Effect of hydrosoluble polysaccharides of *Macrocystis pyrifera* on physiological and metabolic responses of *Litopenaeus vannamei* infected with *Vibrio campbellii*

Efecto de polisacáridos hidrosolubles de *Macrocystis pyrifera* sobre las respuestas fisiológicas y metabólicas de *Litopenaeus vannamei* infectado con *Vibrio campbellii*

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ABSTRACT

Adult white shrimp *Litopenaeus vannamei* between 23 and 32 g of wet weight were injected or submerged in a hydrosoluble polysaccharides extract from *Macrocystis pyrifera* and infected with *Vibrio campbellii*. The infection decreased the oxygen consumption rate to 24 mg O_2 h^{-1} kg^{-1} w.w., in shrimps of the control group, which were only infected with *V. campbellii*. Immunestimulated shrimps did not decrease their oxygen consumption rate at any hour p.i. (46 mg O_2 h^{-1} kg^{-1} w.w.) maintaining it similar to the pre-injection group. Glucose level in the hemolymph of *V. campbellii* infected shrimps at two hours p.i. was significantly higher ($p > 0.05$) than the level of the pre-injection group. *L. vannamei* injected with the extract showed a significant decrease ($p > 0.05$) in their glucose level at 12 hours p.i., but at 24 hours p.i. it returned to normal level. Shrimps submerged in the extract showed no significant glucose level difference ($p < 0.05$). Lactate concentration in the hemolymph of the pre-injection group was 11.4 mg dL^{-1}, but adults injected with the extract had the lowest lactate levels throughout the experiment. Shrimps submerged in the extract decreased lactate levels at 6 and 12 hours p.i. but at 24 hours p.i. the level returned to 9.0 mg dL^{-1}. The total protein concentration in the hemolymph of pre-injection group was 115.3 mg mL^{-1}, shrimp injected with saline solution showed no significant differences ($p < 0.05$) compared to basal control; shrimps injected with *V. campbellii* had the lowest values of total proteins 6 hours p.i ($p > 0.05$) immunoestimulated shrimps showed an increase in their total proteins levels. This study concluded that administration of extract of *M. pyrifera* via injection and immersion in adult white shrimp can be used for immunostimulation purposes.

Key words: Oxygen consumption, metabolites, immunostimulation, *Vibrio campbellii*.

RESUMEN

Adultos de camarón blanco *Litopenaeus vannamei* de entre 23 y 32 g de peso húmedo fueron inyectados o sumergidos en un extracto de polisacáridos hidrosolubles de *Macrocystis pyrifera* e infectados con *Vibrio campbellii.* La infección disminuyó la tasa de consumo de oxígeno a 24 mg O_2 h^{-1} kg^{-1} p.h. en los camarones del grupo control infectados con *V. campbellii.* Los camarones inmunoestimulados vía inyección e inmersión, mantuvieron una tasa de consumo de oxígeno de 46 mg O_2 h^{-1} kg^{-1} p.h., similar a los camarones control. La concentración de glucosa en la hemolinfa de los adultos infectados con *V. campbellii* a las dos horas p.i. fue significativamente más alto ($p > 0.05$) que la del grupo control de pre-inyección. Los inyectados con el extracto e infectados con *V. campbellii* disminuyeron los niveles de glucosa a las 12 horas p.i., pero a las 24 horas p.i. volvió a los niveles basales. Los camarones inmunoestimulados con el extracto vía inmersión no mostraron ninguna diferencia significativa ($p < 0.05$) en sus niveles de glucosa a lo largo de las 24 horas p.i. La concentración de lactato en la hemolinfa de los camarones del grupo pre-inyección fue de 11.4 mg dL^{-1}, pero los adultos inyectados con el extracto tuvieron los niveles más bajos durante todo el experimento. *L. vannamei* sumergidos en el extracto disminuyeron sus niveles de lactato a las 6 y 12 horas p.i. pero a las 24 horas p.i. el nivel regresó a 9 mg dL^{-1}. La concentración de proteínas totales en la hemolinfa de los camarones del control basal fue de 115.3 mg mL^{-1}, los organismos inyectados con *V. campbellii* tuvieron los valores más bajos ($p > 0.05$) respecto a los del control basal. En los adultos infectados sumergidos e inyectados con el extracto se obtuvieron los valores más altos de proteínas totales en hemolinfa a las 24 horas p.i. Se concluye que la administración del extracto de *M. pyrifera* vía inyección e inmersión en adultos de camarón blanco puede ser utilizada para propósitos de inmunoestimulación.

Palabras clave: Consumo de oxígeno, metabolitos, inmunoestimulación, *Vibrio campbellii*.

