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Original Article

Effect of commercial herbal-extract products Tetrayou® and Feed Ad® on the survival of white shrimp, *Penaeus vannamei* challenged with a pathogenic *Vibrio parahaemolyticus*-AHPND strain

Efecto de los extractos herbales comerciales Tetrayou® y Feed Ad® sobre la supervivencia del camarón blanco del Pacífico, *Penaeus vannamei*, expuesto a una cepa patogénica de *Vibrio parahaemolyticus*-AHPND

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ABSTRACT

Herbal remedy is one of the most important eco-friendly strategies because of its content of natural bioactive compounds that can act against bacterial diseases. In the present study, organisms of *P. vannamei* were challenged with a strain of *V. parahaemolyticus*-AHPND. A simple experimental design was performed to compare the survival rate among five different commercial herbal extracts concentrations of Tetrayou® and Feed Ad® against a control treatment. Gross observation and histopathology were carried out on moribund organisms. Significant differences were observed ($p < 0.05$) where organisms fed with T5, which contained a combination of Tetrayou® and Feed Ad® at a concentration of 400 mg Tetrayou®/kg of commercial feed + 1g Feed Ad®/kg of commercial feed, showed the highest survival value (73 ± 5.8 %) in comparison to the rest of experimental treatments. Gross observation showed the characteristic signs of AHPND which were supported by histopathological analysis. It is concluded that, although Tetrayou® and Feed Ad® are suitable products to control the mortality caused by AHPND, it is recommended to use them in combination during a minimum feeding period of six weeks as a prophylactic agent.

Keywords: AHPND; herbal remedy; ecofriendly; histopathology; *V. parahaemolyticus.*

RESUMEN

Una de las estrategias ecológicamente amigables para el tratamiento de enfermedades en acuacultura es la utilización de los compuestos herbales debido a su gran contenido de compuestos bioactivos con efecto antimicrobiano. En este estudio, se evaluó la supervivencia de *P. vannamei* ante una cepa de *V. parhaemolyticus*-AHPND. Se llevó a cabo un diseño experimental simple para comparar la tasa de supervivencia de los organismos de *P. vannamei* alimentados con cinco diferentes concentraciones de los productos herbales Tetrayou® y Feed Ad® contra un control. Adicionalmente, se realizaron análisis en fresco, así como histopatología. Al final del experimento, se observaron diferencias significativas ($p<0.05$) donde el tratamiento T5, el cual contenía una combinación de ambos productos a una concentración de 400 mg Tetrayou®/kg de alimento + 1g Feed Ad®/kg de alimento, mostró el mayor valor de supervivencia (73 ± 5.8 %). La observación en fresco mostró el daño característico de AHPND, los cuales fueron respaldados por histopatología. Se concluye que, si bien Tetrayou® y Feed Ad® son productos efectivos que pueden ser utilizados para combatir la mortalidad causada por AHPND, se recomienda utilizarlos en combinación durante un periodo de al menos seis semanas como agente profiláctico.

Palabras clave: AHPND; compuestos herbales; ecológicamente amigables; *V. parahaemolyticus.*

INTRODUCTION

Acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome (EMS) (Lightner *et al*., 2012), is probably the most critical shrimp disease in Mexico and Asian countries (Shinn *et al*., 2018; Soto-Rodriguez *et al*., 2018), reaching losses up to US$44 billion between 2010 and 2016 in China, Malaysia, Mexico, Thailand, and Vietnam (Tang and Bondad-Reantaso, 2019). The first AHPND outbreak was reported in China in 2009, and subsequently countries such as Mexico, Thailand, Malaysia, Vietnam, the Philippines, Bangladesh, and the USA, have reported outbreaks of the disease, causing a significant reduction of shrimp production in these countries (Nunan *et al*., 2014; Eshik *et al*., 2017; Dhar *et al*., 2019; Muthukrishnan *et al*., 2019; Peña-Navarro *et al*., 2020).

