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Effect the seaweed *Sargassum filipendula* and *Ascophyllum nodosum* on the resistance of the shrimp *Litopenaeus vannamei* to hypothermal shock

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ABSTRACT

This study investigated the resistance of Pacific white shrimp (*L. vannamei*) to hypothermal shock when treated with extracts from the brown seaweeds *Sargassum filipendula* and *Ascophyllum nodosum*, either by immersion for post-larvae or as a dietary supplement for juveniles. Post-larvae were exposed to seaweed extracts at concentrations of 20, 50, 100, and 200 mg·L^{-1}, while juveniles were fed diets with 0.5%, 2.0%, and 4.0% seaweed extracts for 10 days. For juveniles (~12 g), hemato-immunological and microbiological analyses were conducted after feeding with 5% seaweed diets for 15 days, all followed by hypothermal shock. Samples were collected prior to the start of thermal shock, immediately after shock (0 h), and 1 and 24 h after return to initial temperature. The aqueous extract of the seaweeds *A. nodosum* and *S. filipendula* at concentrations 100 mg·L^{-1} and 200 mg·L^{-1}, respectively, improves the survival rates of post-larvae *L. vannamei* submitted to hypothermal stress. For juvenile shrimps, hypothermal shock causes significant immunodepression and reduces the bacterial community in the intestine. The addition of seaweed to the feed did not increase the survival rate and did not affect the intestinal microbiota of *L. vannamei*. In the case of immunological parameters, both species positively affect the agglutination titer, but no other parameters analyzed.

Keywords: Immunology; Microbiology; Seaweed extracts; Shrimp farming; Thermal stress.

Efeito das macroalgas *Sargassum filipendula e Ascophyllum nodosum* na resistência do camarão *Litopenaeus vannamei* ao choque hipotérmico

RESUMO

Este estudo investigou a resistência do camarão-branco-do-Pacífico (*L. vannamei*) ao choque hipotérmico após tratamento com extratos das algas marrons *Sargassum filipendula* e *Ascophyllum nodosum*, aplicados por imersão para pós-larvas ou como suplemento dietético para juvenis. Pós-larvas foram expostas aos extratos de algas em concentrações de 20, 50, 100 e 200 mg·L^{-1}, enquanto juvenis foram alimentados com dietas contendo 0,5%, 2,0% e 4,0% de extrato de alga por 10 dias. Para juvenis (~12 g), análises hematoimunológicas e microbiológicas foram realizadas após 15 dias de alimentação com 5% de algas na dieta, seguidas de choque hipotérmico. Amostras foram coletadas antes do choque, imediatamente após (0 h), e 1 e 24 horas após o retorno à temperatura inicial. Os extratos aquosos de *A. nodosum* (100 mg·L^{-1}) e *Sargassum filipendula* (200 mg·L^{-1}) aumentaram as taxas de sobrevivência das pós-larvas submetidas ao estresse hipotérmico. Para juvenis, o choque hipotérmico causou imunodepressão significativa e reduziu a comunidade bacteriana intestinal. A adição de algas na dieta não aumentou a taxa de sobrevivência nem afetou a microbiota intestinal de *L. vannamei*. Quanto aos parâmetros imunológicos, ambas as espécies aumentaram o título de aglutinação, mas não influenciaram outros parâmetros analisados.

Palavras-chave: Immunologia; Microbiologia; Extratos de algas; Cultivo de camarão; Estresse térmico.

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INTRODUCTION

The marine shrimp *Litopenaeus vannamei* is of great importance in the context of aquaculture, being a species of aquatic animal with the largest production volume worldwide (6.8 million tons) (FAO, 2024b) and the main crustacean cultivated in Brazil (FAO, 2024a). Although *L. vannamei* is a tropical shrimp species, it achieves high growth rates at temperatures above 20°C, with the optimal range between 28 and 32°C (Ponce-Pallafox et al., 1997; Van Wyk & Scarpa, 1999). Its farming has been established in subtropical regions with mean water temperature lower than 18°C (Kumlu et al., 2010; Peixoto Jr. et al., 2003), but characterized by sudden drops in water temperature. In fact, fluctuations may affect the physiology of these ectothermic animals in several ways.

