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Originales

Assessment of homeopathic medicines on survival and antioxidant response in white shrimp *Litopenaeus vannamei*

Evaluación de medicamentos homeopáticos en la supervivencia y respuesta antioxidante del camarón blanco *Litopenaeus vannamei* infectado con *Vibrio parahaemolyticus*

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Abstract:

Objective. Evaluating the effect of homeopathic medicines on survival and activity of the superoxide dismutase (SOD) enzyme in white shrimp *Litopenaeus vannamei* subjected to infection with the pathogenic *Vibrio parahaemolyticus.* **Materials and methods.** The average lethal dosage (LD_{50}) was determined for the pathogen strain in *L. vannamei* juveniles under immersion (Imm), injection (Inj) and incision + immersion (Inc+Imm) methods. Four treatments were applied: (1) Mix CIB®-HOM Heel-Mix (TH1), constituted by equal v/v ratio, of Cyme-Heel®, Gal-Heel®, Hepa-Heel®, Mucs-Heel® and Chol-Heel®; (2) Mix CIB®-HOM Pav-Mix (TH2), constituted by the same v/v ratio of *Passiflora incarnata, Valeriana officinalis, Zincum valerianicum* and *Ignatia amara* (Similia®); (3) Heel-Mix/Pav-Mix (TH3) consisting of a 1:1 v/v combination of the TH1 and TH2 treatments, and (4) ViT-Mix (TH4), constituted by Vidatox®, and a control (not treated/infected). **Results**. While applying the methods Imm, Inj and Inc+Imm, LD_{50} was 0.9×10^6; 0.6×10^6 and 0.5×10^6 UFC.mL^{-1}, respectively. At the end of the challenge, the groups treated with TH2, TH3 and TH4 had a greater survival rate to that of the control group ($p<0.05$). Moreover, these two last treatments showed a greater SOD activity with respect to the control group ($p<0.05$). **Conclusions**. The homeopathic treatments (TH3 and TH4) increased survival and SOD activity in *L. vannamei* juveniles challenged with *V. parahaemolyticus*, which suggests that the homeopathic treatments employed had the potential as an alternative for the control of *V. parahaemolyticus* and its associated diseases, including the early mortality syndrome in shrimp farming.

Keywords: Aquaculture homeopathy, shrimp, immune response, Vibrio parahaemolyticus.

Resumen:

Objetivo. Evaluar el efecto de medicamentos homeopáticos sobre la supervivencia y actividad de la enzima superóxido dismutasa (SOD) del camarón *Litopenaeus vannamei* sometido a infección con *Vibrio parahaemolyticus*. **Materiales y métodos.** Se determinó la dosis letal media (DL_{50}) para la cepa patógena en juveniles de *L. vannamei*, bajo los método de inmersión (Inm), inyección (Iny) e incisión + inmersión (Inc+Inm). Luego el efecto de cuatro medicamentos homeopáticos sobre juveniles de *L. vannamei* retados con *Vibrio parahaemolyticus* fue evaluado usando el índice la supervivencia y la actividad SOD. Se aplicaron cuatro tratamientos: (1) Mezcla CIB®-HOM Heel-Mix (TH1), constituido por igual proporción v/v, de Cyme-Heel®, Gal-Heel®, Hepa-Heel®, Mucs-Heel® y Chol-Heel®; (2) Mezcla CIB®-HOM Pav-Mix (TH2), constituido por igual proporción v/v de *Passiflora incarnata, Valeriana officinalis, Zincum valerianicum* e *Ignatia amara* (Similia®); (3) Heel-Mix/Pav-Mix (TH3) constituido por una combinación 1:1 v/v de los tratamientos TH1 y TH2, y (4) ViT-Mix (TH4), constituido por Vidatox®, y un control (no tratado/infectado). **Resultados**. Al aplicar los método Inm, Iny e Inc+Inm la DL_{50} fue de 0.9×10^6; 0.6×10^6 y 0.5×10^6 UFC.mL^{-1}, respectivamente. Los camarones tratados con TH3 y TH4 presentaron una mayor actividad de SOD con respecto al grupo control ($p<0.05$). Al final del reto, los grupos TH2, TH3 y TH4 tuvieron una supervivencia mayor a la del grupo control ($p<0.05$). **Conclusiones**. Los tratamientos homeopáticos (TH3 y TH4), aumentaron la actividad de la enzima SOD y la supervivencia en juveniles de *L. vannamei,* retados con *V. parahaemolyticus*. Esto sugiere que los tratamientos homeopáticos empleados tienen potencial como alternativa para el control de *V. parahaemolyticus* y sus enfermedades asociadas, incluido el síndrome de mortalidad temprana en el cultivo del camarón

Palabras clave: Camarón, homeopatía acuícola, respuesta inmune, Vibrio parahaemolyticus.

