

Research Article

## Effects of highly-diluted bioactive compounds (HDBC) on growth, survival and physiological condition of *Penaeus vannamei* shrimp reared in a commercial farm

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**ABSTRACT.** This study evaluates the application of highly-diluted bioactive compounds (HDBC) as an alternative to improve the white shrimp *Penaeus vannamei* performance reared during a semi-intensive commercial-scale production cycle (130 days) in arid environmental conditions. The effects of HDBC were analyzed on productive performance, enzyme activity, and energy reserves associated with the nutritional condition. Six 10-ha earthen ponds were used. Two experimental HDBC treatments and one control treatment were evaluated: [T1 = PhA+SiT (phosphoric acid 7C and *Silicea terra* 7C); T2 = PaV+ViT (Passival<sup>®</sup> 7C and Vidatox<sup>®</sup> 31C); T3 = Control (No HDBC)], using 2 ponds × 3 treatments = 6 ponds. Three shrimp samples were taken (S1, S2, S3) at 7, 52, and 130 days. The results indicated that organisms from T2 significantly increased in several productive indicators, such as weight gain, daily weight gain, specific growth rate, total length, feed efficiency, and productive performance. T2's bioactive compounds ingested with the pelleted feed and present in the gut -even at highly diluted concentration- improved energetic reserves, such as lipids in the hepatopancreas and digestive enzyme activity, enhancing the physiological status of shrimp farmed under semi-intensive rearing conditions, suggest that HDBC used in this study could have a beneficial and eco-friendly impact on commercial-scale shrimp production.

**Keywords:** *Penaeus vannamei*; aquacultural homeopathy; shrimp aquaculture; semi-intensive system; physiological performance

### INTRODUCTION

In recent years shrimp aquaculture production has increased due to higher stocking densities, either intensive or semi-intensive rearing systems (Duan et al. 2017). Environmental factors, such as salinity, temperature, and dissolved oxygen, greatly affected the shrimp farm's growth, survival, and physiological performance under semi-intensive and intensive stress conditions (Wang et al. 2019). Commonly, intensification results in failures reflected in postlarvae (PL) survival decrease (PL and bacterial and viral pathogen proliferation with the occurrence of their related diseases, Wang et al. 2019). Highly pathogenic and virulent bacteria, such as *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, and *V.*

*vulnificus* have undermined shrimp aquaculture (Martínez-Porchas & Martínez-Córdova 2012, Tzuc et al. 2014).

The use of antibiotics and other chemotherapeutics has been the most important and effective strategy to prevent, control and treat bacterial infections and their related problems in aquaculture production (Mazón-Suástegui et al. 2018a,b). However, the use and abuse of antibiotics may negatively impact environmental integrity, crop, and human health innocuity (Seenivasan et al. 2012). From the preceding, environmental and economic considerations have driven the need to improve reared shrimp management and performance with natural and eco-friendly alternative treatments, such as highly-diluted bioactive compounds (HDBC).

The poor PL quality and decreasing shrimp prices have resulted in considerable economic pressures on shrimp farming (FAO 2020). If shrimp farmers continue running a profitable business, they must improve organism management and reduce environmental impacts. These problems have motivated the shrimp industry to explore and develop equally good or even better control strategies than antibiotics and other chemotherapeutics. These alternatives could also be respectful of the environment and sustainable application in the medium and long terms.

As part of the search for new alternatives, HDBC has emerged as an optional treatment with a great and sustainable potential for disease control in farming aquatic, marine, and terrestrial organisms, including plants (Ortiz-Cornejo et al. 2017, Mazón-Suástegui et al. 2018a,b, 2019a). HDBCs are very close to the homeopathic medicine concept. They are high serial dilutions (decimal, centesimal, millesimal, and up) of bioactive substances and compounds derived from plants, minerals, or animals. Their application in human or veterinary medicine -even in aquaculture (Mazón-Suástegui et al. 2018a,b) and organic agriculture (Mazón-Suástegui et al. 2019a)- is based on the principle of similarity, hormesis and minimum doses (Khuda-Bukhsh & Pathak 2008, López-Carvallo et al. 2019).

Farmed animals, including shrimp, could benefit because HDBC stimulates and enhance their immune system and induce specific and beneficial organic responses (Vockeroth 1999, Mazón-Suástegui et al. 2018a,b). Because of their close similarity to homeopathic medicines, HDBC can prophylactically reduce stress, use chemotherapeutic agents and antibiotics, and avoid and reduce risks for crop species, consumers, and the environment (Siena et al. 2010, Ortiz-Cornejo et al. 2017).

Several HDBC treatments have been tested in shrimp to evaluate postlarval *Penaeus vannamei* growth and survival before a challenge bioassay against pathogenic and virulent strains of *V. parahaeolyticus* under biosecure laboratory conditions (Mazón-Suástegui et al. 2018a,b). These authors observed significantly higher average survival and superoxide dismutase (SOD) activity, suggesting that these treatments had potential as an alternative to control infections and their associated diseases in farmed shrimp.

The hepatopancreas is a critical organ in metabolism and nutrient absorption; its study evaluates the state of stress and health condition in shrimp. The physiological conditions can be evaluated using metabolic response variables such as energetic reserves (carbohydrates, proteins, and lipids) in muscle and

hepatopancreas (Racotta & Palacios 1998, Cuzón et al. 2000). Furthermore, histological analyses of the hepatopancreas can be used to monitor the impact of the stressed environment in early shrimp stages (Collins 2010, Wang et al. 2019).

This commercial-scale bioassay intends to assess the potential use of HDBC on white shrimp *P. vannamei* aquaculture and their effects on growth, survival, and physiological condition by analyzing the hepatopancreas and other response variables in a semi-intensive commercial farm located in an arid region of northwest Mexico.