Weather plays an important role in shrimp farming, contributing to disease outbreaks in shrimp farms. Abiotic stresses, such as temperature and hypoxia, as well as salinity and ammonia concentrations, can have a significant impact on the physiological homeostasis of aquatic animals, especially immune responses (Zhang et al., 2023). Studies have shown that *L. vannamei*, when exposed to low salinity and challenged with the white spot syndrome virus (WSSV), presents negative regulation of genes related to energy metabolism and the immune system, which can be attributed to the combination of abiotic and biotic stresses (Gonçalves-Soares et al., 2012). Thermal shock also causes pronounced changes in hemocyte lipid and protein profiles that affect molecules involved in membrane fluidity, lipid and energy metabolism, immune response, apoptosis and antioxidant capacity (Schleder et al., 2020).

Several metabolites detected in seaweeds with biological activities have recently given good results in shrimp farming. They have improved antioxidant and immunological activity owing to their antibiotic, antiviral, and anti-inflammatory properties (Smit, 2004; Thanigaivel et al., 2016). These properties are related to molecules detected mainly in brown seaweeds, such as sulfate polysaccharides, proteins, lipids, phenolic compounds, terpenes, carotenoids, volatile halogenated organic compounds, and others (Gupta & Abu-Ghannam, 2011; Holdt & Kraan, 2011). Brown seaweed polysaccharides have antioxidant and immune-stimulant activities (Zhang et al., 2023), enhancing and improving the immunological response of *L. vannamei* (Schleder et al., 2017a, 2017b, 2020), improving balance of the intestinal microbiome, reducing Vibrionaceae count in the digestive tract, and reducing shrimp mortality after acute heat stress (thermal shock) and after WSSV infection associated with thermal fluctuation (Rezende et al., 2021; Rezende et al., 2022). Phenolic compounds have a high antioxidant capacity (Kuda et al., 2005; Lopes et al., 2012), similar to carotenoids, protecting organisms against free radicals and lipid peroxidation (Zhang et al., 2023). Besides having antioxidant, anti-inflammatory and antibiotic activities (Holdt & Kraan, 2011; Pal et al., 2014), the seaweed species *Ascophyllum nodosum* and *Sargassum* spp. can also induce the production of antioxidant enzymes in shrimps (Cornish & Garbary, 2010; Stengel et al., 2011; Thanigaivel et al., 2014; Zhang et al., 2023).

The genus *Sargassum* is widely distributed throughout tropical and temperate regions (Mattio et al., 2015), and previous work reported that *Sargassum* species can act as immune-stimulants in penaeid shrimps, increasing their resistance to viral and bacterial infections and improving molecular response in the wake of thermal stress (Huynh et al., 2011; Immanuel et al., 2012; Rezende et al., 2021, 2022; Schleder et al., 2017a, 2017b). The species *A. nodosum* is native to the North Atlantic Ocean, and some studies have reported that its extracts can increase the tolerance of the nematode *Caenorhabditis elegans* to thermal stress, inducing the production of antioxidant enzymes and heat shock proteins and regulating energy metabolism (Fan et al., 2011; Kandasamy et al., 2011, 2012, 2014). Commercial products manufactured from *A. nodosum* also have immune-modulatory and prebiotic activities in big mammals (Bahar et al., 2016; Ciliberti et al., 2015; O'Sullivan et al., 2010; Saker et al., 2004).

Therefore, brown seaweed species have several metabolites that may improve the resistance of aquatic organisms against environmental stress. In that light, the present work aimed to investigate the resistance of Pacific white shrimp *L. vannamei* to HS when treated with extracts of the brown seaweed species *S. filipendula* and *A. nodosum*, either by immersion bath for post-larvae or dietary supplement for juveniles, by analyzing immunological and intestinal microbiota parameters of the shrimp prior to, during and after stress.

MATERIAL AND METHODS

Biological material

Shrimp nauplii of the species *L. vannamei*, high-performance strain SPEEDLINE HB16, were obtained from AQUATEC (Rio Grande do Norte, Brazil). They were grown in 20,000-L tanks, and 5-day-old post-larvae (PL5) were transferred to a 50-m^3 matrix tank and reared in a super-intensive biofloc system at the Laboratory of Marine Shrimps of the Universidade Federal de Santa Catarina until they weighed between 10 and 12 g. The species *Sargassum filipendula* C. Agardh was

harvested on Ponta do Sambaqui Beach, Florianópolis (27°29' 27.43"S; 48°32'21.51"W), Santa Catarina, Brazil. The specimens were cleaned, quickly washed in fresh water, and dried at room temperature for 4 h. Seaweeds were then placed in a kiln at 40ºC until weight stabilization. Seaweeds were ground and sieved to 600 µm. The species *Ascophyllum nodosum* (L.) Le Jolis, from Canada, was donated by Acadian Seaplants Limited, already dried and ground.

