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ORIGINAL RESEARCH

Increased resistance to thermal shock in pacific white shrimp fed on green algae and its effect in conjunction with probiotics

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Abstract This study aimed to evaluate the *in vivo* effect of the macroalgae *Ulva ohnoi,* alone and combined with the probiotic *Lactobacillus plantarum,* on zootechnical, immunological, and microbiological performance, thermal resistance and survival of *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus.* The shrimp were fed four diets: control, seaweed, probiotic, and a combination of seaweed + probiotic. After six weeks, a significant difference between the control and probiotic, and the control and seaweed treatments was demonstrated in shrimp challenged with *Vibrio*, with the highest percentage of mortality being observed in the group fed only with seaweed, followed by probiotic, seaweed + probiotic, and control. The control and seaweed treatment groups also showed a significant difference in resistance to thermal shock. The animals treated with the control diet had the highest mortality rate, followed by seaweed + probiotic, probiotic, and seaweed. The zootechnical, immunological, and microbiological parameters did not differ significantly among treatments. In conclusion, the use of *U. ohnoi* alone demonstrated a positive effect on thermal shock resistance but did not demonstrate protection against infection caused by *V. parahaemolyticus*. On the other hand, its combined use with *L. plantarum* in the diets did not have a positive effect on the parameters evaluated.

Keywords *Litopenaeus vannamei* . *Ulva ohnoi* . *Lactobacillus plantarum* . *Vibrio* . Thermal shock

Introduction

The pacific white shrimp (*Litopenaeus vannamei*) is considered one of the most important species for shrimp farming and in recent years, production has increased significantly. Marine shrimp are ectothermic organisms that can be stressed due to temperature fluctuations in their culture system. At low temperatures, general metabolic functions such as the immune, antioxidant, and osmoregulation systems, and the behavior and growth of these animals, can be negatively affected (Xu et al. 2019; Ren et al. 2021).

In addition, changes in environmental factors contribute to the immunosuppression of cultured animals and may increase their susceptibility to diseases caused by pathogenic viruses and bacteria (Reverter et al. 2014; Klongklaew et al. 2020; Jiao et al. 2021). Among the infections are those caused by bacteria of the genus *Vibrio*, such as *Vibrio parahaemolyticus*, known to be the etiologic agent of acute hepatopancreatic necrosis disease (AHND) that has caused enormous economic losses in global shrimp farming (Boyd and Phu 2018; Santos et al. 2020).

One of the strategies employed to control these microbial infections in farmed animals is the use of antibiotics. However, their indiscriminate use is not recommended, since they can accumulate in the tissues of

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animals and thus increase the health risks to these animals, and humans, as well as having severe environmental impacts (Burridge et al. 2010; Wang et al. 2021). For these reasons, it is of paramount importance to seek alternatives using biological methods that aim to improve the immunity of animals, control infections, and help their resistance to drops in temperature (Naiel et al. 2021). Among these methods is the use of probiotics, which are described as live microorganisms that, when administered in adequate amounts, confer benefits to the host's health (FAO/WHO 2001; Hill et al. 2014). An example of a probiotic is *Lactobacillus plantarum*, which belongs to the lactic acid bacteria (LAB) group (Zheng et al. 2018). When added as a supplement to shrimp feed, this strain was shown to decrease the Vibrionaceae count in the intestinal tract and improve growth performance, feed efficiency, enzyme activity, and survival of animals challenged with this bacterium (Kongnum and Hongpattarakere 2012; Vieira et al. 2016; Li et al. 2018; Nguyen et al. 2018). However, studies relating the use of probiotics in the diet of *L. vannamei* and their effect on resistance to thermal shock are still scarce.

Another prophylactic measure that supports the mitigation of diseases, reduces the use of chemotherapy in aquaculture and improves the animal's resistance to heat stress is the use of natural products of marine origin (Romero et al. 2012; Rezende et al. 2021). Among these are the green algae, which are part of the *Ulva* genus. They are widely distributed worldwide and have significant amounts of bioactive compounds that have shown potential in improving the immune system in cultured organisms (Tziveleka et al. 2019; Mantri et al. 2020). An example is *Ulva ohnoi*, a species of green macroalgae with high tolerance to abiotic factors and rapid growth, being used in the bioremediation of effluents in aquaculture (Lawton et al. 2013). It comprises polysaccharide ulvan and other by-products such as fatty acids, fibers, and amino acids, which can add value to the production chain, creating interesting economic possibilities for its use as a biomass (Angell et al. 2014; Glasson et al. 2017). In addition, several studies have used macroalgae in the diet of reared animals, as they have no toxic effects on cellular metabolism, are simple to rear, grow fast and have notably low production costs (Vatsos and Rebours 2015). *Ulva fasciata* extract added to the diet of black tiger shrimp (*Pennaeus monodon)* was shown to act as a prophylactic agent in the control of vibriosis (Vatsos and Rebours 2015). Extracts of *Ulva intestinalis*, *Ulva clathrata*, *Ulva rigida*, and *Ulva lactuta* species in the diet of *L. vannamei* improved growth performance and immunological parameters (Akbary and Aminikhoei 2018; Elizondo-González et al. 2018; Klongklaew et al. 2021; Pratiwi and Pratiwy 2021). In addition, research indicates that diets containing brown algae fed to marine shrimp have a positive effect on resistance to thermal shock and this could be related to their bioactive compounds (Schleder et al. 2017b; Rezende et al. 2021). However, this resistance has not yet been proven in *L. vannamei* fed with the green algae *U. ohnoi.*