INTRODUCTION

The shrimp *Litopenaeus vannamei* is a species with rapid growth, high survival and price in the market, which makes this crustacean one of the most important resources at world level (1). Nonetheless, the production of this important resource has been hindered by recurrent epizootic outbreaks and sudden mortalities caused by pathogen microorganisms, particulary to pathogenic bacteria *Vibrio parahaemolyticus* that causes the pathology identified as "Early Mortality Syndrome" (EMS), and later called "Acute Hepatopancreatic Necrosis Syndrome" (AHPNS) caused by a pathogenic strain of *Vibrio parahaemolyticus* was observed (2). This pathology occurs during the first 30 days post-harvest and could cause up to 100% mortality (3).

To face the challenge, several chemical and antibiotic products have usually been applied (4), whose prophylactic application was initially an effective strategy. However, they have caused the development of resistant bacteria making it necessary to reduce their application (5). These problems have led the shrimp culture industry to explore and develop new strategies that would be as effective or better than antibiotics but respectful to the environment and consumers, and above all sustainably applicable in the medium and long term (6). As part of the search for new options, homeopathy has been confirmed as an alternative with the potential of disease control in farming aquatic and terrestrial animals (7). It has been catalogued as a complementary medication that uses high dilutions of substances that derive from plants, minerals, or animals, and it is based on the similarity principle (8). The animals could benefit from the use of homeopathic products because they stimulate their immune system and specific organic responses (9). Besides contributing preventively by reducing stress, homeopathic treatments can also reduce the application of chemotherapeutic agents and antibiotics avoiding risks for cultured animals, consumers, and the environment (10).

Taking this background into account, the objective of this study was to assess the effect of homeopathic medication on survival and antioxidant response in L. vannamei before infection with *V. parahaemolyticus*.

MATERIALS AND METHODS

Study site. The experiment was performed in the Mollusc Laboratory of Centro de Investigaciones Biológicas del Noroeste (CIBNOR) in La Paz, Baja California Sur, México. Juveniles of *L. vannamei* (8 ± 0.05 g) were obtained from the commercial shrimp laboratory of the company Acualcultura Mahr located in Puerto de Pichilingue, La Paz, México. The shrimp were acclimated in 1500-L fiberglass tanks with filtered seawater at 1 μm and sterilized with UV light, salinity of 37 g L^{-1}, continuous airflow and temperature of 29 ± 0.5°C for five days. The organisms were fed *ad libitum* three times a day with a balanced diet (Alimento PIASA®, Planta La Paz, B.C.S., México, 35% protein).

Bacterial strains. For the survival study, the strain *Vibrio parahaemolyticus* (CIAD-CAIM 170) was used; it was cultivated in tryptone soy broth (TSB; #257107, Difco) at 35°C for 48 h. The culture was centrifuged at 4696 g at 4°C for 10 min; the pellet was re-suspended in sterile seawater, and bacterial density was adjusted to 1.0 to obtain a final density of 1 x 10^9 UFC mL^{-1} (11).

Pathogen inoculation and average lethal dosage (LD_{50}) in *Litopenaeus vannamei*. To determine LD_{50}, a batch of shrimp was infected in groups in triplicate by means of three inoculation methods: [immersion (Imm), injection (Inj) and incision + immersion (Inc+Imm)]. For each infection method, three groups (10 shrimp each) were formed with different concentrations (10^3, 10^5, 10^7) of *V. parahaemolyticus*. For Imm, the shrimp were placed in water at the pathogen concentrations previously mentioned. For Inj the shrimp were injected in the third abdominal segment with 30 μL of each one of the bacterial concentrations already mentioned (12). For Inc+Imm, two incisions of approximately two mm in length were performed through the cuticle and the muscle of the third abdominal muscle. Then, the shrimp were immersed in seawater with the pathogen at the concentrations mentioned (13). After infection was performed, mortality was recorded in each group and replicate for the following 48 h to determine the average lethal dosage (LD_{50}) by applying the Probit analysis (14).