In 2013, the causative agent of AHPND was identified as *V. parahaemolyticus*, which is considered as a ubiquitous and opportunistic marine bacterium (Tran *et al*., 2013). Additionally, all AHPND-causing strains of *V. parahaemolyticus* carry a large plasmid which is absent in non-AHPND strains (Han

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et al., 2015b; Sirikharin *et al*., 2015). This plasmid contains the *pir*A and *pir*B genes that encode for binary toxins named Pir A and Pir B in *V. parahaemolyticus*, which are the main virulence factor of the AHPND (Kondo *et al*., 2014; Han *et al*., 2015a). Nowadays, it is known that *V. parahaemolyticus* is not the only species that carries the plasmid-AHPND, but there are some other species such as *Vibrio campbellii* (Han *et al*., 2017), *Vibrio harveyi* (Kondo *et al*., 2015), *Vibrio owensii* (Liu *et al*., 2018) and *Vibrio punensis* (Restrepo *et al*., 2018) that may carry it, and consequently cause AHPND (Aranguren-Caro *et al*., 2020).

Organisms affected by AHPND show visible clinical signs that includes slow growth, anorexia, erratic swimming, melanized spot in the hepatopancreas, lethargy, atrophied pale to white hepatopancreas, empty gastrointestinal tract, red appendages, and soft shells (Tran *et al*., 2013; Soto-Rodriguez *et al*., 2015). Additionally, histological analysis of hepatopancreas collected from infected organisms reveals sloughing of tubule epithelial cells in the early stage of AHPND, while in later stages of infection necrosis of the tubular epithelium and infiltration of the hemocytes are observed (Soto-Rodriguez *et al*., 2015; Kumar *et al*., 2020).

On the other hand, shrimp farmers have been and still are using antibiotics to control bacterial diseases. Nevertheless, due to the potential adverse effect on human health, the utilization of antibiotics in aquaculture has been restricted or even banned in many countries (Lulijwa *et al*., 2019). Morever, the frequent use of antibiotics in aquaculture has led to the emergence of multiple antibiotic-resistant strains, making antibiotics ineffective against bacterial infections (Sivasankar *et al*., 2017; Zago *et al*., 2020; Loo *et al*., 2020).

Herbal remedy is one of the most important eco-friendly strategies to substitute the use of antibiotics (Citarasu *et al*., 2022), since herbal medicine contains bioactive compounds like flavonoids, pigments, steroids, tannins, glycosides, terpenoids and essential oils, that promote a variety of beneficial effects including antimicrobial properties and resistance to disease (Citarasu *et al*., 2002; Citarasu *et al*., 2010; Citarasu *et al*., 2022). Tetrayou® and Fed Ad® are both commercial herbal blends that are mainly used as a prophylactic product in livestock industry in Northwest Mexico, which utilization may be a suitable option to be extended to shrimp culture. Therefore, the present study aimed to evaluate the effect of two commercial herbal blends Tetrayou® and Feed Ad® on the survival of *P. vannamei* challenged with a pathogenic strain of *V. parahaemolyticus*-AHPND.

MATERIALS AND METHODS

Experimental organisms of *P. vannamei* and acclimatization

A total of 210 organisms of *P. vannamei* with a mean weight of 12 g were collected from experimental ponds of University of Sonora located at Kino Bay Experimental Station, Kino Bay, Sonora, Mexico. Organisms were transported alive with continuous aeration to the Aquaculture Laboratory of University of Sonora in Hermosillo, Sonora. Upon arrival, organisms were stocked randomly in seven 250-L tanks, at a density of 30 organisms per tank. Organisms were fed with a commercial feed with 35 % of protein for one week. A composed sample of 15 organisms was taken to be analyzed by PCR to make sure that organisms were not previously infected with *V. parahamolyticus*.

