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Litopenaeus vannamei immunostimulated with *Macrocystis pyrifera* extract: improving the immune response against *Vibrio campbellii*

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ABSTRACT

Objective: To examine the immune responses of *Litopenaeus vannamei* after different treatments with a hot water extract of *Macrocystis pyrifera* (*M. pyrifera*) and a subsequent challenge with *Vibrio campbellii* (*V. campbellii*).

Methods: A total of 184 adult white shrimp that were infected with *V. campbellii* (1×10^6 CFU/shrimp) were immunostimulated by the hot-water extract from *M. pyrifera* via either injection (10 µg) or immersion (350 mg/L), the experimental controls were injected with either saline solution or *V. campbellii* (1×10^6 CFU/shrimp). The bacterial DNA depuration rate, antimicrobial activity and total hemocyte count were evaluated in hemolymph samples at 2, 6, 12, 24, 48 and 72 h post-infection.

Results: Injected shrimp (10 µg *M. pyrifera* extract) demonstrated the best clearance of bacterial infection, with 82% survival at 72 h post-infection (cellular response). Hemolymph from the immersed organisms had the best antimicrobial activity against *Escherichia coli* growth; specifically, the most efficient antimicrobial activity was observed at 24 h post-infection. Both types of immunostimulated shrimp had similar total hemocyte counts at 24 h post-infection (1.63–1.59 million/mL); however, after 72 h, injected shrimp had higher total hemocyte counts than immersed animals (2.59 *v.s.* 0.56 million/mL).

Conclusions: The injection of the *M. pyrifera* hot-water extract facilitated a more efficient response to *V. campbellii* infection due to the stimulation of the hemocytes of the shrimp. In other words, the cellular immune response was more efficient to eliminate bacterial infection than the humoral response in shrimp.

1. Introduction

Aquaculture is an extremely useful tool for economic and social growth, practiced in many countries, but the constant

increase in the demand for its products has resulted in more cultures that are intensive, therefore, aquatic organisms are kept in physiologically stressful environments, which directly affect their immune system, suppressing it, so they become more susceptible to infectious diseases^[1]. It is well known that, in shrimp cultures, bacterial infections constitute a very high risk factor, which many times have caused the total collapse of the production process,

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destroying its great profitability. Physical, chemical and biological variables are determining factors for disease outbreaks that affect shrimp survival and growth^[2].

Therefore, due to the fact that health of cultured organisms is difficult to maintain, bacterial infections are always a potential risk and the use of antibiotics is harmful, immune stimulation has been successfully employed for many years as the best available option for increasing the resistance of invertebrates, because their immune (defense) system lacks immunoglobulins, lymphoid cells or immunological memory, so it does not allow the use of vaccines^[3,4]. An immunostimulant is defined as a chemical, drug, stressor or action that enhances the immune response, thus the organisms become more resistant to infection diseases^[1].

The use of natural immunostimulants is very valuable, because of their biocompatibility, biodegradability, cost effectiveness and safety for the environment and human health^[5], and its use has been increasing rapidly in aquaculture to protect organisms against adverse conditions, avoiding the indiscriminate use of hazardous antibiotics^[1]. Invertebrate immunostimulants are extracted from five different sources: Gram-negative and Gram-positive bacterial cell walls, fungi, yeast and algae^[4,6,7].

The crustacean defense system lacks specificity and immunological memory, but it can be compared with the cellular and humoral components of the vertebrate immune system, which act in concert to eliminate potentially infectious microorganisms. In the crustacean model, the humoral fraction of the defense system is composed of hemolymph (analogous to both the blood and the lymph of vertebrates), in which various important molecules circulate, including hemocyanin, protease inhibitors, recognition proteins, and several lipoproteins, such as the clotting factor. The cellular fraction is composed of hemocytes (that are analogous to vertebrate white blood cells), which eliminate foreign particles and old cells through phagocytosis and are considered to be the most important cells in the crustacean defense system; in addition to phagocytosis, the hemocytes encapsulate and lyse foreign particles, participate in cicatrization through cellular aggregation, liberate the clotting factor, activate the prophenol oxidase enzymatic system and facilitate the synthesis of antimicrobial peptides^[8–10].