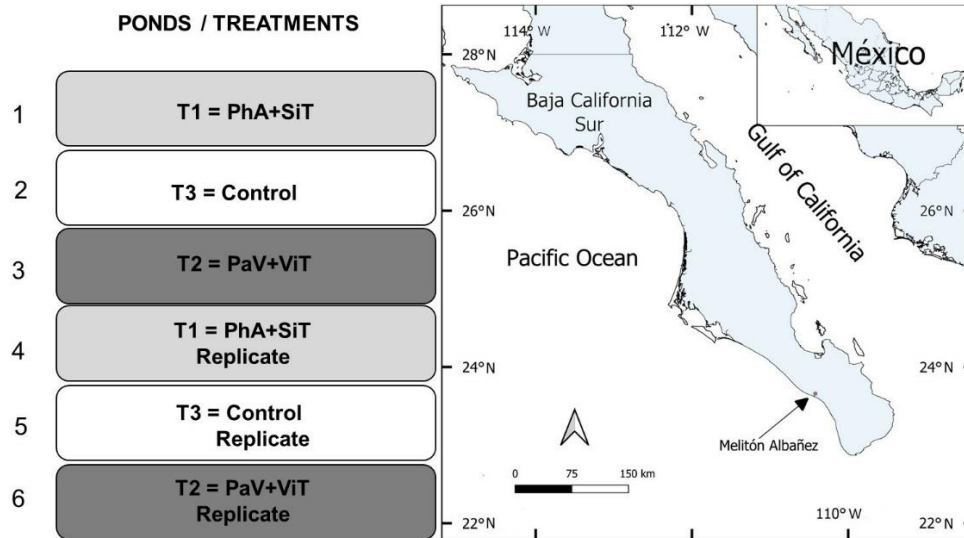
## MATERIALS AND METHODS

### Farm location and experimental conditions

The study was conducted under semi-intensive rearing conditions throughout a commercial aquaculture cycle of *Penaeus vannamei* on a farm established in a semi-desert region, property of "BCS Camarón", a company located in Ejido Melitón Albañez Domínguez (23°39'41"N, 110°25'15"W), a small village of Baja California Sur, Mexico (Fig. 1). The commercial farm has six rustic ponds of 10 ha each. It normally operates in hypersaline conditions, performing a "long rearing cycle" (160 days) with partial harvest depending on shrimp size and market conditions.

The experimental evaluation period began with juveniles with an average body weight of  $2.0 \pm 0.07$  g at a density of 8 ind m<sup>-2</sup>. The six ponds were defined as "Independent Experimental Units of 10 ha each", and this commercial-scale bioassay was conducted for 130 days. The experimental design consisted of two HDBC treatments (T1, T2), each one based on a mixture of two HDBC [T1 = PhA+SiT (phosphoric acid 7CH + *Silicea terra* 7CH); T2 = PaV+ViT (Passival® 7CH+Vidatox® 31CH)] and a control treatment [T3 = Control (no HDBC)]. T2 included Passival® formula (*Passiflora incarnata* 7CH, *Valeriana officinalis* 7CH, *Zincum valerianicum* 7CH, and *Ignatia amara* 7CH) and Vidatox®, formulated from the venom of the Cuban scorpion *Rhopalurus junceus*. Two independent experimental units (10 ha each) per treatment were used, one of which was considered a replicate (Fig. 1). Thus, each treatment was applied in two ponds (2×10 = 20 ha).

As previously stated, T1 and T2 were formulated with two HDBCs in the form of highly diluted and succussed homeopathic medicines. These medicines are authorized for humans by official institutions, such as the Health Ministry and Federal Commission for the Protection Against Sanitary Risks (COFEPRIS, by its Spanish acronym) in Mexico. They must be prepared by an authorized pharmacy in the ethanolic vehicle,



**Figure 1.** Experimental design and location of the shrimp farm “BCS Camarón” (BCS Camarón Company) in Melitón Albañez, La Paz, Baja California Sur, Mexico.

under procedures of the Homeopathic Pharmacopoeia of the Mexican United States (SSA 2015) and its similar entity in Cuba for Vidatox<sup>®</sup>.

T1 (PhA+SiT) was formulated (1:1 v/v) by centesimal (1:99) dilution/succussion in distilled water vehicle from phosphoricum acid 6C (PhA-6C) and *S. terra* 6C (SiT-6C), which have as dilution vehicle ethanol 87 °GL (Similia<sup>®</sup>, Farmacia Homeopática Nacional<sup>®</sup>, CDMX, MX). Therefore, T1 treatment corresponded to a mixture of these two components in the seventh centesimal Hahnemannian dilution (PhA-7C+SiT-7C).

Passival<sup>®</sup> includes homeopathic medicines: *P. incarnata*, *V. officinalis*, *I. amara*, and *Z. valerianicum*. T2 (PaV+ViT) was also formulated (1:1 v/v) utilizing a centesimal dilution (1:99), dilution/succussion in distilled water vehicle from Passival<sup>®</sup> 6C (PaV-6C), which have as dilution vehicle ethanol 87°GL (Farmacia Homeopática Nacional<sup>®</sup>, CDMX, MX), and Vidatox<sup>®</sup> 30C (ViT-30C) vehicle is a 30% hydroalcoholic solution (Labiofam<sup>®</sup>, Havana, CU). Vidatox<sup>®</sup> is produced from the venom of the Cuban blue scorpion *R. junceus*. Therefore, T2 corresponded to a mixture of a seventh Hahnemannian centesimal dilution of PaV and a 31st centesimal Hahnemannian dilution of ViT (PaV-7CH+ViT-31CH).

HDBC administration is based on the hormesis principle, which establishes that high concentrate doses of a substance generate a harmful negative response while ultra-diluted low doses of the same substance induce a contrary and favorable response (Endler et al. 2015, López-Carvalho et al. 2020). During the

preparation of HDBC by a licensed pharmacy, several dilutions must be made from an initial concentrated solution or mother tincture (MT) and alternated with vigorous shaking or succussion. The purpose is to obtain very low doses but sufficient to incite different physiological, genomic, and transcriptomic responses in treated organisms to increase their defenses naturally and strengthen their immune system (Bellavite & Signorini 2002, López-Carvalho et al. 2020).

T1 components (phosphoricum acid and *S. terra*) are used in humans to promote nutrition and food assimilation. However, when applied to juvenile horse mackerel *Seriola rivoliana*, they stimulate intestinal maturation, trypsin activity, and growth (Mazón-Suástegui et al. 2019a). According to these authors, PhA-SiT can be applied in larval stages to improve digestion and assimilation of inert food in juveniles of the species when weaning, a critical moment because live food must be replaced with artificial food. In juvenile Catarina scallop (*Argopecten ventricosus*), *S. terra* and homeopathic phosphoric acid increased growth, survival and several biochemical reserves in the digestive gland, and the number of hemocytes in hemolymph (López-Carvalho et al. 2019), inducing differential expression of genes associated with the immune system and non-self recognition (López-Carvalho et al. 2020).

The T2 (PaV+ViT) includes the formulation of Passival<sup>®</sup>, a commercial homeopathic drug whose main therapeutic use in human medicine is a natural tranquilizer to reduce stress and promote sleep and relaxation. This T2 component may have an anti-stress

effect on shrimp grown under a semi-intensive system and stressful hypersaline conditions (Mazón-Suástegui et al. 2018b). A second element (ViT) contained in T2 was the homeopathic drug Vidatox® (Labiofam®, Habana, CU), to which antitumor effects have been attributed (Díaz-García et al. 2013) because it acts on epithelial cells (Díaz-García et al. 2010), in addition to having immunomodulatory properties that increase white cells and produce interleukins (Hernández-Betancourt et al. 2009).