Due to the negative effects of low temperatures on the metabolism of crustaceans, as well as the need to seek more sustainable approaches that reduce the use of chemotherapy in shrimp farming, the use of dry biomass of *U. ohnoi* together with the probiotic *L. plantarum* to supplement the diet of penaeids shows promise. In addition to serving as sources of nutrients, they can act in synergy and assist in the immunocompetence of animals against pathogens, such as those of the genus *Vibrio*. Therefore, the present study aimed to evaluate the in vivo effect of dry biomass of *U. ohnoi* alone and together with *L. plantarum* on zootechnical, immunological, and microbiological performance, resistance to thermal shock, and survival of *L. vannamei*. challenged with *V. parahaemolyticus*.

Materials and methods

The preparation and implementation of all stages of the experiment were carried out in laboratories belonging to the Federal University of Santa Catarina (UFSC) located in Florianópolis, Santa Catarina, Brazil. The *in vivo* experiment was conducted at the Marine Shrimp Laboratory (LCM). The collection of macroalgae *U. ohnoi* was performed in the tanks at the Marine Fish Laboratory (LAPMAR). The diets were manufactured at the Aquaculture Species Nutrition Laboratory (LABNUTRI) and the diet with added seaweed was prepared at the LCM, thermal shock and the infection with *V. parahaemolyticus* were conducted at the LCM.

Probiotic preparation

For the preparation of the probiotics strain, *L. plantarum* was first seeded in MRS solution (Man Rogosa Sharpe Broth), with 3% NaCl and incubated at 35°C. After 24 h, 10 mL of the probiotic suspension was

inserted into 100 mL of whey (containing 3% NaCl and 2% sugar) and incubated in an oven at 35°C for 24 h. After this period, the pH of the probiotic was measured to confirm bacterial growth, where a pH below 4 indicates good growth. Then the probiotic was stored in the refrigerator and used for a maximum of 48 h. For the measurement of *L. plantarum* in the diet, the probiotic suspension was performed as described above and included in the diet. This was then macerated and serially diluted (1/10) in 3% sterile saline. Thereafter it was seeded in an MRS agar culture medium and incubated in an oven at 35°C for 48 h. The total count of colony-forming units (CFU) obtained was 1.7×10^8 CFU mL^{-1}.

Macroalgae dry biomass

The macroalgae *U. ohnoi* was collected in March 2021 and it was inside the ponds of the culture of mullet (*Mugil liza*) at LAPMAR. The seaweed was transported to the LCM, any encrusted material was removed, and then it was quickly cleaned with fresh water and dried at room temperature for approximately 3 h. Subsequently, it was placed in an oven, with air circulation, and dried for 24 h at 38.5°C. Finally, the dry biomass was ground, sieved, and stored in a freezer at -20°C until use.

Animals

The research was conducted with the marine shrimp *L. vannamei* acquired from Aquatec Aquacultura Ltda. (Rio Grande do Norte, Brazil). The animals were reared in the LCM in a mature biofloc system, with constant aeration and a heating system, with a salinity of 33 mg L^{-1} until reaching the weight of 4.5 ± 0.13 g for the beginning of the experimental tests.

Preparation of experimental diets

The diets were formulated with the aid of Optimal Formula 2000® software, version 19102009, based on the recommendations and nutritional requirements for *L. vannamei* (Gong et al. 2000; NRC - National Research Council. 2011; Zhou et al. 2012)and their potential interaction. A 3 x 4 factorial design consisting of three cholesterol levels (0%, 0.2%, and 0.5% of diet. The dry ingredients were crushed and sieved at 600 µm. Next, the micro-ingredients were homogenized and added to the macro-ingredients. Then, the oils, soy lecithin, and water (200 mL kg^{-1} of the diet) were added to the mixture. The resulting mixture was pelleted in a micro-extruder (Inbramaq MX-40), dried in an oven at 40°C, and the finished feed was refrigerated at 4°C until use.