INTRODUCTION

The Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) is distributed along the Pacific coast, from the Sea of Cortez to Peru. It is the most worldwide cultivated penaeid but in Latin America despite being the most profitable aquatic resource, its culture has moved to second place in recent years due to production losses caused by disease-causing viruses and bacteria (FAO, 2008). The sustainability of shrimp farming depends heavily on disease control (Rodríguez & Le Moullac, 2000), however, the shrimp defense system as in other invertebrates, does not allows the use of vaccines because it does not have specificity or immunological memory and lacks immunoglobulins and lymphoid cells (Muñoz *et al*., 2000; Rodríguez & Le Moullac, 2000; Montaño-Pérez *et al*., 2005, Huang *et al*., 2006).

Immunostimulation has been until now the most viable option to enhance disease resistance in shrimp under culture conditions (Berger, 2000, Vargas-Albores, 2002; Rendón & Balcazar, 2003; Smith *et al.,* 2003). Immunoestimulant are extracted from the cell walls of Gram negative bacteria which contain lipopolysaccharides, Gram positive bacteria with peptidoglycan, as well as fungi, yeasts and algae with β-glucans; their role is to simulate a bacterial infection which activates the cellular and humoral defense system of crustaceans (Rendón *et al*., 2004).

Approximately 80 to 90% of bacteria isolated from penaeid cultures with mass mortalities belong to the genus *Vibrio* (Pacini), which behaves as an opportunistic pathogen that takes advantage of environmental conditions that affect the host organism, such as climate changes, population density, poor nutrition and water quality (Leaño *et al*., 1998). Different species of *Vibrio* affect penaeid shrimps under culture conditions, such as *V. harveyi* (Johnson & Shunk), *V. campbellii* (Baumann), *V. parahaemolyticus* (Fujino), *V. vulnificus* (Reichelt) and *V. alginolyticus* (Miyamoto), which are responsible for the so-called "vibriosis" whose principal symptoms in shrimp are whitening of the thoracic region, lethargy, erratic swimming, loss of appetite, intestine inflammation and necrosis of the appendages (Lavilla *et al*., 1990).

Crustacean gills are not only involved in gas exchange and ionic regulation, they also contribute to the defense system, it is known that they are the main site of foreign particles and bacterial elimination, which, once removed from the hemolymph, they accumulate and are eventually encapsulated in the gills (Martin *et al*., 2000).

In aquatic animals, oxygen consumption measurement is a standard method to assess the effect of environmental factors such as temperature, salinity, exposure to pollutants, light intensity or dissolved oxygen, and to determine the energy costs associated with the physiological stress imposed on the organisms (Lemos *et al*., 2001; Altinok & Grizzle, 2003).

To evaluate the effect of environmental conditions in wild and farmed shrimp, variables which are considered indicators of physiological, nutritional and immunological health of *Litopenaeus vannamei* and *L. setiferus* (Perez-Farfante) have been proposed, such as the metabolites glucose, lactate and total protein in the hemolymph (Racotta & Palacios 1998; Rosas *et al.,* 2001, 2002; Sanchez *et al.,* 2001; Pascual *et al.,* 2003, 2004). These variables have been proposed because they reflect the process of adapta-

tion to stress when the metabolism of an organism is modified to try to counteract the effect of the stressor.

Macrocystis pyrifera (Linnaeus) C. Agardh is a brown algae of the order Laminariales commonly called "kelp" which grows in rocky substrates near the coast, at depths not exceeding 40 meters in temperate or cold waters. It is found mainly along the coasts of North and South America, Australia, New Zealand, Norway, Scotland, Japan, Korea and South Africa (Cruz-Suarez *et al.*, 2000). It is one of the brown seaweed that is exploited commercially as a source of alginates in México; its distribution extends from the border with the United States of America to Punta San Hipolito, Baja California Sur. The estimated harvestable biomass (1 m below the sea surface) varies seasonally from 36,520 tons in the winter to 99,626 tons in the summer (Reyes-Tisnado *et al.*, 2004).

The aim of this study was to assess the effect of hydrosoluble polysaccharides extract of *Macrocystis pyrifera* on adult *Litopenaeus vannamei* immunostimulated by immersion or injection via and infected with *Vibrio campbellii* on some physiological and metabolic responses.

MATERIALS AND METHODS

Macrocystis pyrifera blades were collected at La Mision, B.C., México, during the summer of 2007 and a hydrosoluble extract of polysaccharides was prepared based on the methodology described by Yeh *et al.* (2006).