Preparation of homeopathic medication. The experimental treatments were prepared starting from the commercial homeopathic medicine for humans, ingestible in alcoholic dilution 30 Centesimal Hahnemaniana (30 CH) of the Laboratorios Similia® (México), Labiofam® (Cuba), and Heel® in injectable aqueous dilution of Laboratorios Rubiopharma® (México). The treatments were prepared by combining the required volume with the respective "stock dynamisation" (30 CH) of each one of their components diluted in a proportion of 1:99 to obtain "work dynamisation" (31 CH) utilizing ethanol 30oGL as vehicle, which was prepared by diluting ethanol 87oGL (Similia®, México) in distilled water.

Experimental treatments. Four treatments were used (1) Mix CIB®-HOM Heel-Mix (TH1) constituted by an equal v/v proportion of Cyme-Heel®, Gal-Heel®, Hepa-Heel®, Mucs-Heel® and Chol-Heel®; (2) Mix CIB®-HOM Pav-Mix (TH2) constituted by equal v/v proportion of *Passiflora incarnata, Valeriana officinalis, Zincum valerianicum* and *Ignatia amara* (Similia®); (3) Heel-Mix/Pav-Mix (TH3) constituted by a proportion of 1:1 v/v combination of the treatments TH1 and TH2; and (4) ViT-Mix (TH4) constituted by Vidatox®. Additionally, a fifth experimental group was included as control (non-treated/non-infected). The homeopathic treatments (31CH) prepared with ethanol 30oGL were interspersed (5% volume/weight) in pelletized commercial feed with 35% protein (PIASA®, Planta La Paz, B.C.S. México), provided *ad libitum* for seven days previous to and also during challenge. The same type of food was used for the control group, but in this case it was interspersed with ethanol 30oGL.

During the sequential dynamisation process, the dilution at 1:99 was alternated with shaking in vortex equipment (Benchmark mixer™, Benchmark Scientific Inc. Edson, NJ, U.S.A.) at 3200 rpm for two minutes applying basic principles of homeopathic pharmacopoeia. Independently of its origin, all "work" (31 CH)

dynamisations were prepared utilising ethanol 30oGL as vehicle to homogenise alcoholic content in all treatments since the medication Vidatox® (La Habana, Cuba) vehicle is ethanol 30oGL.

Experimental design. The juveniles of *L. vannamei* were distributed randomly (10 shrimp/group) in 4 L experimental units gauged with 2 L of filtered seawater and disinfected with UV at 29 ± 0.5°C with constant aeration and photoperiod of 12:12 h. These units were covered with lids under pressure fitted with an opening of 5 x 5 cm (with mosquito mesh). Such recipients were placed in a thermoregulated system at 29 ± 1°C within plastic boxes with chlorinated drinking water. The shrimp were fed the interspersed commercial diet *ad libitum* with the respective homeopathic treatments and with 30oGL ethanol for control three times a day. Particulate bottom matter of the tubs was eliminated daily by siphoning, recovering the volume (25%) of eliminated water immediately. The treatments were administered for seven days, and a challenge bioassay started from the eighth day with a total length of 120 h.

Challenge with ***Vibrio parahaemolyticus*****.** After seven days of treatment, we proceeded to challenge the shrimp with the pathogen. For this purpose, five experimental groups were formed (10 shrimp per group) and three replicates, which were placed separately in 4-L experimental units and gauged with 2-L filtered and sterilised seawater. All the groups were challenged with the pathogen V. parahaemolyticus, corresponding to the diet LD_{50} (obtained in the previous assays) except for the control group.

Determination of SOD activity. At 70 h after starting the challenge, nine shrimp from each experimental group were selected at random. Muscular tissue (100 mg) was taken from these juveniles and submerged in 500 µL of phosphate-buffered saline (PBS) pH 7.5 for subsequent determination of superoxide dismutase (SOD) activity. The sampled tissue was processed with a tissue homogeniser (Toption, model JS18, Xi'an, China). The homogenised tissue was centrifuged at 9327 g at 4°C for 10 min; once the supernatant was recovered, it was stored at -20°C for its subsequent assessment. The SOD activity was determined using a commercial kit (SOD Assay Kit-WST #19160, Sigma-Aldrich, St. Louis, MO, U.S.A.), following the instructions of the manufacturer. The results were expressed as inhibition percentage of complex formation WST-1 (water-soluble tetrazolium) formazan (15).