The homeopathic procedure of serial dilution (decimal 1:9; centesimal 1:99, millesimal 1:999, and higher), alternated with vigorous shaking, also called succussion, is known as "dynamization" or "potentization". It is common for these dilutions to be identified as dynamizations or potentizations of a given drug to the general public. In this case, dilution was performed on a centesimal scale ( $C = 1:99$ ), using distilled water as the dilution vehicle to avoid the side effects of alcohol on shrimp.

HDBC and control treatments were sprayed (1.5 L/25 kg) in pelleted commercial feed (Nutrimentos Acuícolas AZTECA MP, Sinaloa, MX; 25% crude protein) using a concrete mixer machine with a new plastic container to avoid contamination from any previous use. The treated (T1, T2) and un-treated (T3) feed was prepared and distributed in shrimp ponds (T1 and T2) first thing in the morning and once a day, from Monday to Saturday, to favor ingestion. In addition to the pelleted feed, the diet of reared shrimp included nutritional components derived from the natural productivity that occurred in each pond. The balanced feed daily rations were recommended for shrimp in the standard feeding tables, according to periodic population evaluations performed by the shrimp company (BCS Camarón) and bimonthly adjustments according to organism growth starting with  $40 \text{ kg pond}^{-1} \text{ d}^{-1}$ .

Daily evaluations of temperature ( $^{\circ}\text{C}$ ), salinity, and dissolved oxygen (DO) by the Winkler titration method (DO,  $\text{mg L}^{-1}$ ) were monitored in each of the ponds. The experimental evaluation period lasted 130 days, during which three samples of organisms were taken: an initial seven-day sampling (S1), a second 52-day sampling (S2), and a third 130-day sampling (S3) after applying the treatments. The total number of individuals evaluated was 1440 ind, distributed in  $80 \text{ ind tank}^{-1} \text{ sampling}^{-1}$ .

All shrimp used in this research were handled following the Official Mexican Standard protocols (NOM-062-ZOO-1999) and the Internal Committee for the Care and Use of Laboratory Animals (CICUAL) to minimize organisms suffering and pain. Organisms were kept in optimal rearing conditions to avoid stress; no harmful effect was detected using HDBC in marine organisms.

## Morphological evaluations

### Growth and survival

Growth biometric measurements corresponded to each sampled shrimp's total weight (TW, g) and total length (TL, cm). TW was obtained in an analytical balance with an accurate 0.01 g (OHAUS® SP602AM, USA) and TL with a measuring ruler (0.1 mm accuracy) from the ocular lobule to the tip of the telson.

The farmers calculated survival (S, %) in each of the six ponds at the end of the experimental period by the following formula:

Survival (%) = (initial number of shrimp - number of dead shrimp) / initial number of shrimp  $\times 100$ .

Additional parameters associated with productive performance were estimated according to Martínez-Antonio et al. (2019), such as relative weight gain (WG, %) = ((final weight (g) - initial weight (g)  $\times 100$ ) / initial weight (g); specific growth rate (SGR,  $\% \text{ d}^{-1}$ ) =  $100 \times (\ln \text{ final weight (g)} - \ln \text{ initial weight (g)}) / \text{days of experiment}$ ; feed conversion ratio (FCR) = feed consumption (dry matter) (g) / WG (g), and finally productive performance (PP) was calculated using the following formula:  $\text{PP} = (\text{S} \times \text{SGR}) / \text{FCR}$ .

Fulton's equation (Chow & Sandifer 1991, Valenzuela-Madriral et al. 2017) was used to determine the condition factor (CF<sub>i</sub>) of each experimental group:

$$\text{CF}_i = (\bar{W}_i \times 100) / \bar{TL}^3$$

where CF<sub>i</sub>: condition factor for treatment i;  $\bar{W}_i$ : average weight (g); and  $\bar{TL}$ : average total length (mm).

## Physiological evaluations

### Tissue processing

From each freshly sampled shrimp, muscle and hepatopancreas tissues were carefully dissected, fixed in liquid nitrogen, and later stored at  $-80^{\circ}\text{C}$  to evaluate the physiological condition of each shrimp through the analyses of different biochemical reserves and activity of digestive enzymes.

### Biochemical analysis of muscle and hepatopancreas

Muscle and hepatopancreas tissues preserved at  $-80^{\circ}\text{C}$  were placed in a VirtTis lyophilizer (BenchTop 3.5, NY, USA) at a maximum temperature of  $-30^{\circ}\text{C}$  and a maximum pressure of 100 mTorr, conditions suitable for an optimal sublimation process of the water present in tissues. Later, they were weighed, re-hydrated in 1 mL of cold distilled water, and homogenized to obtain crude extracts that were used to quantify the content of total protein (TP), total carbohydrate (TCH), and total lipid (TLD). The biochemical composition of each sample was assessed in triplicate and expressed in g dry

weight. Determination of TP was done using a bicinchoninic acid protein (BCA) reagent (Sigma-Aldrich #B9643, St. Louis, MO, USA) and the method of Smith et al. (1985), which uses bovine serum albumin (Sigma-Aldrich #9048-46-8, St. Louis, MO, USA) as the standard to perform a calibration curve; absorbance was read at 562 nm. TCH content was determined using a reagent blank and dextrose solution as the standard (Vedco #3803) to prepare a calibration curve according to the method described by Roe et al. (1961); absorbance was read at 630 nm. TLD content in tissues was determined by the sulphophosphovanillin method following a modified version of Barnes & Blackstock (1973) in a microplate with 20  $\mu$ L supernatant extract previously digested with sulfuric acid, 200  $\mu$ L phosphor-vanillin 0.2%, and sulfuric acid 80% as lipid reactive solution. A solution of 20 mg mL<sup>-1</sup> of lipids was used as a standard solution to prepare a calibration curve.

### Enzymatic analysis of hepatopancreas

To assess the digestive enzyme activity of amylase, protease, and lipase, hepatopancreas samples stored at -80°C were homogenized in distilled water, followed by double centrifugation at 12,000 rpm at 4°C for 10 min. The supernatants were transferred to new tubes and used as crude enzyme sources. The protein content of crude extracts was determined by Bradford's method (Bradford 1976), using bovine serum albumin as standard. Protease activity was determined with azocasein (1% in 50 mM Tris-HCl, pH 7.5) as substrate, according to Vega-Villasante et al. (1995). Lipase activity was analyzed using  $\beta$ -naphthyl-caprylate as substrate, according to Versaw et al. (1989). Amylase activity was estimated using starch (1%) as substrate, according to Nolasco & Vega-Villasante (1992). All digestive enzyme activities are expressed as specific activity (U mg<sup>-1</sup> protein).