A total of 270 adult *Litopenaeus vannamei* shrimps (previously dried with absorbent paper and weighted on an Ohaus Explore balance) ranging from 23 to 32 g of wet weight (w.w.) from the laboratory La Gloria located in Nayarit, México were kept for two months in 2000 L tanks with continuous replacement of seawater (35 ‰), at a temperature of 26 ± 1 °C, with constant aeration and food satiation (Rangen© with 40% protein). A pathogenic strain of *V. campbellii* was cultured in Zobell marine medium at 28 °C for 18 hours, then harvested and resuspended in saline solution (9 ‰), at a concentration of 1×10^7 colony-forming units cfu mL^{-1}. Shrimps were injected with 100 µL of this bacterial suspension, the final concentration was 1×10^6 colony-forming units cfu $shrimp^{-1}$.

To measure the shrimp's oxygen consumption rate, a semi-open respirometer with 21 respirometric chambers of 2800 mL each was used; it was connected to a system similar to that described by Díaz *et al.* (2007). Twenty shrimp were placed individually in 20 respirometric chambers and leaving one chamber without any organism in order to calculate the oxygen consumption of microorganisms present in the water. Water temperature of the entire system was maintained at 26 ± 1 °C using 1000 watts titanium heater connected to programmable temperature controller. The dissolved oxygen concentration was measured by an oximeter YSI 52 B equipped with a polarographic sensor accuracy ± 0.03 mg L^{-1}, which was inside an acrylic hermetic chamber of 10 mL with adequate stirring magnet, through tubing system to prevent the water sample had direct contact with air. Before closing the chamber, a seawater sample was taken to measure the initial concentration of oxygen, respirometric chambers were closed for 1 hour because according to Stern *et al.* (1984) is the adequate time in which dissolved oxygen will not decrease below the 30% of saturation, so it does not cause stress on the organisms.

Oxygen consumption rate (OCR) was calculated according to the following equation (Cerezo Valverde *et al.*, 2006; Zheng *et al.*, 2008)

$$OCR = (C_t - C_0)\, V / (W \times T)$$

were C_t is change in the oxygen content (mg O_2) in the respirometric chambers before and after the test

C_0 is the change of the oxygen content, in the blank (control)

V is the volume of the chamber

W and T are the weight of shrimps in kg of wet biomass, and the time duration in hours, respectively.

Lyophilized hydrosoluble polysaccharides extract (6 mg) of *M. pyrifera* was dissolved in 6 mL of saline solution (9 ‰), and 60 organisms were injected in the abdominal muscle with 100 µL of the prepared extract, the final concentration was 10 µg g^{-1}; 24 hours later the same shrimps were injected with 100 µL (1×10^6 cfu $shrimp^{-1}$) of the bacterial suspension of *V. campbellii* into abdominal muscle and 20 organisms were placed individually in each respirometric chamber two hours before starting the oxygen consumption measurements which were conducted at two, four, six, eight, 12 and 24 hours post injection (p.i).

For the immersion test, 3.5 g of the lyophilized hydrosoluble polysaccharides extract of *M. pyrifera* were dissolved in 10 L of seawater; (final concentration of 350 mg L^{-1}). Sixty *L. vannamei* adults were kept for 3 hours in aquariums containing the extract with constant aeration. Subsequently, they were injected with 100 µL of the bacterial suspension of *V. campbellii* (1×10^6 cfu $shrimp^{-1}$) and 20 organisms were placed individually in each respirometric chamber two hours before the begin of the oxygen consumption measurements which were conducted for two, four, six, eight, 12 and 24 hours p.i.

As control 150 shrimps were used, 60 were injected with 100 µL of saline solution, 20 were placed individually in each respirometric chamber two hours before starting the measurements at two, four, six, eight, 12 and 24 hours p.i. Other 60 shrimps were injected with 100 µL of the bacterial suspension (1×10^6 cfu $shrimp^{-1}$), 20 of which were placed inside the respirometric chambers and the oxygen consumption was measured in the same way as described above.

The remaining 30 organisms were used as basal control (pre-injection), these organisms were not subjected to any treatment or infection but the oxygen consumption rate was measured with one repetition of the experiment.

Hemolymph was extracted from 8 shrimps from each experimental condition at two, six, and 12, 24 hours after treatment, a 2:1 ratio anticoagulant solution (Vargas-Albores *et al*., 1993) was used. All samples were centrifuged at 800 g for 3 min at 4 °C, the supernatant was separated for quantification of each metabolite through commercial kits (Lopez *et al*., 2003; Pascual *et al*., 2004; Rosas *et al*., 2004).