Statistical analysis. The data obtained were used in Kolmogorov-Smirnov test to verify normality, and Levene's test was used to analyse equality of variances. Arcsine transformation was applied to the data expressed as percentage (survival and SOD) before performing ANOVA. Tukey multiple comparison of means tests were performed to detect significant differences among the enzymatic SOD activity and survival values obtained in each one of the treatments. The differences were considered significant for $P < 0.05$. All the analyses were performed by the statistical program SPSS version 21 for Windows (SPSS Inc., Chicago II).

RESULTS

During the determination of LD_{50}, the percentage of accumulated mortality of *L. vannamei* was obtained 48 h post-infection by using different methods of infection and charge of *V. parahaemolyticus* (Table 1). The LD50 results were 0.9×10^6 CFU mL^{-1} with the immersion method; 0.6×10^6 CFU mL^{-1} with the injection method; and 0.5 x 106 CFU mL-1 with the incision + immersion method (Table 1). The dosage selected for the experimental challenge was the one that showed a mortality of 50% by the immersion method to avoid possible collateral effects of the incision.

TABLE 1

Table 1. Mortality accumulated and LD_{50} in *Litopenaeus vannamei* juveniles challenged with *Vibrio parahaemolyticus* at 48 h post-infection, using different methods to determine average lethal dosage.

Method of Infection	Charge of V. parahaemolyticus (CFU mL^{-1})	Mortality (%)	LD50 (CFU mL^{-1})
Immersion	107	67	
	105	36	0.9×10^6
	103	17	
Incision + Immersion	107	73	
	105	27	0.5×10^6
	103	27	
Injection	107	66	
	105	36	0.6×10^6
	103	16	

At the end of the challenge, 120 h post-infection, the groups treated with TH2, TH3 and TH4 showed significantly higher survival rates ($P < 0.05$) than the control group (Figure 1). The highest survival rates corresponded to TH3 and TH4 64.43% and 56%, respectively. The group treated with TH1 and the control group, showed an accumulated survival of 0% when the pathogen challenge concluded at 120 h (Figure 1).

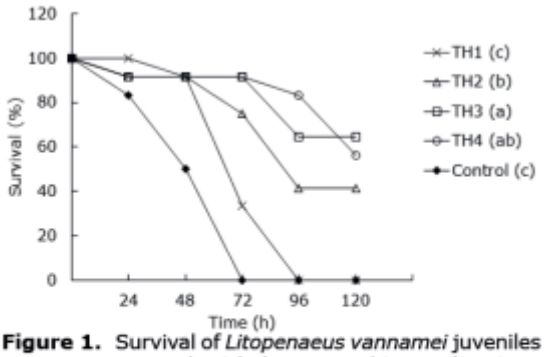

Figure 1. Survival of *Litopenaeus vannamei* juveniles treated with homeopathic medications during challenge (120 h) with *Vibrio parahaemolyticus*. Letters a, b, c indicate statistical differences between treatments and control.

FIGURE 1

Figure 1

With respect to SOD activity in shrimp *L. vannamei* tissue at 72 h after challenge, the groups treated with TH1 and TH2 did not show significant differences (34.48 ± 1.87 and 16.32 ± 1.22, respectively) in relation to the control group (41.63 ± 2.59); whereas TH3 and TH4 showed a SOD activity of 86.43 ± 1.02 and 83.47 ± 5.54, respectively, with significantly higher values ($P < 0.05$) with respect to the control group (41.63 ± 2.59) after challenge with *V. parahaemolyticus* (Figure 2).

