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Effects of a co-culture of marine algae and shrimp (*Litopenaeus vannamei*) on the growth, survival and immune response of shrimp infected with *Vibrio parahaemolyticus* and white spot virus (WSSV)

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ABSTRACT

In aquaculture, fighting infectious diseases is a necessity. This study measured the immuno-stimulating effect of live macroalgae consumption on *Litopenaeus vannamei* against *Vibrio parahaemolyticus* and WSSV infection in two independent bioassays. Shrimps and macroalgae were cultivated in a co-culture with two species of macroalgae separately (*Gracilaria vermiculophylla* and *Dictyota dichotoma*), and later, shrimp were infected with *V. parahaemolyticus*. In another bioassay, shrimp and macroalgae (*G. vermiculophylla, D. dichotoma* and *Ulva lactuca*) were grown and subsequently infected with WSSV. For both bioassays, survival after 120 h was determined, the total hemocyte count (TCH) was measured and the activity of superoxide dismutase (SOD) and catalase (CAT) in tissue were measured. The results indicate that the use of macroalgae in co-culture with *L. vannamei* provides a nutritional benefit that achieves higher growth than the control organisms, as well as improvements of the ammonium concentration and immune response after infection with *V. parahaemolyticus* and WSSV. A better immune response was obtained in organisms cultured with macroalgae in both bioassays at a ratio of 1.6–1.9 for organisms infected with bacteria and 1.4 to 1.6 times for organisms infected with the virus. In turn, the enzymatic activity of SOD and CAT were higher in the treated organisms relative to the controls in both experiments.

1. Introduction

Global production of cultivated crustaceans is one of the fastest growing and most important aquaculture industries, in which penaeid shrimp stands out as the most widely cultivated crustacean [1]. The rapid growth of the shrimp industry has led to outbreaks of infectious diseases, which cause epizootics in almost all areas where shrimp are grown, with global losses accounting for approximately 40% of production [2].

The need to remedy or mitigate health problems and high mortality in production units has led to a search for adequate mechanisms to improve the survival and growth of shrimp in cultivation combined with an effort to improve growth, the feed conversion rate, and the overall yield [3].

Shrimp have an innate system of defense against infectious diseases, without recognition or long-term memory [4]. These diseases are essentially viral and bacterial, such as White Spot Virus Syndrome (WSSV) and *V. parahaemolyticus* [5].

The immune response of shrimp is expressed in the production of hyaline and granular and semi-granular hemocytes, which through their cytotoxic capacity and intercellular communication, facilitate coagulation, recognition, phagocytosis, melanization, nodule formation and encapsulation [6,7]. Shrimp also possess a series of compounds synthesized by these cells that are transported by hemolymph, the prophenoloxidase system and the coagulation cascade that destroy pathogens [6].

This characteristic of penaeid shrimp is relevant for the feasibility of using compounds that help stimulate the immune system to combat disease. The use of natural products of a plant origin is a feasible alternative in the prevention and treatment of disease since plants store chemical compounds with important biological activities in their cells [8].

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Macroalgae contain secondary metabolites with biological activity. Recently, information has been published regarding compounds derived from macroalgae that have antibacterial, antiviral, antioxidant and anti-inflammatory activity [9,10]. The antioxidant capacity of some species of brown algae has been identified, and brown algae are associated with high contents of phenolic compounds. This antioxidant activity contributes to the ability of macroalgae to neutralize the consequences of oxidative stress associated with aging in living organisms [11,12].

The use of immunostimulants has been a common practice in combating infectious diseases [13]. Some of the compounds that are most commonly used in this practice are β-glucans [6,14,and15]], lactic acid bacteria [16], extracts from macroalgae [12], macroalgae and yeast flours [17], and aqueous herbal extracts [18].

Some macroalgae, such as *Fucus vesiulosus* (Phaeophyceae), contain sulfated polysaccharides, such as fucoidan, which has been implicated in the inhibition of human immunodeficiency virus (HIV) in humans [19]. Green algae, such as *U. fasciata*, have been tested for their antiviral potential, which is associated with sulfated polysaccharides, which inhibit virus introduction into cells [11].

The work performed by Chotigeat et al. [19], in which fucoidan was extracted from *Sargassum polycystum*, shows the effect of this compound on improving the survival of *Penaeus monodon* infected with WSSV by immersion in a viral solution. Control organisms died between 3 and 5 days post infection, while organisms fed a diet containing fucoidan had between 46 and 93% improved survival.

In addition to these studies, we highlight those performed in the control of infectious diseases, such as those performed by Layse et al. [20] on the characterization of bioactive compounds of the genus *Gracilaria*. Wefky and Ghobrial [21] studied the bioactivity of extracts of marine macroalgae, and Rebecca et al. [22] studied the effect of an extract of *Ulva* sp. on bacterial pathogens of fish. *U. fasciata* was evaluated for its ability to control bacterial infections in shrimp by Selvin et al. [23].

Activation of the defense mechanisms of penaeid shrimp is possible through administration of immunostimulating compounds [6]. In this sense, marine macroalgae are an important source because, unlike terrestrial plants, their cell wall has sulfated polysaccharides [10,24,25], which in the presence of a pathogen, have the ability to activate defense mechanisms that produce hemocytes, and these, in turn, synthesize antioxidant enzymes of the prophenoloxidase system (proPO) [9,11,19,20]. These sulfated polysaccharides are known as carrageenans in red algae, fucans in brown algae and ulvan in green algae [24,25].