Herbal products feeding trial and daily maintenance

After acclimatization, organisms stocked in tanks from 1 to 5 began to be fed with commercial pellets previously impregnated with herbal commercial products Tetrayou® and Feed Ad® at different concentrations (Table 1) (using molasses as binder), and subsequently dried at room temperature (25 °C) during 24 h. Additionality, organisms contained in tanks 6 and 7 were fed with commercial diet (non-herbal products added) as control. Organisms were fed for 42 days based on 3 % of its body weight, dividing the daily ration into four equal feedings provided at 08:00, 12:00, 16:00 and 20:00 h. Daily maintenance also included cleaning and siphoning the remaining uneaten feeds and feces out of the tanks. Dissolved oxygen (D.O.) was maintained above 6.0 mg L^{-1} throughout the experiment while water temperature and pH were maintained at 27.5 °C ± 1.4 °C and 7.7 ± 0.1, respectively.

AHPND-causing strain of *V. parahaemolyticus*

The pathogenic strain of *V. parahaemolyticus*-AHPND used in the present study was isolated from moribounds white shrimps, *P. vannamei* cultured in Sonora, Mexico during a mortality event with visible signs of AHPND infection. The bacteria was subsequently verified as possessing the *pirA* and *pirB* genes, which are responsible for causing AHPND (Han *et al*., 2015b; Sirikharin *et al*., 2015). The *V. parahaemolyticus*-AHPND strain was cultured on tryptic soy agar (TSA) medium added with 2.5 % sodium chloride during 24 h at 30 °C, and from this plate, another six plates were cultured on TSA under the same conditions. Bacteria biomass produced in plates were washed and collected in a 2-L flask using a 2.5 % sterile saline solution and filled with saline solution to reach a final volume of 1.5 L. Inoculum concentration was determined by turbidimetry using a microplate and solution was read at 550 nm (Alvárez-Cirerol *et al*., 2019).

Challenge test for *P. vannamei* with a *V. parahaemolyticus*-AHPND strain

Inmediatelly after the feeding trial, a challenge test of *P. vannamei* with *V. parahaemolyticus*-AHPND was performed in containers with a 10 L capacity filled with 7 L of seawater, previously filtered, and sterilized. As shown in Table 1, an experimental design consisting of five herbal treatments at different concentrations (containers 1 to 5), a positive control which contained the pathogen (container 6), and a negative control without pathogen (not included in statistical analysis) (container 7) was carried out. All treatments were performed in triplicate (21 containers). A total of 10 organisms taken from feeding treatments (Tetrayou®, Feed Ad®, and commercial diet with non-herbal products added), as explained above, were washed thrice by immersion in sterile seawater

Table 1. Concentration of Tetrayou® and Feed Ad® added (per kg of commercial feed) to each experimental treatment.
Tabla 1. Concentración de Tetrayou® y Feed Ad® adicionada (por kg de alimento comercial) a cada tratamiento experimental.

Experimental treatment	Tetrayou® mg/kg of commercial feed	Feed Ad® g/kg of commercial feed
T1	300	0
T2	400	0
T3	500	0
T4	300	1
T5	400	1
T6 (Positive Control)	0	0
T7 (Negative Control)	0	0

to reduce surface bacteria and stocked in each container (30 organisms per treatment). Each experimental container was added with 60 mL of bacterial inoculum, which corresponded to 6x10^4 CFU/mL of *V.parahaemolyticus*-AHPND. During the bioassay, mortality was recorded daily; moribund and dead organisms were removed. Experiment lasted seven days and the remaining organisms were sacrificed to carry out histological and molecular analysis.

Gross observation and histology

Prior the histological analysis, a meticulously gross examination of remaining organisms was performed. Subsequently, hepatopancreas of 15 organisms per treatment was carefully excised and stored at -20 °C to perform molecular analysis, while a tissue sample was preserved in Davidson's fixative for 48 h before processing by routine histology. The tissue sections were stained with the Hematoxylin-Eosin technique (H&E). Gross observation and histological interpretation were based on de Souza-Valente and Whan (2021) and Le Nguyen *et al.* (2023).

