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Research Article

Dietary supplementation effect of three microalgae on *Penaeus vannamei* growth, biochemical composition, and resistance to *Vibrio parahaemolyticus* (AHPND)

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ABSTRACT. Microalgae are a promising solution in shrimp farming overexploitation. This study assessed the efficacy of microalgae as a food additive for *Penaeus vannamei* growth and their effect on immune system stimulation against *Vibrio parahaemolyticus*. Three diets were formulated with 0, 1, and 3% (DC, D1, and D3, respectively) of a mixture of three different microalga species, *Tetraselmis suecica*, *Dunaliella salina*, and *Chaetoceros muelleri*, and growth, survival, and post-harvest quality in shrimp culture were evaluated. Two bioassays were performed: a 60-day feeding trial and an immersion infection bioassay against *V. parahaemolyticus*. D1 was the best treatment and significantly different to DC ($P \leq 0.05$), achieving a food conversion factor of 1.24 ± 0.11 and 1.85 ± 0.38; specific growth rate 1.58 ± 0.03% d^{-1} and 1.35 ± 0.11% d^{-1}; and weight gain of 5.68 ± 0.32 g and 4.79 ± 0.33 g, respectively. Protein content in shrimp muscle was positively increased by microalgae inclusion, achieving 20.8 ± 0.2% (D1) to 21.7 ± 0.3% (D3), 19.2 ± 0.1% (DC). In the infection bioassay, D1 and D3 reached a 100% survival rate, and histological damage in the hepatopancreas was not observed, suggesting immune system stimulation. These results indicated that microalgae added to food are an excellent source of proteins, carbohydrates, lipids, and promoters of antimicrobial activity that allowed additional protection against mortality caused by *V. parahaemolyticus*.

Keywords: *Penaeus vannamei*; *Vibrio parahaemolyticus*; feed; marine microalgae; aquaculture

INTRODUCTION

Penaeus vannamei is the most valuable shrimp species (FAO 2018). Increases in world population have caused overexploitation of shrimp farming, leading to intensive farming practices to meet market demands. This situation has caused the emergence of viral and bacterial diseases linked to intensive production, inappropriate use of antibiotics, and underutilization of natural diets (Pérez-Sánchez et al. 2018). Future aquaculture profitability requires new production techniques, such as improving diet formulation and efficiency, representing about 50 to 80% of the operational cost of the shrimp farming industry. Several research studies have proposed the use of bioactive or immunostimulant compounds, such asβ-glucan, β-carotene, probiotics, and yeasts as additives in a balanced food formula leading to better shrimp development and production, improving growth rate, biochemical muscle composition, and stimulating the innate immune system (Li et al. 2016, Debasis et al. 2018).

In aquaculture, an alternative is the use of microalgae because of their optimal nutritional and functional compounds, including highly digestible proteins and poly-unsaturated fatty acids (PUFA) necessary for early development and carotenoid pigments that give the desirable reddish color to crustaceans

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(Yaakob et al. 2014). Microalgae also provide antioxidant activity related to enhancing shrimp immune system (Wang et al. 2017, Shah et al. 2018). Therefore, adding microalgae to diets improves survival, growth, and food efficiency and induces an immunonutrition effect on organisms (Pakravan et al. 2017, Allen et al. 2019).

Recent research has focused on testing different levels of microalgae in shrimp diets. Evidence suggests that adding just a small amount of microalgae positively affects immune response and increases shrimp survival since immunostimulants do not show a linear dose-effect relationship (Sajeevan et al. 2009, Medina et al. 2019, Pratiwi 2020). Therefore, low percentages have been chosen to reduce microalgae in shrimp feed and thus the costs of producing microalgal biomass. The main microalgae used in aquaculture are diatoms *Skeletonema*, *Chaetoceros* and chlorophytes *Dunaliella*, *Tetraselmis*, and *Chlorella*. *Dunaliella salina* may produce antiviral and antibacterial activity compounds so that it may be an excellent additive as a shrimp feed ingredient.

On the other hand, the potential use of *Chaetoceros muelleri* and *Tetraselmis suecica* as a feed additive for juvenile *P. vannamei* has not been extensively investigated. Although research has shown that these microalgae have various nutritional benefits for aquatic organisms and different microalga species have been widely used as part of *P. vannamei* larviculture operations, the use of mixtures of microalgal biomass as a dietary additive for juveniles remains relatively unexplored. Hence, this study aimed to evaluate the effect of diets enriched with a mixture of microalgae on *P. vannamei* juvenile growth, post-harvest quality, and resistance to *V. parahaemolyticus*.