The present study measures the immune-stimulating effect on the shrimp *Litopenaeus vannamei* from the consumption of three seaweeds found on the coast of southern Sonora, Mexico. The species studied are *Gracilaria vermiculophylla*, *Dictyota dichotoma* and *Ulva lactuca* (red, brown and green macroalgae, respectively). Using an integrated shrimp culture containing macroalgae, shrimp were subsequently infected with *V. parahaemolyticus* and white spot syndrome virus (WSSV) in two independent bioassays. The immune response was measured by the total count of hemocytes (TCH), and the antioxidant response was measured by quantifying the concentration of soluble protein and activity of superoxide dismutase (SOD) and catalase (CAT) in tissue.

2. Materials and methods

2.1. Algae collection

Macroalgae were collected in southern Sonora, Mexico; *U. lactuca* in the Bay of Agiabampo (26° 22′ 31" N and 109° 13′ 37" O); and *G. vermiculophylla* and *D. dichotoma* on the beach of Camahuiroa (26° 29′ 23" N and 109° 15′ 53" O). They were transported in sealed containers at 20 °C, washed with fresh water to remove epibionts and acclimated to laboratory conditions for one week until their seeding in the system and the beginning of each bioassay.

2.2. Effect of a co-culture of shrimp and macroalgae on the survival, growth and immune response of L. vannamei in the presence of Vibrio parahaemolyticus

2.2.1. Co-culture of shrimp and macroalgae

The co-culture system consisted of 16 aquaria of 60 L each, and in each aquarium, 18 shrimps (63 org·m^{-2}), with a weight of 1.7 ± 0.17 g, were placed. Two species of macroalgae, *G. vermiculophylla* and *D. dichotoma*, were used. Four aquariums were used as controls, without macroalgae. Six aquariums (three with 2 g L^{-1} and three with 4 g L^{-1} of live algae) were planted with each macroalga.

The culture lasted 30 days. The temperature and dissolved oxygen were measured once daily using an YSI 55 digital oximeter (Yellow Spring Instrument, Yellow Spring, Ohio, USA), and the salinity was measured with an Aquafauna® refractometer with a compensated temperature. The concentration of nitrogen compounds (N-NH_4, N-NO_2, and N-NO_3) was measured once weekly with a Biotek ™ Mod. 131030C microplate reader. Feces and residual food were extracted each morning with a siphon after recording the physical-chemical factors and before starting the first food ration.

For algae, fluorescence light was provided at an intensity of 50 μMol·m^{-2}s^{-1}, with a 24:0 photoperiod [26]. Camaronina® 35% PC micropellet balanced feed was supplied to all aquaria twice a day (8:00 and 18:00 h), recording the daily consumption and adjusting the dose according to the demand of the shrimp.

2.2.2. Bacterial challenge with V. parahaemolyticus

The *V. parahaemolyticus* strain was obtained from the CIBNOR microbiology laboratory in La Paz, BCS, Mexico, which was activated in a trypticase soybean broth culture for a period of 24 h at 37 °C. After the co-culture, 10 shrimp from each aquarium were used, injecting the bacteria into the ventral sinus of the cephalothorax with a dose of 10 μL g^{-1} of bacterial inoculum (1×10^6 CFU mL^{-1}) for each organism. Mortality was recorded every 6 h for the first 24 h and thereafter every 24 h up to 120 h post infection.

Three organisms from each aquarium were used to perform the TCH. Three shrimp were used from each aquarium; one tissue sample of 100 mg was taken from each shrimp, and 500 μL of a 0.05 N phosphate buffer solution (pH 7.8) was added and stored at −60 °C. The tissue samples were processed by thawing and were kept below 4 °C; they were then macerated using a PowerGen brand tissue homogenizer and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was placed in Eppendorf ™ tubes and stored at −60 °C for further analyses of the soluble protein and enzymatic activity of superoxide dismutase and catalase [3].

2.3. Effect of a co-culture of shrimp and macroalgae on the survival, growth and immune response of L. vannamei in the presence of white spot virus

2.3.1. Co-cultivation of shrimp and macroalgae for viral challenge

Twelve aquaria that were 60 L each were used for the co-cultivation (three of each species of macroalgae and three as controls). *U. lactuca* was incorporated into this experiment. Four treatments were used with three replicates each, including a control treatment in which no macroalgae species were integrated as well as three treatments with a live macroalgae species in co-cultivation with shrimp. Each co-culture had a macroalgae density of 2 g L^{-1} fresh weight.

Prior to the planting of organisms, algae were placed in each aquarium according to the treatments and were randomly distributed in the bioassay area. The shrimp were selected with a weight of 0.75 ± 0.12 g and were planted in the aquarium at a rate of 20 organisms per aquarium (70 org·m^{-2}). The culture lasted for 30 days. They were fed to satiety twice a day (8:00 and 18:00 h) with Camaronina® 35% PC micropellets.

Temperature and dissolved oxygen were measured once daily using aYSI 55 digital oximeter, and salinity was measured with an Aquafauna® refractometer with a compensated temperature. The concentration of nitrogen compounds (N-NH_4, N-NO_2, and N-NO_3) was measured once weekly with a Biotek ™ Mod. 131030C microplate reader. Feces and residual food were extracted with a siphon after recording the physical-chemical factors and before starting the first food ration. The same photoperiod and light intensity were used as in the previous bioassay [26].