***V. parahaemolitycus* confirmation**

To confirm *V. parahaemolyticus* infection by the PCR procedure, 20 mg of hepatopancreas were obtained from two organisms of each treatment for genomic DNA (gDNA) extraction, using the QIAamp tissue mini kit (Qiagen, Westburg b.v., The Netherlands) according to the manufacturer's protocol. For PCR assay, each PCR reaction contained 0.2 µM of each oligo (forward and reverse), 7.5 µL of Crystal Master mix (Jena Bioscience), 6.1 µL of nuclease-free water and 100 ng of gDNA. To detect *V. parahemolyticus* in organisms, oligos were used for the *V. parahemolyticus* thermolabile hemolysin (TLH) encoding gen: tlF (5′ AAAGCGGATTATGCAGAAGCACTG 3′) and tlR (5′ GTCACTTTCTAGCATTTTCTCTGC 3′) targeting a 450 bp amplicon (Taniguchi *et al*., 1985, 1986; Ward and Bej, 2006). The amplification conditions were as follows: 95 °C for 3 min, followed by 30 cycles at 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and a final step at 72 °C for 6 min. In addition, to detect *pirA* and *pirB* genes of *V. parahaemolyticus* in the bacterial culture, primers used were PIRF: 5′ TGACTATTCACGATTGGACTG 3′ and AP4R1: 5′ CACGACTAGCCATTGTTA 3′ for *pirA*, and PIRF2: 5′ TGATGAAGTGATGGGTGCTC 3′ and PIRR2: 5′TGTAAGCGCCGTTTAACTCA 3′for *pirB* (Han *et al*., 2015b). The cycling reactions were conducted at 95 °C for 5 min, followed by 30 cycles at 94 °C for 30 sec, at 54 °C for 30 sec and 72 °C for 60 sec, plus a final extension at 72 °C for 10 min. Amplified PCR products (5 µL) were verified by electrophoresis using 1.5 % agarose gels in 1× TAE buffer stained with GelRed, and visualized under UV transillumination and documented by a digital photography system (DNR minibis pro).

***PirA* and *PirB* confirmation**

PCR analysis of bacteria used to infect the organisms in the present experiment revealed a 450 bp band (Fig. 1A), this gene encodes for the TLH, which is considered as a signature molecular for *V. parahaemolyticus*. Additionally, PCR analysis to detect *pirA* and *pirB* genes revealed a pair of bands of ~ 300 bp and ~400 bp (Fig. 1B) respectively, all bands were sequenced (data not shown). Therefore, molecular analysis confirmed that the bacteria used in the present experiment was a causative-AHPND strain of *V. parahaemolyticus*.

Statistical Analysis

Survival rate (%) was subjected to one-way analysis of variance and Duncan's multiple range test for comparison of means ($P < 0.05$) using the XLSTAT software package. All percentage data were arcsine-transformed prior to analysis.

RESULTS AND DISCUSSION

At the end of the challenge, the highest survival value (73 ± 5.8 %) was observed in treatment T5 and the lowest in treatment T6 where 100 % mortality was registered (Fig. 2). Additionally, all survival rates where *P. vannamei* was fed with commercial herbal-extracts Tetrayou® and Feed Ad® were statistically higher ($p<0.05$) in comparison to those fed with treatment T6 (positive control-non-herbal products added), except when it was compared with treatment T2 (17 ± 11.5 %) where no significant differences were found (Fig. 2). It is evident that survival rate for treatment T2 did not behave as expected, since mortality in this treatment showed high values compared to other treatments where herbal extracts were evaluated. One possible explanation for this is that lixiviation may have occurred, since Tetrayou® and Feed Ad® herbal extracts utilized in the present study were bound by impregnation utilizing molasses and water. It is known that in aquafeeds preparation, it is crucial to supply the whole amount of nutrients and additives (Volpe *et al*., 2012). In this regard, to reduce lixiviation and enhance the stability of herbal products in water, other methods of binding must be explored. Pelleting is considered the most common thermal processing method in the production of animal feed. This method consists in agglomerating smaller feed particles by the use of mechanical pressure, moisture and heat (Abdollahi *et al*., 2013). Mišljenović *et al.* (2016) evaluated the effects of sugar beet molasses on pellet quality, concluding that molasses as a binder is effective when pelleting is performed at certain temperature. Therefore, adding herbal products

