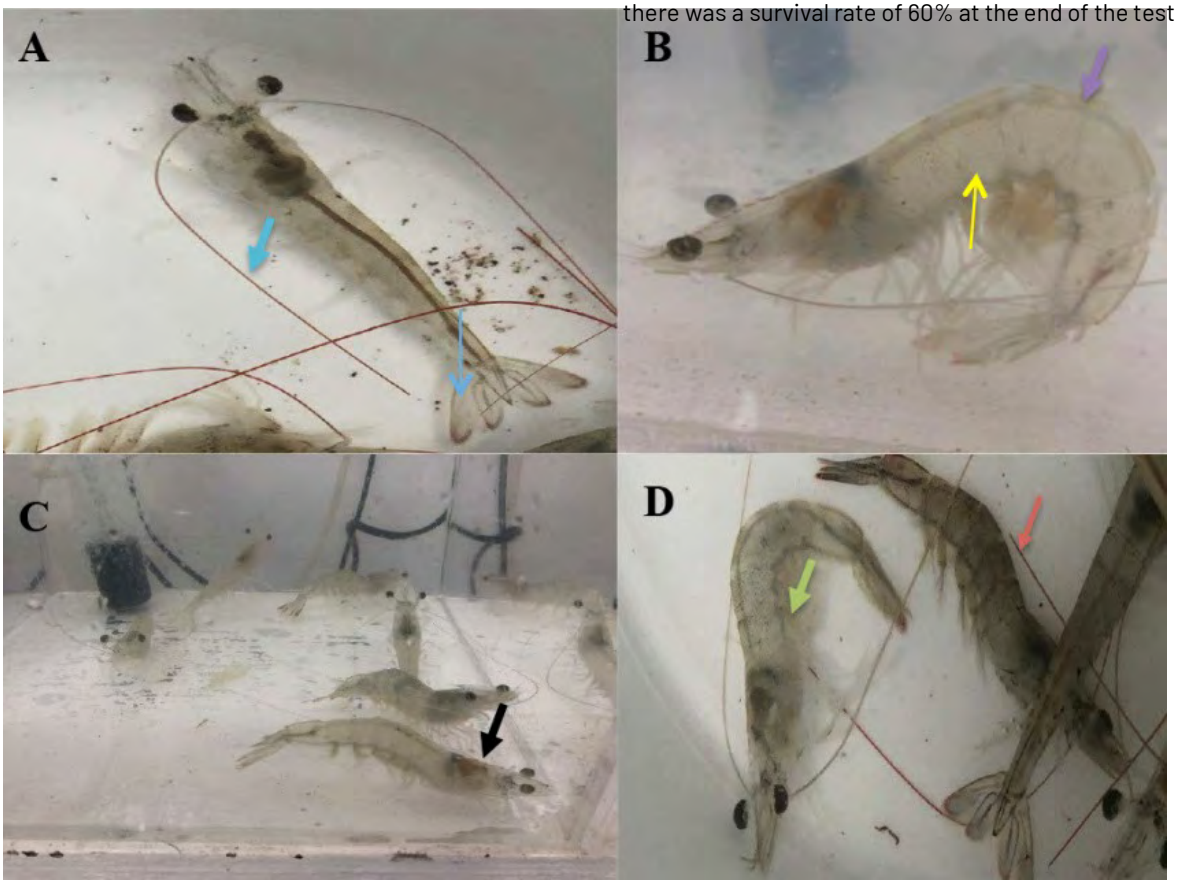

FIGURE 4. Positive control shrimps with red antennae and uropods (FIG. 4A), muscular opacity, cramping, erratic swimming with permanence at the bottom of the tank (FIG. 4B), pale hepatopancreas (FIG. 4C), and swimming in the decubitus position with static lapses (FIG. 4D)

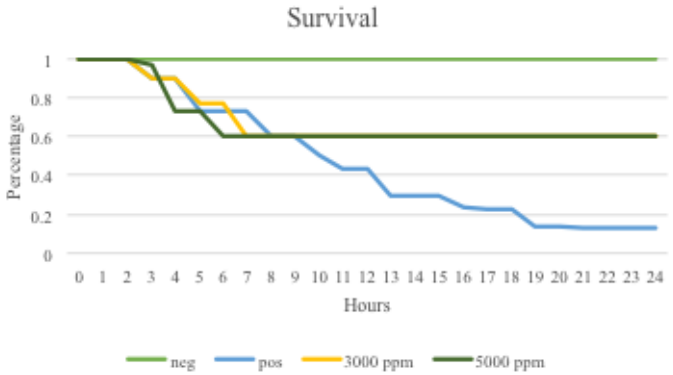

FIGURE 5. Rates of survival at the end of the 24 h bioassay

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).