For the experiment, four diets were tested: a) Control diet (without additives); b) Diet with seaweed added; c) Diet containing probiotic, and d) Diet with a combination of seaweed + probiotic. The dry biomass of *U. ohnoi* was added during the preparation of the ration at a concentration of 2%, as described in the study by Legarda et al. (2021). *L. plantarum* (prepared as previously described) was pipetted into the ration 30 minutes before feedings with a volume of 100 mL of probiotic per kilogram in the diet, according to the methodology described by Vieira et al. (2008) and the relation of total haemocyte count and serum phenol oxidase activity of shrimp challenged with Vibrio harveyi. Shrimps were fed with a probiotic-supplemented diet, for eight days, then shifted to a commercial diet. Shrimps fed only with the commercial diet served as control. Evaluations were made on the 8(th, each gram of feed containing probiotic at a concentration of 1.7×10^8 CFU mL^{-1}. The percentage composition of the feed was analyzed as described by AOAC (2005) and the ingredients used are listed in Table 1.

Experimental design

The experiment lasted six weeks and evaluated the effect of the diets previously described for *L. vannamei* shrimp kept in clear water. The design was entirely random, with four replications, totaling sixteen tanks. The experimental units consisted of polyethylene tanks, containing 400 L of water, which was renewed, completely removing leftover food, feces, and seedlings, on alternate days.

The experimental units had constant aeration and heaters with a thermostat maintaining the temperature at 28.3 ± 0.6 ºC. These units were populated with thirty-five shrimps with an initial average weight of 4.5 ± 0.13 g. Feeds occurred four times a day and were adjusted according to weekly biometrics and survival,

Table 1 Formulation and percentage composition of the feed used

Ingredients (g kg^{-1})	Control	Seaweed	Probiotic	Seaweed + Probiotic
Soybean meal	324.63	324.63	324.63	324.63
Wheat flour	150.00	150.00	150.00	150.00
Fish residue	150.00	150.00	150.00	150.00
Offal flour	125.67	125.67	125.67	125.67
Carboxymethylcellulose	5.00	5.00	5.00	5.00
Soy lecithin	25.00	25.00	25.00	25.00
Monocalcium phosphate	25.00	25.00	25.00	25.00
Soy oil	10.00	10.00	10.00	10.00
Vitamin C	0.70	0.70	0.70	0.70
Vitamin premix[1]	5.00	5.00	5.00	5.00
Mineral premix[2]	17.00	17.00	17.00	17.00
Magnesium sulphate	15.00	15.00	15.00	15.00
Kaolin	100.00	100.00	100.00	100.00
Sodium chloride	12.00	12.00	12.00	12.00
Potassium chloride	10.00	10.00	10.00	10.00
Methionine	5.00	5.00	5.00	5.00
Fish oil	20.00	20.00	20.00	20.00
Ulva ohnoi	0%	2%	0%	2%
Lactobacillus plantarum	0	0	1.7x10^{8} CFU mL^{-1} per gram of feed	1.7x10^{8} CFU mL^{-1} per gram of feed
Moisture	9.04			
Crude protein	39.60			
Ethereal extract	8.71			
Crude fibre	1.87			
Ash	17.65			

[1]Vitamin premix: vit. A - 900 mg kg^{-1}; vit. D3 - 25 mg kg^{-1}; vit. E – 46,900 mg kg^{-1}; vit. K3 – 1,400 mg kg^{-1}; cobalamin (B12) – 50 mg kg^{-1}; pyridoxine (B6) – 33,000 mg kg^{-1}; riboflavin – 20,000 mg kg^{-1}; nicotinic acid – 70,000 mg kg^{-1}; pantothenic acid – 40,000 mg kg^{-1}; biotin – 750 mg kg^{-1}; and folic acid – 3,000 mg kg-^{1}.
[2]Mineral premix: copper – 2,330 mg kg^{-1}; zinc – 10,000 mg kg^{-1}; manganese – 6,500 mg kg^{-1}; selenium - 125 mg kg^{-1}; iodine – 1,000 mg kg^{-1}; cobalt – 50 mg kg^{-1}; magnesium – 20 g kg^{-1}; and potassium – 6,1 g kg^{-1}.

following an estimated programmed conversion (Ray et al. 2010). In addition, throughout the experiment, water quality parameters, such as dissolved oxygen and temperature, were monitored once a day (YSI 55, YSI Incorporated, Yellow Springs, OH, EUA). Once a week, analyses of pH (pHmetro Tecnal®), salinity (Eco-Sense YSI EC30), alkalinity (APHA, 2005), nitrite, and total ammonia were performed according to Strickland and Parsons (1972).

