Immune response of *Litopenaeus vannamei* juveniles immersed in *Gracilariopsis heteroclada* hot-water extract

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Abstract

The emergence of diseases such as Acute Hepatopancreatic Necrosis Disease and White Spot Syndrome has compelled researchers in shrimp aquaculture to develop more innovative methods in improving shrimp health management for the prevention of diseases. One of these methods involves the use of immunostimulants, which enhance the innate immunity of shrimp. Polysaccharides extracted from red seaweeds such as *Gracilariopsis heteroclada*, locally known as "Gulaman", present a great source of immunostimulants. Thus, this study aimed to evaluate *Gracilariopsis heteroclada* as an immunostimulant for *Litopenaeus vannamei*, or Pacific white shrimp. Hot-water extracts of *Gracilariopsis heteroclada* were administered through immersion. Results showed no significant changes in total haemocyte count, ambiguous fluctuations in phenoloxidase activity, and a late temporary increase in respiratory burst activity. Overall, these indicate a lack of significant immunostimulation in *Litopenaeus vannamei* juveniles when immersed in *Gracilariopsis heteroclada* hot-water extract.

Keywords: *Gracilariopsis heteroclada, Litopenaeus vannamei, immunostimulants*

Introduction. Immunostimulation can increase the resistance of farmed aquatic animals to pathogens during exposure to stress, such as handling, crowding, sampling, transport, vaccination, reproduction, and the rearing larval stages when high levels of mortality occur. Common immunostimulants include beta-glucans, lipopolysaccharides, and seaweed polysaccharides such as carrageenan, and other sulfated galactans [1,2,3,4]. Many of these compounds are derived from seaweed hot-water extracts.

Hot-water extracts of seaweeds have been demonstrated to be capable of improving the immune response of cultured shrimp. These hot-water extracts contain polysaccharides such as carrageenan, fucoidan, and other sulfated galactans from the seaweeds, and are responsible for their immunostimulating properties against cultured shrimp [5]. Specifically, hot-water extracts of *Sargassum duplicatum, Gelidium amansii, Gracilaria edulis,* and *Gracilaria tenuistipitata* have been reported to possess immunostimulating activities in various cultured shrimp species [6,7,8,9].

Administration via injection, immersion, and dietary supplements are all capable of and effective in administering the immunostimulant. As reported by Fu et al. [6], *L. vannamei* have enhanced innate immunity when immersed in hot-water extract at 400 mg/L, injected with hot-water extract at 6 mg/g shrimp, and fed hot-water extract of *G. amansii* at 2.0 g/kg or less. Also, Yeh et al. [9] observed that *L. vannamei* immersed in hot-water extract of *S. duplicatum* at 300 mg/L, and the shrimp that were injected with hot-water extract at 10 ug/g shrimp or less showed both improved innate immunities. *M. rosenbergii* that were immersed in 0.1 g/L *G. edulis* hot-water extract also exhibited an increase in immune response [7]. These improvements were indicated by immune parameter assays such as total haemocyte count, phenoloxidase activity, and respiratory burst activity.

Overall, these studies have established the immunostimulatory properties of seaweed hot-water extracts against cultured shrimp species. None of these studies, though, have validated, if any, the potential of *G. heteroclada* hot-water extracts as immunostimulants for *L. vannamei* by immersion; hence the need for this study. In addition, *G. heteroclada* is an extensively cultured seaweed species in the Philippines commonly used in food, fertilizer, and medicine [10]. This, along with its abundance, makes it viable as a source of immunostimulants.

This study aimed to evaluate the immune response of *Litopenaeus vannamei* immersed in *Gracilariopsis heteroclada* hot-water extract. Specifically, the aim of this study was to:

(i) Determine the total haemocyte count of *Litopenaeus vannamei* juveniles immersed in *Gracilariopsis heteroclada* hot-water extract at different concentrations (0, 100, 200 mg/L) at different time periods (1, 3, 4, 24, 72, 120 hrs).

