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# Increase in the survival of whiteleg shrimp (*Litopenaeus vannamei*) infected with white spot virus and fed a diet supplemented with coconut (*Cocos nucifera*) oil

Incremento de la supervivencia de camarón blanco (*Litopenaeus vannamei*) infectado con el virus de la mancha blanca y alimentado con una dieta suplementada con aceite de coco (*Cocos nucifera*)

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**ABSTRACT**

The aim of this study was to evaluate the antiviral potential of coconut (*Cocos nucifera*) oil supplemented in the diet of whiteleg shrimp (*Litopenaeus vannamei*) to reduce the mortality caused by the white spot syndrome virus (WSSV) under shrimp culture. Two bioassays were conducted for shrimp juveniles. The first bioassay (20 days) consisted of supplying the following feeds: I) commercial feed (CF); II) CF + 2.5% of extra virgin coconut oil (EVCO); III) CF + 5% EVCO and the second bioassay (27 days), the following feeds were supplied: I) CF + WSSV; II) CF + 2.5% EVCO + WSSV; III) CF; IV) CF + 2.5% EVCO. Specific growth rate (EGR), survival, and total hemocyte count (THC) were determined in both bioassays. The prevalence of WSSV was only determined for the second bioassay. The results of EGR, THC and prevalence of WSSV indicate that there were no significant differences among treatments in both bioassays nor in survival during the first bioassay. However, in the second bioassay, differences were found ($p = 0.0030$) between I (83.3%) and the rest of treatments, which showed higher survival values. We conclude that the supplementation of 2.5-5.0% of EVCO in shrimp feed promoted a decrease in the prevalence and viral load of WSSV in juveniles of *L. vannamei*. In addition, this supplement did not show negative effects in survival and EGR. Finally, no immunosuppression or reduction of the immune response of THC was detected among treatments in both bioassays.

**Keywords**: *Cocos nucifera*, *Litopenaeus vannamei*, fatty acids, WSSV, immunostimulants, antiviral activity.

**RESUMEN**

El aceite de coco (*Cocos nucifera*) es considerado una sustancia inmunoestimulante natural que contiene compuestos bioactivos con la capacidad de regular o modificar la respuesta inmune e incrementar la resistencia contra agentes patógenos. Es por ello que el objetivo de esta investigación fue evaluar el aceite de *C. nucifera* suplementado en la dieta como potencial antiviral para reducir las mortalidades ocasionadas por el virus de la mancha blanca (WSSV, white spot syndrome virus) en el cultivo de camarón *Litopenaeus vannamei*. Se realizaron dos bioensayos con juveniles de camarón: el primero (20 días) consistió en

alimentar con I) alimento comercial (AC); II) AC + 2.5 % de aceite de coco extra virgen (ACEV); III) AC + 5 % ACEV y el segundo bioensayo (27 días) con I) AC + WSSV; II) AC + 2.5 % ACEV + WSSV; III) AC y IV) AC + 2.5 % ACEV. Al final se determinó la tasa de crecimiento específico (TCE), la supervivencia, el conteo total de hemocitos (CTH) y únicamente en el bioensayo II se determinó la prevalencia del WSSV. Los resultados de TCE, CTH para ambos bioensayos no mostraron diferencias significativas entre tratamientos, al igual que la supervivencia en el bioensayo I. Sin embargo, si existieron diferencias significativas en la supervivencia del bioensayo II ($p = 0.0030$) entre el tratamiento I (83.3 %) y el resto de los tratamientos que presentaron supervivencias más altas. Se concluye que los resultados de prevalencia de WSSV del bioensayo II mostraron que la adición con 2.5 % de ACEV en el alimento presentó un efecto en la disminución de la prevalencia y la carga viral de WSSV en juveniles de *L. vannamei*, sin embargo, estos resultados no presentaron diferencias significativas con el resto de los tratamientos, no mostró efectos negativos en cuanto a la supervivencia y la TCE, así como tampoco se registró un efecto en el CTH entre los tratamientos de ambos bioensayos.
**Palabras clave**: *Cocos nucífera*, *Litopenaeus vannamei*, ácidos grasos, WSSV, inmunoestimulantes, actividad antiviral.

## INTRODUCTION

Shrimp farming in developing countries contributes to their economic growth and provides sources of employment (Patil *et al*., 2021); within this activity, the production of *L. vannamei* shrimp worldwide (4,966241 t) exceeds that of other penaeid shrimp (154,615 t) (FAO, 2018); however, infectious diseases and different environmental conditions have been a continuous challenge in shrimp farming of any species (FAO, 2018), generating large economic losses and instability in the shrimp farming industry (Varela *et al*., 2017). Viral diseases are the most difficult to control because of their great power of dissemination, their multiple routes of infection and the wide spectrum of both wild and cultivated hosts (Wang *et al*., 1997). White spot syndrome virus (WSSV) is one of the most pathogenic shrimp viruses infecting a wide range of decapod crustaceans worldwide; some of these have the role of vectors or reservoirs (Flegel & Alday, 1998; OIE, 2019). This virus can also cause massive mortalities reaching cases up to 100% during a period of three to ten days after the first clinical signs of the disease (Marks, 2005).

Despite treatments for the management and control of the disease and mortality caused by the WSSV presence, the only effective alternative so far in shrimp production is the implementation of biosecurity or exclusion practices such as filtration and disinfection (Esparza-Leal *et al*., 2009). However, scientific research is currently focused on improving management practices and reducing stress conditions through the evaluation and development of immunostimulant additives, which have become a novel and promising strategy to establish the basis of resistance and prophylactic measures for *L. vannamei* against pathogens (Barracco *et al*., 2014).
Immunostimulant substances are obtained through natural sources and also by chemical synthesis based on the molecular structure of natural products, these substances have the ability to regulate or modify the immune response; therefore they are also known as immunomodulators or immunopotentiators, and can be defined as a natural component that regulates the immune system and increases host resistance against diseases caused by pathogens (Bricknell & Dalmo, 2005).