Preparation of extract with seaweed

The experiment with post-larvae comprised the removal of an aqueous extract for each dry and ground seaweed (*S. filipendula* and *A. nodosum*), following Yeh et al.'s (2006) method, 10 g of seaweed were added to 300 mL of filtered water (1 µm) and boiled for 3 h. The solution was filtered through nylon mesh, and the filtrate was lyophilized.

Preparation of feed with seaweed

For the experiment with juveniles, the feed was prepared according to Table 1 (control). For treatments, *S. filipendula* and *A. nodosum* were added to this diet at concentrations of 0.5, 2, and 4% (pelleted).

Table 1. Bromatological composition and analysis (dry matter) of diet employed in the assay with *Litopenaeus vannamei* juveniles.

Ingredients	g·kg^{-1}
Soybean bran	424
Fish waste flour	232
Wheat flour	150
Kaolin	84
Lecithin	30
Monocalcium phosphate	20
Mineral-vitamin premix	15
Sodium chloride	12
Soybean oil	10
Cod liver oil	10
Magnesium sulfate	7.7
Agglutinant (CMC)	5.0
Vitamin C	0.6
Humidity (%)	10.8
Crude protein (%)	39.5
Ether extract (%)	8.9
ADF (%)	6.3
Ashes (%)	21.7

CMC: carboxymethyl cellulose; ADF: acid detergent fibers.

Analysis of post-larvae mortality

Approximately 1,000 post-larvae (PLs) of 16-day-old *L. vannamei* (PL16) were transferred from larva-rearing tanks to a 20-L aquarium and maintained at 28ºC, salinity = 32‰ and constant aeration during 24 h for acclimatization.

Seaweed extracts (*S. filipendula* and *A. nodosum*) (described above) were diluted in 500-mL Erlenmeyer flasks filled with seawater (32‰) at concentrations of 20, 50, 100, and 200 mg·L^{-1} for the total of four treatments and control. As control, seawater with no seaweed extract was used. All treatments and control were done in triplicate. Erlenmeyer flasks with aeration were randomly placed in three tanks filled with water up to the middle of the flasks. The tanks were furnished with heaters, thermostats and strong aeration for control and temperature homogenization under the conditions described above.

After acclimatization, 25 post-larvae were transferred to control and Erlenmeyer flasks with extracts at the same temperature conditions as those for acclimatization and kept there for 3 hours. After this period, the water temperature was decreased using ice, following Samocha et al.'s (1993) methodology, at the rate of 1ºC every 15 min until 18ºC and at 1ºC every 30 min until 12.9ºC, the lethal temperature for 50% of the population (TL50), according to Pontinha et al. (2018).

After 72 h, the flasks were removed from the tanks. After a rise in the temperature, live post-larvae were counted, and mortality rate was calculated according to the Eq. 1:

$$\text{Mortality (\%)} = [(AI - AF) \times AI\text{-}1] \times 100 \quad (1)$$

Where: AI = number of live animals at the start of the assay; AF = number of live animals at the end of the assay.

Post-larvae which moved by mechanical stimulus were considered alive.

Analysis of juvenile mortality

Thirty shrimp weighing between 10 and 12 g were transferred to aquariums with 60 L of sea water, kept at 28ºC and salinity of 32‰, without feeding for 12 h for acclimatization.

After this period, the animals were fed, with the control diet or treatments with seaweed (as described before), for 10 days and at different concentrations (0.5, 2, and 4%) (Schleder et al. 2017b). They were fed four times a day (9 a.m., 12 p.m., 2 p.m., and 5 p.m.) with a daily amount of biomass of 3%. Control and treatments were done in triplicate (n = 3). Shrimps were transferred to aquariums with seawater, at 11.7°C (TL50 suggested by Pontinha et al., 2018) for 1 h, and then returned to initial temperature. As soon as death

was confirmed by lack of movements when stimulated, animals were removed and counted. Mortality rate was calculated after 24 h, according to the Eq. 1.