2.3.2. Determination of the 50% lethal viral dose (LD_{50})

Positive cryopreserved WSSV organisms were obtained from the laboratory of the Department of Biotechnological Research of ITSON, Cd. Obregón, Sonora. To prepare the viral inoculum, the modified method of White et al. [27] was used. One gram of shrimp tissue was macerated with 5 mL of a saline solution (2.5%) and centrifuged at 3000 g for 5 min at 4 °C, and the supernatant was stored at −60 °C.

Fifteen 60 L aquaria were used, and 1.2 ± 0.2 g of 10 organisms was placed in aquarium. Four viral inoculums (1:1, 1:10, 1:100 and 1:1000) were prepared from the 1:1 inoculum and randomly distributed in triplicate treatments. Control organisms (3 aquaria) were injected with a saline solution (2.5%), and the rest of the organisms were injected with the previously mentioned inoculums (three aquaria per inoculum) at a dose of 10 μL g^{-1} of living organisms. WSSV were injected into the ventral sinus of the cephalothorax.

After five days (120 h), the total mortality of each treatment was recorded, reaching 0% for the saline solution and 1:1000 for the inoculum, while for inoculations of 1:1, 1:10 and 1:100, the total mortality was 100, 80 and 20%, respectively, and the LD50 with the 1:16 inoculum was determined using a logarithmic equation, which was used in the viral challenge.

2.3.3. Viral challenge with white spot syndrome virus (WSSV)

A system of 14 60 L aquaria containing filtered and chlorinated sea water was used. The aquarium system had constant aeration and physical-chemical control. A daily water change of 20% was performed. All shrimp from each aquarium were injected with an inoculum dose (1:16) of 10 μL g^{-1} of live weight and with saline (2.5%) for the negative control organisms. They were observed for 120 h, recording mortality, and were fed satisfactorily twice a day. At the end of the experiment, three organisms were taken from each aquarium for hemolymph extraction and three for tissue extraction to determine the soluble protein as well as the enzymatic activity of SOD and CAT.

2.4. Immune response and antioxidant activity

To determine the immune response and antioxidant activity, hemolymph and tissue samples were used, which were obtained from both experiments and were analyzed separately for each bioassay.

2.4.1. Total count of hemocytes

Three organisms were taken from each aquarium, and each was extracted from 100 to 200 μL of hemolymph with 300 μL of an anticoagulant solution based on NaCl (450 mM), EDTA (10 mM), KCl (10 mM) and HEPES (4- (2-hydroxyethyl) -1-piperazineethanesulfonic acid) [3], adjusted to a pH of 7.3. This solution was filtered using a 1.4 μm micropore membrane and stored at 4 °C for counting. Anticoagulant solution (0.3 mL) was placed in a 1 mL hypodermic syringe, and the hemolymph was extracted from each organism at the beginning of the 5th pereiopod, homogenized and placed in 2.0 mL Eppendorf ™ tubes. These already labeled samples were immediately placed in an ice bath to maintain them at 4 °C for immediate analysis. A hemocytometer that was 0.1 mm deep was used to count the hemocytes with the aid of a compound microscope, and their density was calculated, with the results in cel·mL^{-1}.

2.4.2. Determination of soluble protein in tissue

Samples of three shrimp were taken from each aquarium. The Bradford method (1976) was used. Tissue samples were prepared by taking 100 mg of tissue homogenized in 500 μL of a 50 mM phosphate buffer solution, pH 7.8 (K_2HPO_4/KH_2PO_4), and centrifuged at 3000 rpm for 10 min at 4 °C, and the supernatant was stored at −60 °C until use.

To determine the soluble protein in the tissue extract, a 1:100 (supernatant: buffer) dilution was made. Then, 160 μL of sample and 40 μL of Bradford reagent (BioRad 500-0006) were taken in triplicate, placed in the microplate and incubated at room temperature for 10 min. Absorbance was read at 595 nm, and the protein concentration was calculated using a standard bovine albumin curve [28].

2.4.3. Determination of superoxide dismutase (SOD)

The SOD activity was determined according to the method of Beauchamp and Fridovich [29], using nitro blue tetrazolium (NBT) in the presence of riboflavin. Activity was expressed in units per milligrams of tissue soluble protein (U SOD·mg^{-1}).

2.4.4. Determination of catalase

The catalase activity was determined according to Aebi [30]. The reduction of the hydrogen peroxide (H_2O_2) concentration at 240 nm was recorded based on an extinction coefficient of 40 M^{-1} cm^{-1}, where a unit of catalase decomposes 1 μmol H_2O_2·min^{-1} and a change in the optical density per unit time is a measure of catalase activity.

2.5. Statistical analysis

One-way analysis of variance was used to analyze the differences between different treatments ($p < 0.05$), and Tukey's test was used to compare the means of each treatment. Previously, tests of normality and the homogeneity of variance were performed.

3. Results

3.1. Effect of a co-culture of shrimp and macroalgae on the survival, growth and immune response of L. vannamei in the presence of Vibrio parahaemolyticus

Table 1 shows the physicochemical factors of a water co-cultivation of shrimp and macroalgae. The temperature remained stable during the 30 days of the culture, between 29.7 and 29.9 °C, whereas the oxygen values were between 5.70 and 5.78 mg L^{-1}. The salinity did not vary, and 35 PSU (Practical Salinity Unit) was recorded without variation,

Table 1
Record of physical-chemical factors of *L. vannamei* bioassay in co-cultivation with macroalgae.