Niu *et al*. (2018) mention that using the macroalga *Porphyra haitanensis* (2.51 and 3.14 %) as a supplement in aquafeeds improves the immune response of *L. vannamei*, increasing resistance against viruses and bacteria, generating an improvement in intestinal function and a resistance to oxidative stress.

DebMandal & Mandal (2011) review the bioactive compounds of *C. nucifera* in traditional Indian medicine, in terms of health promotion and disease prevention. Among these bioactive compounds of coconut are mainly medium-chain fatty acids, such as lauric, myristic and palmitic, phenolic compounds and vitamin E (Montoya, 2021). Given the negative impact of white spot syndrome in shrimp farming, it is highlighted that coconut can present antiviral, antibacterial, antiparasitic activity and also has an immunostimulatory effect in various animal models, including a potential antiviral effect against SARS-CoV-2 (DebMandal & Mandal, 2011; Elsbaey *et al*., 2021; Angeles-Agdeppa *et al*., 2021).
In the present work, coconut oil (*C. nucifera*), supplemented in the diet with antiviral potential to reduce mortalities caused by white spot virus in white shrimp *L. vannamei* culture, was evaluated.

## MATERIAL AND METHODS

**Ethical considerations.** This study complies with Mexican Official Standard NOM-062-ZOO-1999, technical specifications for the production, care and use of laboratory animals. *L. vannamei* is not considered an endangered or protected species.

**Acclimatization of shrimp to culture conditions**. For the bioassays, presumptively healthy shrimp were collected from aquaculture farms in San Blas, Nayarit, and immediately transported to the of Experimental Molecular Biotechnology Laboratory (LABME) of the National School of Fisheries Engineering (ENIP), in 100 L plastic containers, with water from the culture ponds and aeration (= 5.4 ± 0.12 mg/L). Shrimp acclimatization to culture conditions was carried out for 48 h, adjusting salinity 2 ‰/h until reaching 35 ‰. A plastic tank with a capacity of 1000 L containing 800 L of filtered seawater (20 µm) was used; the organisms were maintained with continuous aeration and in turn were fed with a daily ration corresponding to 5 % of their total biomass twice a day 08:00 and 17:00 h with commercial feed (AC, 35 % protein).

**Preparation of the experimental diets with coconut oil (*C. nucifera*):** The EVCO used for the preparation of the experimental diets was subjected to a fatty acid profile analysis, according to the method proposed by AOAC (2012). For the inclusion of extra virgin coconut oil (cold pressed), AC was pulverized in a food processor (Oster® model FPSTFP1355); AC was mixed with coconut oil (EVCO) at 2.5 % and 5 % per 0.5 $kg^{-1}$ of feed. Once the mixture was made, a solution of grenetin (4 %) in 180 mL of distilled water was added until a paste was formed. The pellet was made from the resulting paste using a meat grinder (Torrey model 22), equipped with a 3/32" (3 mm) die. The formed pellets were placed in trays for dehydration and drying at room temperature for 24 h; the feed was then stored at 4 °C.

**Proximal analysis of experimental diets.** Proximal analysis of AC and AC with 2.5 % EVCO and AC with 5 % EVCO was performed. The analyses performed were moisture with method NMX-F-083-1986, ash with method NMX-F-607-NORMEX-2013, lipids (ethereal extract) with method NOM-086-SSA1-1994 (normative appendix C, numeral 1), protein (NX6. 25) with method NMX-F-608-NORMEX-2011, crude fiber with method NMX-F-613-NORMEX-2017 and total carbohydrates with method NOM-051-SCFI/SSA1-2010 (by difference of proximate analysis).

**WSSV viral inoculum and its molecular detection by PCR.** The WSSV viral inoculum (251018) was donated by the Interdisciplinary Center for Regional Integral Development (Centro Interdisciplinario para el Desarrollo Integral Regional, CIIDIR IPN Unidad Sinaloa), and prior to use was analyzed using simple PCR and analyzed (Kimura *et al*., 1996). The detection of WSSV in the inoculum and organisms was performed by end-point PCR in two phases; first the samples were analyzed with simple PCR, and those that were negative were analyzed by nested PCR with the oligonucleotides proposed by Kimura *et al*. (1996).

**Analysis of WSSV prevalence.** Prevalence was determined according to the percentage of diseased and/or WSSV-infected organisms among the number of organisms analyzed according to Margolis *et al.* (1982).

**Hemolymph extraction and total hemocyte count (THC).** Hemolymph extraction was performed before the first food ration (7:00-8:00 a.m.), to avoid differences in the circadian cycle; hemolymph was obtained with a sterile syringe (1 mL) in the ventral abdominal artery (Fisher *et al*., 1995). The syringe was pre-loaded with 200 µL of isotonic SIC-EDTA anticoagulant solution (450 mM NaCl, 10 mM KCl, 10 mM EDTA-Na2, 10 mM HEPES, pH 7.3 and 850 mOsm $kg^{-1}$) pre-cooled (4 °C) for shrimp (Vargas-Albores *et al*.,1993), deposited in sterile 1.5 mL tubes on ice. Fifty µL of the 2:1 dilution of SIC-EDTA-hemolymph was taken and mixed with 150 µL (1:3 v/v) of a 4% pre-chilled formaldehyde solution to fix the hemocytes. From this dilution, individual counting was performed using a Neubauer camera with a 0.01 mm reticle under a binocular microscope (Leica DM300). The immune response of the shrimp was evaluated by total hemocyte count according to Cabrera-Pérez *et al.* (2019):

$$(\mathbf{THC})\ (\mathbf{cells/mL}) \ = \ (\mathbf{Hc} * \mathbf{D} * \mathbf{C})/\mathbf{0.4},$$

Where: Hc is the total number of hemocytes counted; D is the hemolymph dilution factor and C is the conversion factor x 0.1 $mm^3$ x mL (1000).