### Histological analysis of hepatopancreas

The entire cephalothoracic region up to the first abdominal segment of 30 shrimp of each treatment was individually fixed in Davidson solution (Humanson 1972) for 24 h, dehydrated, cleared, and embedded in a paraffin-paraplast mixture (Bell & Lightner 1988). Transverse sections of the hepatopancreas (6  $\mu$ m in thickness) from the cephalothoracic region located at the approximate midpoint (anterior-posterior) were obtained using a rotary microtome (Leica RM 2155 Leica Microsystems, GmbH, Wetzlar, DE) mounted on glass slides and stained with Harris-haematoxylin-alcoholic eosin solution (SJV Grupo Bioquímico Médico, MX) with a multistainer (Leica ST5020; Leica Microsystems, GmbH, Wetzlar, DE). Each slide was

examined under a microscope (Olympus BX50, Olympus Optical, JP) connected to a video camera (CoolSNAP-ProColor, Olympus Optical, JP) for quantitative histological analyses. Images were digitalized at high resolution (600 dpi; 40x) using the Image-Pro Plus version 9.0 software (Media Cybernetics, Bethesda, MD, USA). Four transects (vertical and horizontal) on each histological image were traced to obtain a square surface containing the complete hepatopancreas tissue. The hepatopancreas coverage index (HCI) represents the total area ( $\mu$ m<sup>2</sup>) occupied by the hepatopancreas in the cephalothorax. It was obtained and estimated by the hepatopancreas (HCA) and cephalothorax (CCA) coverage areas that form the complete surface area ( $\mu$ m<sup>2</sup>) of the cephalothorax. Therefore, HCI was obtained by using the following formula: HCI (%) = (HCA / CCA)  $\times$  100.

### Histochemical analysis of hepatopancreas

Hepatopancreas tissue slides were also stained with Sudan black B (Bayliss 1984), Sudan black stained triglycerides (TG) in shades ranging from dark blue to black, and phospholipids (ph) in shades of gray (Bayliss & Adams 1972, Rodríguez-MoscOSO & Arnaiz 1998). Coverslips were applied with Entellan mounting media (Sheehan & Hrapchak 1980). Slides were observed under a standard light microscope equipped with a CoolSNAP-pro color imaging camera (Roper Scientific, Tucson, AR, USA). Images were obtained with the camera and digitalized at high resolution (600 dpi; 40x) with Image-Pro Premier 9.0 software (Media Cybernetics, Rockville, MD, USA). For each hepatopancreas slide, three images were obtained and processed. Digital image analysis was based on pixel-specific color staining. For each shrimp, a lipid index (LI) was estimated by the formula of Rodríguez-Jaramillo et al. (2008): LI = (SAC / AT)  $\times$  100; where SAC is the total coverage area of hepatopancreas, and AT is the total area of each cell type in the hepatopancreas, both expressed in  $\mu$ m<sup>2</sup>. The pixel-specific color proportion of the total area of each cell type represented a proportion of the lipids in each hepatopancreas section.

### Statistical analyses

All data were subjected to a Shapiro-Wilk normality test ( $P < 0.05$ ) and Levene's homoscedasticity test ( $P < 0.05$ ). Quantitative data that did not meet the assumptions of normality and homoscedasticity were transformed using the log<sub>10</sub> function. Data in percentage (LI) were transformed using the arcsine function (Zar 2010). With the transformed data, a one-way analysis of variance (ANOVA) was performed using GLM (generalized linear model), considering the treatments (T1, T2, T3) as a factor, and as dependent

variables, morphometric data, parameters associated with productive performance, biochemical and histochemical reserve values, and enzymatic activity data obtained of shrimp from each pond (independent experimental unit). The significance level was preset at  $P < 0.05$  for all analyses. A Tukey test is *a posteriori* test applied for cases with significant statistical differences. All data were analyzed using Statistical software version 10 (StatSoft, Tulsa, OK, USA). The results are shown as mean  $\pm$  standard deviation.

## RESULTS

### Morphological evaluations

#### Growth

The ANOVA showed significant differences ( $P < 0.05$ ) in TW (g), WG (%), and SGR (% d<sup>-1</sup>) among treatments in the second (S2; 52 days) and third (S3; 130 days) samplings. In terms of WG, shrimp that received T2 (PaV+ViT) had reached an average gain of 16.31 g at the second (S2) and third (S3) samplings, a better average gain of 19.75 g (T2) (Table 1, Fig. 2).

Regarding growth in size (TL) for S1 (7 days) and S2 (52 days) samplings, no differences were found among treatments, but significant differences ( $P < 0.05$ ) were observed at the third (S3; 130 days) sampling. The shrimp that received T2 showed greater TL with an average of 15.81 cm (Fig. 2).

#### Survival

Survival was significantly ( $P < 0.05$ ) higher in ponds of T3 (Control) (67.47%) than those of T2 (66.53%) and T1 (PhA+SiT) (55.14%), (Fig. 2).

### Productive performance parameters

At the end of the 130 days evaluation (S3), the results of productive performance parameters (Table 1) indicated that shrimp from T2 and T1 had a significant high ( $P < 0.05$ ) SGR, FCR and CFi. With respect to these results, SGR of shrimp from T2 was higher (1.67) compared to T1 (1.62) and T3 (1.56). FCR was significantly higher in shrimp from T2 (1.49) with respect to those of T1 (1.33) and T3 (1.24). CFi was higher in shrimp from T2 (0.60) and T1 (0.59) compared to shrimp treated with T3 (0.49).

The productive performance (PP) of shrimp treated with T2 was also higher (97.47) than the one obtained for shrimp of T1 (74.28) and T3 (73.85) (Fig. 2).

### Physiological evaluations

#### Biochemical analyses

Table 2 shows the values of the biochemical reserves in the hepatopancreas and muscle. TCH and TP levels in

the hepatopancreas of organisms subjected to the treatments did not differ at sampling times. Regarding TLD content, the ANOVA results indicated that shrimp from T3 had significantly ( $P < 0.05$ ) lower content ( $276.98 \pm 58.21$  mg g<sup>-1</sup>) than that from T1 ( $386.41 \pm 45.48$  mg g<sup>-1</sup>) and T2 ( $480.67 \pm 64.67$  mg g<sup>-1</sup>) at the initial S1 (7 days) sampling.

At the second S2 (52 days) sampling shrimp treated with T2 had also higher levels ( $417.30 \pm 61.58$  mg g<sup>-1</sup>) than those from T1 ( $411.71 \pm 56.35$  mg g<sup>-1</sup>) and T3 ( $316.57 \pm 50.58$  mg g<sup>-1</sup>). No significant differences among treatments were recorded at the third S3 (130 days) sampling

Regarding the biochemical reserves analyzed in muscle (Table 2), no significant differences were observed in TCH and TLD contents among shrimp of the three treatments. For TP, only at the third S3 (130 days) sampling differences ( $P < 0.05$ ) were observed; shrimp from T2 showed high content ( $683.79 \pm 82.81$  mg g<sup>-1</sup>) compared to those of T1 ( $641.60 \pm 76.15$  mg g<sup>-1</sup>) and T3 ( $626.02 \pm 73.20$  mg g<sup>-1</sup>) treatments.