The total hemocyte count (THC) in the hemolymph is increased when the organism is subjected to environmental stress or infections (a similar process occurs in vertebrates with the cellular system), as well as during the molt cycle; THC is considered to be a useful parameter for evaluating

the effects of immunostimulants in crustaceans, including shrimps^[11,12].

White shrimp *Litopenaeus vannamei* (*L. vannamei*), naturally distributes along the Pacific coast, from the Sea of Cortés to Peru. Actually, white shrimp is the main cultured crustacean over the world, but in Latin America due to disease caused by virus and bacteria, today is in second place, even when the most valuable species^[13].

Between 80% and 90% of the bacteria isolated from the shrimp farms that report massive deaths are from the genus *Vibrio*, and the most common species found in culture are *Vibrio campbellii* (*V. campbellii*), *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio alginolyticus*^[14,15].

In the present study, we used the brown seaweed *Macrocystis pyrifera* (*M. pyrifera*), commonly known as “kelp”, as a source of immunostimulants. This macroalga grows in temperate or cold rocky shores from California (USA) to Baja California Sur (México), with an estimated biomass crop that reaches 99 626 tons in summer^[16].

PCR is a new, popular molecular biology technique for enzymatically replicating DNA without using a living organism; it allows a small amount of the DNA molecule to be amplified many times in an exponential manner, making its analysis much easier. It is commonly used in medical and biological labs and the most widely accepted technique, for diagnostic modalities with very high specificity and sensitivity for detection of hereditary diseases, the identification of genetic fingerprints, the diagnosis of infectious diseases, the cloning of genes and parent testing^[17].

The objective of this study was to examine the cellular and humoral immune responses of *L. vannamei* after different treatments with the hot water extract of *M. pyrifera* and a subsequent challenge with *V. campbellii*.

2. Materials and methods

Healthy adult organisms of *L. vannamei* with a wet weight of between 23 and 32 g were obtained from a hatchery in Nayarit, México, and kept for 2 months in 2 000 L tanks with constant aeration and the continuous replacement of seawater (35‰). The tanks were maintained at a temperature of (26±1) °C, which is the preferred temperature for these organisms^[18], and the organisms were provided with food to satiation (Rangen feed with 40% protein).

Algal leaves were collected from the “La Misión” coast in Baja California, México, during the summer of 2007, and a hot water extract was prepared based on the method previously described^[19].

V. campbellii (CICESE 559; CAIM 416) was isolated from the seawater of a shrimp broodstock tank from a hatchery in Sonora, México; its peak pathogenicity to crustaceans has been reported^[20]. *V. campbellii* was cultured on Zobell medium for 18 h at 28 °C, then harvested and re-suspended in saline solution (9 mg/L) at 1×10^7 CFU/mL; this bacterial suspension was used for all of the infections.

For the injection test, 60 shrimp were injected in the third abdominal segment with the hot water extract of *M. pyrifera* at 10 µg/g and were infected 24 h later with 100 µL of the bacterial suspension (1×10^7 CFU/mL), resulting in a final concentration of 1×10^6 CFU/shrimp.

For the immersion test, 60 shrimp were immersed in two aquariums containing seawater and 350 mg/L of the hot water extract of *M. pyrifera*; 3 h later, the animals were injected in the third abdominal segment with 100 µL of the bacterial suspension (final concentration, 1×10^6 CFU/shrimp).

The pre-injection control group was composed of a total of 30 animals that did not receive an injection either of the hot water extract or *V. campbellii*. The saline control group was composed of 60 animals that were injected with 100 µL of saline solution (9 mg/L), and the *V. campbellii* control group was composed of 60 animals that were injected with 100 µL of the bacterial suspension (final concentration of 1×10^6 CFU/shrimp).