(ii) Quantify phenoloxidase activity of *Litopenaeus vannamei* juveniles immersed in *Gracilariopsis heteroclada* hot-water extract at different concentrations (0, 100, 200 mg/L) at different time periods (1, 3, 4, 24, 72, 120 hrs).

(iii) Measure respiratory burst activity of *Litopenaeus vannamei* juveniles immersed in *Gracilariopsis heteroclada* hot-water extract at different concentrations (0, 100, 200 mg/L) at different time periods (1, 3, 4, 24, 72, 120 hrs).

(iv) Compare the total haemocyte count of *Litopenaeus vannamei* juveniles immersed in *Gracilariopsis heteroclada* hot-water extract at

different concentrations (0, 100, 200 mg/L) at different time periods (1, 3, 4, 24, 72, 120 hrs)

(v) Compare the phenoloxidase activity of *Litopenaeus vannamei* juveniles immersed in *Gracilariopsis heteroclada* hot-water extract at different concentrations (0, 100, 200 mg/L) at different time periods (1, 3, 4, 24, 72, 120 hrs)

(vi) Compare the respiratory burst activity of *Litopenaeus vannamei* juveniles immersed in *Gracilariopsis heteroclada* hot-water extract at different concentrations (0, 100, 200 mg/L) at different time periods (1, 3, 4, 24, 72, 120 hrs)

Methods. In order to test the immune responses of *L. vannamei* juveniles, an experimental study was conducted. Juvenile *L. vannamei* cultures and *G. heteroclada* hot-water extracts were prepared, and the shrimp cultures were immersed in *G. heteroclada* hot-water extract as an immunostimulant. Thereafter, various immune parameters — Total Haemocyte Count, Phenoloxidase Activity, and Respiratory Burst — were assessed to determine the immune responses of the shrimp.

Preparation of materials. Four (4) kilograms' worth of fresh samples of *G. heteroclada* were bought from Iloilo Central Market and identified by Ms. Hananiah Sollesta from the SEAFDEC FishWorld. One hundred eighty (180) *L. vannamei* juveniles with an average body weight of 13 grams were purchased from IJY Farms, Zarraga and were brought to the Infection Building of Southeast Asian Fisheries Development Center/Aquaculture Department (SEAFDEC/AQD) for acclimatization. Chemicals were purchased and obtained from SEAFDEC/AQD.

Hot-water extraction of G. heteroclada. *G. heteroclada* samples were sun-dried for 48 hours. After, it was powderized in a corn mill grinder and was sifted using a 250 um mesh. Thirty (30) grams of powder were boiled in 900 mL of distilled water for three hours at 100°C. The solution was filtered using a cloth and a sieve of 250 um mesh size into the metal trays. The trays were frozen overnight at -80°C then lyophilized for 24 hours thereafter at 2 mmHg.

Experimental set-up. For the maintenance of the shrimp culture for the experiment, the seawater in the tanks were monitored with the following the conditions: pH of 7.7 to 8.2, salinity of 33±1 ppt, temperature of 30±1°C, and constant aeration. Commercial feeds were used and administered twice a day. Ten (10) shrimps were placed into separate 50 L tanks filled with 30 L of filtered seawater. Also, tanks used for each treatment (0, 100, and 200 mg/L hot-water extract) were in triplicate.

Immune response assays. Haemolymph samples were withdrawn from one shrimp randomly selected from each tank 0, 1, 3, 4, 24, 72, and 120 hours after immersion has commenced. The haemolymph mixtures taken were of the following ratios: 100uL haemolymph: 200uL buffered formalin solution; 300uL haemolymph: 300uL anticoagulant solution; and 100uL haemolymph: 200uL anticoagulant solution for total haemocyte count, phenoloxidase activity, and respiratory burst activity, respectively. For total haemocyte count 10 uL of haemolymph-buffered formalin mixture was pipetted into the space formed between Neubauer haemocytometer and coverslip while avoiding bubble formation. This was viewed under a compound light microscope for counting. The calculation of the total haemocyte count was based on the formula:

$$Total\ Haemocyte\ Count = \frac{N \times D \times 10^3}{A \times 10} cells \cdot mL^{-1}$$

where N = total number of cells counted; D = dilution of haemolymph; A = total area counted (in mm^2, 10^3 = conversion factor from uL to mL, 10 = conversion factor from mm^2 to uL). Phenoloxidase activity was performed on the extracted 600 uL of haemolymph mixture (300uL haemolymph: 300uL anticoagulant). This mixture was centrifuged for 15 min at 1000*g* at 5°C and the supernatant was removed. The pellet was rinsed and suspended in 200uL cacodylate buffer. This was centrifuged again and resuspended in cacodylate buffer. Afterward, 200 uL of 0.1% zymosan in cacodylate buffer was added and the cell suspension was incubated for 1 hr at room temperature. The mixture was centrifuged at 1000*g* for 5 min and 60 uL of the supernatant was placed in microtiter plate wells. 25 uL of L-DOPA was added to the wells and incubated for 10 min before adding 300 uL of cacodylate buffer. The optical density was measured at 492 nm using a microplate reader. Three hundred uL of cacodylate buffer was used as the blank control reaction for subtraction from the absorbances of the samples. The formula used for the final absorbance was:

$$A_s = A_o - A_b$$

where A_s = final absorbance of the sample, A_o = observed absorbance of the sample, and A_b = absorbance of the blank sample (solvent). Respiratory burst activity was assessed using the extracted 300 uL of haemolymph mixture (200uL haemolymph: 100uL anticoagulant). The mixture was centrifuged at 1000g for 10 min at room temperature. The supernatant was removed and 100 uL of HBSS was added. The resulting solution was incubated for 30 min at 37°C. After which the sample was washed three times with HBSS, stained with 100 uL of 0.3% NBT solution, and incubated for 30 min at 37°C. Then, the NBT was removed before adding 100 uL of absolute methanol. The methanol was removed, and the solution was washed three times with 70% methanol. The pellet was air dried for 5 min before adding 120 uL of 2M KOH and 140 uL DMSO. The solution was transferred into microtiter plate wells and the optical density was read at 620 nm using a microplate reader. 120 uL of 2M KOH and 140 uL DMSO was used as the blank control reaction for subtraction from the absorbances of the samples. The formula used for the final absorbance was:

$$A_s = A_o - A_b$$

where A_s = final absorbance of the sample, A_o = observed absorbance of the sample, and A_b = absorbance of the blank sample (solvent).

Data Analysis. R programming language in the RStudio software was used to analyze the data. First,

parametric data assumptions were validated. Specifically, homogeneity of variances was assessed through Bartlett or Levene test and normal distribution was assessed through Shapiro-Wilk test. When either of the two assumptions were violated in a data set, the data set would undergo a Box-Cox transformation to meet the said assumptions. After this, one-way ANOVA along each factor (treatment and time) was performed at p=0.05. Tukey's test was then used to check for specific significant difference among groups.

Safety Procedures. Gloves and lab gowns were used throughout the conduct of the experiment to avoid contact with chemicals and other harmful substances. The shrimps were placed inside a cooler with ice for anesthesia. The needles were recapped when not in use. After extraction, the syringes were placed in a biohazard container for disposal. The shrimp carcasses were thrown into a garbage container. Both were turned over to SEAFDEC/AQD for their disposal protocol.

Results and Discussion. *L. vannamei* immersed in *G. heteroclada* hot-water extracts showed no significant differences in total haemocyte count ($p>0.05$) all throughout the 120 hours of immersion (Figure 1) along each treatment. Also, no significant differences in total haemocyte count ($p>0.05$) occurred between the treatment groups and control group along each time point.