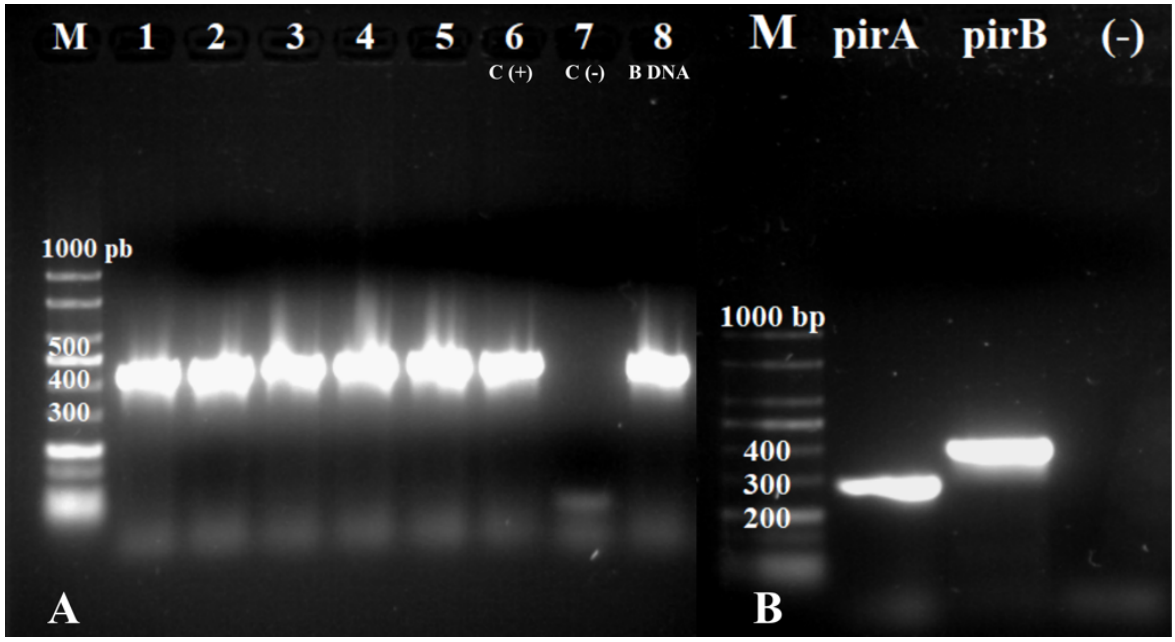

Figure 1. PCR detection of the thermolabile hemolysin (TLH), *pirA* and *pirB* genes, from *V. parahaemolyticus* used in the bioassay. A. Detection of the TLH gene from *V. parahaemolyticus*. M. Low Range (Jena Bioscience). Lines 1-6 TLH detection in organisms of experimental treatments. Line 7 negative control. Line 8 *V. parahaemolyticus* gDNA bacterial culture used in the bioassay. B. PCR assay targeting toxigenic genes *pirA* and *pirB* in *V. parahaemolyticus*. Lane (-) negative control.
Figura 1. Detección por PCR del gen de la hemolisina termolábil (TLH), y de los genes pirA y pirB de *V. parahaemolyticus* utilizados en el bioensayo. A. Detección del gen TLH de *V. parahaemolyticus*. M. Low Range (Jena Bioscience). Líneas 1-6 detección de TLH en organismos de los tratamientos experimentales. Línea 7 control negativo. Línea 8 cultivo bacteriano de ADN genómico de *V. parahaemolyticus* utilizado en el bioensayo. B. Ensayo PCR especificos a los genes toxigénicos pirA y pirB en *V. parahaemolyticus*. Línea (-) control negativo.

through pelletizing method may be a suitable option to reduce lixiviation. However, caution should be taken, since it is reported that bioactive compounds found in herbal products responsible for their beneficial effects on shrimps, including resistance to disease, anti-stress, appetite stimulant, immunostimulation and antimicrobial properties (Citarasu, 2022), may be altered when exposed to high temperatures (Maurus *et al*., 2023).