MATERIALS AND METHODS

Algal culture

Three microalgal strains were previously investigated according literature and screened against each other to select the species with the best productivity and biochemical composition (data not shown). *Tetraselmis suecica* (TES-2) for high protein content, *Chaetoceros muelleri* (CIB 37) for high lipid content (both obtained from Centro de Investigaciones Biológicas del Noroeste, La Paz, Baja California Sur, México) and *Dunaliella salina* (DUS1, obtained from Centro de Investigación Científica y de Educación Superior de Ensenada, Ensenada, Baja California, México) for high polyunsaturated fatty acid, linoleic, linolenic, octadecenoic and eicosanoid acid contents.

The microalgal strains (*T. suecica*, *C. muelleri*, and *D. salina*) were cultured in 6 L photobioreactors, each one inoculated with an initial cell density of 2×10^6 cell mL^{-1} in sterile seawater with a salinity of 35 and enriched with a sterile nutrient solution (Ulloa et al. 2012). Microalgal proximate composition was determined, and carbohydrate and protein contents were analyzed according to Dubois et al. (1956) and Herbert et al. (1971), respectively. Lipid was extracted and analyzed following Bligh & Dyer (1959) and Marsh & Weinstein (1966) carbonization methods, and fatty acids by Sato & Murata (1988) method using heptadecanoic acid as an internal standard. The Jeffrey & Humphrey (1975) method measured total carotenoids and chlorophyll content. Briefly, 3 mg sample with 5 mL of acetone-methanol (2:1 v/v) solvents was separately homogenized by an Ultrasonic homogenizer (4710 Series, Cole-Parmer Instrument Co., Chicago, IL, USA) at room temperature in darkness. The supernatant was separated, and absorbance was measured at 400-700 nm using an ultraviolet-visible (UV-Vis) spectrophotometer (Spectroquant Pharo 300, Merck, Darmstadt, Germany). All the experiments were performed in triplicate. This study also used three different equations in calculating chlorophyll-*a*, *b*, and total carotene levels.

Chlorophyll-*a* ($\mu g\ mL^{-1}$) = $11.93\ A_{664} - 1.93\ A_{647}$

Chlorophyll-*b* ($\mu g\ mL^{-1}$) = $20.36\ A_{647} - 5.50\ A_{664}$

Total carotenoids ($\mu g\ mL^{-1}$) = $4\ A_{480}$

where A is the sample absorbance at different wavelengths.

Upon harvest, microalgal biomass was partially dewatered using a centrifuge process at 3100 rpm for 10 min, then dried at ~55°C and processed with a mortar until a microalgal powder meal was obtained.

Experimental diets

Two experimental diets (D1 and D3) and control (DC) were calculated in a spreadsheet, so their composition met the nutritional requirements of penaeid shrimp. The diets were isoproteic, isolipidic, and isocaloric (Table 1). DC (positive control) was formulated without microalga biomass, and D1 and D3 were formulated with the addition of microalgal biomass mixtures (in equal portions each) to feedstuffs at 10 and 30 g kg^{-1} dry weight of feed, respectively. According to Achupallas et al. (2016), the diet preparation procedure was used, and vitamins and minerals were added according to the manufacturer's specifications (PIASA™, La Paz, BCS, México). Cornstarch, fish oil, soy, and wheat flour were adjusted to obtain balanced diets. All dry ingredients (including microalgal meal) were sieved using a 250 µm mesh and mixed for 15 min

Table 1. Ingredient and proximate compositions of experimental diets. *Mean values ± standard deviation (n = 3). *Gross energy was estimated using the following coefficients: 23.6 kJ g^{-1} for crude protein, 39.5 kJ g^{-1} for crude fat, and 17.2 kJ g^{-1} for carbohydrates (NCR 1993). D1: diet 1, D3: diet 3, DC: control diet.

Ingredients (g kg^{-1})	D1	D3	DC
Microalgae	11.95	31.87	0.00
Soybean meal	378.41	374.43	390.36
Fishmeal	294.76	294.76	294.76
Wheat flour	111.53	95.60	111.53
Cornstarch	203.78	209.07	204.13
Fish oil	17.48	15.06	17.05
Soybean lecithin	39.17	39.17	39.17
Mineral premix	10.00	10.00	10.00
Vitamin premix	10.00	10.00	10.00
Alginic acid	20.00	20.00	20.00
Ash	3.80	0.00	5.90
Butylhydroxytoluene	0.015	0.015	0.015
Proximate composition value* (% of wet basis)			
Crude protein	46.0 ± 0.8	48.0 ± 0.21	47.0 ± 0.2
Ether extract	10.0 ± 0.25	9.9 ± 0.25	10.2 ± 0.09
Carbohydrate	14.9 ± 0.8	15.8 ± 0.62	15.8 ± 0.62
Fiber	18.6 ± 0.0	19.3 ± 0.01	18.9 ± 0.05
Ash	4.63 ± 0.1	3.81 ± 0.5	4.13 ± 0.8
Moisture	5.7 ± 0.89	3.8 ± 0.33	3.8 ± 0.56
Gross energy* (MJ kg^{-1})	17.36 ± 0.42	17.99 ± 0.25	17.87 ± 0.19