Evaluation of zootechnical parameters

At the end of the experiment, the final biometrics of all shrimps reared in each experimental unit were calculated to obtain the following zootechnical parameters:
- Weekly growth (g week^{-1}) = (final mean weight - initial weight)/ weeks of rearing;
- Total weight (g) of the animals at the end of the experiment;
- Feed Conversion Ratio (FCR) = feed consumed (kg) / shrimp biomass produced (kg);
- Survival (%) = (final number of shrimp / initial number of shrimp) × 100.

Analysis of immunological parameters

At the end of the experiment, the hemolymph was collected from the ventral sinus of ten prawns per tank (four pools per treatment). For this purpose, sterile 1 mL syringes cooled to 4°C were used. From the collected hemolymph, 50 µL was fixed in modified Alsever anticoagulant solution (MAS, Modified Alsever Solution - 27 mM sodium citrate, 9 mM EDTA, 115 mM glucose, 336 mM NaCl, pH 7.2) with 4% formaldehyde for total hemocyte count (THC). The remainder was coagulated at 4°C, macerated, and centrifuged at 6,000 × *g* for 10 min to obtain the serum, which was then aliquoted and stored at -20°C. The number of hemocytes per milliliter of hemolymph was estimated by direct counting in a Neubauer chamber. The calculation of total serum protein concentration was performed in a flat-bottomed 96-well microplate and estimated using Bradford (1976) with

bovine serum albumin as a standard. Initially, the serum was diluted in distilled water (1:99) and pipetted 20 µL in the wells of the plate, in triplicate. Distilled water was used as the control. After the serum and control sample distribution was added to all wells, 200 µL of the Bradford solution was prepared with Coomassie Brilliant Blue G-250, Sigma dye. After incubating at room temperature for 15 min, the reading was performed by spectrophotometry (595 nm). All tests were carried out in triplicate.

The activity of the phenoloxidase (PO) enzyme was determined by spectrophotometry (490 nm) based on the formation of DOPA-chromium pigment after the oxidation of the substrate L-dihydroxyphenylalanine (L-DOPA). The serum samples were diluted (1:15) in TBS-1 (1 mM Tris, 336 mM NaCl, 5 mM CaCl2, 10 mM MgCl2, pH 7.6). Of this solution, 50 µL was incubated in an equal volume of trypsin (Sigma-Aldrich, 1 mg mL-1) enzyme inducer in a flat-bottomed 96-well microplate for 5 min at room temperature. After incubation, 50 µL of L-DOPA (Sigma-Aldrich, 3 mg mL-1) was added to all wells. In the controls, trypsin was replaced by TBS. The formation of DOPA-chromium was monitored after 5, 10, and 15 min. PO activity was expressed in units of enzymatic activity (U) by varying 0.001 in absorption/minute/milligram of protein (Söderhäll and Häll 1984). For the analysis of serum agglutination titer, 50 µL of TBS-2 solution (50 mM Tris, 5 mM MgCl2, 10 mM CaCl2, 150 mM NaCl, pH 7.4) was initially added to all wells of a U-shaped bottom microplate. Then, 50 µL of the serum diluted (1:15) in TBS-2 was added to the first well, and serial dilutions were performed until the 12th well. Then, 50 µL of 2% canine erythrocyte solution was added to each well, mixed, and incubated for 2 h in a humid chamber at room temperature. In the control wells, the serum from the shrimp was replaced by TBS-2. Serum agglutination titer was defined as the reciprocal of the last dilution capable of agglutinating erythrocytes (Maggioni et al. 2004).

Intestinal tract bacteriological count

For this analysis, the intestines of ten shrimp per tank were sampled and pooled (four pools per treatment), totaling forty shrimp per treatment. The intestinal tracts were aseptically extracted, homogenized in a grail, and diluted serially (1/10) in sterile 3% saline. Then they were sown in TSA (tryptic soy agar), TCBS (thiosulfate citrate bile-salts sucrose agar), and MRS (Man Rogosa Sharpe Agar) for total count of heterotrophic bacteria, total count of *Vibrio* and total count of lactic acid bacteria, respectively. The intestines sown in the TSA and TCBS plates were incubated in an oven at 30°C, for 24 h, the MRS plates were incubated in an oven at 35°C, for 48 h and later the total counts of colony forming units (CFU) were performed.