The hemolymph from eight shrimp were individually sampled with a pre-chilled syringe inserted at the base of the fifth pereopod. Samples were taken at 2, 6, 12, 24, 48 and 72 h post-infection. SIC-EDTA was used as an anticoagulant in a 2:1 ratio (400 µL of anticoagulant: 200 µL of hemolymph). Some authors have asserted that the use of this anticoagulant solution prevents the lysis, degranulation and clotting of hemocytes^[21].

To perform a THC, 10 µL of hemolymph from each of the experimental and control groups was placed in a Neubauer chamber, and hemocytes were counted under a microscope (10×).

To extract *V. campbellii* DNA from the hemolymph by the thermal shock technique, each sample was centrifuged at 12 000 r/min for 15 min, after which the supernatant was discarded and the resultant cellular pellet was re-suspended in 100 µL of sterile distilled water; subsequently, the samples were subjected to 5 cycles of thermal shock at 80 °C and –80 °C. The samples were then centrifuged once

again at 12 000 r/min for 15 min, and the DNA was recovered from the resulting supernatant.

Forward and reverse primers for PCR were designed with MacVector 7.2.2 based on the nucleotide sequences of a ribonucleoside–diphosphate reductase protein from the genome of *V. campbellii*, resulting in the next pair of oligonucleotids:

5′–TCG GCG AAA TCT GGC ACA AC–3′

5′–GCG TGA AAT ACT CTG ACC GCA G–3′

The DNA of *V. campbellii* was amplified by PCR in a 25 µL reaction mixture (GoTaq, Promega) containing 100 ng of chromosomal DNA. The amplifications were performed in a PerkinElmer thermocycler, using 30 cycles with an annealing temperature of 55 °C; each cycle consisted of 30 seconds at 94 °C, 30 seconds at 55 °C and 30 seconds at 72 °C. The amplified products (400 bp) were electrophoresed in 2% agarose gels to assess the depuration rate of the bacterial DNA.

Dilutions of the *V. campbellii* suspension were prepared with saline solution (10^7 to 10^1); these samples were plated on TCBS in duplicate and incubated for 24 h at 28 °C for posterior counting. Following the previously described thermal shock, DNA was extracted from each dilution, amplified and analyzed in a 2% agarose gel.

The antimicrobial activity of the hemolymph samples was tested using *Escherichia coli* (*E. coli*) (1×10^7 CFU/mL). First, the minimum inhibitory concentration of *E. coli* with carbenicillin was standardized; once this value was determined, tests with different hemolymph protein concentrations. Using the samples from each experimental condition to determine the standard protein concentration (1.25 mg/mL) and then, the total protein concentration of each sample was adjusted to the desired standard concentration with phosphate buffered saline (PBS 1×). The quantification of the protein was performed using a biuret reagent kit (Pointe Scientific Inc., Canton MI, USA). Finally, the antimicrobial activity tests, which used 200 µL of *E. coli* in LB medium and 10 µL of each sample in duplicate, were performed in a 96-well microplate. LB medium, anticoagulant solution and PBS 1× were used as negative controls, and carbenicillin (1 mg/mL) was used as a positive control. All of the microplates were incubated at 37 °C for 18 h; subsequently, the O.D. of the microplates was measured at 655 nm.

The Sigma Stat software package was used to conduct a Kruskal–Wallis test to determine the significant differences in the THC and antimicrobial activity of the hemolymph among the treatments. A statistical significance level of $P < 0.05$ was employed in this study.