in a Hobart mixer (Hobart A-200, Troy, OH, USA). Then a combination of soybean lecithin and fish oil, which had been previously mixed, was added to the dry ingredient mix. Subsequently, warm (~55°C) water was added to the mix to bring the moisture content of the resulting mash to ~35%, which was ground through a Torrey® grinder (Model MV-22R-SS, Torrey, SA de CV, Nuevo León, México) fitted with a 3 mm diameter die. The resulting moist pellets were dried in a forced-air oven at room temperature (~28-32°C) until moisture below 10% was achieved. Processed pellets were stored in hermetic containers at 2°C and protected from light for usage within 21 days of trial.

The physical and chemical quality of the diets was determined by the contents of protein, fat, carbohydrates, and fiber, according to the Official Methods of Analysis (AOAC 2005), and moisture and leaching by Obaldo et al. (2002). The contents of chlorophyll and total carotenoids in the feed and feces were estimated using Jeffrey & Humphrey (1975) method.

Experimental design and feeding trial

Penaeus vannamei juveniles were provided by Larvas Genesis, SA de CV Sonora, Mexico (3.78 ± 0.41 g). A pleopod of the last pair was removed and analyzed to rule out virus-carrying organisms before bioassays were initiated. Then the diagnosis of Infectious Hypodermal and Hematopoietic Necrosis Virus and White Spot Syndrome Virus was determined by real-time PCR following the kit IQ REA™ Quantitative System (GeneReach Biotechnology Corp. Taichung, Taiwan). The Research Ethics and Animal Welfare Committee reviewed and approved this project of the Instituto Tecnológico de Sonora.

The feeding trial was conducted indoors in circular fiberglass ponds (286 L: 0.6 m^2 round bottom area) that were filled with 115 L of filtered seawater (salinity at 35); a blower provided continuous aeration to maintain dissolved oxygen at 5.5 ± 0.23 mg L^{-1}, the temperature at 27 ± 2°C, and pH 7.9 ± 23. The photoperiod was provided by overhead fluorescent ceiling lights set on at 14:10 h light-dark cycle. The shrimp were randomly selected and weighed to determine an initial average weight and distributed into nine tanks at 16 shrimp per tank density. All triplicate groups of shrimps were hand-fed with one of the three diets with 5% of their weight in feeders, twice a day for 60 days. The feeding rate was adjusted every 10 days, and the amount of diet fed per day was recorded for each tank. All tanks were siphoned daily to remove uneaten diet, feces, and molts before feeding each morning. All treatments were made by triplicate.

At the end of the experiment, shrimp were weighed to determine growth performance (Duan et al. 2017). Response parameters, feed conversion ratio (FCR), feed efficiency (FE), weight gain percentage (PWG),

specific growth rate (SGR), and weight gain (WG) were calculated using the following equations:

$$FCR = \frac{Wf}{W2 - W1}$$

$$FE\ (\%) = WG\left(\frac{100}{Wd}\right)$$

$$PWG\ (\%) = \frac{W2 - W1}{W1} \times 100$$

$$SGR\ \%\ d^{-1} = \frac{\left[ln\ \frac{W2}{W1}\right]}{t} \times 100$$

$$WG\ (g) = W2 - W1$$

where W_f is total feed consumption as dry matter (g); W2 is final shrimp weight (g); W1 is the initial weight (g); t is time (days) for growth assessment, and W_d is dry weight feed offered.

On the other hand, some shrimp from each group were harvested, chill sacrificed in an ice-water bath to drastically reduce body temperature, then weighed, placed into plastic storage bags, and frozen at 20°C for subsequent whole-body proximate analysis. Moisture, crude protein, and lipid content were determined according to AOAC procedures (AOAC 2005).

Infection bioassay with *Vibrio parahaemolyticus*

At the end of the feeding trial, 24 organisms from each treatment were randomly selected and transferred to three glass tanks (20 L) under the same conditions to initiate the infection bioassay- each tank contained eight organisms. Treatments and two controls, positive DC and negative DC (-), were assayed by triplicate. First, organisms were acclimatized for 24 h prior to the challenge with *V. parahaemolyticus*. The bacterial inoculum was obtained from a shrimp-positive organism and identified using conventional morphological and molecular tests. The bacteria were recovered and inoculated in 30 mL Tryptic Soy Broth (TSB, Becton Dickinson, France) supplemented with 1.5% NaCl and incubated at 28°C overnight. The TSB suspensions were checked using a spectrophotometer at an optical density of 600 nm to determine bacterial density; then, these suspensions were plated onto TCBS to determine the colony-forming units CFU mL^{-1} of the isolates (Galaviz-Silva et al. 2021). Infection was carried out by immersion with *V. parahaemolyticus* in each tank with a final bacterial density of 2.24×10^6 CFU mL^{-1} (Joshi et al. 2014). The organisms were continuously monitored for six days during the bioassay. Every dead organism was immediately removed to avoid system contamination.