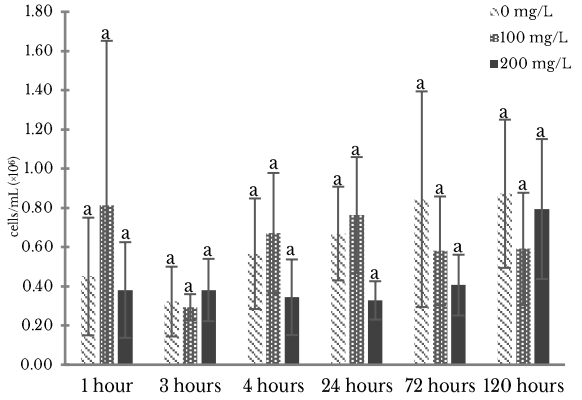

Figure 1. Total Haemocyte Count of *L. vannamei* juveniles immersed in 0, 100, 200 mg/L of *G. heteroclada* hot-water extract at 1, 3, 4, 24, 72, 120 hours after immersion. Bars with different superscripts show significant mean differences at $p<0.05$.

As shown in Figure 2, all *L. vannamei* treatment groups showed increased phenoloxidase activity ($p<0.05$) in the 1st hour relative to the succeeding hours; however, the control group (0 mg/L) did not exhibit a significantly lower phenoloxidase activity as compared to the treatment groups. Overall, it shows ambiguous, and therefore inconclusive, fluctuations in the phenoloxidase activity of *L. vannamei* juveniles immersed in *G. heteroclada* hot-water extract.

For respiratory burst activity, among all treatments and time points, only *L. vannamei* treated at 200 mg/L showed increased respiratory burst activity at the 4th hour ($p<0.05$) but returned to its background values on the 72nd hour (Figure 3). The rest of the measurements showed no significant changes in respiratory burst ($p>0.05$) between treatments along each time point and between time points along each treatment. This suggests the late and temporary onset of immunostimulation of *L. vannamei* immersed in *G. heteroclada* hot-water extract.

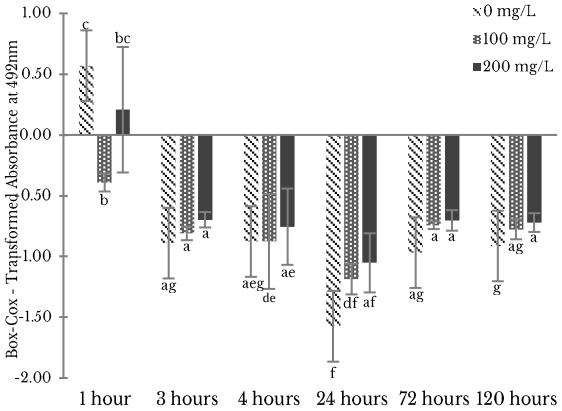

Figure 2. Phenoloxidase Activity of *L. vannamei* juveniles immersed in 0, 100, 200 mg/L of *G. heteroclada* hot-water extract at 1, 3, 4, 24, 72, 120 hours after immersion. Bars with different superscripts show significant mean differences at $p<0.05$.

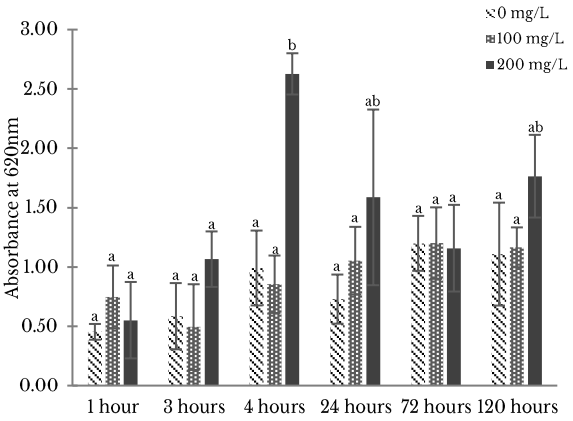

Figure 3. Respiratory Burst Activity of *L. vannamei* juveniles immersed in 0, 100, 200 mg/L of *G. heteroclada* hot-water extract at 1, 3, 4, 24, 72, 120 hours after immersion. Bars with different superscripts show significant mean differences at $p<0.05$.