#### Enzymatic analyses

The results related to the digestive enzymes are shown in Table 3. No significant differences were observed in shrimp's protease, amylase, and lipase activities with the treatments at the three samplings performed.

### Histological analyses of hepatopancreas

Figure 3 shows the HCI estimated in shrimp subjected to HDBC treatments and control (T1, T2, and T3). The initial sampling (S1: 7 days) did not show significant differences. However, at the second sampling (S2: 52 days) the average HCI value was significantly higher ( $P < 0.05$ ) in shrimp treated with T2 ( $32.34 \pm 0.64\%$ ) with respect to those of T1 ( $28.05 \pm 0.85\%$ ) and T3 ( $26.58 \pm 0.54\%$ ). At the third sampling (S3: 130 days), the HCI of shrimp treated with T2 ( $30.72 \pm 0.80\%$ ) and T1 ( $30.80 \pm 0.71\%$ ) were significantly higher than those of T3 ( $25.75 \pm 0.46\%$ ).

### Histochemical analyses of hepatopancreas

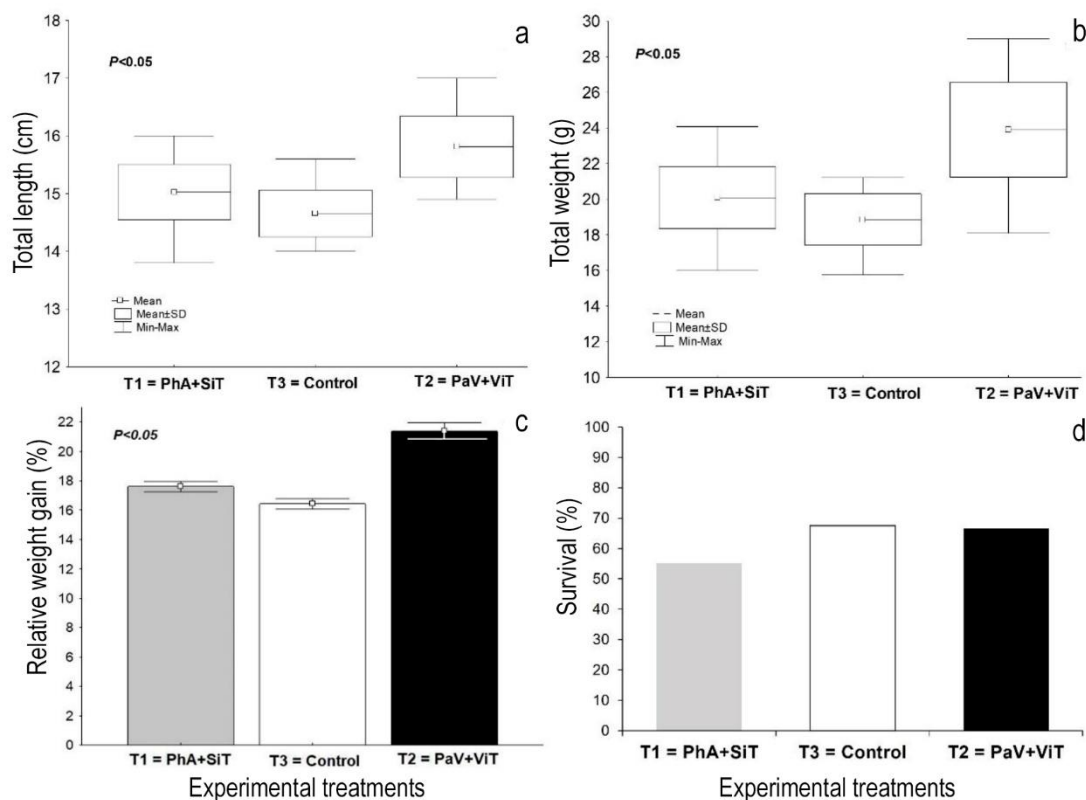
Regarding the LI (%) index, the results (Table 2) indicated that shrimp from the three treatments did not show differences at the initial sampling (S1: 7 days), but at the second (S2: 52 day) and third (S3: 130 days) samplings, LI index was significantly higher ( $P < 0.05$ ) in shrimp from T2 ( $45.06 \pm 5.92\%$ ;  $47.44 \pm 5.41\%$ , respectively) and T1 ( $41.33 \pm 5.54\%$ ;  $45.05 \pm 5.17\%$ , respectively) compared to T3 ( $34.11 \pm 5.92\%$ ;  $38.79 \pm 6.00\%$  respectively).

### Water quality parameters of shrimp ponds

Changes in water quality parameters of shrimp ponds during the experimental period (130 days) of the

**Table 1.** Productive performance parameters (mean  $\pm$  standard deviation) of *Penaeus vannamei* shrimp treated with highly diluted bioactive compounds (HDBC) under commercial semi-intensive farming conditions. Two ponds were being used (2 $\times$ 3 treatment = 6 ponds) and a total area of 20 ha per treatment. After Tukey's test, different letters indicate significant differences ( $P < 0.05$ ).

Parameters	Sample time (d)	Experimental groups			ANOVA ( $P$ -value)
		T1 = PhA-SiT	T2 = PaV-ViT	T3 = Control	
Specific growth rate (% d <sup>-1</sup> )	S3(130)	1.62 $\pm$ 0.14 <sup>ab</sup>	1.67 $\pm$ 0.17 <sup>a</sup>	1.56 $\pm$ 0.12 <sup>c</sup>	0.021
Feed conversion rate	S3(130)	1.33 $\pm$ 0.04 <sup>b</sup>	1.49 $\pm$ 0.04 <sup>a</sup>	1.24 $\pm$ 0.21 <sup>c</sup>	0.025
Condition factor	S3(130)	0.59 $\pm$ 0.05 <sup>a</sup>	0.60 $\pm$ 0.03 <sup>a</sup>	0.49 $\pm$ 0.03 <sup>b</sup>	0.014



**Figure 2.** Parameters associated with growth: a) total length and b) total weight, productive performance, c) relative weight gain, and d) survival, recorded at the end of the experimental period (S3: 130 days) in *Penaeus vannamei* shrimp subjected to treatments of highly diluted bioactive compounds (HDBC) in a semi-intensive commercial farm. After Tukey's test, different letters indicate significant differences ( $P < 0.05$ ).

production cycle are shown (Fig. 4). The temperature ( $^{\circ}\text{C}$ ), water in all ponds remained below the optimum range during the first 40 days and subsequently remained within the optimum range ( $28^{\circ}\text{C}$ - $32^{\circ}\text{C}$ ), except for days 119 to 122, during which the temperature was below the optimum range; daily oscillation was of about  $4^{\circ}\text{C}$ . Dissolved oxygen concentration had a highly dynamic fluctuation, but levels in all ponds remained within the optimum range ( $4$ - $10\text{ mg L}^{-1}$ ). Salinity had a significant progressive

increase in all ponds, reaching 75. No statistical differences ( $P < 0.05$ ) were observed in the levels of water physicochemical parameters among rearing ponds.