Organs from infected and controlled shrimp were examined by histology. Davidson's fixative solution was prepared (330 mL 95% ethyl alcohol, 220 mL 100% formalin, 115 mL glacial acetic acid, and 335 mL distilled water) and injected into the surviving organisms hepatopancreas, posterior and anterior abdominal regions. The fixed organisms were preserved in a Davidson fixative solution container for 48 h and then transferred to a 70% alcohol solution. The specimens were cross-sectional cut between the cephalothorax and abdomen and longitudinal sections along the median lines of the cephalothorax. The tissues were then paraffin-embedded, and slices of 5 mm in thickness were obtained using a microtome and stained with hematoxylin-eosin, according to Bell & Lightner (1988) to perform the histological study.

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA). Mean comparisons were performed using Tukey's tests. The Pearson correlation and regression analyses were applied to identify significant correlations among the different diet effects on body composition. All statistical analyses were performed by SPSS Statistics Base 22.0 software (IBM, Armonk, NY, USA).

RESULTS

Diet formulation and quality

The biochemical profile of each ingredient from the balanced diet was required. Table 2 shows the proximal analysis of the three cultured microalgae, in which *Tetraselmis suecica* had the highest protein and chlorophyll content, *Chaetoceros muelleri* the highest carbohydrate and lipid content, and *Dunaliella salina* the highest carotenoids and fatty acid content. The main fatty acids were saturated and mono-unsaturated (16:0 and 18:1) in the three species (Table 3). The fatty acids ω-3 and ω-6 concentrations, essential for shrimp growth, were higher in *D. salina* and *C. muelleri* than *T. suecica*. Moreover, leaching was analyzed as a quality parameter of the diets to verify that all ingredients remained in the pellets immersed for two hours in saltwater. The results showed values for leaching of 5.3 ± 0.4% for DC, 5.6 ± 0.09% for D1, and 5.3 ± 0.3% for D3.

Feeding trial

After feeding the shrimp for 60 days, the response variables were calculated. The results are shown in Table 4. WG is one of the main response variables in aquaculture because it directly affects productivity. After day 14 of the bioassay, an increase in weight was observed in D3 organisms; it showed a greater gain in weight, followed by D1 and DC diets. The response variables of the feeding bioassays are shown in Table 4.

Table 2. Biochemical composition of *Tetraselmis suecica*, *Chaetoceros muelleri*, and *Dunaliella salina* biomass produced in 6-L photobioreactors using "algal" medium. Mean values ± standard deviation (n = 3). Values within the same row with different superscript letters are significantly different ($P < 0.05$).

Strains	Chlorophyll (μg mg^{-1})	Carotenoid (μg mg^{-1})	Protein (%)	Carbohydrate (%)	Lipid (%)
T. suecica	12.9 ± 0.8^{b}	2.64 ± 0.1^{ab}	43 ± 0.7^{c}	17 ± 0.5^{a}	7 ± 0.8^{a}
C. muelleri	8.3 ± 0.4^{a}	2.5 ± 0.1^{a}	35 ± 0.9^{b}	23 ± 0.7^{b}	34 ± 0.5^{c}
D. salina	15.11 ± 0.4^{c}	2.76 ± 0.1^{b}	25 ± 1.8^{a}	17 ± 0.6^{a}	12 ± 0.3^{b}

Table 3. Fatty acid profile (%) of lipid extracted from microalgae biomass (*Tetraselmis suecica*, *Chaetoceros muelleri* and *Dunaliella salina*). ND: not detected; SFA: saturated fatty acid; MUFA: mono-unsaturated fatty acid; PUFA: poly-unsaturated fatty acid; values are given as a percentage (%) of total fatty acids.

Fatty acid	*T. suecica*	*C. muelleri*	*D. salina*
14:0	1.1	18.2	1.8
14:1	0.6	ND	1
15:0	4.4	ND	ND
15:1	2.8	0.3	0.8
16:0	36.2	20.4	33.8
16:1	1.9	19.5	1.7
17:1	0.9	2.3	1.3
18:1	8.8	7.3	10.3
18:1ω9	20.8	2.5	1
18:2ω6	2.5	0.8	12
20:0	9.1	0.9	ND
18:3ω6	3.6	0.2	28.1
20:1	1.1	0.6	ND
18:3ω3	0.2	0.2	2.2
20:3ω3	ND	9.5	ND
20:2	3.9	9.5	ND
20:3ω6	1.3	1	2.4
22:1ω9	ND	2.3	0.3
20:5ω3	ND	ND	2
22:6ω3	0.8	0.6	0.8
SFA	50.8	39.5	35.6
MUFA	40.8	44.3	16.4
PUFA	8.4	12.3	47.5