The glucose samples were read on a spectrophotometer (Hach) at 520 nm, the lactate at 546 nm and total proteins at 540 nm, finally, the concentration of each metabolite was calculated from respectively standards (Pointe Scientific Inc.).

Statistical analysis of data (oxygen consumption rate, glucose, lactate and total proteins concentrations in the hemolymph) was carried out using the Sigma Stat program, Kruskal-Wallis (Zar, 1999) nonparametric test was used previous determination of the normality and homoscedasticity of the data, to determine if there were significant differences between the control and experimental shrimps.

RESULTS

The oxygen consumption rate of shrimps from the pre-injection control was 46 mg O_2 h^{-1} kg^{-1} w.w. Saline control organisms increased their oxygen consumption rate at two and 12 hours p.i. (62.1 and 63.8 mg O_2 h^{-1} kg^{-1} w.w., respectively) however, this increase was not significantly different ($p < 0.05$) compared to pre-injection control (Fig. 1A).

Shrimps injected with *V. campbellii* had an oxygen consumption rate increase at two, four and six hours p.i. of 65.6, 67 and 65.4 mg O_2 h^{-1} kg^{-1} w.w. respectively; while at 8 to 12 hours p.i. the oxygen consumption rate initiate to decline (54.3 and 31 mg O_2