Physiological parameters

Shrimps weighing between 10 and 12 g were transferred to 12 70-L aquariums with seawater. Each aquarium contained 35 animals. Only control and the ones with the 0.5% concentration of both seaweeds were used, or rather, diets closest to the composition when compared to control. Each treatment and control were performed in quadruplicate (n=4), shrimps were transferred to aquariums filled with seawater maintained at 11.7°C. They were exposed to this temperature for 1 hour, simulating HS, and then returned to normal conditions for 24 hours. Three shrimp samples from each aquarium were taken at different times. Hemolymph was removed for immunological analysis. Intestine was removed for microbiological analysis prior to and after thermal shock.

Hemato-immunological analysis

Approximately 500 µL of hemolymph were collected from the first abdominal segment of each shrimp, using a 1-mL syringe and 21G needle, and kept in ice. Hemolymph from three shrimps from each aquarium was collected. Further, 50 µL of collected hemolymph were fixed in a modified anticoagulant isotonic balanced salt solution (Alsever's, consisting of sodium citrate 27 mM, EDTA 9 mM, glucose 115 mM, and NaCl 336 mM; pH 7.2) with 4% formaldehyde at 5:1 and stored at 4°C for total hemocyte count (THC). The remaining hemolymph was left to coagulate at 4ºC and then centrifuged at 10,000 × g for 10 min. Supernatant (serum) was divided into aliquots and stored at -20ºC for later use.

THC was estimated by the number of hemocytes per milliliter of hemolymph through direct counting in a Neubauer chamber. The serum's protein concentration (PC) was determined, following Bradford (1976), by employing bovine serum albumin (BSA) as standard. Analysis was done in triplicate.

Agglutination titer was determined by serially diluting 50 µL of serum in TBS-1 (Tris 50 mM, NaCl 150 mM, $CaCl_2$ 10 mM, and $MgCl_2$ 5 mM; pH 7.4) in wells of a 96-well microplate and incubating with 50 µL of 2% dog erythrocyte suspension in TBS-1 for 3 h at 25ºC in a humid chamber. Serum was replaced by TBS-1 in control. The agglutinating titer was defined as the reciprocal of the highest dilution capable of presenting erythrocyte agglutination (Maggioni et al., 2004).

Phenoloxidase enzyme activity (PO) was determined by spectrophotometry with the formation of coral-red DOPA-chromo pigment derived from the oxidation of enzymatic substrate L-dihydroxyphenylalanine (L-DOPA) by PO. Serum samples were diluted (1:9) in TBS-1 (1 mM Tris, 336 mM NaCl, 5 mM $CaCl_2$, and 10 mM $MgCl_2$ at pH 7.6), and a 50-µL aliquot of this solution was incubated with the same volume of the enzyme inducer trypsin (Sigma, 1 mg·mL^{-1}) in wells of a 96-well microplate (flat bottom) for 15 min at 20°C. In control, serum and trypsin were replaced by TBS-1 in equivalent volumes. After incubation, 50 µL of L-DOPA (Sigma, 3 mg·mL^{-1}) were added to each well, and the formation of DOPA-chrome was monitored in a microplate reader (490 nm) after 2-, 4-, 6-, 8- and 10-min. PO activity was expressed by the variation of absorbance per minute and per milligram of total proteins in samples. An enzyme activity unit (U) is equivalent to the variation 0.001 in absorbance minute-1 mg-1 of protein at 20°C (Söderhaäll & Häll, 1984). Analysis was done in triplicate.

Microbiological analysis of the intestine

The digestive tracts of a pool of three shrimps from each replication of treatments and control were sampled, weighed and homogenized in a vessel. They were then diluted in series (1:10) in 3% sterile saline solution (SSS) and seeded in marine agar culture medium for counts of total heterotrophic bacteria (THB) and in agar thiosulfate citrate bile sucrose (TCBS) for counts of *Vibrio* spp. The intestines seeded in Petri dishes were incubated in a buffer at 30°C. Colony forming units (CFUs) were counted after 24-h incubation.