**Bioassay I. Evaluation of the concentration of *C. nucifera* oil as an immunostimulant**. The duration of this bioassay was 20 days; it was carried out in plastic tanks with a capacity of 200 L, containing 180 L of seawater (35 ‰) filtered (20 µm) and constant aeration. The bioassay consisted of three treatments each in triplicate (30 shrimp per tank with an average initial weight of 5.46 ± 0.19 g): I) AC control; II) AC + 2.5 % EVCO; III) AC + 5 % EVCO. The experimental organisms were fed twice a day (8:00 a.m.

and 17:00 p.m.). During the bioassay, physicochemical variables were monitored twice a day: pH was measured with a Hanna Hi98130 potentiometer, salinity with an ATAGO refractometer model 2491, temperature and dissolved oxygen with an YSI pro20i multiparameter.
Ammonium, nitrite and nitrate were determined every 10 days with a YSI 9500 photometer. At the end of each bioassay, survival was determined using the equation:

$$\boldsymbol{S = \ (N° \ ind \ f \ / \ N° \ ind. i) \ * \ 100}$$

Where N° ind f is the number of live individuals at the end of the bioassay and N° ind i is the number of live individuals at the beginning of the bioassay (Escobar-Gil *et al*., 2017). The specific growth rate (SGR) was determined by the equation:

$$\mathbf{SGR \ (\%day - 1) \ = \ [100 * (LnW2 - LnW1)]t}$$

Where: W2 is the final weight, W1 is the initial weight and t is the number of days of culture (Ziaei-Nejad *et al*., 2006).

The immune response of shrimp was evaluated by total hemocyte count as described by Cabrera-Pérez *et al.* (2019). Individual (27) organisms were analyzed in the THC, 9 organisms per treatment representing 30 % of the total population of each treatment.

**Bioassay II. Evaluation of the antiviral activity of coconut oil supplemented in the diet of WSSV-infected *L. vannamei*.** The duration of the bioassay was 27 days, where on day 23 the shrimp were infected with the WSSV inoculum. It was carried out in aquaria with a capacity of 80 L, which were filled with 50 L of seawater (35 ‰) filtered (20 µm). The bioassay consisted of four treatments, each in triplicate (10 shrimp per aquarium with an average weight of 7.7 ± 0.25 g): I) CF + WSSV; II) CF + 2.5 % EVCO + WSSV; III) CF control; IV) CF control + 2.5 % EVCO. Experimental organisms were fed twice daily (8:00 a.m. and 17:00 p.m.). During the bioassay, physicochemical variables were monitored twice a day in the same way as in biennial test I.

At the end of each bioassay, survival was determined according to Escobar-Gil *et al*. (2017) and EGR according to Ziaei-Nejad *et al. (*2006). Prior to infection, an initial WSSV prevalence analysis of shrimp from the farm was performed which was 100 % (determined by nested PCR). On day 23, the shrimp in treatments I and II were reinfected with 10 µL of inoculum from shrimp previously infected with WSSV, which was injected into the dorsal part of the second abdominal segment with a 0.3 Ml insulin syringe; in addition, the organisms from these treatments (I and II) were fed with paste from WSSV-infected shrimp.

The prevalence of WSSV in shrimp was quantified as previously described (Margolis *et al.,* 1982). To determine the prevalence of WSSV, infected shrimp were kept under observation until they showed obvious signs of white spot disease. Thirty organisms per treatment were individually tested; in addition, moribund organisms were collected and sacrificed to verify the presence of WSSV with single and nested PCR; they were stored at -80 ºC. Shrimp immune response was also evaluated by total hemocyte count (Cabrera-Pérez *et al*., 2019. Three organisms per replicate were individually analyzed for THC, 9 organisms per treatment representing 30 % of the total population of each treatment.

**Statistical analysis.** A normality (Kolmogorov Smirnov) and homoscedasticity (Bartlett) test, one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test (HSD) were performed to identify the nature of these differences in survival, specific growth rate, total hemocyte count and WSSV prevalence; using the STATISTICA Version 6 program (StatSoft 2003). Values of $p<0.05$ were considered significantly different. Survival and prevalence data were transformed using an arcsine function according to Font *et al.* (2007).

## RESULTS

**Fatty acid profile of coconut oil (*C. nucifera*).** A total of 36 fatty acids were identified, of which 12 presented percentage values (g/100 g fat) (Table 1). The rest of the fatty acids presented values <0.01 below the minimum detectable calibration limit. Within this determination 94.52 % corresponds to saturated fats, 4.90 % to monounsaturated fats and 0.83 % to polyunsaturated fats**.**

**Chemical-proximal composition of the experimental diets.** The results of proximal analysis of treatment I (CF), treatment II (CF + 2.5 % EVCO) and treatment III (CF + 5 % EVCO) are presented in Table 2. The moisture percentage was lowest in treatment III with a value of 10.91 %, followed by treatment II with a value of 12.30 % and treatment I with 12.37 %. The ash percentage was 8.12 %, 8.34 % and 8.52 % for treatment I, III and II, respectively. As for lipids, a percentage of 5.26 % was obtained for treatment I, followed by 7.64 % for treatment II and 10.09 % for treatment III. The protein percentage was 32.89 %, 34.53 % and 35.28 %, corresponding to treatment III, II and I, respectively. The percentage of crude fiber was 4.53 % for treatment II, 6.86 % for treatment III and 9.67 % for treatment I; while for total carbohydrates the lowest percentage was for treatment II with 37.01 %, followed by treatment III with 37.77 % and treatment I with 38.97 %. The percentages of protein, lipids and carbohydrates with respect to the total diet in each of the treatments were within the recommended nutritional requirements for *L. vannamei* according to Martínez-Córdova *et al*. (2014).