In general, the organisms fed with D1 diet obtained the highest FE (80.16 ± 5.4%), SGR (1.58 ± 0.03% d^{-1}), WG (5.68 ± 0.32 g), and PWG (160.3 ± 13.5%) values, in contrast to DC diet. Treatments D1 and D3 increased shrimp weight by 60.3 and 45.3%, respectively, with significant differences ($P \leq 0.05$) in DC (25.7%). Concerning the FCR (one of the most important variables to determine feed efficiency in aquaculture), a significant difference ($P \leq 0.05$) was obtained among treatments D1, D3, and DC, with values of 1.24 ± 0.11, 1.26 ± 0.17, and 1.85 ± 0.38, respectively, keeping the behavior of WG and PWG, due to these variables are related to each other.

Chlorophyll and carotenoid content in experimental diets and organism feces were measured because of the importance of knowing if the organism metabolizes these main microalgae pigments. As shown in Table 5, pigments were not detected in shrimps and feces fed with a DC diet. However, by increasing the percentage of microalgal inclusion in diets, the chlorophyll concentration increased significantly ($P \leq 0.05$) and in the feces of shrimp fed with the respective diets.

Proximate composition of *Penaeus vannamei*

At the end of the feeding trial, the biochemical muscle composition of shrimp fed with the experimental diets was analyzed since diet composition impacts post-harvest quality. It is expressed in protein, lipid, and moisture contents (Table 6). In post-harvest analysis, the muscle protein contents of *P. vannamei* fed with DC, D1, and D3 were 19.4, 20.8, and 21.7%, respectively. Protein content on shrimp muscle was associated with the linear model 90% and showed a positive correlation ($r = 0.96$) with the addition of microalgal mixtures on diets. Moisture, lipid, and ash content were neither significantly different nor significantly correlated ($P > 0.05$).

Infection bioassay

Feed formulation is the most important factor for disease prevention in shrimp aquaculture. Therefore, to investigate the effect of adding the microalgal mixture in feed against infection by vibriosis, shrimp were infected with *V. parahaemolyticus* by immersion. The signs in shrimp challenged with *V. parahaemolyticus* included similar characteristics of infected wild or farmed shrimp. The challenged organisms showed visible expansion of chromatophores and reddish coloration in the pereiopods, pleopods, appendages, erratic swimming, anorexia, and lethargy. However, shrimp fed with D1 and D3 reached a 100% survival rate, which was significantly different ($P \leq 0.05$) to DC (Fig. 1). Mortality in positive DC was confirmed 24 h post-infection (HPI), revealing the presence of vibriosis. During the test period, no mortality was observed in the negative DC (-).

Table 4. *Penaeus vannamei* growth results after 60 days of feeding trial. DC: control diet, D1: diet 1, D3: diet 3, W1: initial weight; W2: final weight; FCR: feed conversion ratio; FE: feed efficiency; SGR: specific growth rate; WG: weight gain; and PWG: percentage weight gain of *Penaeus vannamei*. Mean values ± standard deviation (n = 3). Data with different letters showed significant differences ($P \leq 0.05$) among treatments.

Diet	Response variables						
	W1 (g)	W2 (g)	FCR	FE (%)	SGR (%) d^{-1}	WG (g)	PWG (%)
DC	3.81 ± 0.9^a	8.6 ± 1.23^a	1.85 ± 0.38	53.84 ± 3.5^a	1.35 ± 0.11^a	4.79 ± 0.33^a	125.7 ± 22.6^a
D1	3.57 ± 0.5^a	9.25 ± 0.82^a	1.24 ± 0.11	80.16 ± 5.4^b	1.58 ± 0.03^b	5.68 ± 0.32^b	160.3 ± 13.5^b
D3	4.19 ± 0.83^a	10.21 ± 1.45^a	1.26 ± 0.17	74.89 ± 4.2^b	1.48 ± 0.14^b	6.02 ± 0.62^b	145.3 ± 14.3^b

Table 5. Pigment content in experimental diets and shrimp feces (μg mg^{-1}). Mean values ± standard deviation (n = 3). Data with different letters show significant differences ($P \leq 0.05$) among treatments. DC: control diet, D1: diet 1, D3: diet 3, ND: not detected.