In the present study, despite survival rate registered for treatment T2, the rest of experimental treatments containing Tetrayou® and Feed Ad® showed higher survival values (ranging from 33 ± 15.3 % to 73 ± 5.8 %) compared to control treatment, suggesting that these herbal extracts contain bioactive compounds with a potent antimicrobial effect against *V. parahaemolitycus*-AHPND. Similarly, several studies carried out under laboratory conditions have demonstrated the effectiveness of plant-based compounds against AHPND-causing strains of *V. parahaemolyticus*, either increasing survival rate or enhancing the immunity response of shrimps. Maurus *et al*. (2023) determined that the extract of *Scutellaria baicalensis* at a concentration of 1 % enhances the resistance and survival rate of *P. vannamei* against *V. parahaemolyticus*-AHPND after four weeks of feeding the extract, while Nguyen *et al*. (2024) reported that the inclusion of *Galla chinensis* at 1 g/kg, not only promotes growth but also reduce *P. vannamei* mortality during *V. parahaemolyticus* infection. Linh *et al*. (2024) and Dewi *et al*. (2021) determined that *Annona glabra* and *Psidium guajava* leaf increase the immunity response and resistance of *P. vannamei* against *V. parahaemolyticus*-AHPND infection.

Interestingly, Martínez-Aguilar *et al*. (2013) and Más *et al*. (2017) have reported that medicinal plants are more effective when they are used in blends with other plants than individually, since bioactive compounds found in different plants can act in synergy. In the present study, it was observed that the survival rate was higher in treatments where the herbal products Tetrayou® and Feed Ad® were used in combination (40-73 %), compared to treatments where Tetrayou® was evaluated alone (17-33 %) (Fig. 2). It is important to mention that, Tetrayou® and Feed Ad® are commercial blends manufactured by the extraction of several plants known by their antiseptical properties, including *Cuminum* sp (Sharifi *et al*., 2021) and *Cinnamomum* sp. (Bandeira-Junior *et al*., 2022), which may act in synergy to control bacterial diseases, specifically against *V. parahaemolyticus*-AHPND as it was observed in the present study and as it is suggested by literature. Based on the promising results obtained against strains of *V. parahaemolyticus*-AHPND under laboratory conditions, plant-based compounds seem to be a realistic option to substitute the use of antibiotics in aquaculture in the short-term. Therefore, in our view, future research must be focused on evaluating plant-based compounds in semi-commercial ponds, in order to evaluate how these herbal compounds behave under variable conditions. It is worth mentioning that some studies have reported that results utilizing plant-based products may vary depending on certain factors (Maurus *et*