Statistical analysis of data

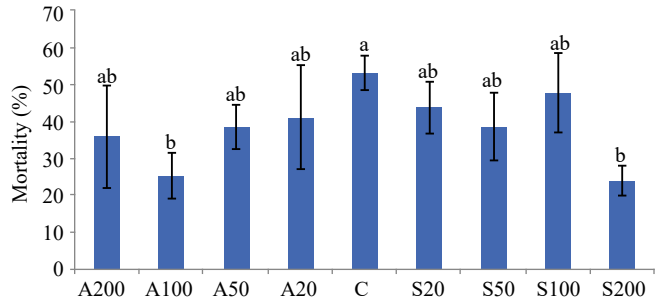
Agglutination titer data were transformed into log2, while microbiological data were transformed into log10 for the homogenization of variance. All data were verified by Shapiro-Wilk's test ($\alpha = 0.05$), and homoscedasticity was confirmed by Levene's test ($\alpha = 0.05$). Unifactorial analysis of variance (ANOVA) tested the mortality data for juveniles and post-larvae, while bifactorial ANOVA tested data for physiological and microbiological parameters (period, regarding thermal shock × diets used). When ANOVA showed significant difference, Tukey's HSD test ($\alpha = 0.05$) was applied to separate means.

RESULTS

Mortality

Seaweed aqueous extract improved the survival of shrimp PLs under HS (Fig. 1). The mortality rates of treatments with 100 mg·L^{-1} of *A. nodosum* and 200 mg·mL^{-1} of *S. filipendula* were significantly lower than those of control.

In the case of juveniles, no significant differences in mortality rates were observed between treatments and control (Fig. 2).



A: *Ascophyllum nodosum* (20, 50, 100 and 200 mg·L^{-1}); S: *Sargassum filipendula* (20, 50, 100, 200 and 400 mg·L^{-1}); C: control.

Figure 1. Mortality of *Litopenaeus vannamei* post-larvae treated with seaweed aqueous solution after 24 h thermal shock. Different letters indicate significant difference between treatments and control (α = 0.05) (means and standard deviation).

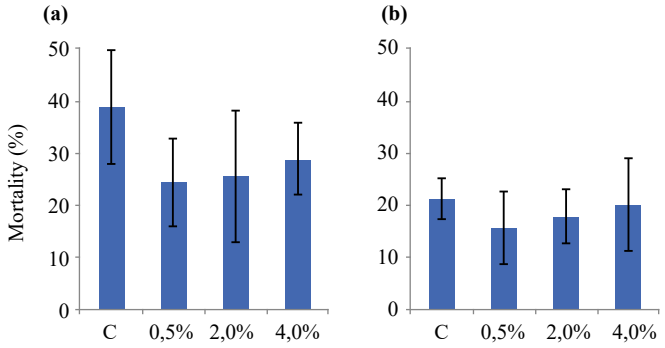


Figure 2. Mortality of *Litopenaeus vannamei* after being fed with commercial diet (C) or commercial diet with addition of concentrations of seaweed (0.5, 2, and 4%) and then submitted to thermal shock after 24 h. (a) *Sargassum filipendula*; (b) *Ascophyllum nodosum* (means and standard deviation).

Immunological analysis

Immunological analysis demonstrated a significant immunodepression in shrimps submitted to HS, at least during the first hour after stress, with return to normal conditions in 24 h (Table 2). The number of hemocytes, along with agglutinating and phenoloxidase activities, fell significantly after HS, but normalized in 1 hour after returning the animal to a comfortable temperature. However, phenoloxidase activity returned to normal level 24 h after HS. Statistical differences in protein concentration were observed only during the period from 0 to 1 hour after shock.

Data presented in mean ± standard deviation; *significant differences by two-factor analysis of variance ($p < 0.05$), with feed (ff) and period (t) as factors and their interaction expressed as t × ff. Values followed by lowercase letters (A or B) in the same column indicate significant difference when considering only the effect of feed addition. Values followed by uppercase letters (a or b) in the same row indicate significant difference when considering only the effect of period (t). THC: total hemocyte count; PC: protein concentration; PO: phenoloxidase; S. f.: *Sargassum filipendula*; A. n.: *Ascophyllum nodosum*.

The inclusion of seaweed in the diet affected only the agglutination titer of shrimp serum. The two seaweed treatments had significant effects on this parameter relative to control when compared to the period prior to shock.

Microbiological analysis

Thermal shock reduced THB and *Vibrio* spp. in the shrimp's intestine (Table 3). One hour later, the total bacteria counts were significantly lower than those prior to shock. However, seaweed treatments did not affect the number of microorganisms in shrimp intestine.