**Table 1. Fatty acid profile of pure coconut oil**

| COMMON NAME | TYPE OF FAT | CONCENTRATION (%) (g/100g fat g/100g of fat) |
|---|---|---|
| Lignoceric acid | Saturated | 0.03 |
| Arachidic acid | Saturated | 0.08 |
| Caproic acid | Saturated | 0.77 |
| Stearic acid | Saturated | 2.92 |
| Capric acid | Saturated | 6.38 |
| Palmitic acid | Saturated | 8.03 |
| Caprylic acid | Saturated | 8.60 |
| Myristic acid | Saturated | 17.97 |
| Lauric acid | Saturated | 49.47 |
| Butyric acid, pentadecanoic acid, pentadecanoic acid, heptadecanoic acid, heneicosanoic acid, behenic acid, tricosanoic acid | Saturated | <0.01* |
| cis-11- Eicosenoic acid | Monounsaturated | 0.03 |
| Oleic acid (omega 9 group) | Monounsaturated | 4.87 |
| Myristoleic acid, cis-10- pentadecenoic acid, palmitoleic acid, cis-10- heptadecenoic acid, nervonic acid (Omega 9 Group), erucic acid (Omega 9 Group) | Monounsaturated | <0.01* |
| Linoleic acid (Omega 6 Group) | Polyunsaturated | 0.83 |
| g- linolenic acid (Omega 6 Group), linolenic acid (Omega 3 Group), cis-4,7,10,13,16,19-docosahexaenoic acid (Omega 3 Group), cis-5,8,11,14,17- eicosapentaenoic acid (Omega 3 Group), cis-11, 14- eicosadienoic acid, cis-8,11,14-eicosatrienoic acid (Omega Group 6), cis-11-14-17-'eicosatrienoic acid (Omega Group 3), arachidonic acid (Omega Group 6), cis-13,16- docosadienoic acid (Omega Group 6) | Polyunsaturated | <0.01* |
| elaidic acid (C18:1 TFA), linolelaidic acid (C18:2 TFA), C18:3 TFA | Trans | <0.01* |

*Below the most critical calibration level

Bioassay I.

**Physicochemical parameters.** The physicochemical parameters evaluated were maintained within the optimal ranges for the culture of *L. vannamei* during the 20 days of the bioassay, according to Brock & Main (1994).

**Water quality (ammonium, nitrite and nitrate).** During the culture, the concentrations of nitrogen compounds were within the optimal range for white shrimp culture, according to Boyd (2001) and SENASICA (2003).

**Table 2. Chemical-proximal composition of the experimental diets**

| Proximal composition (% by weight) | Treatments | | |
|---|---|---|---|
| | I | II | III |
| Moisture | 12.37 | 12.30 | 10.91 |
| Ash | 8.12 | 8.52 | 8.34 |
| Lipids (ethereal extract) | 5.26 | 7.64 | 10.09 |
| Proteins (N-6.25) | 35.28 | 34.53 | 32.89 |
| Crude fiber | 9.67 | 4.53 | 6.86 |
| Total carbohydrates | 38.97 | 37.01 | 37.77 |

Treatments: I) CF (35 % protein); II) CF + 2.5 % EVCO; III) CF + 5 % EVCO

**Survival and specific growth rate (SGR).** No significant differences were found in survival among treatments (p = 0.256); however, survival (Fig. 1) in treatment I was 100 %, while in treatments II and III with 2.5 % and 5 % EVCO, values of 93.33 % and 98.89 % were obtained, respectively.

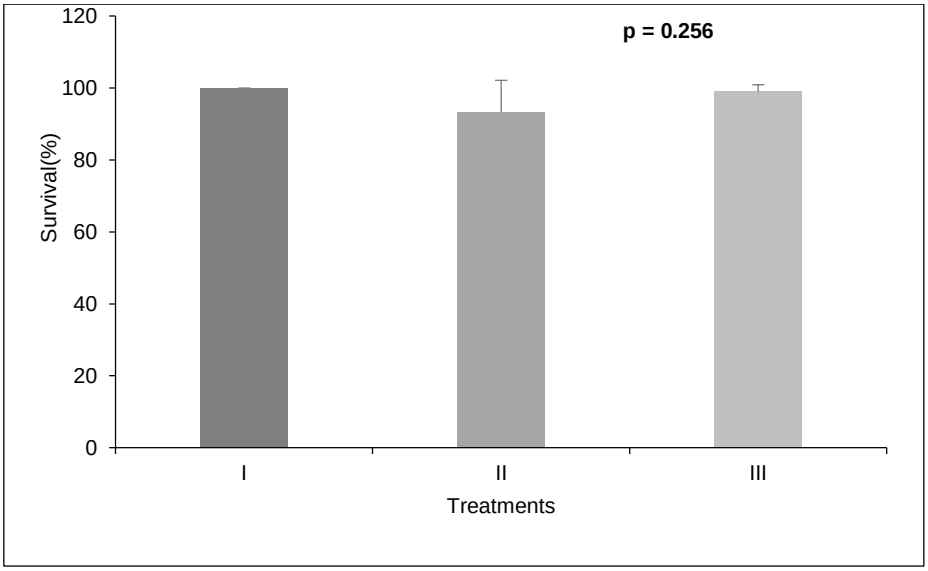


**Figure 1. Survival of *L. vannamei*.** Treatments: I) CF control (35 % protein); II) CF + 2.5 % EVCO; III) CF + 5 % EVCO

Statistical analysis showed no significant differences in EGR (% day-1), among treatments (p = 0.668); however, EGR was higher in treatment I with 1.44 ± 0.32 (%day$^{-1}$), with respect to treatment II with 1.41 ± 0.13 (%day$^{-1}$) and treatment III with 1.28 ± 0.11 (%day$^{-1}$) (fig. 2).

**Total hemocyte count (THC).** The average value of total hemocyte count per milliliter of hemolymph in treatment I was 3.83 x $10^6$ ± 0.52 x $10^6$ cells /mL. In treatments II and III it was 4.11 x $10^6$ ± 0.36 x $10^6$ cells /mL and 3.92 x $10^6$ ± 0.50 x $10^6$ cells/mL, respectively. Analysis of variance (ANOVA) showed no significant differences (p = 0.449) between treatments (Fig. 3).