## DISCUSSION

To avoid the use and abuse of chemotherapeutics, such as antibiotics in aquaculture facilities, scientists and technologists have been searching for sustainable

**Table 2.** Biochemical reserves (mean  $\pm$  standard deviation) in hepatopancreas and muscle of *Penaeus vannamei* shrimp, subjected to highly diluted bioactive compound (HDBC) treatments in a commercial semi-intensive farm. After Tukey's test, different letters in the same raw indicate significant differences ( $P < 0.05$ ).

Biochemical reserves	Sample time (d)	Experimental groups			ANOVA ( $P$ -value)
		T1 = PhA-SiT	T2 = PaV-ViT	T3 = Control	
<b>Hepatopancreas</b>					
Total carbohydrates (mg g <sup>-1</sup> )	S1(7)	29.69 $\pm$ 6.23	32.61 $\pm$ 5.88	29.89 $\pm$ 7.79	0.424
	S2(52)	45.02 $\pm$ 8.88	41.18 $\pm$ 5.99	42.39 $\pm$ 6.83	0.700
	S3(130)	39.98 $\pm$ 4.42	35.33 $\pm$ 5.44	42.82 $\pm$ 5.47	0.256
Total protein (mg g <sup>-1</sup> )	S1(7)	246 $\pm$ 48.93	240.00 $\pm$ 56.69	238.90 $\pm$ 36.30	0.997
	S2(52)	257.23 $\pm$ 44.69	263.12 $\pm$ 47.75	290.16 $\pm$ 54.96	0.318
	S3(130)	241.09 $\pm$ 51.74	251.73 $\pm$ 53.32	244.41 $\pm$ 59.67	0.728
Total lipids content (mg g <sup>-1</sup> )	S1(7)	386.41 $\pm$ 45.48 <sup>ab</sup>	480.67 $\pm$ 64.41 <sup>a</sup>	276.98 $\pm$ 58.21 <sup>b</sup>	0.001
	S2(52)	411.71 $\pm$ 56.35 <sup>b</sup>	417.30 $\pm$ 61.58 <sup>b</sup>	316.57 $\pm$ 50.58 <sup>a</sup>	0.021
	S3(130)	431.06 $\pm$ 48.22	407.12 $\pm$ 69.67	428.49 $\pm$ 51.38	0.753
Lipid index (%)	S1(7)	42.56 $\pm$ 6.65	42.82 $\pm$ 7.23	39.49 $\pm$ 6.57	0.424
	S2(52)	41.33 $\pm$ 5.54 <sup>b</sup>	45.06 $\pm$ 5.92 <sup>b</sup>	34.11 $\pm$ 5.92 <sup>a</sup>	0.021
	S3(130)	45.05 $\pm$ 5.17 <sup>b</sup>	47.44 $\pm$ 5.41 <sup>b</sup>	38.79 $\pm$ 6.00 <sup>a</sup>	0.026
<b>Muscle</b>					
Total carbohydrates (mg g <sup>-1</sup> )	S1(7)	44.45 $\pm$ 8.92	47.04 $\pm$ 11.51	42.88 $\pm$ 11.06	0.952
	S2(52)	41.53 $\pm$ 8.98	45.91 $\pm$ 10.99	49.76 $\pm$ 9.05	0.445
	S3(130)	50.51 $\pm$ 14.06	50.38 $\pm$ 11.70	47.73 $\pm$ 12.02	0.612
Total protein (mg g <sup>-1</sup> )	S1(7)	521.63 $\pm$ 31.76	509.04 $\pm$ 42.35	515.26 $\pm$ 47.11	0.887
	S2(52)	595.07 $\pm$ 62.21	580.61 $\pm$ 58.27	605.99 $\pm$ 57.85	0.871
	S3(130)	641.60 $\pm$ 76.15 <sup>b</sup>	683.79 $\pm$ 82.81 <sup>a</sup>	626.02 $\pm$ 73.20 <sup>b</sup>	0.040
Total lipids content (mg g <sup>-1</sup> )	S1(7)	29.09 $\pm$ 6.07	28.35 $\pm$ 11.59	29.70 $\pm$ 8.60	0.830
	S2(52)	42.75 $\pm$ 9.98	38.58 $\pm$ 10.59	38.21 $\pm$ 12.72	0.209
	S3(130)	35.18 $\pm$ 10.79	33.88 $\pm$ 12.88	35.54 $\pm$ 16.90	0.935

alternatives as immune stimulants (Wang et al. 2017), probiotics (Prado et al. 2010, Abasolo-Pacheco et al. 2017, García-Bernal et al. 2018, 2020, Mazón-Suástegui et al. 2019b), antimicrobial essential oils (Romero et al. 2012). Recently a new alternative has been proposed: aquacultural homeopathy (Mazón-Suástegui et al. 2017, 2018a,b,c,d, 2019c,d, Rosero-García et al. 2019, López-Carvalho et al. 2019), which precisely consists of the application of HDBC (López-Carvalho et al. 2020).

HDBC is a natural but highly-diluted and agitated (succussed) substance of animal, plant, or mineral origin (Ortiz-Cornejo et al. 2017, López-Carvalho et al. 2020). Thus, as homeopathic medicines, they can activate specific responses in treated organisms (Khuda-Bukhsh & Pathak 2008). HDBC has been used to enhance aquaculture production and reduce the stress associated with intensification, a trend in modern aquacultural systems to increase biomass production and financial results (Merlini et al. 2014, Mazón-Suástegui et al. 2017). This type of stress that reared shrimp usually suffer under poor farm conditions and

bad zootechnical management affects growth, survival, and natural immune resistance and opens the door to pathogens and their related diseases (Gómez et al. 2001, Mazón-Suástegui et al. 2018d).