Experimental challenge with *Vibrio parahaemolyticus*

At the end of the rearing, forty prawns of each treatment were transferred to another experimental room and distributed in sixteen experimental units of 50 L with constant aeration. Through the dorsal part of the first abdominal segment, the shrimps were inoculated with 100 µL *V. parahaemolyticus* solution at a concentration of 3×10^8 CFU mL^{-1} according to the LD_{50} test previously performed. The animals were monitored for 48 h, without feeding, and during this period mortality was evaluated.

Thermal shock

After six weeks of the experiment, forty shrimp from each treatment were transferred from the tanks with water at a temperature of 28.3 ± 0.6°C to another experimental room containing 60 L aquariums with salt water at a temperature of 11.5 ± 0.1°C (according to TL_{50} test carried out previously) and with constant aeration. The animals were kept at this temperature for 1 h and then transferred to tanks with salt water at a temperature of 28.7 ± 0.3°C. Shrimp were monitored for 48 h without food and during this period mortality was assessed.

Statistical analysis

Bacterial count data from the intestinal tract were transformed to $\log^{10}$, and those with agglutinating titer were transformed to $\log^2$ before being subjected to statistical analysis. The homoscedasticity and normality of all data were assessed using the Bartlett and Shapiro-Wilk tests, respectively. They were then subjected to Factorial analysis of variance (ANOVA) (Probiotic × Seaweed), followed by the Tukey test. All statistical

tests were performed in the Statistica 13.5 program (TIBCO Software Inc.), using a significance level of 5%.

Results

Water quality parameters

Temperature and dissolved oxygen were maintained at 28.3 ± 0.63°C and 5.69 ± 0.57 mg L-1 respectively and remained constant throughout the experiment. The mean for salinity was 32.51 ± 0.74 g L-1, pH 8 ± 0.14, alkalinity 123.06 ± 4.47 mg CaCO3 L-1, total ammonia 1.02 ± 0.6 mg L-1 and nitrite 0.03 ± 0.05 mg L-1. According to Van Wyk and Scarpa (1999), the parameters remained within the appropriate standards for the species.

Evaluation of zootechnical parameters

After the six weeks, no significant differences ($P > 0.05$) were observed in any of the zootechnical parameters analyzed, such as weekly weight gain (WWG), initial weight, final weight, and feed conversion ratio (FCR), and survival, as listed in Table 2.

Analysis of immunological parameters

It was not possible to observe significant differences ($P > 0.05$) in any of the immunological parameters analyzed, such as THC, total serum protein concentration, PO, and serum agglutination titer between treatments, as described in Table 3.

Intestinal tract bacteriological count

No significant differences were demonstrated ($P > 0.05$) in the bacteria count from the intestinal tract of *L. vannamei* fed supplemented diets and a control diet, as shown in Fig. 1.

Table 2 Zootechnical parameters of *Litopenaeus vannamei vannamei* fed with a control diet, a diet containing seaweed, a diet with probiotic, and a diet with the combination of Seaweed + Probiotic

Treatment	WWG(g)	Initial weight (g)	Final weight (g)	FCR	Survival (%)
Control	1.63 ± 0.33	4.57 ± 0.10	14.34 ± 0.14	1.47 ± 0.06	96.43 ± 2.74
Seaweed[a]	1.47 ± 0.30	4.41 ± 0.21	13.22 ± 0.24	1.56 ± 0.08	99.29 ± 1.43
Probiotic[b]	1.64 ± 0.29	4.47 ± 0.11	14.29 ± 0.25	1.43 ± 0.07	99.29 ± 1.43
Seaweed + Probiotic[c]	1.48 ± 0.34	4.56 ± 0.15	13.46 ± 0.16	1.56 ± 0.04	99.29 ± 1.43
[a] p-value	0.514	0.585	0.185	0.516	0.147
[b] p-value	0.832	0.718	0.356	0.554	0.147
[c] p-value	0.976	0.120	0.178	0.589	0.147

The results are presented as mean ± standard deviation of quadruplicates. WWG: weekly weight gain; FCR: feed conversion ratio. Different letters indicate: a= p-value for Seaweed treatment, b= p-value for Probiotic treatment and c= *p*-value for interaction between Seaweed + probiotic.