Treatment	Tempe-rature (°C)	dissolved oxygen (mg·L^{-1})	pH	Salinity (PSU)	Ammo-nium (mg·L^{-1})	Nitrites (mg·L^{-1})	Nitrates (mg·L^{-1})
Control	29.86 ± 0.18	5.70 ± 0.11	7.92 ± 0.04	35.0 ± 0.0	0.88^{a} ± 0.13	0.15^{a} ± 0.08	2.99 ± 1.51
G. vermiculophylla (2 g L^{-1})	29.78 ± 0.08	5.77 ± 0.14	7.88 ± 0.04	35.0 ± 0.0	0.23^{c} ± 0.09	0.11^{b} ± 0.01	3.30 ± 1.27
G. vermiculophylla (4 g L^{-1})	29.79 ± 0.06	5.86 ± 0.04	7.80 ± 0.12	35.0 ± 0.0	0.32^{bc} ± 0.10	0.10^{b} ± 0.04	3.21 ± 1.70
D. dichotoma (2 g L^{-1})	29.73 ± 0.07	5.79 ± 0.07	7.88 ± 0.04	35.0 ± 0.0	0.45^{b} ± 0.11	0.13^{b} ± 0.04	2.34 ± 1.23
D. dichotoma (4 g L^{-1})	29.90 ± 0.05	5.78 ± 0.05	7.89 ± 0.03	35.0 ± 0.0	0.51^{b} ± 0.16	0.15^{b} ± 0.05	2.73 ± 1.26

Mean ± standard deviation of three replicates. Different letters in the columns indicate significant differences between the means of the treatments ($p < 0.05$).

Table 2
Zootechnical parameters of the immunostimulation bioassay for *L. vannamei*, in co-cultivation with macroalgae and challenged with *V. parahaemolyticus*.

Treatment	Wi (g)	Wf (g)	S%	FCR	FAC
Control	1.726 ± 0.18	3.90^{b} ± 0.66	100 ± 0.0	1.16 ± 0.04	84.60 ± 2.68
G. vermiculophylla 2 g L^{-1}	1.725 ± 0.18	4.02^{ab} ± 0.58	94.4 ± 0.05	1.20 ± 0.14	83.23 ± 2.96
G. vermiculophylla 4 g L^{-1}	1.711 ± 0.16	3.92^{b} ± 0.56	96.3 ± 0.06	1.21 ± 0.07	82.43 ± 2.24
D. dichotoma 2 g L^{-1}	1.727 ± 0.17	3.93^{b} ± 0.55	96.3 ± 0.06	1.26 ± 0.14	85.37 ± 2.32
D. dichotoma 4 g L^{-1}	1.725 ± 0.18	4.30^{a} ± 0.70	96.3 ± 0.03	1.13 ± 0.05	83.58 ± 1.80

Mean ± standard deviation of three replicates. Different letters in the columns indicate significant differences between the means of the treatments ($p < 0.05$). Wi = initial weight, Wf = final weight, S% = survival percentage, FCR = Feed conversion rate, FAC = Food apparently consumed.

whereas the pH ranged from 7.80 to 7.92. There were no significant differences ($p < 0.05$) between the means of the treatments for any of the mentioned parameters. The ammonium content (Table 1) clearly showed that aquariums that had macroalgae in co-culture with shrimp recorded a lower concentration compared to the control. The ammonium concentration of the control was the highest at 0.88 mg L^{-1}, whereas the lowest concentration was obtained with *G. vermiculophylla* (2 g L^{-1}) 0.23 mg L^{-1}. *D. dichotoma* (in both densities: 2 and 4 g L^{-1}) removed less ammonia from the water compared to *G. vermiculophylla*, registering between 0.45 and 0.51 mg L^{-1} on average during the culture.

The zootechnical parameters of the co-culture bioassay are presented in Table 2. These data show that shrimp cultured with *D. dichotoma* (at a density of 4 g L^{-1}) obtained significantly higher growth ($p < 0.05$) than the rest of the treatments and controls. Survival ranged from 96 to 100%, with no significant differences between treatments. The feed conversion rate resulted in values from 1.13 to 1.26.

Fig. 1 shows the TCH obtained from the immune response of shrimp infected with *V. parahaemolyticus* at 120 h post infection. Analysis of these results showed an increase of 1.4–1.6 times in organisms treated with macroalgae (*G. vermiculophylla* and *D. dichotoma* in both densities) with respect to the control organisms (positive and negative).

The soluble protein found in shrimp treated with *V. parahaemolyticus* was approximately 1.5 times higher for treatments than for controls (Table 3). The values of the SOD enzyme activity were higher for treated organisms and significantly lower for controls (2.36 and 1.57 U SOD·mg $Prot^{-1}$, respectively). The enzymatic activity of catalase was higher for *D. dichotoma* in the two densities and *G. vermiculophylla* at 4 g L^{-1}, with values ranging from 25.67 to 33.27 U CAT·mg $Prot^{-1}$, whereas the mean activity was obtained with *G. vermiculophylla* at 2 g L^{-1} (15.76 U Cat·mg $Prot^{-1}$), and the positive and negative controls had the lowest activity (8.6 and 9.6 U Cat·mg $Prot^{-1}$, respectively).