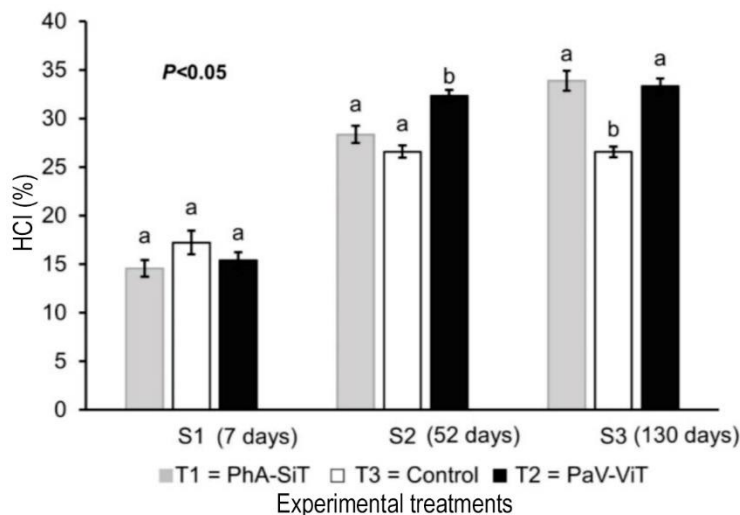
This study is the first to evaluate the response of white shrimp *Penaeus vannamei* to the application of treatments based on HDBC in a commercial shrimp farm. The BCS Camarón Company (Baja California Sur, Mexico) commercially operates 60 ha of a rustic ranch with a natural earth bottom; it applies a semi-intensive cultivation system with a long cycle and from two to three partial harvests during an annual cycle. Under these productive conditions and from the results obtained and previously described, this commercial-scale research has demonstrated that shrimp with T2 had a significant increase in several productive indicators, such as WG, SGR, FCR, and CFI, compared to T3, that did not receive HDBC.

The results revealed a positive effect of the commercial homeopathic medicines Passival<sup>®</sup> and Vidatox<sup>®</sup> for humans contained in T2 and their potential applicability to improve *P. vannamei*



**Table 3.** Digestive enzyme (protease, amylase, lipase) activity (mean  $\pm$  standard deviation) in *Penaeus vannamei* shrimp, subjected to treatments of highly diluted bioactive compounds (HDBC) in a commercial semi-intensive farm. After Tukey's test, different letters indicate significant differences ( $P < 0.05$ ).

Digestive enzyme activity	Sample time (d)	Experimental groups			ANOVA ( $P$ -value)
		T1 = PhA-SiT	T2 = PaV-ViT	T3 = Control	
Protease (U mg <sup>-1</sup> protein)	S1(7)	2.93 $\pm$ 0.48	2.96 $\pm$ 0.70	2.91 $\pm$ 0.80	0.708
	S2(52)	3.62 $\pm$ 0.95	3.67 $\pm$ 1.26	3.34 $\pm$ 0.95	0.655
	S3(130)	3.53 $\pm$ 1.09	3.57 $\pm$ 1.02	3.43 $\pm$ 1.20	0.889
Amylase (U mg <sup>-1</sup> protein)	S1(7)	159.65 $\pm$ 29.85	162.10 $\pm$ 37.37	148.54 $\pm$ 36.84	0.596
	S2(52)	166.09 $\pm$ 40.44	167.92 $\pm$ 38.54	154.25 $\pm$ 43.47	0.992
	S3(130)	173.20 $\pm$ 35.80	172.08 $\pm$ 35.78	162.11 $\pm$ 49.14	0.816
Lipase (U mg <sup>-1</sup> protein)	S1(7)	7.44 $\pm$ 4.14	7.51 $\pm$ 3.57	7.14 $\pm$ 4.68	0.970
	S2(52)	7.50 $\pm$ 4.21	7.57 $\pm$ 3.89	7.35 $\pm$ 3.81	0.737
	S3(130)	8.47 $\pm$ 3.80	8.35 $\pm$ 3.81	8.23 $\pm$ 4.44	0.711

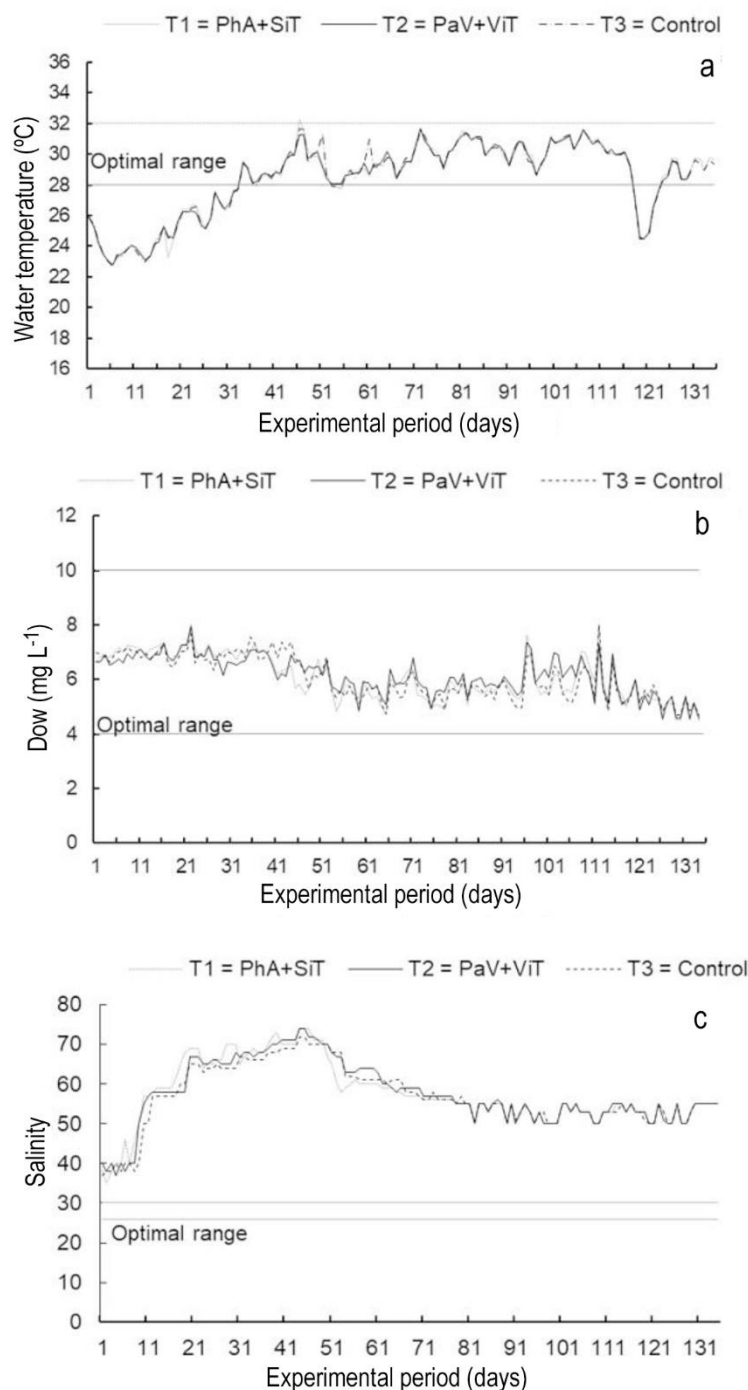


**Figure 3.** Hepatopancreas coverage index (HCI, %) of *Penaeus vannamei* shrimp treated with highly diluted bioactive compounds (HDBC) in a commercial semi-intensive farm. S1 (7 days), S2 (52 days), and S3 (130 days) are samplings. After Tukey's test, different letters indicate significant differences ( $P < 0.05$ ).

productive performance (in terms of growth and productive performance) when cultivated under stressing hypersalinity conditions (35 to 75 range in salinity), which were recorded during the production cycle (Fig. 4). T1 and T2 favored FCR, SGR, and WG percentage despite this abiotic stressor.