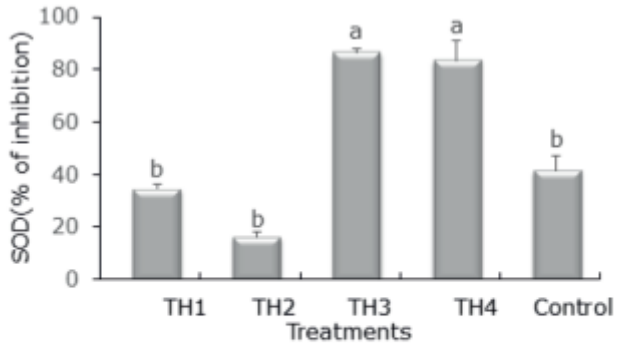

Figure 2. Activity of SOD enzyme in white shrimp *Litopenaeus vannamei* juveniles treated with homeopathic treatments at 72 h after starting challenge with *Vibrio parahaemolyticus*. Data were expressed as media ± standard deviation. Columns with different letters show difference ($p<0.05$).

FIGURE 2
Figure 2

DISCUSSION

Homeopathic medications are natural substances of animal, plant or mineral origin, highly diluted and agitated, and thus sufficiently innocuous (7), which could activate specific sensibility mechanisms in living organisms, and they do not contravene their natural defence reactions (8). Homeopathy has been used with success in aquaculture (16,17), and it has the applicability to reduce stress associated to a gradual and progressive intensification, which is habitual in modern aquaculture production systems (7,16).

The lesions and stress that cultured shrimp may suffer under adverse environmental conditions reduce their natural resistance, making them susceptible to viral and/or bacterial infections (18). In shrimp, natural infection by virulent bacteria is made by different routes: oral, transcuticular or by lesions, and it could be associated with an imbalance of their normal intestinal microbiota (19). The average lethal dosage (LD_{50}) was established to determine virulence of the pathogen strain, and it has been used to evaluate shrimp resistance or susceptibility to vibriosis (11). This study selected the LD_{50} of 0.9×10^6 UFC mL^{-1} by the immersion method because it was the least aggressive method of infection with less collateral effects that imitates the natural conditions that shrimp are exposed to the bacteria present in the marine environment (20).

The marine invertebrates, including shrimp, have developed an immune innate system based mainly in phagocytosis and generation of antimicrobial peptides and reactive oxygen species (ROS) (21). Because ROS, in their majority superoxide anions, can also affect the host tissues; invertebrates counterattack their effect by producing diverse compounds, including SOD enzymes, catalase and glutathione peroxidase (22). The activity of SOD has been referenced as a parameter to evaluate the potential of the immune system in some shrimp species (23,24).

This study has demonstrated that in the *L. vannamei* juveniles treated with the homeopathic treatments TH3 and TH4, the SOD enzyme activity increased significantly at 70 h post-infection with *V. parahaemolyticus* with respect to the control group. Whereas shrimp treated with TH1 and TH2, their response was similar to that of the control group. Treatment TH3 included the formulation of the medication Passival®, used in human medicine as tranquiliser to reduce stress and improve sleep besides the mixture Heel-Mix, which included medication used for enzymatic disorder, infectious diseases and

stimulation of organism defence. Treatment TH4 is a homeopathic medication whose active principle is the venom of the *Rhopalurus junceus* scorpion, an endemic species to Cuba. Anti-tumour properties have been attributed to this medicine shown through some preclinical toxicity studies (25) on tumour cells of epithelial origin (26) and an immunomodulatory effect that increases production of white cells and interleukins (27).

The greatest survival recorded in animals that received treatments TH2, TH3 and TH4 after *V. parahaemolyticus* challenge indicated a protector effect, which shows that the homeopathic medications assessed could be an alternative tool for controlling bacterial diseases in shrimp farming. In relation to this study, the bacteria of the genus *Vibrio* are well known as pathogens of marine shrimp, so it is possible to assume that a greater survival of the shrimp treated with homeopathic medications is related to the stimulation of their immunological systems, and in consequence to a greater resistance to acute infectious diseases associated to this genus, for example, the early mortality syndrome or acute hepatopancreatic necrosis syndrome (EMS/AHNPS) (28,29).

In conclusion the homeopathic medicine included in the treatments TH3 and TH4, increased survival and immunity of the shrimp *L. vannamei* juveniles when infected with *V. parahaemolyticus.* These results contribute to the generation of new knowledge that helps to confirm that aquaculture homeopathy is a potential and eco-sustainable alternative facing the use and abuse of chemotherapeutic agents and antibiotics used to attack certain pathogens, including EMS/AHNPS increasing productivity in shrimp industry at world level.

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