Data presented in mean ± standard deviation; *significant differences by two-factor analysis of variance (ANOVA) ($p < 0.05$), with feed (ff) and period (t) as factors and their interaction expressed as t × ff. Values followed by uppercase letters (a or b) in the same row indicate significant difference when considering only the effect of period (t); THB: total heterotrophic bacteria; CFU: colony forming units.

DISCUSSION

Previous studies have confirmed the inclusion of seaweed in the diet promotes the growth performance of shrimps, including weight gain, specific growth rate, and condition factors (Niu et al., 2019; Zhang et al., 2023). The addition of seaweed to the diet also has the potential to improve immune response and physiological performance of marine shrimps under stress situations (Schleder et al., 2020).

In the present work, the use of *S. filipendula* and *A. nodosum* aqueous extract improved the survival of *L. vannamei* shrimp PLs submitted to hypothermic stress. Mulyadi and Wa (2020) reported greater survival with the use of seaweed extract (*Sargassum* spp.) with shrimp juveniles (*L. vannamei*) infected by *Vibrio alginolyticus*. However, in our work with juveniles of *L. vannamei* shrimps, the use of feed with seaweed concentrations included had no effect on survival after thermal shock. These results corroborate those of Schleder et al. (2017b), who also used this shrimp species, as well as *S. filipendula* for inclusion in the diet, for HS experiments. However, inconclusiveness of the results across the literature is highlighted by recent studies which have

Table 2. Immunological parameters of *Litopenaeus vannamei* submitted to thermal shock, including prior to shock and 0, 1 and 24 h after shock, fed on control diet or commercial diet with 0.5% inclusion of *Sargassum filipendula* and 0.5% inclusion of *Ascophyllum nodosum*.

Variables	Feed ff	Period (t)				Mean	Two-Factor ANOVA		
		After	0 hour	1 hour	24 hours		t	ff	t x ff
THC ($\times 10^6$ cells·mL^{-1})	Control	42.4 ± 1.11	15.4 ± 1.08	40.9 ± 1.19	38.6 ± 0.77	3.4 ± 1.4			
	0.5% *S. f.*	42.8 ± 1.01	17.6 ± 0.96	37.7 ± 1.37	55.6 ± 1.12	3.8 ± 1.7	F = 19.78	F = 0.86	F = 1.33
	0.5% *A. n.*	34.7 ± 0.39	21.0 ± 0.80	32.9 ± 1.27	49.9 ± 1.33	3.5 ± 1.4	$p < 0.001$*	$p = 0.430$	$p = 0.268$
	Mean	40.0 ± 0.9 a	18.0 ± 0.7 b	37.0 ± 1.1 a	48.0 ± 1.2 a				
PC (mg·mL^{-1})	Control	165.9 ± 43.9	205.9 ± 38.8	121.1 ± 26.8	164.1 ± 61.8	164.2 ± 54.5			
	0.5% *S. f.*	189.0 ± 61.4	173.9 ± 64.3	146.6 ± 52.4	226.9 ± 27.0	184.1 ± 65.6	F = 3.69	F = 0.53	F = 1.09
	0.5% *A. n.*	189.4 ± 56.1	235.5 ± 62.8	129.4 ± 30.1	153.1 ± 34.4	176.9 ± 59.9	$p = 0.020$*	$p = 0.592$	$p = 0.387$
	Mean	181.4 ± 50.4 ab	205.1 ± 70.0 a	132.4 ± 33.7 b	181.4 ± 58.0 ab				
Agglutination titer Log_2 (x)	Control	10.50 ± 0.58	9.25 ± 0.50	10.25 ± 0.50	10.75 ± 0.50	10.2 ± 0.7 B			
	0.5% *S. f.*	11.50 ± 0.58	9.75 ± 0.50	11.25 ± 0.50	10.75 ± 0.50	10.8 ± 0.8 A	F = 15.33	F = 5.86	F = 1.02
	0.5% *A. n.*	11.25 ± 0.50	10.00 ± 0.82	10.75 ± 0.50	10.75 ± 0.50	10.7 ± 0.7 A	$p < 0.001$*	$p = 0.006$*	$p = 0.46$
	Mean	11.1 ± 0.7 a	9.7 ± 0.6 b	10.8 ± 0.6 a	10.8 ± 0.4 a				
PO activity (U min^{-1}·mg^{-1} prot)	Control	33.9 ± 5.80	18.6 ± 3.80	13.5 ± 2.40 [c]	38.0 ± 9.20	26.0 ± 13.6			
	0.5% *S. f.*	39.1 ± 1.60	16.0 ± 9.40	16.2 ± 3.00	35.6 ± 2.60	26.8 ± 13.9	F= 41.47	F = 0.14	F = 0.82
	0.5% *A. n.*	32.6 ± 1.50	16.9 ± 2.70	17.9 ± 3.20	41.2 ± 9.40	27.1 ± 13.3	$p < 0.001$*	$p = 0.872$	$p = 0.561$
	Mean	35.2 ± 4.3 a	17.2 ± 5.4 b	15.9 ± 3.2 b	38.3 ± 7.1 a				