	Food		Feces	
	Total chlorophyll	Total carotenoids	Total chlorophyll	Total carotenoids
DC	ND	ND	ND	ND
D1	2.2 ± 0.05^a	0.23 ± 0.04^a	0.52 ± 0.00^a	ND
D3	4.0 ± 0.02^b	1.20 ± 0.00^b	3.0 ± 0.01^b	0.08 ± 0.00^a

Table 6. Proximate composition of *Penaeus vannamei* muscle when shrimp were fed with DC: control diet, D1: diet 1, and D3: diet 3 after 60 days of the rearing period. Mean values ± standard deviation (n = 6). Data with different superscript letters show significantly differences ($P \leq 0.05$) among treatments. *When regression was significant, $R^2 = 0.96$.

Diet	Nutrient (% wet basis)			
	Protein*	Lipid	Moisture	Ash
DC	19.4 ± 0.1^a	0.8 ± 0.07^a	74.9 ± 0.5^a	1.4 ± 0.2^a
D1	20.8 ± 0.2^b	0.8 ± 0.09^a	75.5 ± 1.3^a	1.3 ± 0.2^a
D3	21.7 ± 0.3^c	0.86 ± 0.06^a	75.6 ± 0.6^a	1.3 ± 0.2^a

Histopathological tests from the hepatopancreas of surviving organisms were performed to confirm the results from the *V. parahaemolyticus* infection bioassays (considering that survival in DC (-), D1, and D3 were 100%). The histopathologic cuts showed healthy hepatopancreatic tissues due to the absence of vibriosis lesions (Fig. 2a). Hepatopancreatic tissue from surviving shrimp of groups D1 and D3 showed normal hepatopancreatic histology, same as DC (-) (Fig. 2b). In contrast, the histopathological analysis of dying shrimp from DC showed pathognomonic lesions of acute hepatopancreatic necrosis disease (AHPND) with the massive detachment of epithelial cells from hepatopancreatic tubules and presence of nodules in dying tissue (indicated by black arrows in Figs. 2c-d).

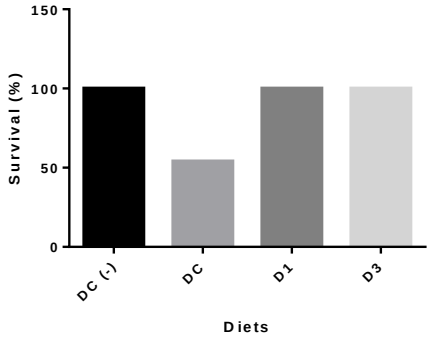

Figure 1. Survival of *Penaeus vannamei* infected with *Vibrio parahaemolyticus*. DC: control diet, D1: diet 1, D3: diet 3.

DISCUSSION

The results of this study indicate that microalgae may be used as a dietary additive for *Penaeus vannamei* juveniles at levels of 1 to 3%, with significant positive effects on FCR, FE, SGR, WG, PWG, muscle protein composition, and immune protection against *Vibrio parahaemolyticus*. Three balanced diets were formulated, and the biochemical composition of microalgae (protein, lipid, fatty acid, and carbohydrate content) was analyzed according to literature (Roy & Pal 2015). Proteins are indispensable in diet formulation because they are responsible for shrimp tissue synthesis and increased growth rate (Tantikitti 2014). However, increases in protein requirements de crease the carbohy-

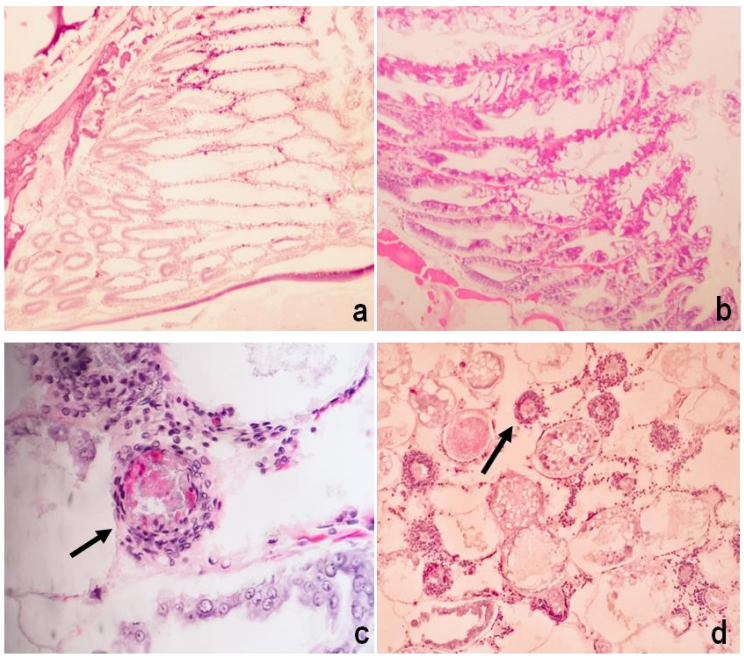

Figure 2. Micrographics of hepatopancreas tissues stained with hematoxylin-eosin of organisms in the different treatments. a) Hepatopancreas of negative DC shrimp fixed at the end of the experiment showing normal histology, b) histology of normal hepatopancreas of surviving shrimp at the end of the experiment fed D3 diet, c-d) lesions of AHPND (arrows: hemocytic nodules) in the hepatopancreas of challenged shrimp for 48 h post-infection of the positive DC group.