Table 3 Immunological parameters of *Litopenaeus vannamei* fed with a control diet, a diet containing seaweed, a diet with probiotic, and a diet with the combination of Seaweed + Probiotic

Treatment	THC(x10^6 Cells mL^{-1})	Protein Concentration(mg mL^{-1})	PO Activity(Unit min^{-1} mg^{-1} Protein)	Agglutination Titer(log^2)
Control	33.47 ± 13.24	484.81 ± 4.39	5.26 ± 0.96	10.93 ± 0.13
Seaweed[a]	36.93 ± 18.58	484.94 ± 3.99	4.76 ± 0.43	10.39 ± 1.02
Probiotic[b]	38.95 ± 11.18	484.21 ± 2.65	3.57 ± 0.87	10.18 ± 0.90
Seaweed + Probiotic[c]	37.1 ± 9.93	482.05 ± 0.75	3.37 ± 0.73	10.43 ± 0.66
[a] p-value	0.280	0.544	0.385	0.706
[b] p-value	0.860	0.305	0.180	0.368
[c] p-value	0.148	0.496	0.691	0.315

The results are presented as mean ± standard deviation of quadruplicates. THC: Total Hemocyte Count; PO: Phenoloxidase Activity. Different letters indicate: a= p-value for Seaweed treatment, b= *p*-value for Probiotic treatment and c= p-value for interaction between Seaweed + probiotic.

Experimental challenge with *Vibrio parahaemolyticus*

After 48 hours, the animals challenged with *V. parahaemolyticus* showed a significant difference between the treatments control and seaweed (P = 0.014579) and control and probiotic (P = 0.0262) as shown in Fig. 2. It was observed that the animals treated with the diet with seaweed had a higher mortality rate, with 92.5%, followed by treatments with probiotic with 90%, probiotic + seaweed with 87.5%, and control with 65%.

Thermal shock

At the end of monitoring, a significant difference between the control and seaweed treatments (P = 0.0485) was demonstrated in animals subjected to thermal shock, as shown in Fig. 3. It was observed that the animals treated with the control diet had a higher mortality rate, at 37.5%, followed by treatments with seaweed + probiotic at 32.5%, probiotic at 27.5%, and seaweed at 22.5%.

Discussion

Food supplementation with macroalgae and probiotics has generated increasing interest in recent years

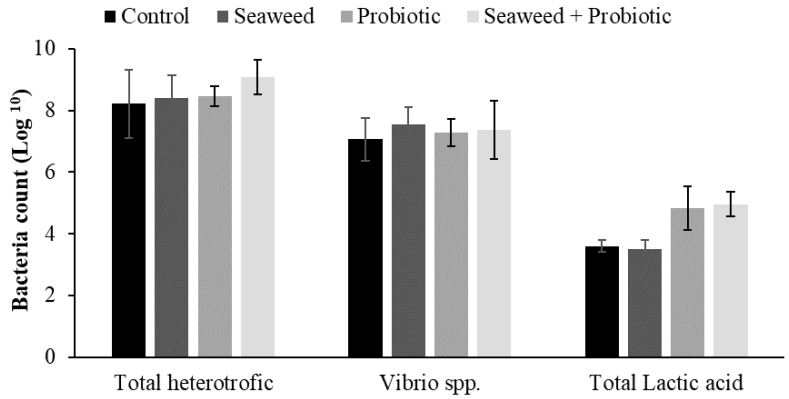

Fig 1. Bacteria count from the intestinal tract of *Litopenaeus vannamei* fed with a control diet, a diet containing seaweed, a diet with probiotic, and a diet with Seaweed + Probiotic. The results are presented as mean ± standard deviation of quadruplicates and the bars indicate the standard deviation of the mean. The p-value refers to the interaction between the treatments Probiotic × Seaweed: Total heterotrophic P = 0.563. *Vibrio* spp. P = 0.575. Total lactic acid p = 0,635.

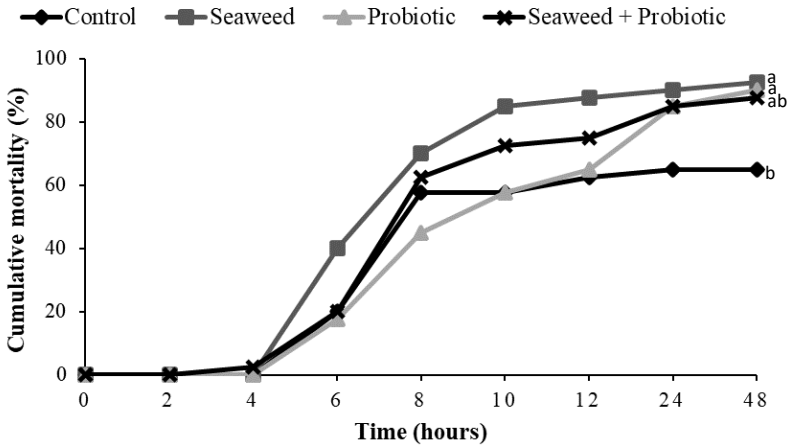

Fig 2. Cumulative mortality of *Litopenaeus vannamei* fed with a control diet. a diet containing seaweed, a diet with probiotic, and a diet with a combination of Seaweed + Probiotic after a challenge with *V. parahaemolyticus*. The results are presented as mean ± standard deviation of quadruplicates. The p-value refers to the interaction between the treatments Probiotic × Seaweed. Statistical differences are indicated by different letters (^{ab}) (P < 0,05).