3. Results

The THC of the pre-injection control group (498 750 cells/mL (was significantly lower than the THC of the saline control group at 2, 6 and 12 h) post injection (1 092 000; 1 140 000 and 1 305 000 cells/mL, respectively). The THC of the *V. campbellii* control group was significantly higher than that of the pre-injection group at 6 and 48 h post-infection (1 983 750 and 1 616 250 cells/mL, respectively). Shrimp injected with a hot water extract of *M. pyrifer*a exhibited significantly higher THCs than the pre-injection shrimp throughout the 72 experimental h (1 627 500 *v.s.* 2 752 500 cells/mL). The THC of *L. vannamei* submerged in a hot water extract of *M. pyrifer*a was also significantly higher than the THC of the pre-injection group throughout the experiment (566 250 *v.s.* 3 228 750 cells/mL) (Figure 1).

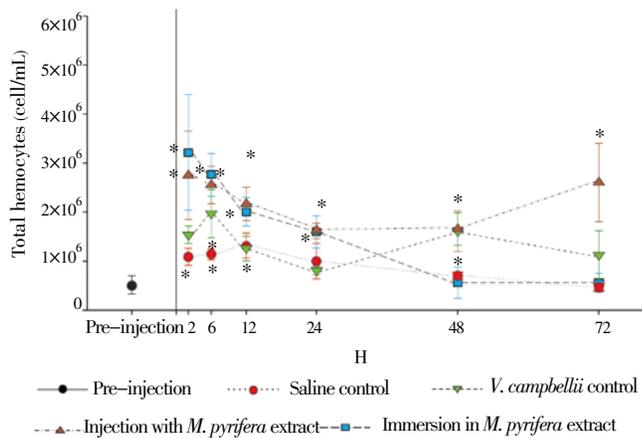


Figure 1. The THCs (cell/mL) of control and experimental organisms. * Indicates significant differences between the pre-injection control and the samples (*P* < 0.05).

Different concentrations of extracted bacterial DNA were detected in 2% agarose gels (Figure 2); the first undiluted sample (10⁷) contained 8 × 10⁷ CFU/mL of *V. campbellii*, whereas the minimum concentration of *V. campbellii* detected in the agarose gels was 14 CFU/mL (for the 10¹ sample).

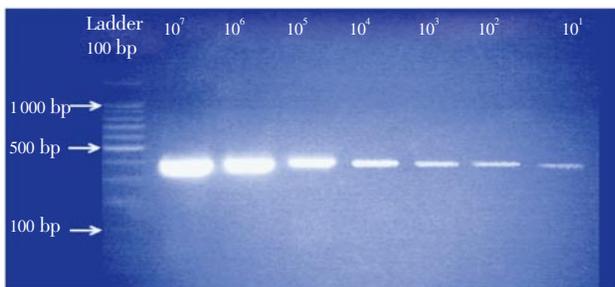


Figure 2. Detection of different *V. campbellii* DNA concentrations amplified through PCR. Bacterial concentrations range from 8 × 10⁷ CFU/mL to 14 CFU/mL.

The bacterial DNA depuration rate of the *V. campbellii* control group indicates that the hemocytes were inefficient

to eliminate the bacteria, as only 50% of the infected shrimp survived at 72 h post-infection. *V. campbellii* DNA was not detected in the agarose gel (Figure 3).

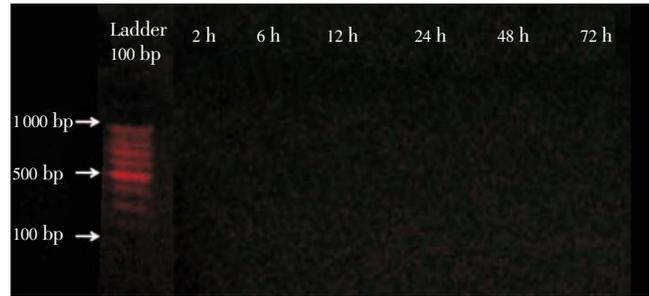


Figure 3. Bacterial DNA depuration rate of the *V. campbellii* control group, as detected by PCR.