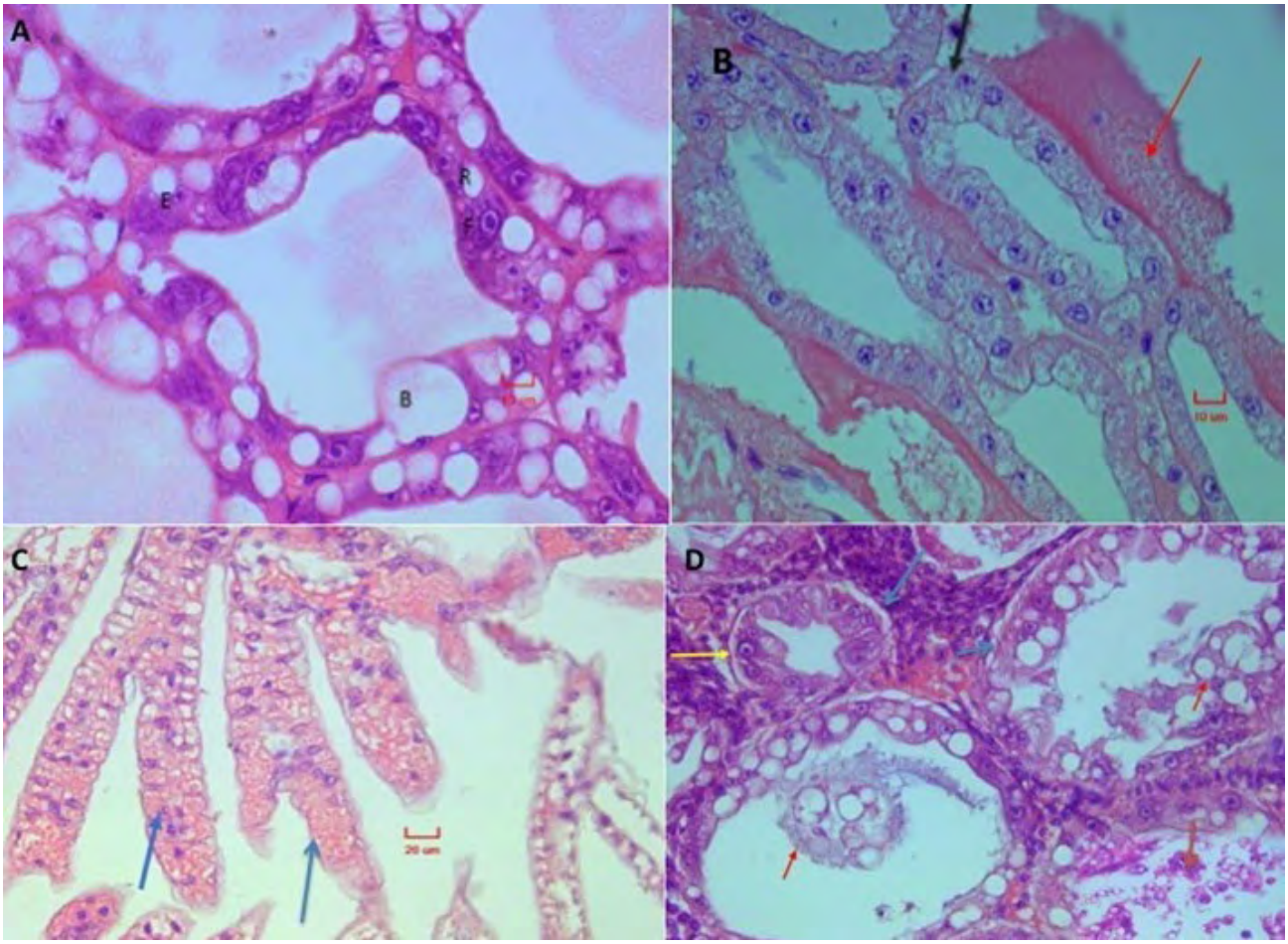

FIGURE 6. Histological sections of tissues of *Litopenaeus vannamei* showing normal, moderate to severe changes with different grades. 6A). Hepatopancreas with normal hepatopancreatic tubules, with embryonic (E), fibrillar (F), reserve (R), and secretory or globular cells (B); 6B); Antennal gland with 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

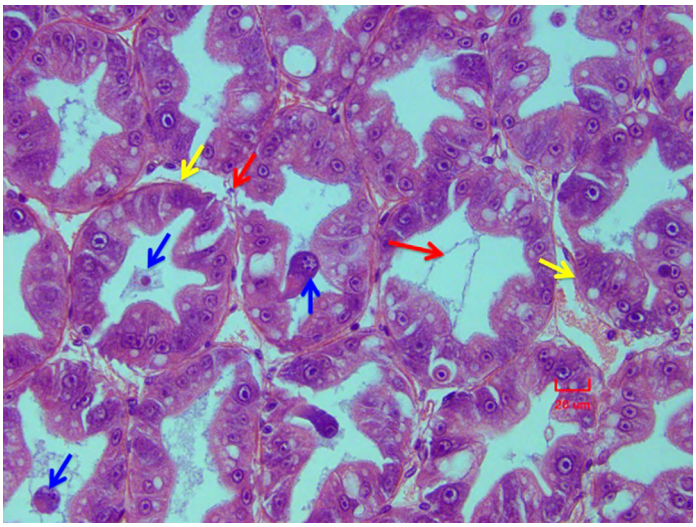

FIGURE 7. *Litopenaeus vannamei* histological section of hepatopancreas with separation of the epithelial membrane (red arrow) and epithelium (yellow arrow). Detachment of the hepatopancreatic cells (blue arrows) from proximal área to the intestine. Staining method: hematoxylin-eosin. scale bar = 20 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 feed, 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 feed showed beneficial properties that can help reduce the dependence on antibiotics in shrimp farming and, therefore, produce an antibiotic-free product.

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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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