as a means of growth promotion, immunomodulation, and reduction in the use of chemotherapy in the production of aquatic animals (Vidhya Hindu et al. 2019). In addition, some studies have shown that the use of seaweed as a food additive can help animals to resist adverse weather conditions. For this reason, mortality after the thermal shock was evaluated and it was possible to observe that shrimp fed with *U. ohnoi* demonstrated a significantly lower mortality rate when compared to the control group. These findings are consistent with those of other studies that confirmed that the use of dry biomass (0.5 and 2%) of the algae *Sargassum filipendula* (S) alone and in combination (1%S:2%U) with *Undaria pinnatifida* (U) significantly improved the thermal shock resistance of shrimp (Schleder et al. 2017b; Rezende et al. 2021). Thus, it is suggested that the bioactive compounds present in the seaweed used in this study may have contributed to the improvement of the thermal resistance of the animals. However, analyses were not performed to confirm this hypothesis.

The temperature fluctuations that occur in aquaculture crops, such as those found in the southern region of Brazil in the winter period, are considered a limiting factor for the growth and development of animals (Shields 2019). According to the review by Ren et al. (2021), the shrimp *L. vannamei* has a temperature tolerance range between 16 and 38ºC, but optimal growth is from 28 to 32ºC (Ponce-Palafox et al. 1997; Van Wyk and Scarpa 1999). However, when the water temperature exceeds the shrimp's regulatory capacity, low temperatures slow the growth rate and can even cause mortalities (Ren et al. 2021). Because of this problem, several studies have evaluated the tolerance of these crustaceans to cold and explored the effects that it causes (Pontinha et al. 2018; Xu et al. 2019).

Among the numerous effects are changes in the neuroendocrine response and its signaling molecules, such as dopamine and norepinephrine, in addition to oxidative and antioxidant responses in shrimps (Pan et al. 2008; Mapanao et al. 2018; Xu et al. 2018). Low temperatures can also cause immunological changes, such as the reduction of THC found in *L. vannamei* when the temperature decreases from 28 to 13°C, as well as suppressing antibacterial activity, making animals more susceptible to pathogens (Powell and Watts 2006; Fan et al. 2013; Xu et al. 2019). Changes in the energy metabolism of crustaceans are also found, as energy consumption increases and the activity of digestive enzymes decreases, causing a metabolic disorder with behavioral and physical changes (Anestis et al. 2008). Another effect is on the fatty acid metabolism of crustaceans, which are sensitive to cold and can modify their cellular composition, decreasing the proportion of saturated fatty acids, leading to a rapid increase in the proportion of unsaturated fatty acids, which could affect the fluidity of the cell membrane (Fan et al. 2019; Meng et al. 2019; Azra et al. 2020a, b; Ren et al. 2020).

The alteration in the cell membrane caused by cold has already been highlighted by Schleder et al. (2017a) who used the brown seaweed Sargassum filipendula (0.5%) in the diet of *L. vannamei*. They observed increases in polyunsaturated phospholipids which are related to greater fluidity of the membrane

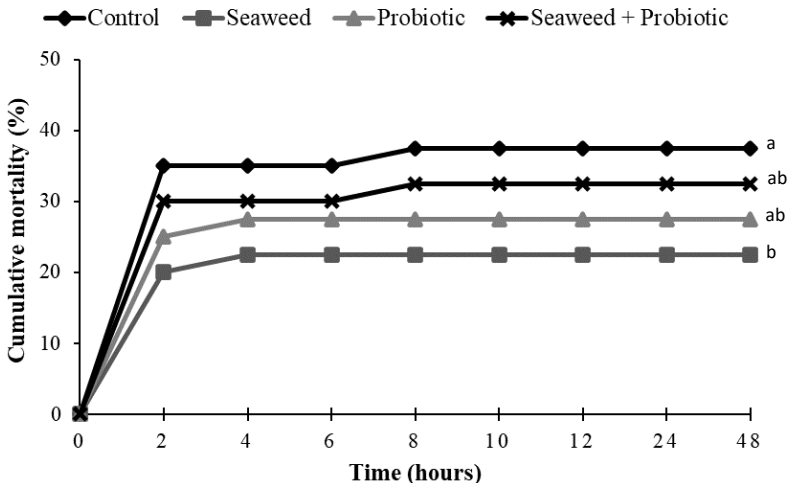

Fig 3. Cumulative mortality of *Litopenaeus vannamei* fed with a control diet, a diet containing seaweed, a diet with probiotic, and a diet with the combination of Seaweed + Probiotic after thermal shock. The results are presented as mean ± standard deviation of quadruplicates. The p-value refers to the interaction between the treatments Probiotic × Seaweed. Statistical differences are indicated by different letters (^{ab}) ($P < 0,05$).