Table 3. Microbiological parameters of *Litopenaeus vannamei* intestines submitted to thermal shock, in two periods (prior to shock and 1 h after shock), and fed with three types of diet (control; 0,5% inclusion of *Sargassum filipendula*; and 0.5% inclusion of *Ascophyllum nodosum*).

Variables	Feed ff	Period (t)		Mean	Two-Factor ANOVA		
		After	1 hour		t	ff	t x ff
THB (Log CFU·g^{-1})	Control	8.38 ± 0.67	7.06 ± 0.70	7.7 ± 0.9			
	0.5% *S. f.*	8.93 ± 0.54	5.49 ± 1.60	7.4 ± 1.9	F = 37.03	F = 0.24	F = 1.85
	0.5% *A. n.*	8.77 ± 0.54	6.57 ± 0.71	7.7 ± 1.3	$p < 0.001$*	$p = 0.787$	$p = 0.286$
	Mean	8.7 ± 0.6 a	6.5 ± 1.1 b				
PC (mg·mL^{-1})	Control	6.20 ± 0.57	3.25 ± 1.06	4.7 ± 1.8			
	0.5% *S. f.*	6.54 ± 0.95	3.28 ± 1.19	4.9 ± 2.0	F = 56.11	F = 3.45	F = 1.08
	0.5% *A. n.*	6.84 ± 0.72	4.82 ± 0.66	5.8 ± 1.2	$p < 0.001$*	$p = 0.054$	$p = 0.359$
	Mean	6.5 ± 0.7 a	3.8 ± 1.2 b				

reported differences in the survival rate of PLs (Rezende et al., 2021) in experiments with thermal stress and in juvenile shrimps (Rezende et al., 2022; Schleder et al., 2020) in experiments with WSSV. These studies also combined the use *S. filipendula* and *Undaria pinnatifida* in association with thermal fluctuation.

Environmental stress can significantly alter shrimp defense, resulting in a reduction of THC and other immune parameters (Le Moullac & Haffner, 2000). In our work, HS caused immunosuppression with changes in THC and agglutinating activity, which only returned to normal levels after 1 hour, while changes in PO activity only returned to normal after 24 hours. Some works report that low temperatures modify immunological patterns. When the temperature drops from 28 to 13°C, *L. vannamei* can experience reduction of THC. Such

drop in temperature also affects the binding of hemocyanin, along with suppressing antibacterial activity, making the animals more resistant to pathogens (Xu et al., 2019). Using MALDI-TOF MS data, Schleder et al. (2020) demonstrated that HS alone has a significant impact on the lipid and protein profile of shrimp (*L. vannamei*), particularly affecting molecules involved in membrane fluidity, lipid and energy metabolism, immune response, apoptosis and antioxidant capacity.

Moreover, when using the brown seaweed *S. filipendula* (0.5%) in the diet of *L. vannamei*, Schleder et al. (2017b) demonstrated an increase in the fluidity of membrane and antimicrobial defenses and a decrease in apoptosis signals, thereby modulating the regulation of DNA and lipid energy metabolism in shrimp hemocytes. In this work, we observed that *L. vannamei* shrimps treated with a diet of *S. filipendula* extract presented a higher agglutination activity before, during and 1 hour after thermal shock.