Table 3 shows the values obtained from the survival of organisms postinfection with *V. parahaemolyticus*. The highest survival was obtained by the organisms treated in co-culture with the two macroalgae and their respective densities, with values of 100% for macroalgae at 4 g L^{-1}, followed by treatments with a macroalgae density of 2 g L^{-1} and negative control organisms (87.5–95.8%), while the lowest survival was obtained with positive control shrimp (62.5%).

3.2. Effect of a co-culture of shrimp and macroalgae on the survival, growth and immune response of L. vannamei in the presence of white spot syndrome virus

The physicochemical factors are shown in Table 4. The environmental conditions during culture were stable and within the recommended intervals for the species [31], with no significant differences between the treatments and controls. Significant differences were only observed in the concentration of ammonium in water, where treatments with *U. lactuca* registered the lowest concentration (0.0 mg L^{-1}), followed by *G. vermiculophylla* and *D. dichotoma* (0.04 and 0.12 mg L^{-1}, respectively). The highest concentration was recorded in the control aquaria (0.21 mg L^{-1}).

The zootechnical parameters of growth, survival, and the feed conversion rate (FCR) are presented in Table 5. There were no significant differences in the survival of treatments or controls ($p < 0.05$).

Differences were found in the final weight of shrimp, with the treatments with *D. dichotoma* and *U. lactuca* having the largest size (3.30 and 3.18 g, respectively), followed by *G. vermiculophylla* and controls, which had the smallest size (3.03 and 2.68 g, respectively). The values of the feed conversion rate coincide with the growth, in relation to the trend recorded, where the best factor was treatments with D. dichotoma and U. lactuca (0.90 and 0.99, respectively), and G. vermiculophylla and controls 1.04 and 1.15, respectively). The immunological response expressed as the total count of hemocytes found in WSSV-infected shrimp showed that organisms treated in co-culture with the three algae had a greater response (1.4–1.6 times) than infected and uninfected controls, registering 3.82 to 4.21 × 10^{6} cel·mL^{-1} and 2.65 × 10^{6} cel·mL^{-1}, respectively (Fig. 2).

In the bioassay in which shrimp were infected with WSSV, the

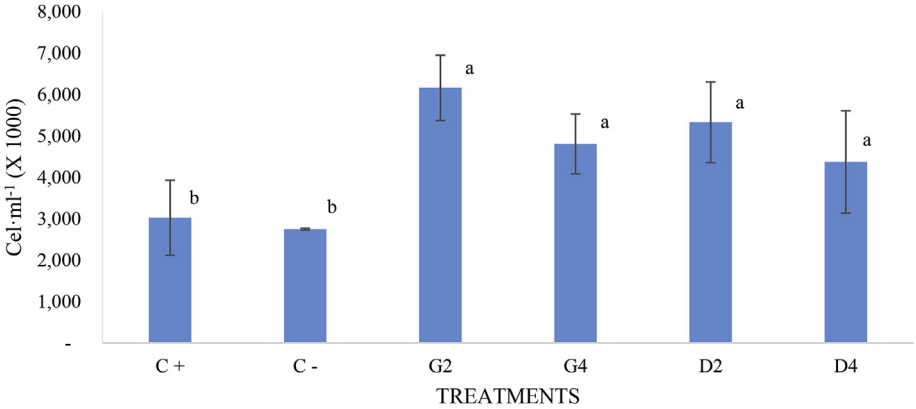

Fig. 1. Total count of hemocytes in *L. vannamei* in co-cultivation with macroalgae and infected with *V. parahaemolyticus*. (C+: positive control, C-: negative control, G: *G. vermiculophylla*, D: *D. dichotoma*, 2: 2 g L^{-1} macroalgae, 4: 4 g L^{-1} macroalgae). Different letters in the bars indicate significant statistical differences ($p < 0.05$).

Table 3
Antioxidant response and survival of *L. vannamei* in co-cultivation with macroalgae and infected with *V. parahaemolitycus*.

Treatment	Soluble protein (mg·mL^{-1})	SOD (U·mg Prot^{-1})	CAT (U·mg Prot^{-1})	Survival %
Control +	16.37^{b} ± 3.23	1.55^{b} ± 0.19	8.67^{c} ± 2.11	62.5^{c} ± 17.6
Control -	17.92^{b} ± 2.25	1.60^{b} ± 0.12	9.64^{c} ± 2.30	87.5^{b} ± 0.00
G. vermiculophylla 2 g L^{-1}	20.37^{ab} ± 1.85	2.07^{ab} ± 0.53	15.76^{b} ± 3.45	95.8^{ab} ± 7.22
G. vermiculophylla 4 g L^{-1}	22.79^{a} ± 1.77	2.95^{a} ± 0.56	25.67^{a} ± 5.67	100.0^{a} ± 0.00
D. dichotoma 2 g L^{-1}	25.63^{a} ± 2.47	2.22^{a} ± 0.53	32.45^{a} ± 3.45	91.7^{b} ± 7.22
D. dichotoma 4 g L^{-1}	24.40^{a} ± 2.72	2.21^{a} ± 0.39	33.27^{a} ± 6.98	100.0^{a} ± 0.00

Mean ± standard deviation of three replicates. Different letters in the columns indicate significant differences between the means of the treatments ($p < 0.05$).