and in proteins related to microbial defenses. Furthermore, Legarda et al. (2021) when supplementing with *U. fasciata* (10 g kg−1) in the diet of yellowtail amberjack (*Seriola Dorsalis*) found an increase of approximately 49% in docosahexaenoic acid (DHA) in fish muscle tissue. Based on these studies, it is suggested that the increase in unsaturated fatty acids may be due to their incorporation into the cell membrane, thus increasing its fluidity and this could explain, in part, the greater resistance to thermal shock in animals fed diets supplemented with algae.

Seaweeds, when included at low levels in the diet, can behave as immunostimulant sources and improve the immune response of animals. This has also been demonstrated with the use of probiotics (Vidhya Hindu et al. 2019). However, when evaluating the survival of shrimp fed supplemented diets and challenged with Vibrio parahaemolyticus, a negative effect on their resistance was observed. Even the animals fed the diet containing only *U. ohnoi* had the highest mortality rate, demonstrating a significant difference from the control group. In addition, the animals supplemented with probiotics showed a higher mortality rate when compared to the control group. In contrast to these results, Akbary and Aminikhoei (2018) observed that the use of *U. rigida* extract in L. vannamei diets improved animal resistance after bacterial challenge with Photobacterium damselae, and the same was verified in red seabream (*Pagrus major*) fed with Ulva pertusa and infected with *Photobacterium damselae* subsp. Piscicide (Satoh et al. 1987). Results divergent from those obtained in the present study were also reported by several studies in which the use of *Lactobacillus* sp. increased the resistance of shrimp when challenged with *V. harveyi* (Vieira et al. 2010; Kongnum and Hongpattarakere 2012; Li et al. 2018). One explanation for these contradictory results is that probiotics are influenced by factors such as culture conditions, the method of administration and concentration, the probiotic strain, and the species of fish or shrimp used (Toledo et al. 2019). In relation to experiments using algae, some authors, such as Fumanal et al. (2020), demonstrated that the proportion of algae can influence the final result, and it is necessary to test its specific effects for each species of animal and diet formulation, always considering that its compounds may vary in different species of algae (Martínez-Antequera et al. 2021). Furthermore, the results obtained in the present study suggest that the inoculum of *V. parahaemolyticus*, used at a concentration of 3 × 108 CFU mL-1 (obtained in a previous LD50 test), may have been very high, and the control group reached 65% mortality, slightly above the expected 50%.

The addition of 2% dry seaweed biomass alone and together with the probiotic in the diets did not show significant differences in the counts of intestinal tract bacteria, such as lactic acid bacteria (LAB), which indicates that there was no colonization of these bacteria in the intestines of shrimps, the expected prebiotic effect not occurring. There were also no statistical differences in the evaluated immunological parameters, which is consistent with findings of other studies (Fumanal et al. 2020). In addition, the zootechnical parameters of the animals fed the supplemented diets were not affected, suggesting that there was no interference from possible anti-nutritional factors present in the algae, as demonstrated in other studies (Bandara 2018; Vizcaíno et al. 2019, 2020; Sáez et al. 2020).

Although several studies demonstrate that seaweed and probiotics have many benefits for the health of farmed animals, there are divergences in the results found in the literature. These differences may be due to the variation of the farmed species and its physiology, the macroalgae and the probiotic strain used, the method of inclusion, the dosage administered, the place and season of the year in which the algae were harvested, and the form of drying them (Vatsos and Rebours 2015; Araújo et al. 2016; Uribe et al. 2019; Vizcaíno et al. 2019). Consequently, the results obtained in the present study reveal the importance of evaluating the effects of the use of different species of *Ulva* in shrimp feeding and their combination with probiotics. To our knowledge, this is the first report on the inclusion of *U. ohnoi* alone and together with *L. plantarum* in *L. vannamei* diets.

Conclusion

The inclusion of *U. ohnoi* in the diet of *L. vannamei* demonstrated a positive effect on resistance to thermal shock but did not demonstrate protection against infection caused by *V. parahaemolyticus*. The use of this macroalgae together with *L. plantarum* in the diets did not exert any synergistic or antagonistic effect on the zootechnical, immunological, and microbiological performance of the animals.

Conflict of interest The authors have no competing interests to declare.

Authors' contributions All authors have contributed equally to data collection, data analysis, writing, reviewing, editing, and finally, approving the final manuscript.

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