Since *P. vannamei* is a euryhaline species with a wide salinity tolerance range (0.5-78) (Zhang 1990, Zhao et al. 2018), it is clear that the species can grow better in salinities below the isosmotic point. In this sense, their salt tolerance is an extremely complex process that involves physiological, biochemical, and genetic processes (Li et al. 2020). Ayaz et al. (2015)

reported that a higher growth rate of *P. vannamei* was observed at 40 salinity but lower growth at 50. Additionally, its higher weight gain and better survival are favored at 35 and 40 salinity against 45 and 50 salinity (Rizk et al. 2002, Maicá et al. 2014). The importance and potential applicability of the highly-diluted bioactive compounds contained in T1 and T2 treatments take advantage because high salinity can induce oxidative stress, reduce antioxidants, and achieve changing the process of nutrient absorption and secretion, which could decrease energetic reserves (Chen et al. 2015, Yu et al. 2020). In this sense, HDBC could reduce those damages when applied prophylactically in hypersaline shrimp farms.



**Figure 4.** Physicochemical parameters of *Penaeus vannamei* white shrimp production ponds subjected to highly dilute bioactive compound (HDBC) treatments (T1, T2 and T3) in a commercial semi-intensive farm. The optimal range is indicated. a) Water temperature, b) dissolved oxygen, c) Salinity.

A possible synergic action has increased the overall performance of shrimp treated with T2. Particularly, the PaV component in T2 seems to have had an anti-stress effect under hypersaline culture conditions (Mazón-Suástegui et al. 2018b), while immunomodulatory properties are attributed to Vidatox<sup>®</sup> that increase white cells and produce interleukins (Hernández-Betancourt

et al. 2009). The greatest growth and productive performance recorded in shrimp receiving T2 suggest that this treatment could be an alternative tool for enhancing performance and overall zootechnical production in shrimp farming. On the other hand, greater growth of the shrimp treated with HDBC could be assumed and also related to enhancing their

capability for food assimilation (Mazón-Suástegui et al. 2017, Ortiz-Cornejo et al. 2017) and stimulation of their immunological system for greater related resistance to diseases associated to *Vibrio* and other pathogenic bacteria (Mazón-Suástegui et al. 2018a,b). The bacterium of the genus *Vibrio* is well known as pathogenic and virulent for marine shrimp, so the early mortality syndrome or acute hepatopancreatic necrosis syndrome (EMS/AHNPS) is related (Akazawa & Eguchi 2013, Hoanh et al. 2013).

Several authors have reported that T1 can improve the energetics reserves as lipids in the hepatopancreas, digestive enzyme activity, and increase immune response, which translates into organisms that resist more biotic and abiotic stressors with the potential capability to activate immunomodulatory response and antioxidant system in marine organisms (Genard et al. 2013, Mazón-Suástegui et al. 2017, López-Carvalho et al. 2019).

Shrimp survival has also increased after being treated therapeutically with HDHC and then challenged against highly pathogenic and virulent agents, such as *Vibrio parahaemolyticus* (Mazón-Suástegui et al. 2018a). López-Carvalho et al. 2020 have evidenced that HDHC at low concentrations acts rapidly in the metabolism of juvenile scallop *Argopecten ventricosus*, with modulation differences in gene expression. In this farm-scale study, the LI evaluated histochemically in shrimp hepatopancreas from T1 and T2 were significantly higher than from the T3. This result is in accordance with López-Carvalho et al. (2019) because T1 increased energetic reserves in *A. ventricosus*. In this sense, carbohydrates, protein, and lipids are basic macronutrients whose content can influence digestive enzyme activities in shrimp (Gamboa-Delgado et al. 2003).

In shrimp species, the hepatopancreas occupies a large part of the cephalothorax. This organ is relevant for crustaceans since it is directly involved in synthesizing and secretion of digestive enzymes and nutrient absorption, assimilation, and waste excretion (Vogt 1993). Hepatopancreas store important nutrients, such as lipids, glycogen, and organic and inorganic compounds (Felgenhauer 1992). These compounds are transported to other organs and used for body growth and the development and maturation of sexual structures (Al-Mohanna & Nott 1989).

The hepatopancreas could be used to investigate the nutritional status of shrimp species, as some authors have recommended applying histology in nutritional studies (Al-Mohanna & Nott 1987, Vogt 1993). Figure 3 shows that HDHC treatments increased HCI in this commercial-scale study, evaluating the specific conditions under which organisms are developing and

valuable to culture system management. HCI may be usually considered an indicator of change in the physiological well-being of the individuals under study. In addition, the hepatopancreas provides energy and has many important physiological functions, including those of immunity and digestion.

The attained results related to HDHC application in commercial shrimp farms demonstrated that the presence of T2's bioactive compounds -even at low concentration in the gut- can improve energetic reserves as lipids in the hepatopancreas. Therefore, enhancing the overall physiological status of shrimp reared under a semi-intensive system on a commercial farm. The digestive enzymes of penaeid shrimp have been studied for various nutritional physiology and biochemistry applications over the last decades. Vega-Villasante et al. (1999) and Carrillo-Farnés et al. (2007) suggested that the differences in the activities of the digestive enzymes could be related to the specific necessities of nutrients and energy during the different stages of the molting cycle. Moreover, Casillas-Hernández et al. (2007) reported that digestive enzyme activities in *Penaeus stylirostris* are strongly affected by molting stages, as a probable physiological response towards metabolic energy and nutrient requirements that high digestive enzyme activity can promote through digestion and absorption of dietary protein, thereby, shrimp growth.

This commercial-scale investigation has generated new scientific knowledge and demonstrated the relevance of highly-diluted bioactive compounds and their potential application's effects and viability at commercial *P. vannamei* farms. The results obtained suggest that the application of HDHC could have a beneficial impact on overall shrimp performance, biological productivity, and their related economic profitability in the shrimp industry.

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