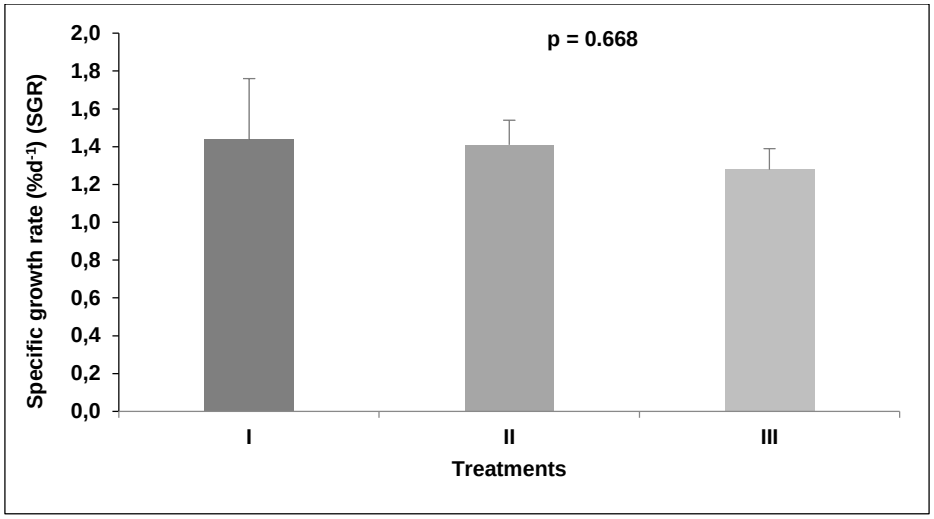


**Figure 2. Specific growth rate of *L. vannamei*.** Treatments: (I) CF control (35 % protein); (II) CF + 2.5 % EVCO; (III) CF + 5 % EVCO. Bars indicate mean value ± standard deviation and (P) ANOVA analysis of variance

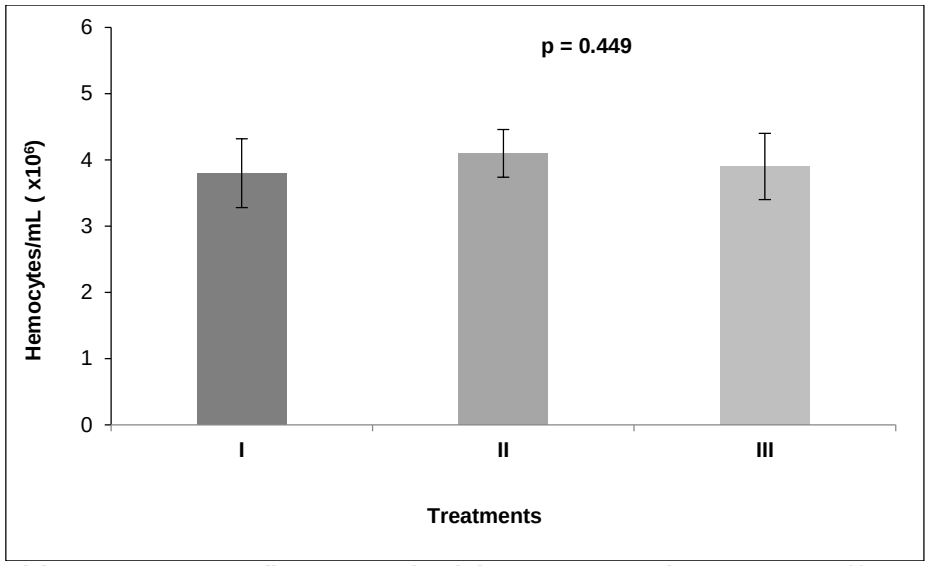


**Figure 3. Total hemocyte count (hemocytes/mL) in *L. vannamei*.** Treatments: (I) CF control (35 % protein); (II) CF + 2.5 % EVCO; (III) CF + 5 % EVCO. Bars indicate mean value ± standard deviation and (P) ANOVA analysis of variance

Based on the results obtained, although no significant differences were found between EVCO treatments, treatment II (2.5 % EVCO) was selected for the following bioassay, because it was the one where the least amount of EVCO was used and also the results of EGR and THC in this treatment were higher than in the other treatments.

**Bioassay II.**

**Physicochemical parameters.** The physicochemical parameters evaluated were maintained within the optimal ranges for the culture of *L. vannamei* during the 27 days of the bioassay, according to Brock & Main (1994).

**Water quality (ammonium, nitrite and nitrate).** During the culture, the concentration of nitrogen compounds was within the optimal range for white shrimp culture, according to Boyd (2001) and SENASICA (2003).

**Specific growth rate (SGR).** SGR (%day$^{-1}$) did not show significant differences (p = 0.644) among treatments (Fig. 4). Treatment I presented 0.60 ± 0.10 %day$^{-1}$, treatment II presented 0.58 ± 0.08 %day$^{-1}$; while treatment III presented the highest specific growth rate per day with 0.73 ± 0 .29 (%day$^{-1}$), and treatment IV presented 0.69 ± 0.10 %day$^{-1}$. These results indicate that EVCO did not have an effect on SRG (%day$^{-1}$).