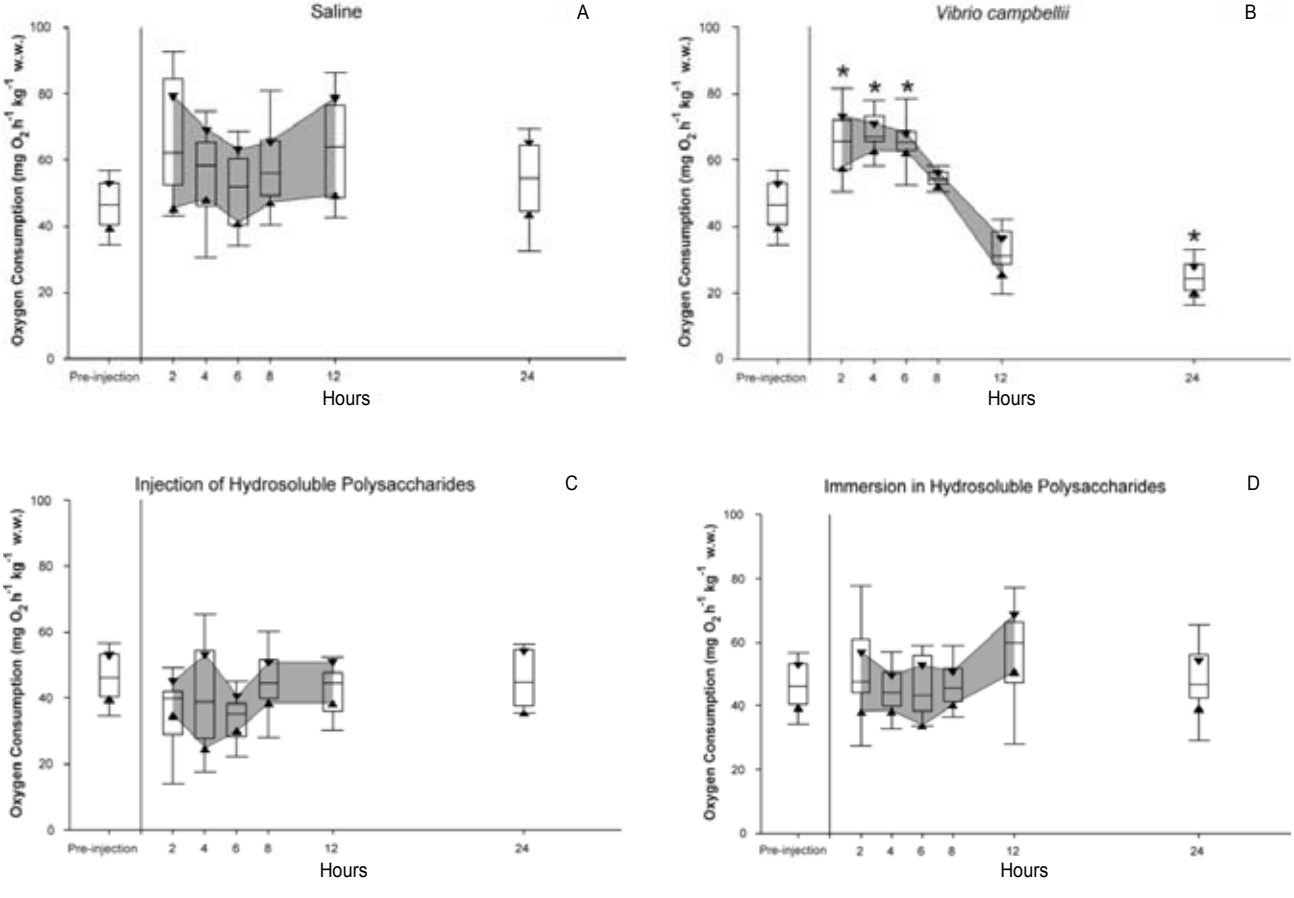

Figure 1 A-D. Oxygen consumption rate (mg O_2 h^{-1} kg^{-1} w.w.) of saline injected *Litopenaeus vannamei*. Pre-injection values represent the median oxygen consumption rate of 20 organisms 2 hours before saline injection (A), injected with *Vibrio campbellii* (B), injected with the hydrosoluble polysaccharides extract of *Macrocystis pyrifera* and infected with *Vibrio campbellii* (C), submerged in the hydrosoluble polysaccharides extract of *Macrocystis pyrifera* and infected with *Vibrio campbellii* (D). Shaded area represents the 95% confidence intervals of median, the bars include 50% of the distribution and the vertical lines represent the quartiles.

h^{-1} kg^{-1} w.w., respectively), and at 24 hours p.i. the lowest oxygen consumption rate was registered (24.15 mg O_2 h^{-1} kg^{-1} w.w.) (Fig. 1B).

The oxygen consumption rate of pre-injection shrimps and that of those injected with the hydrosoluble polysaccharides extract of *M. pyrifera* and infected with *V. campbellii* was not significantly different (two to 24 hr p.i.), with a range of 35.3 to 44.7 mg O_2 h^{-1} kg^{-1} w.w. ($p < 0.05$) (Fig. 1C).

There were no significant differences ($p < 0.05$) between the oxygen consumption rate in the pre-injection shrimps and p.i. measurements in organisms that were submerged in the hydrosoluble polysaccharides extract of *M. pyrifera* and infected with *V. campbellii* which had an oxygen consumption rate of 43.3 to 59.8 mg O_2 h^{-1} kg^{-1} w.w. (Fig. 1D).

Glucose concentration on the shrimps controls was 10 mg dL^{-1}. The adults injected with *V. campbellii* had the highest glucose concentration at two hours p.i. (32.7 mg dL^{-1}). Shrimps injected with the hydrosoluble polysaccharides extract of *M. pyrifera* and infected with *V. campbellii* showed a decrease in hemolymph glucose concentration at 12 hours p.i. (2.3 mg dL^{-1}), however, at 24 hours p.i. their glucose concentration returned to normal level (11.7 mg dL^{-1}). Adults exposed to immersion treatment with the hydrosoluble polysaccharides extract of *M. pyrifera* and infected with *V. campbellii* showed no significant differences in their hemolymph glucose concentration, compared to pre-injection control shrimps ($p < 0.05$) (Fig. 2).

Lactate concentration in the hemolymph of pre-injection *L. vannamei* was 11.4 mg dL^{-1}. Adults injected with saline solution and *V. campbellii* showed no significant differences ($p < 0.05$) over the pre-injection control, however, the organisms injected with the hydrosoluble polysaccharides extract of *M. pyrifera* and infected, had the lowest concentrations of lactate in the hemolymph ranging from 0.9 to 3.4 mg dL^{-1} at 24 hours p.i. Shrimps submerged in the hydrosoluble polysaccharides extract of *M. pyrifera* had an hemolymph lactate concentration similar to that of pre-injection shrimps. Lactate concentration decreased to 3.8 and 3.1 mg dL^{-1} at six and 12 h p.i., and later, at 24 hours p.i. the level returned to the normal concentration of 9.0 mg dL^{-1} (Fig. 3).

Total proteins concentration in the hemolymph of pre-injection adults was 115.3 mg mL^{-1}, shrimp injected with saline showed no significant differences ($p < 0.05$) compared to the pre-injection organisms, however, the shrimps infected with *V. campbellii* had the lowest values of total proteins, compared to pre-injection organisms, with a range of 57 to 86 mg mL^{-1} ($p > 0.05$) (Fig. 4).