drate level, the main dietary energy source (Molina-Poveda et al. 2015). Further-more, lipids are essential for crustacean metabolism because they are a source of essential fatty acids, sterols, and phospholipids. An optimal lipid level was obtained in experimental diets (9.9 to 10.2%) enriched with PUFA, especially the ω-3 and ω-6 fatty acids, which are essential for the tested organisms (Gong et al. 2004). The moisture of the diets was less than 14%, preventing the possible appearance of aflatoxins generated by the fungus *Aspergillus flavus* (Yu et al. 2018). Moreover, leaching of the feedstuffs was less than 15%, which suggests no loss of nutrients in the water (Ly et al. 2019), and that organisms had complete access to all the nutrients present in the feed pellets. In this study, shrimp fed with microalgae-enriched diets grew slightly faster than those in control, with the following descending order: D1, D3, and DC. The results of the feeding trial indicated that diets with microalgal mixtures showed a significant difference ($P \leq 0.05$) in FCR, FE, SGR, WG, and PWG concerning the control, indicating that the greater shrimp growth effect observed in the trial could be attributable to known nutritional factors in the microalgae biomass. These enhanced effects using microalgae-enriched diets may be due to the high level of bioactive compounds as shown in Tables 2 and 3 (ω-3 and ω-6 fatty acids). In addition, algal proteins are considered good quality protein; protein foods improve weight because of the content of essential amino acids.

Treatment D1 and D3 had the best FCR value. FCR is one of the main response variables sought in a feeding trial, related to greater protein synthesis and increased growth. Thus, results are consistent with others reports in which shrimp-fed microalgae-rich diets showed significantly better feed conversion rate and weight gain than the control (Venkateswarlu 2019). Bioactive compounds from microalgae could improve the digestibility and availability of nutrients, which results in greater use and assimilation of these. Subsequently, a greater synthesis of proteins, that is necessary for maintenance and maximum weight gain for the organism (Kureshy & Davis 2002) and therefore greater growth, and productivity in shrimp cultures were observed, affecting FE variable directly. In this sense, in our work, juvenile shrimp fed with D1 y D3 exhibited significantly higher FE than juveniles fed the DC. The lower response resulted from feeding the non-microalgae meal diet.

Likewise, Rahman et al. (2017) reported that *T. chuii* improved growth performance and SRG percentage in *P. vannamei*. Similarly, Ju et al. (2012) found that the inclusion of microalgae meal in four levels (3, 6, 9, and 12%) in the shrimp diet registered the highest ($P \leq 0.05$) utilization of nutrients, the

highest growth and yield of shrimp fed a diet with 3% inclusion *vs*. control. Probably related to the rapid assimilation of the proteins in the microalgae, accelerating the organism's growth, obtaining a higher WG and, therefore, a better PWG.

In this study, D1 was the best diet for production parameters in shrimp rearing and lowered costs because less microalga addition was needed. In markets related to human consumption, the production cost of microalga biomass is greater than 5 € kg^{-1}, but new technologies are required to reduce costs and allow an increase in several orders of magnitude of the current production capacity (Fernández et al. 2019).

One of the most interesting characteristics of the algae is their wide range of pigment content like chlorophylls, carotenoids, phycobiliproteins, and xanthophylls. The three microalga species used in this work contained lipophilic pigments such as chlorophylls and carotenoids. These pigments help animal species combat stressful environments, and although shrimp cannot synthesize carotenoid pigments, they can metabolize them from food (Wade et al. 2017b). The use of carotenoids as an additive in shrimp diets is well documented, and their functions include the role of antioxidant and provitamin A inducer and immune response, growth, reproduction, and maturation enhancer (Wade et al. 2017a). In this study, carotenoids in feces were not detected; hence, they could have been metabolized; in the same way, chlorophyll content in shrimp feces was lower than in food, suggesting organisms could assimilate and digest it too. On the other hand, chlorophyll regulates the gut microbiota, which influences nutrient assimilation and the shrimp immune system (Li et al. 2018), and chlorophylls may act as interferents in the antibacterial activity (Saeed et al. 2019).