The hemocytes of the immersed shrimp were also inefficient to eliminate *V. campbellii*, given that a very small amount of bacterial DNA was detected in the agarose gel. Only 17% of the immersed shrimp survived at 72 h post-infection (Figure 4).

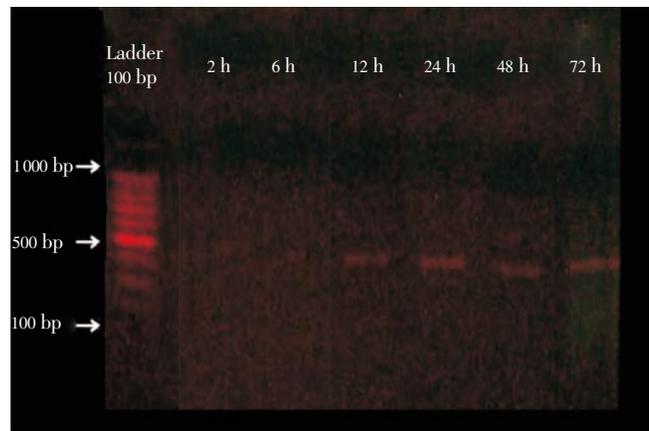


Figure 4. Bacterial DNA depuration rate of shrimp immersed in a *M. pyrifer*a hot water extract (350 mg/L), as detected by PCR.

The hemocytes of the shrimp injected with a hot water extract of *M. pyrifer*a were the most efficient to eliminate bacteria from the hemolymph. There was more bacterial DNA detected in the agarose gel of samples from these injected shrimp, and 82% of the shrimp survived at 72 h post-infection (Figure 5).

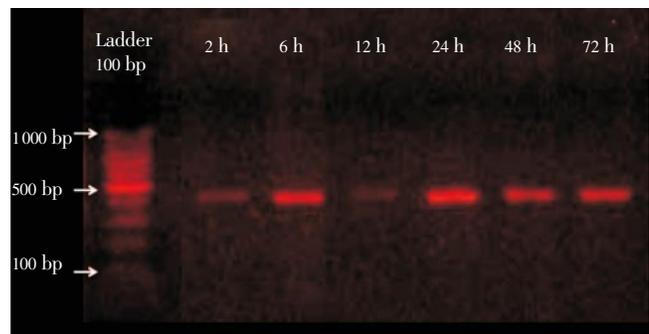


Figure 5. Bacterial DNA depuration rate of shrimp injected with a *M. pyrifer*a hot water extract (10 µg/g), as detected by PCR.

The hemolymph from the immersed organisms had the best antimicrobial activity against *E. coli* growth, specifically at 24 h post-infection (Figure 6). This treatment was, in general, statistically the most effective of the examined methods for decreasing *E. coli* growth.

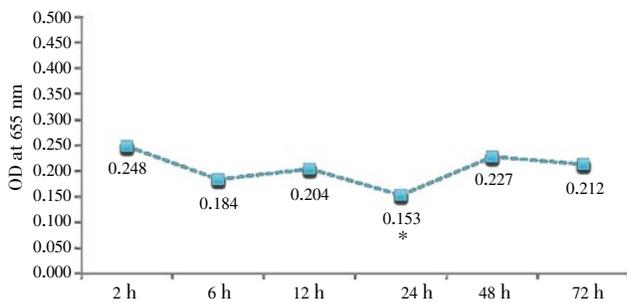


Figure 6. Antimicrobial activity against *E. coli* growth of the hemolymph, from the shrimp immersed in the *M. pyrifera* hot water extract (350 mg/L).

The total proteins in the hemolymph of each sample were adjusted to equal values (1.25 mg/mL), and the best antimicrobial activity was detected in the 24 h post-infection samples. * Indicates significant differences between samples at different hours ($P < 0.05$).

4. Discussion

The use of natural products extracted from certain organisms as strong stimulants of the immune systems of diverse species (earthworms, shrimp, fish, mice, rats, rabbits, guinea pigs, sheep, pigs, cattle and even humans) is well known[22].