Shrimps injected with the hydrosoluble polysaccharides extract of *M. pyrifera* and infected with *V. campbellii* had signifi-

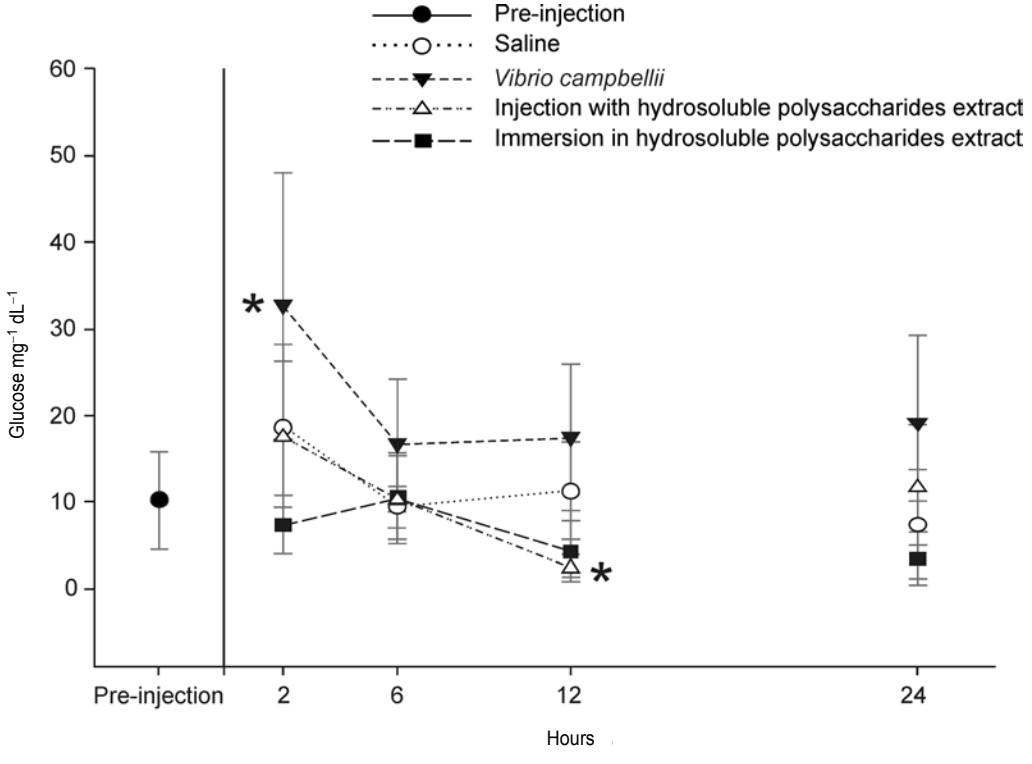

Figure 2. Glucose concentration in the hemolymph of *Litopenaeus vannamei*, each point represents the median glucose levels of 8 shrimps and its confidence intervals (95%), * significant differences ($p > 0.05$).

cantly higher values of total proteins in hemolymph at 24 hours (p.i. 184 mg mL^{-1}). Adults that were immersed in the extract and infected with *V. campbellii*, had values of total proteins in hemolymph significantly higher ($p > 0.05$) at six and 24 hours p.i. (167.4 and 168.3 mg mL^{-1}, respectively).

DISCUSSION

Shrimps injected only with *V. campbellii* increased their oxygen consumption rate during the first six hours p.i., and decreased their rate later on, Scholnick *et al.* (2006) reported a similar effect in *L. vannamei* also infected with *V. campbellii*. Burnett *et al.* (2006) studied the effect of the presence of bacteria and hemocytes in the gills of *Callinectes sapidus* (Rathbun) and proved that oxygen uptake decreased. Martin *et al.* (1993, 1998, 2000) reported that hemocytes bind to circulating particles in the hemolymph and form nodules which, due to normal mechanical processes, accumulate in the gills. These nodules may affect the ability of the gills to perform their function gas exchange and ion regulation function, causing hypoxia in the infected shrimp.

The formation of these nodules in the gills of shrimp infected with *V. campbellii*, was probably the cause of oxygen consumption rate had significant reduction at the time lapses (eight, 12 and 24 hours p.i.). The effect of the *M. pyrifera* extract in the injection and immersion treatments on shrimp challenged with *V. campbellii*, prevented the reduction in the oxygen consumption rate as the infection progressed over time, as opposed to the results in the control shrimps only injected with the bacteria.

Several authors mention that immunostimulation via immersion improves the immune response of shrimp (Itami *et al.*, 1998; Alabi *et al.*, 1999; Campa- Córdova *et al.*, 2005; Hou & Chen, 2005; Yeh *et al.*, 2006; Fu *et al.*, 2007) and by injection (Cheng *et al.*, 2004; Hou & Chen, 2005; Yeh *et al.*, 2006; Fu *et al.*, 2007), in the present study, both methods of immunostimulation were effective in fighting infection of *V. campbellii*, in both methods, the organisms resisted the bacterial infection and the presence of these microorganisms did not reduce the oxygen consumption rate in shrimps exposed to hydrosoluble polysaccharides extract via immersion and injection, as not nodules were formed.