Table 4
Record of physical-chemical factors of *L. vannamei* bioassay in co-cultivation with macroalgae.

Treatment	Tempe-rature (°C)	dissolved oxygen (mg·L-1)	pH	Salinity (PSU)	Ammo-nium (mg·L-1)	Nitrites (mg·L-1)	Nitrates (mg·L-1)
Control	27.47 ± 1.14	5.87 ± 0.20	8.04 ± 0.19	35.3 ± 0.4	0.21^{a} ± 0.04	0.19 ± 0.43	3.43 ± 2.10
G. vermiculophylla	27.46 ± 1.01	5.90 ± 0.16	8.04 ± 0.14	35.4 ± 0.4	0.04^{bc} ± 0.09	0.55 ± 0.69	2.85 ± 1.47
D. dichotoma	27.46 ± 1.01	5.87 ± 0.15	8.01 ± 0.14	35.3 ± 0.4	0.12^{ab} ± 0.16	0.73 ± 0.62	3.03 ± 1.60
U. lactuca	27.47 ± 0.98	5.90 ± 0.18	8.03 ± 0.14	35.4 ± 0.4	0.00^{c} ± 0.0	0.29 ± 0.30	1.74 ± 1.01

Mean ± standard deviation of three replicates. Different letters in the columns indicate significant differences between the means of the treatments ($p < 0.05$).

results of the soluble tissue protein concentration did not show significant differences ($p < 0.05$), but some differences were found for the SOD and CAT activities (Table 6). The highest activity of SOD was obtained in organisms treated with the three macroalgae (5.26 ± 0.08 U SOD mg Prot·mL^{-1}), without differences between treatments, but with differences compared to the controls (3.025 ± 0.5 U SOD mg Prot·mL^{-1}). The highest activity of CAT was obtained in shrimp treated in co-culture with *D. dichotoma*, for which a value of 43.49 U Cat mg Prot·mL^{-1} was obtained, followed by treatments with G. *vermiculophylla* and *U. lactuca*, which had values of 21.74 and 25.56 U Cat mg Prot·mL^{-1}, respectively. The lowest activity of catalase was obtained for the positive and negative controls, with values of 9.96 and 8.86 U Cat mg Prot·mL^{-1}, respectively ($p < 0.05$).

The survival of organisms challenged with WSSV is shown in Table 6. The highest survival was obtained by the negative control organisms and those treated in co-culture with *G. vermiculophylla* (93.7 and 80.0%, respectively), whereas organisms in co-culture with *D. dichotoma* and *U. lactuca* survived at a rate of 73.3% in both treatments. The lowest survival was obtained in control positive organisms, with 56.2%.

4. Discussion

4.1. Effect of a co-culture of shrimp and macroalgae on the survival, growth and immune response of L. vannamei in the presence of Vibrio parahaemolyticus

The immune response in shrimp is an important parameter for measuring the immuno-stimulating effect of certain compounds, such as β-glucans, probiotics, sulfated polysaccharides, and yeasts, among others [6].

4.1.1. Physicochemical factors

The environmental conditions were kept within the range of preference for *L. vannamei* [31], without significant differences, assuming that they were not a determining factor to obtain better growth in the treated organisms with respect to the controls. At the same time, the conditions of temperature and salinity were within the preferences of the macroalgae [26,32].

No publications were found that establish the environmental conditions at which major species *D. dichotoma* and *U. lactuca* develop, but according to Ochoa-Izaguirre et al. [33], the three species studied share the same habitat, as they are found in Gulf of California as well as in tropical and subtropical zones around the world. It is considered that, like *G. vermiculophylla*, the other two species were under optimal conditions and developed properly in the removal of the ammonium.

4.1.2. Zootechnical parameters

The greater weight gain of the organisms treated with macroalgae suggests that macroalgae are a viable alternative for animal nutrition. Benjama and Masniyom [34] mention that *Ulva* sp. have a profile of amino acids, fatty acids, and physico-chemical properties that allow them to be used as food.

Cruz-Suárez et al. [35], in their bibliographic review work on the use of macroalgae in shrimp feed and co-culture, found that these species provide many benefits related to growth, the feed conversion rate, disease resistance, greater food stability in water, better quality of postharvest shrimp, among others.

Although no reduction in FCR was observed in this bioassay, there are studies, such as those performed by Cruz-Suárez et al. [36], in which co-cultivation of *U. clathrata* and *L. vannamei* was performed, leading to a reduction of the use of balanced feed between 10 and 45% with respect to control organisms, which is why it is considered one of the major benefits of shrimp culture.

Table 5
Zootechnical parameters of the immunostimulation bioassay for *L. vannamei*, in co-cultivation with macroalgae and challenged with white spot virus.

Treatment	Wi (g)	Wf (g)	S%	FCR	FAC
Control	0.750 ± 0.12	2.68^{c} ± 031	90.0 ± 10.0	1.15^{a} ± 0.07	55.7 ± 14.7
G. vermiculophylla	0.745 ± 0.12	3.03^{b} ± 0.38	95.0 ± 5.0	1.04^{ab} ± 0.06	59.7 ± 4.95
D. dichotoma	0.747 ± 0.12	3.30^{a} ± 0.12	96.7 ± 2.9	0.90^{c} ± 0.05	57.4 ± 2.27
U. lactuca	0.747 ± 0.10	3.18^{ab} ± 0.04	95.0 ± 8.7	0.99^{bc} ± 0.05	58.9 ± 6.72

Mean ± standard deviation of three replicates. Different letters in the columns indicate significant differences between the means of the treatments ($p < 0.05$). Wi = initial weight, Wf = final weight, S% = survival percentage, FCR = Feed conversion rate, FAC = Food apparently consumed.