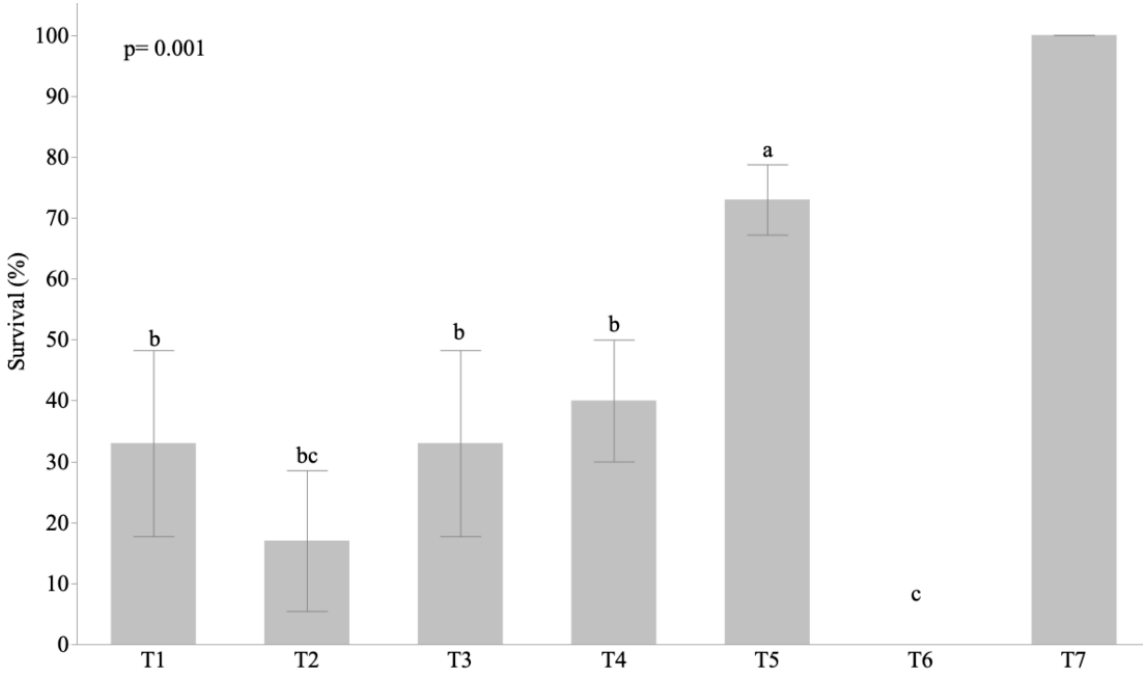

Figure 2. Survival of *P. vannamei* fed with different concentration of Tetrayou® and Feed Ad®. Data are means of 3 replicates ± S.D. (Standard Deviation). Different letters indicate significant differences (Duncan's alpha = 0.05). T7: Negative control without pathogen used as reference (not included in statistical analysis).

Figura 2. Supervivencia de *P. vannamei* fed alimentado con diferentes concentraciones de Tetrayou® y Feed Ad®. Los datos mostrados son resultados de triplicados ± D.S. (Desviación estándar). Letras diferentes indican diferencias significativas ((Duncan's alpha=0.05). T7: control negativo sin patógeno utilizado sólo como referencia (no incluido en el análisis estadístico).

al., 2023) such as extraction method, exposure time, concentration and dosage applied, which also needs to be explored in future research.

On the other hand, visible signs and gross examination of moribund shrimps revealed lethargy, melanization, red appendages, pale body, sloughed hepatopancreas, and soft shells (Fig. 3). These findings are consistent with gross observations reported by Tran *et al.* (2013) and Soto-Rodriguez *et al.* (2015), as well as those documented in mortality events of *P. vannamei* caused by AHPND infection in Asia and Mexico (Nunan *et al*., 2014; Eshik *et al*., 2017; Dhar *et al*., 2019; Muthukrishnan *et al*., 2019; Peña-Navarro *et al*., 2020). Additionally, histological analysis of infected organisms supported previous gross observations revealing necrosis

Figure 3. Gross observation of *P. vannamei*. A-B. Healthy shrimp: (b) bright body, normal hepatopancreas (nh) and brown midguts (i). C-D. AHPND infected organisms: (m) melanization, (ra) red appendages, (p) pale body, (sh) sloughed hepatopancreas and (s) soft shell.

Figura 3. Observación en fresco de *P. vannamei*. A-B. Camarón sano: (b) cuerpo con brillo, hepatopáncreas con aspecto normal (nh) y vísceras marrón (i). C-D. organismos infectados con AHPND: (m) melanización, (ra) apéndices rojizos, (p) cuerpo pálido, (sh) desprendimiento del hepatopáncreas y (s) cuerpo blando.

and severe disorganization of hepatopancreatic tissue due to the sloughing of tubular epithelium (Fig. 4), which is considered a specific sign of AHPND infection (Soto-Rodriguez *et al*., 2015; Kumar *et al*., 2020). Therefore, based on the damage observed in hepatopancreatic tissue, organisms of *P. vannamei* infected in this study with a pathogenic strain of *V. parahaemolyticus*-AHPND, were classified either in acute or terminal stage of AHPND infection based on de Souza-Valente and Whan (2021).