Recent research has pointed to new uses of the bioactive molecules extracted from green, brown and red seaweed like: cytostatic, antiviral, antihelminthic, antifungal and antibacterial activities, since they are rich in vitamins, minerals, proteins, steroids, dietary fibers and because they synthesize various compounds such as carotenoids, terpenoids, xanthophylls, chlorophyll, fatty acids, amino acids, acetogenins, antioxidants and many polysaccharides which have been used to immunostimulation purposes such as agar, carrageenan, proteoglycans, alginates, laminaran and fucoidan. With their results, they concluded that the polysaccharides play an important role in the defense against pathogens and mentioned that seaweeds are an interesting source for biologically active compounds that can be used for prevention and therapy of bacterial diseases and recommend their use for immunostimulation in aquaculture[23,24].

In the present study, we noticed a slight increase in the THC in the saline control at 2, 6 and 12 h post injection, which can be due to injury caused by the needle at the injection site, and as well, the THC of the white shrimp significantly

increased after the administration of a *M. pyrifera* extract, via either injection or immersion. Diverse authors report an increase in the THC after using immunostimulants in penaeid shrimps[19,25–31].

After displaying a significant increase in THC during the first 24 h post-infection, the immersed shrimp decreased their THC to pre-injection levels at 48 h post-infection; this effect persisted until 72 h post-infection. The THC of the shrimp injected with the *M. pyrifera* extract returned to the original immunostimulated THC at 72 h post-infection; this result is consistent with the similar patterns that have been noticed by other authors in penaeid shrimps[32–34]. This THC decrease could be induced by the recruitment of the circulating stimulated hemocytes to the hematopoietic tissue, which can generate mature hemocytes[35].

Some studies regarding the effect of immunostimulants in white shrimp infected with *Vibrio*, showed that water extracts can maintain physiologic and metabolic homeostasis[36] and enhance immunity against the *Vibrio* infection[37].

The results of the depuration rate, obtained using PCR and assessments of the survival of the organisms at 72 h post-infection, indicate that the phagocytic activity of the hemocytes of the shrimp injected with the *M. pyrifera* hot water extract was the most effective against *V. campbellii*. This result indicates that we can detect bacterial DNA in hemocytes at 6 h post-infection. In addition, we have found a high number of surviving shrimp from this group (82% of the infected shrimp survived). This combined information indicates that the amplified bacterial DNA corresponds to dead bacteria that had already been phagocytized by hemocytes at the time of hemolymph extraction.

We could not detect phagocytized bacterial DNA in the infected control group, and we detected a small quantity of phagocytized bacterial DNA at 12 h post-infection in the immersed group. The survival rates of these two groups of shrimp were less than 50%.

In the present study, the depuration rate could more practically be determined through the detection of bacterial DNA, than through the culture of viable bacteria from hemolymph samples of infected shrimp, a metric that has been used to quantify clearance efficiency[19,29,30]. In this work, we indirectly detect the number of bacteria phagocytosed by activated hemocytes. We were able to detect a wide range of bacterial concentrations, from as low as 1.4 bacteria/ μ L to 8 million bacteria/ μ L.

To date, few studies have used techniques similar to

those of this study, to analyze the depuration rate through the amplification of DNA. Lin, *et al.* used real-time PCR to amplify WSSV DNA in the Japanese shrimp *Marsupenaeus japonicus*, and Pathak, *et al.* used the same amplification technique to quantify mycobacterial DNA in infected human macrophage cultures and in mouse tissues^[38,39]. These previous studies concluded that PCR methods offer advantages in biosafety, time-saving, assay range and reproducibility relative to colony counting procedures.

The present study is the first report in which a bacterial DNA depuration rate was determined through PCR in shrimp, and the study results demonstrated that this technique was a reliable method of detecting whether immunostimulants are sufficiently efficient to clear or eliminate a bacterial infection.