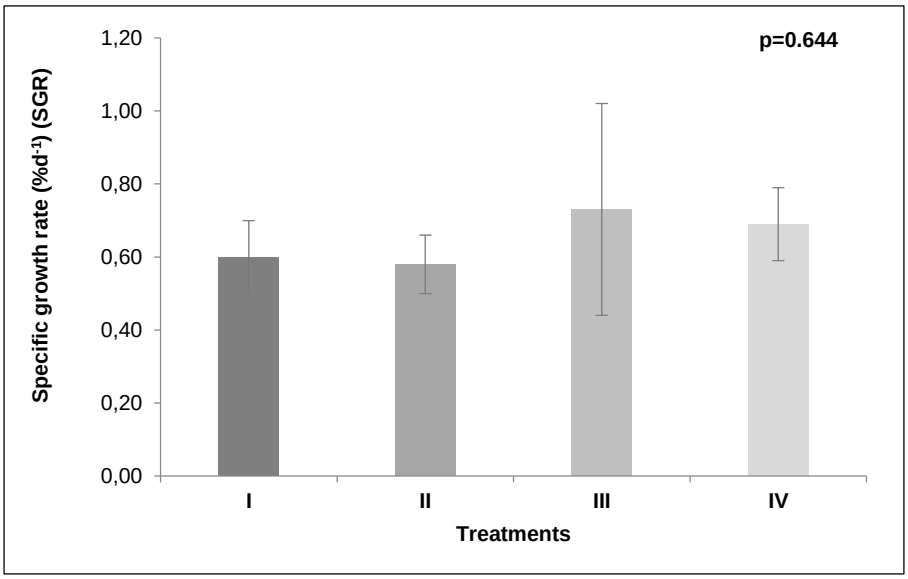


**Figure 4. Specific growth rate of *L. vannamei***. Treatments: TI) CF + WSSV; TII) CF + 2.5 % EVCO + WSSV; TIII) CF control; TIV) CF control + 2.5 % EVCO. Bars indicate mean value ± standard deviation and (P) ANOVA analysis of variance.

**Survival and prevalence of WSSV.** Survival (Fig. 5) showed significant differences (p = 0.0030) between treatment I and the rest of the treatments. In treatment I (control-infected with WSSV) survival was lower with 83.3 %, followed by treatment II (infected with WSSV) with 93.3 %. In treatments III and IV, survival was 100 %. The results show that despite being infected with WSSV, the organisms in treatment II with 2.5 % EVCO showed higher survival than in treatment I also infected with WSSV, so it could be considered a beneficial effect of EVCO against WSSV.

The initial WSSV prevalence of shrimp from the farm was 100 % (determined by nested PCR), and with reinfection only in treatments I and II, the prevalence was 100 % (determined by single PCR). However, at the end of the bioassay, the WSSV prevalence of all treatments was analyzed and no significant differences were found (p = 0.2192).
The final prevalence of WSSV in shrimp of treatment I was 100% (determined by simple PCR), in shrimp of treatment II it was 96.67% (of which 86.21% were determined by simple PCR and 13.79% determined by nested PCR), where 93.33% were infected alive, 3.33% were infected dead and the remaining 3.33% were not infected. In shrimp of treatment III the prevalence was 100 % (determined by nested PCR) and in shrimp of treatment IV it was 93.33 %, (of which 100 % were determined by nested PCR), where 93.33 % were infected alive and 6.66 % were alive, but without the presence of virus.
It was observed that in treatment II not only the prevalence was reduced to 3.33 %, but also the viral load (determined by nested PCR); while for treatment IV the prevalence decreased 6.67 % (determined by nested PCR). The results show that treatments with 2.5 % EVCO had an effect in decreasing the prevalence and viral load of WSSV (Fig. 5).

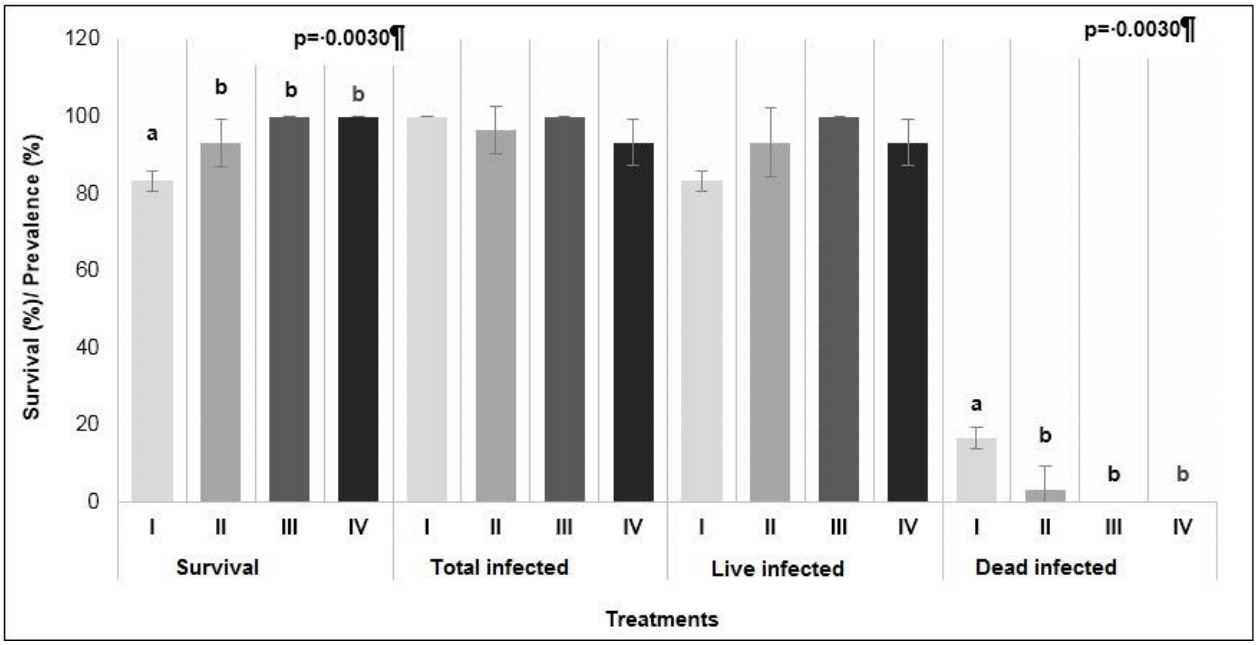


**Figure 5. Survival (%), prevalence (%), live infected (%), dead infected (%) of *L. vannamei*.** Treatments: I) CF + WSSV; II) CF + 2.5 % EVCO + WSSV; III) CF control; IV) CF control + 2.5 % EVCO. Bars indicate mean value ± standard error and (P) ANOVA analysis of variance. Letters indicate significant differences between treatments according to the Tukey multiple range test