At the same time, the proximal composition of shrimp muscle was significantly affected by the inclusion of the microalgal mixture in the diets showing a positive correlation ($R^2 = 0.96$; $P \leq 0.05$). The average moisture content was 74.9 to 75.6%, slightly lower values than those reported by Basri et al. (2015), who evaluated the composition of *P. vannamei* muscle fed with freshwater microalgae as additive. The findings from this study showed a linear relationship between diet microalga percentage and protein content of shrimp muscle with lower values (16.13-17.67%) than those in this study (19.4-21.7%). Furthermore, lipid content was lower (0.39-0.49%) than that obtained in shrimp muscle of the evaluated treatments (0.8 to 0.86%). The evidence suggests a relationship between microalga addition in feed and increase of shrimp muscle protein content. This result can be explained by comparing soybean protein and wheat flour; microalga protein has better quality and all essential amino acids for crustaceans, which are close to shrimp biochemical composition, making it a viable protein source for diet results, a major weight gain for shrimp and a greater economic profit for producers.

On the other hand, AHPND is a disease that can cause up to 100% mortality and losses in shrimp productivity, so a good supplemented food that provides both the nutritional requirements for shrimp health and antimicrobial activity could help the survival of organisms. In the infection assay using *V. parahaemolyticus*, no mortality was recorded for shrimp fed with D1 and D3. In contrast, mortality in DC occurred at 12 HPI and reached above 40% at the end of the challenge. Previous research by Soto-Rodríguez et al. (2015) with infected *P. vannamei* challenged against the same bacterium by immersion (2.2×10^6 CFU mL^{-1}) reported mortality reaching 100% after 17 HPI. Another research from Nguyen et al. (2019) with immersed shrimp in bacterial broth showed that the first mortality occurred within 18 HPI and reached 100% after day 4 of the challenge. In both studies, the methodology was similar. These differences may have been due to the type of bacterial strain, shrimp weight, size and stage, and the size of the rearing tanks.

In addition to the nutritional benefits, the high content of bioactive compounds and the immunostimulant effect improve shrimps' innate defense system (Falaise et al. 2016, Shah et al. 2018). The growth of microalgae in the co-culture system in aquaculture could produce and release antimicrobial compounds, which adversely affect *Vibrio* and prevent the growth of pathogenic (D'Alvise et al. 2012). Our results suggested that microalgae-enriched diets improved survival by reducing the bacterial load in the bioassay against *V. parahaemolyticus*, which could have been related to the antimicrobial compounds (e.g. PUFAs and pigments) of microalga diets that were greater than in DC tested *in vitro* (data not shown). Therefore, the antimicrobial activity of microalgae-enriched diets must be tested both *in vitro* and *in vivo* (Monteiro et al. 2021). The possibility of the diets enriched with microalga has an antibacterial activity due to no-polar molecules, which is proven to be efficient against gram-negative bacteria (Shannon & Abu-Ghannam 2016). Other antimicrobial compounds have been found in microalgae biomass, like indol derivatives, beta-ionone, neophytadiene unsaturated fatty acid-containing lipidic fractions (triglycerides and docosapentaenoic acid), poly-unsaturated fatty acids (Falaise et al. 2016). The mechanism of fatty acids against bacteria is not yet fully clarified, but there is evidence of the effects that fatty acids cause on the bacterial cell membrane, causing cell leakage, and inhibiting bacterial fatty acid synthesis (Falaise et al. 2016).

Furthermore, this antimicrobial activity was verified by histopathological sections, without lesions in the tissues of the hepatopancreas in shrimp fed with diets enriched with microalgae, in contrast to the hepatopancreas of positive control shrimp. Similar findings by González-Colunga et al. (2019) reported that marine algae metabolites could be used as additives in shrimp diets and as an antibiotic agent against *V. Parahaemolyticus*.

Furthermore, another explanation is that molecules composing microalgal biomass can stimulate "protection" against the disease caused by the bacteria activating the immune system through fatty acids, pigments, beta-glucans, silica, and antioxidants to successfully protect shrimp (López-Elías et al. 2016, Charoonnart et al. 2018). Alternatively, marine microalgae have been reported to produce a variety of bioactive compounds, including inhibitors of *quorum sensing* without affecting cell density, which is the bacterial-bacterial communication capable of inducing bacterial pathogenicity (biofilms, proteases, invasion, and virulence factors), in this case *V. parahaemolyticus* (Kalia 2018). Anti-*quorum sensing activity* on *T. suecica* was observed, inhibiting the communication of biosensor bacteria (data not shown). In this sense, the experimental diets had an effect against *V. parahaemolyticus* through inhibition of the *quorum sensing* mechanism of the microalgae.

CONCLUSION

This study demonstrated that the inclusion of a microalgal mixture of *T. suecica, C. muelleri, and D. salina* in shrimp feed as an additive affected growth performance with higher SGR and PWG values, as well as showing complete survival and immune response of *P. vannamei* against *V. parahaemolyticus*. Furthermore, diets supplemented with microalga mixtures positively correlated to protein percentage in shrimp muscle and showed antimicrobial activity. The findings of this study are fundamental for the development of diets with high nutritional value- additives for shrimp farming.

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