Seaweed hot-water extracts are a known source of immunostimulatory polysaccharides such as carrageenan, fucoidan, and alginates, among others [11]. These polysaccharides induce immune responses in penaeid shrimp by stimulating physiological functions such as haemocyte production and activating pathways such as the prophenoloxidase (proPO) cascade and respiratory burst [11].

In this study, *L. vannamei* juveniles immersed in *G. heteroclada* hot-water extract showed no significant changes in total haemocyte count, ambiguous fluctuations in phenoloxidase activity, and a temporary increase in respiratory burst. These suggest the lack of immunostimulation by the said hot-water extract. These are contradictory to the study of Maningas et al. [7], which showed that the total haemocyte count and phenoloxidase activity of *Macrobrachium rosenbergii* immersed in 100mg/L *Gracilaria edulis* hot-water extract were significantly

increased. Also, Yeh et al. [9] showed the immune parameters also exhibit an increase, specifically the phenoloxidase activity and the respiratory burst activity of *L. vannamei.* The phenoloxidase activity of the shrimps immersed in 100mg/L *Sargassum duplicatum* hot-water extracts was significantly higher than the control after 1 hour and the respiratory burst of the shrimps immersed in 100 mg/L seaweed hot-water extracts was significantly higher than the control after 4 hours [9]. Both of these studies indicate that there is an increase in immune parameters after being immersed at 100 mg/L seaweed hot-water extracts.

The results of the phenoloxidase and respiratory burst assay may be explained by the lack of changes in the number of haemocytes, the cells in shrimp primarily responsible for its innate immunity [13], throughout the experiment. This overall phenomenon may be explained by two possible reasons: the low administration dosage of the extract and the lack of immunostimulatory polysaccharides in the extract. The former is suggested by the temporary increase in respiratory burst activity in *L. vannamei* immersed in 200 mg/L hot-water extract which, had the dosage been higher, could have probably increased the respiratory burst, and possibly the other immune parameters, at an earlier onset and at a sustained pace. This is also supported by Muñoz et al. [12], which showed that increases in respiratory burst, and other immune pathways, were dependent on the dosage of the administered immunostimulant. On the other hand, the presence and composition of polysaccharides in the hot-water extracts were not evaluated. However, though *G. heteroclada,* as a red alga, is known to have beta-glucans, and sulfated polysaccharides such as carrageenan [14], which are known immunostimulatory polysaccharides [14], its structure, composition, and abundance in the extract could have been affected by seasonality and other environmental factors in its culture. How these changes would result to changes in functional bioactivity is unknown.

Taken all together, the findings suggest that no significant effects in the innate immune response of *L. vannamei* occured when immersed in *G. heteroclada* hot-water extract. This may be due to either the lack of immunostimulatory polysaccharides or the lack of sufficient dosage.

Error Analysis. Transportation and handling of the *G. heteroclada* samples were questionable, for the acquisition and purchase was done in the Central Market of Iloilo City, and not directly from the seaweed farms. These samples should have been monitored from the time it was collected to the time it is processed in the lab. Also, the samples were not characterized therefore it was not determined whether the needed polysaccharides were present in the species. The haemolymph samples were not processed directly after it was extracted. Processing of samples was delayed due to the distance between the labs, where assays were being done, and the location of the set-ups.

Conclusion. Based on the findings on haemocyte count, respiratory burst, and phenoloxidase activity, it appears that there is no immunostimulation of *L. vannamei* upon immersion in *G. heteroclada* hot-water extract.

Recommendations. It is recommended that *G. heteroclada* hot-water extracts be further evaluated as to confirm the lack of polysaccharides known to have immunostimulant properties. *G. heteroclada* must also be bought directly from the farms to ensure freshness of samples, and seasonality and other environmental and culture conditions affect the presence, structure, and composition of these immunostimulatory polysaccharides in *G. heteroclada* must also be investigated. Moreover, higher doses of the hot-water extract may be evaluated for their immunostimulatory properties in *L. vannamei.* Also, a positive control treatment must be included in the conduct of the experiments.

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