Alterations in the agglutination activity of shrimp under stress conditions have been poorly investigated, and few studies have reported that this activity is induced by the presence of microorganisms or foreign particles. One hemolymph protein capable of cellular agglutination is lectin (Marques & Barracco, 2000). Lectins can recognize a specific saccharide by agglutinating the hemocyte through binding to cell surface glycoproteins and glycoconjugates. C-type lectins belong to a lectin superfamily that is used against pathogenic invasion in shrimp (Yan et al., 2022). For example, a significant increase in the expression of the C-type lectin gene was observed in *L. vannamei* shrimp upon oral administration of alginate and fucoidan extracted from the seaweed *Cystoseira trinodis* (Salehpour et al., 2021). Zhang et al. (2023) showed that the addition of seaweed powder to feed significantly increased hemagglutination and the expression of the C-type lectin gene in *L. vannamei*, likely related to the abundance of sodium alginate in seaweed.

Humoral immunity contains the proPO system, lectins, crustin, and lysozyme, all of which can trigger various immune responses through intercellular communication, and thus play an essential role in immune defense (Wang et al., 2020). The inclusion of *S. filipendula* in shrimp diet may have induced the production of antimicrobial peptides and inhibited lysophosphatidic acid, a lipid related to cell apoptosis (Schleder et al., 2017a). Shrimp supplied with this diet became more resistant to pathogens. Several works reported that the inclusion of *Sargassum* extracts in shrimp diets increased the animals' immunological response when compared to THC, PO activity of serum, phagocyte activity of hemocytes, release of oxygen reactive species, antioxidant enzyme and others. This finding suggests the efficacy of *Sargassum* extracts when coupled to survival after exposure to *Vibrios* and white-spot virus (Huang et al., 2006; Immanuel et al., 2012; Yeh et al., 2006). Kitikiew et al. (2013) suggest that fucoidan can cause the degranulation of hemocytes by activating the proPO system.

However, seaweed treatments did not affect the number of microorganisms in shrimp intestine, corroborating by Schleder et al.'s (2017b) results, which did not detect any difference in THB counts in shrimps fed on *S. filipendula* at concentrations 0.5, 2, and 4%. However, Rezende et al. (2021) observed that the number of Vibrionaceae in Pacific white shrimp post-larvae was lower in shrimps fed diets containing 1S:4U (*Sargassum*:*Undaria*).

Low temperatures may naturally decrease the number of potential pathogens and their virulence. Shrimps are less sensitive to WSPS when maintained at low temperatures; consequently, virus replication decreases (Gao et al., 2011). However, the virus is not eliminated, and high mortality rates still occur when shrimp return to higher temperatures (Gunalan et al., 2010; Moser et al., 2012). Therefore, the development of products with immune-stimulating factors, or those that decrease pathogenicity and pathogen concentration, is highly relevant in shrimp farming in subtropical regions.

CONCLUSION

Aqueous extract of the seaweeds *A. nodosum* and *Sargassum filipendula* at concentrations of 100 and 200 $mg \cdot L^{-1}$, respectively, improves the survival rates of post-larvae *L. vannamei* shrimps submitted to HS.

Sargassum filipendula and *A. nodosum* included in the diet of juvenile shrimps did not affect the intestinal microbiota of *L. vannamei*. In the case of immunological parameters, both species positively affected only the agglutination titer, before and after HS, with no influence on other parameters analyzed.

HS causes significant immunodepression and reduces the bacterial community found in the intestine of juvenile shrimps. Immunological parameters (THC, PC, agglutination titer, and PO), however, returned to normal level between 1 and 24 h after shock, followed by reestablishment of the animals' defense.

CONFLICT OF INTERESTS

Nothing to declare.

DATA AVAILABILITY STATEMENT

All datasets were generated or analyzed in the current study.

AUTHORS' CONTRIBUTION

Conceptualization: Pontinha VA, Vieira FN, Hayashi L; **Investigation:** Pontinha VA**,** Schleder DA, Vieira FN, Hayashi L; **Resources:** Pontinha VA; **Data curation:** Schleder D.; Simioni C; **Formal Analysis:** Pontinha VA, Schleder DA, Seiffert WQ; Vieira FN, Hayashi L; **Validation:** Hayashi L.; **Supervision:** Vieira FN, Hayashi L; **Writing – original draft**: Pontinha VA, Schleder DA, Seiffert WQ; **Writing – review & editing:** Seiffert WQ, Simioni C, Hayashi L; **Final approval:** Simioni C, Vieira FN, Hayashi L.

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