Glucose concentration of pre-injection shrimps was similar to that reported by Racotta and Palacios (1998) in the hemolymph of *L. vannamei* (15 mg dL^{-1}), who also proved that hemolymph glucose increased in response to the stress of repeated hemolymph sampling. In this study, the control organisms injected only with *V. campbellii* showed the highest glucose concentration at two hours p.i. due to the stress caused by infection, the hydrosoluble

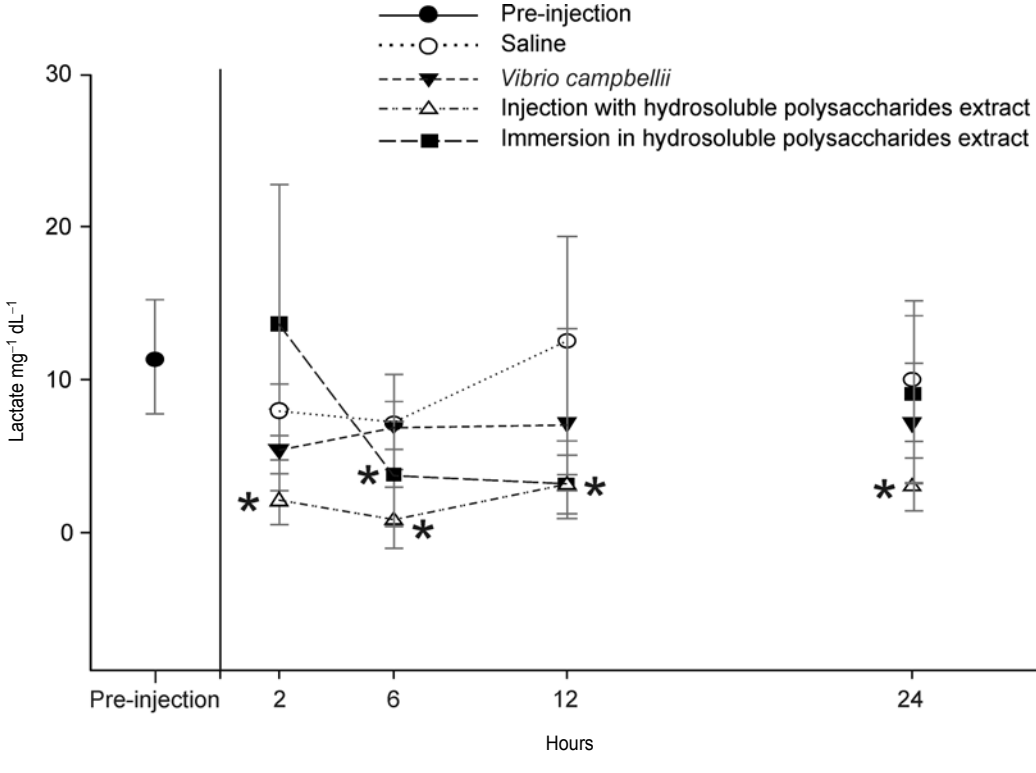

Figure 3. Lactate concentration in the hemolymph of *Litopenaeus vannamei*, each point represents the median lactate levels of 8 shrimps and its confidence intervals (95%), ∗ significant differences ($p > 0.05$).