**Total hemocyte count (THC).** Statistical analysis of total hemocyte count showed no significant differences (p = 0.413) between treatments. The total hemocyte count per milliliter of hemolymph was higher in treatment III with an average of 4.37 x $10^6$ ± 0.52 x $10^6$ cells /mL, followed by treatment IV with 4.27 x $10^6$ ± 0.46 x $10^6$ cells /mL, treatment II with an average of 4.10 x $10^6$ ± 0.46 x $10^6$ cells /mL and treatment I with an average of 3.99 x $10^6$ ± 0.57 x $10^6$ cells /mL (Figura. 6).

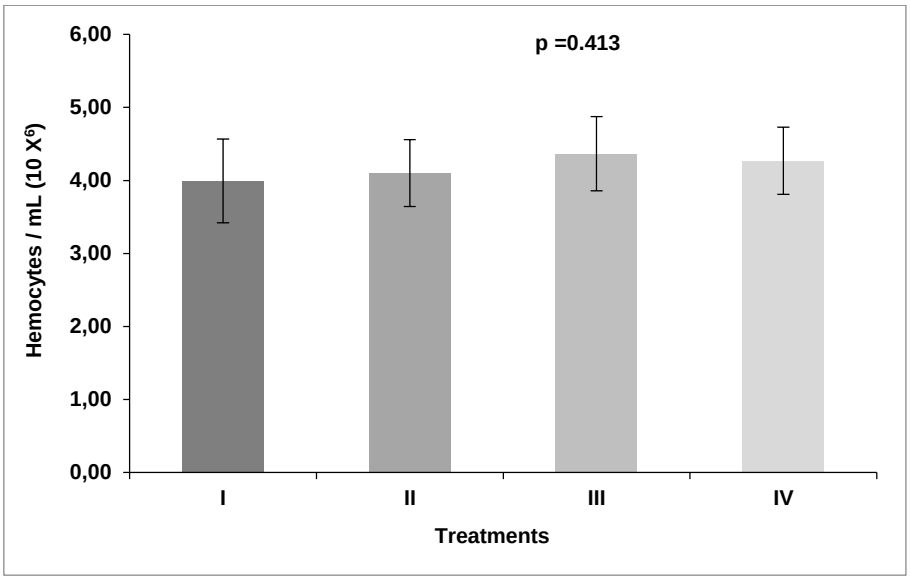


**Figure 6. Total hemocyte count (hemocytes/mL) in *L. vannamei*.** Treatments: (I) CF + WSSV; (II) CF + 2.5 % EVCO + WSSV; (III) CF control; (IV) CF control + 2.5 % EVCO. Bars indicate mean value ± standard deviation and (P) ANOVA analysis of variance

## DISCUSSION

Due to the negative impact generated by diseases caused by different etiological agents such as viruses and bacteria, as well as their resistance to antibiotics used for treatment and control of infectious diseases (Lieberman *et al.,* 2006), there is a need to implement new strategies to improve the immunity of species of aquaculture importance and to increase resistance against viruses and bacteria, also promoting resistance to stress by supplementing aquafeeds with natural immunostimulant additives (Berger, 2000; Rendón & Balcázar, 2003).

According to Turchini *et al.* (2009) and Turchini *et al.* (2011), one of the main alternatives being applied as a dietary additive are vegetable oils, as they offer a wide range of medium chain fatty acids (MCFA) with low molecular weight. According to Kapilan & Reddy (2008) and Akinnuga *et al.* (2014), coconut oil is mainly composed of MCFA; in addition to a wide variety of phenolic compounds, which according to Nevin & Rajamohan (2009) enhance the activity of antioxidant enzymes and eliminate excess free radicals in cells. On the other hand, Lu *et al.* (2018) point out that in the case of lauric acid, it stimulates the growth and health of some aquatic species such as *Pelteobagrus fulvidraco*. In the fatty acid profile analysis of EVCO used in this work, lauric acid presented a concentration of 49.47 % (g/100g of fat), where the high concentration suggests that this oil could be used as an alternative treatment to the current problem against pathogens in shrimp cultures, since it can be added to the feed to combat WSSV or decrease its viral load.

According to Niu *et al.* (2018), substances applied in culture systems or feed should not alter the nutritional value or negatively affect the productive performance of the organisms. This coincides with the present results on the chemical-proximal composition, where it is shown that adding in EVCO did not affect the nutritional values of experimental diets and

were found within the optimal values for the development of *L. vannamei* reported by Martínez-Córdova *et al.* (2014). With respect to EGR, in Bioassay 1 and 2 no affectation was observed in shrimp when EVCO was used at different concentrations. In Bioassay 1, it was observed that as the lipid concentration (% by weight) increased, the EGR showed a tendency to decrease. According to Cahu (1994), the optimum lipid levels recommended for commercial shrimp feed vary from 6 to 7.5 %, without exceeding 10 %; however, in the results of this research, the levels were found to be between 5.26 % (CF), 7.64 % (2.5 % EVCO) and 10.09 % (5 % EVCO), despite being within the optimum level of total lipids (5-10 %) in a shrimp diet. According to Martínez-Córdova *et al.* (2014) and López-Marcos (2020), it could be considered that the lower EGRs are due to an excess in lipid levels (Cahu, 1994).