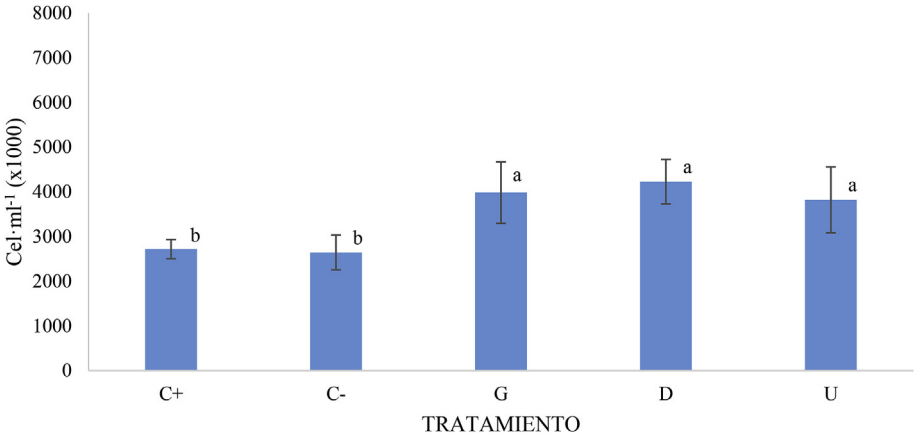

Fig. 2. Total count hemocytes in *L. vannamei* in co-cultivation with macroalgae and challenged with white spot virus. (C+: positive control, C-: control negative, G: *G. vermiculophylla*, D: *D. dichotoma*, U: *U. lactuca*). Different letters in the bars indicate significant differences ($p < 0.05$).

Table 6
Antioxidant response and survival of *L. vannamei* in co-culture with three macroalgae and infected with WSSV.

Treatment	Proteína soluble (mg·mL^{-1})	U SOD (U·mg Prot^{-1})	U CAT (U·mg Prot^{-1})	Supervivencia %
Control +	19.2 ± 2.43	3.02^{b} ± 0.45	9.96^{c} ± 4.32	56.2^{c} ± 8.84
Control -	18.6 ± 2.10	3.03^{b} ± 0.68	8.86^{c} ± 4.49	93.7^{a} ± 8.84
G. vermiculophylla	18.9 ± 3.10	5.29^{a} ± 1.50	21.74^{b} ± 6.58	80.0^{ab} ± 13.13
D. dichotoma	22.0 ± 3.66	5.34^{a} ± 1.32	43.49^{a} ± 9.04	73.3^{b} ± 6.67
U. lactuca	18.8 ± 3.30	5.17^{a} ± 1.25	25.56^{b} ± 6.79	73.3^{b} ± 6.67

Mean ± standard deviation of three replicates. Different letters in the columns indicate significant differences between the means of the treatments ($p < 0.05$).

4.1.3. Survival

One of the main parameters in the commercial production of cultured shrimp is survival. The results shown in this bioassay were expected since the lowest survival was obtained by shrimp that were not treated and were infected in comparison with the negative controls and organisms treated with macroalgae. This is a reflection of the benefit of macroalgae in the fight against disease in shrimp, as discussed by Thanigaivel et al. [10], who showed that used macroalgae to fight against diseases of aquatic organisms and found that macroalgae have a great antibacterial capacity and antiviral and immuno-stimulating properties, which greatly improve the survival of aquatic organisms in the presence of some pathogens.

One of the recent studies on this subject that by Esquer-Miranda et al. [12], in which the survival of *L. vannamei* was improved using methanolic extracts of flour from *U. lactuca* and *Caulerpa sertularioides* after infecting shrimp with a *V. parahaemolyticus* inoculum. On the other hand, administration of *G. corticata* and *S. cerevisiae* in diets of *L. vannamei* improved the immune response (TCH) and oxidative stress response (SOD) when shrimp were infected with WSSV [17].

4.1.4. Immune response and antioxidant activity

Another benefit of the use of macroalgae as food in shrimp culture is the improvement in the immune response and greater enzymatic activity in response to oxidative stress, which are products of the fight against pathogenic organisms. This is due to the incorporation of bioactive compounds, mainly sulfated polysaccharides from the cell wall, phenolic compounds and antioxidants. Studies indicate that these compounds have antibacterial activity in *L. vannamei* and *P. monodon* [12,19,20,22,23] and improve the immune response and survival relative to control organisms.

A study by Niu et al. [37] to measure the effect of the inclusion of wakame meal (*Undaria pinnatifida*) on the immune response showed that an inclusion of only 6% of meal in the diet can improve the response to oxidative stress (SOD) and the nutritional quality of shrimp.

The efficacy of the bioactive compounds of the two macroalgae that were used in this bioassay is demonstrated in the TCH as well as the enzymatic activity of SOD and CAT reported here. In this bioassay, we did not find significant differences in the TCH between treatments, but differences existed when compared against the control organisms. The TCH values of this experiment are very low, approximately half those of Campa-Cordova et al. [6], but proportionally, the differences in treatments are similar compared to the control groups.