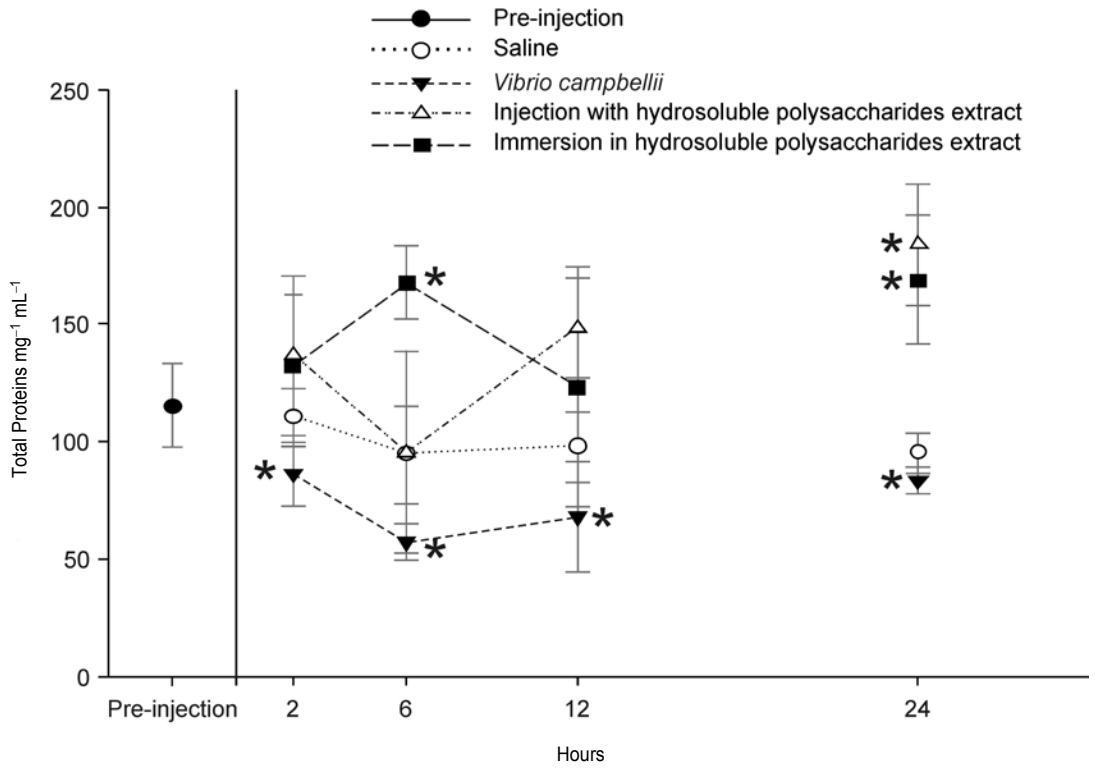

Figure 4. Total Proteins concentration in the hemolymph of *Litopenaeus vannamei*, each point represents the median protein levels of 8 shrimps and its confidence intervals (95%), ∗ significant differences ($p > 0.05$).

polysaccharides extract used in this study had a positive effect as none of the organisms of the experimental treatments presented abnormal values of glucose concentration, the only change recorded was at 12 hours p.i. in the injection of the extract treatment, however this effect did not persist, as at the following sampling of hemolymph glucose levels remained normal, indicating that all organisms were no stressed, except for the infected with *V. campbellii* control group at two hours p.i.

Regarding lactate levels in the hemolymph of shrimp, all organisms from treatments and controls remained below the baseline level, which coincided with the values reported by Racotta and Palacios (1998) of 11 mg dL^{-1}, these authors conclude that despite lactate is being used as an indicator of stress, their results suggest that the hemolymph glucose level, was more sensitive as an indicator of stress in *L. vannamei*, because lactate levels of juvenile *L. vannamei* were less affected by manipulation. The use of the hydrosoluble polysaccharides extract by both methods of immunostimulation in the present study, as evidence by the lactate levels, showed that none of the organisms under treatment were under stress, as to raise their glucose and subsequently lactate levels, upon entering the anaerobic cycle of energy production; Hsieh *et al.* (2008) reported that juveniles of the same species subjected to infection with *V. alginolyticus* increased their levels of glucose and lactate, which supports our hypothesis that the extract works to maintain those levels at normal values, even while the organisms were under the effects of the infection with *V. campbellii*.

The basal concentration of total proteins in the hemolymph of pre-injection shrimps was 117.7 mg mL^{-1}, similar values of 130, 127 and 103 mg mL^{-1} have been reported in the hemolymph of *L. vannamei* by Racotta and Palacios (1998), Rodriguez *et al.* (2000) and Pascual *et al.* (2003), respectively. The levels of protein in the hemolymph are affected by nutritional stress, rather than manipulation stress, suggesting that hemolymph protein levels may reflect changes in the shrimp's health status, including modification of the immune response (Pascual *et al.,* 2003).

Destomieux *et al.* (2001), Pascual *et al.* (2004) and Pérez *et al.* (2006) mentioned that the use of immunostimulants increased the protein levels because of the production of antimicrobial peptides, using hemocyanin as the precursor of these peptides. Crustacean hemocyanin can be processed by a cysteine-type protease to generate antimicrobial peptides under acidic conditions and its production can be induced by injection of lipopolysaccharides and glucans in the organisms (Lee *et al.*, 2003).

In the experiments in which we used the hydrosoluble polysaccharides immunostimulating extract of *M. pyrifera* via injection and immersion, shrimps had protein levels above the pre-injection group, the highest level of total proteins was observed at 24 hours

p.i. in both treatments, probably due to increased production of antimicrobial peptides from the hemocyanin to fight against infection. The organisms from the control group infected with *V. campbellii* showed the lowest levels of protein in all p.i. hemolymph sampling, thus suggesting that those shrimps had a lower amount of proteins involved in immune responses.

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