The scientific literature on the effect of coconut oil on *L. vannamei* is scarce; however, the EGR results of this study are consistent with those reported by Apraku *et al.* (2017), who evaluated the mixture of virgin coconut oil and fish oil on growth and resistance to *Streptococcus iniae* in Nile tilapia (*Oreochromis niloticus*), finding that growth was not affected when partially and completely substituting fish oil for virgin coconut oil. Also, these results are in agreement with other works, where vegetable oils have been used, such as Mozanzadeh *et al.* (2015); who indicate that the growth performance of juvenile *Sparidentex*, even fed with alternative diets of vegetable oil (canola and sunflower oil), was not compromised when partially or completely replacing the fish oil diet.

Regarding survival, in Bioassay 1 no significant differences were found between treatments; while in Bioassay 2, 100 % of the organisms came from shrimp farm ponds infected with WSSV (low viral load), but additionally a reinfection was performed in treatments I and II, resulting with a high viral load at the end of the bioassay and the lowest survival (83. 3 %) observed in treatment I (CF with WSSV), compared to the rest of the treatments II (EVCO with WSSV), III (CF) and IV (EVCO). These results agree with Raa (1996), who mentions that the purpose of using immunostimulants in the aquaculture sector is to improve the survival and immunity of cultured organisms. This is consistent with the fact that when EVCO was added to shrimp feed, the survival rates of challenged shrimp with high and low viral load (WSSV) were significantly higher. This could be due to Fife (2013), who points out that by using lauric acid through the daily intake of coconut oil, it can protect and prevent infections caused by different viruses, such as human immunodeficiency virus (HIV), measles, *Herpesviridae*, influenza virus, among other diseases.

Hemocytes are responsible for coagulation, hardening of the exoskeleton and removal of foreign materials (AftabUddin *et al.,* 2017). In bioassay I of this study, a higher number in the total hemocyte count was obtained in the treatments (II and III), which contained the EVCO additive with respect to treatment I (CF); indicating that there was an increase in the immune response of shrimp fed with this additive. In this regard, Sequeira *et al.* (1996) mention that hemocyte proliferation can increase up to three times when shrimp are fed with an immunostimulant; however, although in bioassay II no significant differences were found between treatments, a decrease in THC of treatment I and II (infected with WSSV) was observed with respect to treatments III and IV. These results agree with what was

reported by Niu *et al.* (2018), who fed shrimp with different dietary values immunostimulates of macroalgae, and upon challenge with WSSV the number of hemocytes was significantly reduced. The decrease in total hemocyte count may be attributed to hemocytes migrating to the site of infection (Söderhäll *et al*., 2003) or where there is tissue damage (Niu *et al.,* 2018).
White spot disease has caused severe mortalities in shrimp cultures worldwide (Jiang *et al.,* 2006), being responsible for large economic losses in this industry (Sahul *et al*., 2006), so it becomes necessary to prevent or control white spot disease (Huynh *et al*., 2011).
In the development of bioassay II, it was observed that the initial prevalence was 100 % WSSV (determined by nested PCR) with low viral load, which meant that all organisms were from infected organisms from the farm. It should be noted that treatments I and II were re-infected with viral inoculum to conduct the experiment and at the end of the bioassay it was observed that in treatment I the final prevalence was 100 % (determined by single PCR) with high viral load, in treatment III the prevalence was 100 % (determined by nested PCR); while in the EVCO treatments, treatment II (2.5 % EVCO + WSSV) and treatment IV (2.5 % EVCO). The prevalence decreased to 96.67 % and 93.33 %, respectively; however, it was observed that in treatment II not only the virus prevalence decreased (96.67 %), but also the viral load; of which 86.21 % were determined by single PCR and 13.79 % by nested PCR.

The decrease in prevalence and viral load in EVCO treatments suggests that its application as a dietary supplement exerts antiviral activity against WSSV and reduces viral loads in a similar way as reported by Lieberman *et al*. (2006) and Fife (2013), pointing out that EVCO is composed of MCFA such as: lauric acid with about 48-50 % of it in its composition, followed by myristic acid with approximately 15 % (Fife, 2013; Ruiz *et al*., 2016), caprylic acid (8 %) (Fife, 2013; Akinnuga *et al*., 2014) and capric acid (7 %) (Fife, 2013). These compounds have been shown to be effective against bacteria, fungi and viruses (Esquenazi *et al*., 2002). In the case of viruses, Oyi *et al*. (2010) mentioned that a component of coconut oil called monocaprine destroyed HIV and *herpes simplex* virus (HSV) within one minute. Similarly, Fife (2013) reported that MCFA present in coconut oil can inactivate viruses such as HIV, coronavirus associated with acute respiratory syndrome, measles virus, rubella virus, *Herpesviridae*, sarcoma virus, respiratory syncytial virus, human limphotropic virus (type 1), vesicular stomatitis virus, Visna virus, human cytomegalovirus (CMV), Epstein-Barr virus (EBV), influenza virus, hepatitis C virus (HCV) and Coxsackie B4 virus. In this regard, Murray (1994) and Dayrit (2000) recorded a decrease in HIV viral load to undetectable levels in people infected with this same virus and a marked improvement in their health after consuming coconut. Therefore, it is suggested that the EVCO composed of MCFA such as lauric acid offers the possibility of decreasing the viral load, which in turn can be used as a form of immunotherapeutic and preventive therapy in situations of WSSV infections.

## CONCLUSIONS

According to the results obtained, the addition of EVCO 2.5% in the feed promoted a decrease in the prevalence and viral load of WSSV in *L. vannamei* juveniles; it did not show negative effects on survival and THC of *L. vannamei*. Additionally, there was no immunosuppression or reduction of the immune response, since there were no significant differences in the THC between the treatments of both bioassays.
This research work is the first report on the effect of *Cocos nucifera* EVCO on the survival of white shrimp (*Litopenaeus vannamei*) challenged with WSSV. Therefore, it is recommended to carry out studies on the use of EVCO in feed under different formulation schemes and application times in shrimp feeding. In addition, it is essential to further study the immunonutritional responses of EVCO when it is included in the feed formulation.

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Errata Erratum

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