Immunostimulants can be administered in aquatic organisms by injection, immersion or orally, and although the administration through injection can be considered a laborious process, it allows the immunostimulant to be quickly absorbed and functional^[40]. The immunostimulant effect of the hot water extract of *M. pyrifera* that was utilized in this study was very efficient in increasing the resistance of *L. vannamei* infected with *V. campbellii*, especially when the extract was administered via injection.

The same antimicrobial activity technique used in the present study, was previously applied by Lee *et al.*^[41] to analyze the effect of a peptide (astacidin 1) from the freshwater crayfish *Pacifastacus leniusculus* on the inhibition of the growth of nine different bacteria (including *E. coli*). This previous investigation determined that the release of astacidin 1 was enhanced when crayfish were injected with either lipopolysaccharide or glucan. Other authors reported that the methanol extracts of *Sargassum vulgare* did not show antibacterial activity against *E. coli* growth, but in contrast, Thirunavukkarasu *et al.* screened the antibacterial activity of nine marine seaweeds against *V. alginolyticus* and found that the methanolic extract of *Sargassum wightii* produced the maximum zone of inhibition^[42,24].

Regarding to the antimicrobial activity assays of the present study, the results of the hemolymph's antimicrobial activity from the shrimp immunostimulated via immersion, demonstrated the strongest growth inhibition of the *E. coli* bacteria, suggesting that the hemocytes of these shrimp degranulate more efficiently than the hemocytes of the injected shrimp. This result could explain the low THC in this group after 24 h post-infection; due to that efficient degranulation, many hemocytes may have been lysed in the

organisms of this group. This assertion that degranulation may be followed by cell lysis, which causes a number of hemocytes to be lost during degranulation, is in accordance with results that were previously reported by Van de Braak^[35]. Generally, our antimicrobial activity results indicate that the hemolymph of shrimp immunostimulated via immersion was more efficient at inhibiting *E. coli* growth; however, the survival results indicate that the phagocytic capacity of the hemocytes (the cellular immune response) was more important for the shrimp, given that only 17% of the shrimps immersed in the *M. pyrifera* hot water extract survived. Alternatively, a lower dose of immunostimulant could reduce the high mortality of immersed shrimps, due to the high energy cost invested in producing the effectors of the immune response.

A possible explanation for the assumption that the hemocytes of shrimp immunostimulated via injection were more efficient at eliminating *V. campbellii* than the hemocytes of the immersed shrimp, could be that the shrimp injected with the extract were given 24 h to mature their hemocytes, prior to bacterial infection. By contrast, the immersed shrimp were given only 3 h for hemocyte maturation. In other study, immersed white shrimps (during 6 h), were given 10 d to process 4 distinct immunostimulant solutions, and then were infected with *Vibrio penaeicida* to evaluate the differences in the antioxidant activity of their cells. Remarkable results were achieved from the first sampling cycle, 48 h after the immersion, but the most effective antioxidant response was obtained in the second sampling cycle 12 d later, at 48 h, after the bacterial infection^[27].

In this work, we demonstrate that the hot water extract of *M. pyrifera* can be used for the immunostimulation of adult white shrimp via injection and immersion, and we conclude that, in terms of survival, the shrimp immunostimulated via injection, resist bacterial infection more efficiently due to the higher phagocytic capacity of their hemocytes, that resulted from stimulation by the hot water extract. Shrimp hemocytes (the cellular response), can depurate the bacterial infection by means of phagocytosis 6 h post-infection if they are appropriately stimulated. By contrast, the antimicrobial activity efficiency (the humoral response), which occurs by means of peptides or different soluble compounds in the hemolymph, demonstrated the best activity 24 h post-infection. In other words, the cellular immune response was more efficient to eliminate bacterial infection than the humoral response in adult white shrimps.

Conflict of interest statement

We declare that we have no conflict of interest.

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