It is concluded that the combination of commercial plant-based extracts Tetrayou® and Feed Ad®, at a concentration of 400 mg Tetrayou®/kg of commercial feed + 1g Feed Ad®/kg of commercial feed, is effective to be used not only as a prophylactic on *P. vannamei*, but to control the mortality caused by *V. parahaemolitycus*-AHPND after a minimum feeding period of six weeks. Finally, further studies are required to elucidate how these plant-based products interact with the immunology mechanisms of *P. vannamei* in order to enhance its efficiency when used to control AHPND.

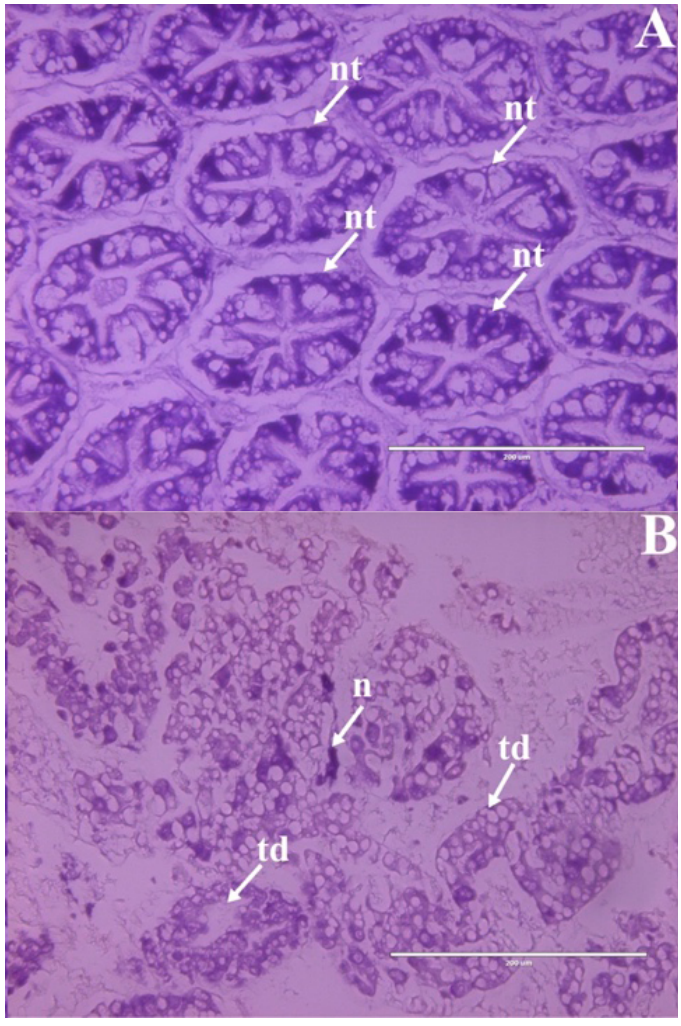

Figure 4. Histopathological examination of *P. vannamei*. A. non-infected organism: normal hepatopancreatic tissue configuration (nt). B. Acute-terminal stage of AHPND infection: necrosis (n) and severe hepatopancreatic tissue disorganization (td). Stained with hematoxylin-eosin. Scale bar: 200 µm.

Figura 4. Examinación histopatológica de *P. vannamei.* A. organismo no infectado: configuración normal del tejido hepatopáncreatico (nt). B. Etapa aguda-terminal de la infección causada por AHPND: necrosis (n) y desorganización severa del tejido hepatopáncreatico (td). Tinción hematoxilina-eosina. Escala: 200 µm.

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CONFLICTS OF INTERESTS

The authors declare no conflict of interest.

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