4.2. Effect of a co-culture of shrimp and macroalgae on the survival, growth and immune response of L. vannamei in the presence of white spot virus

4.2.1. Physicochemical factors

No effects of the zootechnical parameters, immunological response, antioxidant response or survival on the physicochemical factors recorded in this bioassay were found because no significant differences ($p < 0.05$) were found regarding the temperature, oxygen, salinity or pH. The treatments were considered to be maintained under the same conditions. These conditions were recommended for *L. vannamei* [31] and in turn for the three species of macroalgae studied [26,32].

The resulting ammonium concentrations demonstrate that macroalgae are a viable alternative to reduce this ion in aquatic organisms [38–42].

4.2.2. Zootechnical parameters

Weight gain and the feed conversion rate are two of the most important zootechnical parameters in shrimp production. The first represents a reduction in the time of cultivation and the second the use of a less balanced feed, with the economic benefits that these factors represents.

Previous research, in which different species of macroalgae were included in the balanced diet, has shown the viability of using macroalgae to improve growth, survival, and feed conversion rate [36,43,44].

The results of this experiment show that shrimp that fed on the macroalgae were provided the necessary nutrients to improve their growth and FCR, mainly for *D. dichotoma* with respect to the rest of the macroalgae and the controls.

4.2.3. Survival

For a bioassay of this nature, the results were as expected. The lower survival rate obtained by WSSV-infected control shrimp is a reflection of the antiviral activity of bioactive compounds of macroalgae [10,12]. In turn, uninfected control shrimp showed the highest survival, and although there were significant differences between the treatments and negative control, the survival of the negative controls was very high with respect to the infected controls.

Considering that worldwide WSSV has devastated the industry, with mortality close to 100% in most growing areas, the organisms treated in this experiment when infected with the virus obtained very high survival with respect to current production standards.

The viability of the use of macroalgae as an immuno-stimulant in its different presentations (extracts, flours, immersions, etc.) is a viable alternative [10,45].

4.2.4. Immune response and antioxidant activity

As in the experiment in which shrimp were grown with macroalgae and were infected with *V. parahaemolyticus*, shrimp that were infected with WSSV had a better immune response than the controls. Although there were no significant differences between treatments ($p < 0.05$), a higher TCH was observed in *D. dichotoma* than in *G. vermiculophylla* and *U. lactuca*. The context of previous studies demonstrates the effectiveness of the supply of macroalgae, either in extracts of different types or included in the diet, in the immune response and activity of antioxidant enzymes, such as SOD and CAT.

It has been shown that brown algae (Phaeophyceae) that possess fucoidan as a sulfated polysaccharide [9,19,37] help improve the response of hemocytes to oxidative stress for battling pathogens [4]. The sulfated polysaccharides of *Ulva clathrata* and *M. pyrifera* act in the initial stages of viral infection, preventing the entry of the virus to the cell, disabling the events of viral fusion (virus - cell and cell - cell) and, as a consequence, disabling dissemination [45].

The polysaccharides isolated from *Gracilaria fisheri* and supplied to *L. vannamei* improve the immune response and resistance to WSSV and, in turn, the responses to oxidative stress [46]. Polysaccharides had values close to 40×10^6 cel·mL^{-1}, well above that reported in this experiment ($3.8–4.2 \times 10^6$ cel·mL^{-1}), but equal to the significant differences between control organisms.

No previous or recent studies on immuno-stimulation in shrimp penaeids have been found using live macroalgae in co-culture, but there are reports of investigations using macroalgae flour [17] and methanolic extracts [12], as well as the use of lactic acid bacteria [16], β-glucans and vitamin C [15], sulfated red algae polysaccharides [46], sulfated polysaccharides of brown algae [19], and the aqueous extract of *G. bicolor* [18], among others. These investigators evaluated the immuno-stimulating effect, oxidative stress response and survival of each respective treatment on the *L. vannamei* response to infection by WSSV, *V. parahaemolyticus* or *V. alginolyticus*.

In all of the mentioned studies, it has been demonstrated, to a lesser or greater degree, that if there is an immunostimulating effect in *L. vannamei* when treated with macroalgae and its bioactive compounds, survival has the same pattern, in which negative control organisms that were not treated or infected with any pathogens recorded the greatest survival in the challenge, while infected positive control organisms showed the lowest. Also, organisms treated with macroalgae, their extracts or some other immunostimulating compounds, recorded a slightly lower survival to the control negative organisms, but with values that were attractive on a commercial scale, where the survival rates are much lower.

A common practice in shrimp farming is the excessive use of antibiotics and artificial antimicrobials, which cause pathogen resistance to drugs [10,23], so it is advisable to use natural products that improve the resistance of shrimp to bacterial and viral diseases [9,10,45,47].

5. Conclusion

Of the three algae studied in the immunostimulation bioassays infected with *V. parahaemolyticus* and WSSV, a better immune response was obtained from treated organisms compared to the controls, suggesting that algae provides bioactive compounds to improve the immune response through production of hemocytes in response to oxidative stress, as expressed in the quantification of soluble protein and activity of SOD and CAT enzymes.

Co-cultivation of macroalgae and shrimp provides them with adequate environmental conditions to reduce stress due to the low concentration of ammonium, thus allowing shrimp to direct their energy to combat infectious diseases, such as *V. parahaemolyticus* or WSSV.

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