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Research Article

Immune-related gene expression in *Penaeus vannamei* fed *Aloe vera*

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ABSTRACT. The immune response is critical in cultured shrimp facing pathogens that are serious concerns. Therefore, immune-related gene expression was investigated in *Penaeus vannamei* fed with *Aloe vera*. Shrimp weighing 2.9 ± 0.8 g were fed with 1 g of *A. vera* kg feed^{-1}. Six shrimp were sampled at 0, 6, 12, 24, 48, and 72 h for gene expression. Samples of time zero were taken just before feeding with aloe. After feeding with aloe during 24 h, uneaten food and waste material were removed and then shrimp were fed only with commercial feed. Quantitative real-time PCR measured time series expression of four genes (translationally controlled tumor protein [TCTP], superoxide dismutase [SOD], heat shock protein 70 [HSP70], and penaeidin4). The expression of TCTP was down-regulated, and the expression of SOD, HSP70, and penaeidin4 was up-regulated. This is the first report showing the effect of *A. vera* on the modulation of shrimp immune response. The immunostimulant properties of *A. vera* could be useful for cultured shrimp health.

Keywords: *Penaeus vannamei*, shrimp, *Aloe vera*, gene expression, immune response, aquaculture.

INTRODUCTION

Worldwide shrimp production has decreased mainly due to viral and bacterial diseases such as acute hepatopancreatic necrotizing disease (AHPND) and white spot disease (WSD) (Tran *et al.*, 2013; Nunan *et al.*, 2014; Joseph *et al.*, 2015). Therefore, in the last years, the use of natural products such as probiotics, prebiotics, immunostimulants, and medicinal plants have become relevant to reduce mortalities (Peraza-Gómez *et al.*, 2009, 2014; Huynh *et al.*, 2011; Medina-Beltrán *et al.*, 2012; Akhter *et al.*, 2015). Natural products have potential as immunostimulants to prevent and cure diseases (Shukla *et al.*, 2014). Crustaceans do not have a specific immune system with memory capacity (Barraco *et al.*, 2008). Therefore, the innate immune response is based on reactive cells (hemocytes) and humoral molecular effectors. Hemocytes participate in phagocytosis, cytotoxicity, adhesion, cellular communication, recognition, encapsulation, and nodule formation (Chisholm & Smith, 1995; Johansson *et al.*, 2000; Jiravanichpaisal *et al.*, 2006; Lin *et al.*, 2006; Yeh *et al.*, 2009; Sivakamavalli *et al.*, 2014). Humoral effectors include the prophenoloxidase cascade, clotting mechanism, lectins, lysosomal hydrolytic enzymes, lysozymes, antimicrobial peptides, and respiratory burst (Destoumieux *et al.*, 2000; Campa-Córdova *et al.*, 2005; Jiravanichpaisal *et al.*, 2006; Cerenius *et al.*, 2008).

Several plants have been found to have immunostimulant properties to increase resistance in shrimp against microbial diseases, such as *Cardiospermum halicacubum* in *Penaeus monodon* (Rajasekar *et al.*, 2011) and *Magnifera indica* in *Penaeus indicus* (Harikrishnan *et al.*, 2015). Among medicinal plants, *Aloe vera* has active constituents, including vitamins, minerals, polysaccharides (pectins, cellulose, hemicellulose, glucomannan, acemannan and mannose derivatives), amino acids, anthraquinones, enzymes, lignin, saponins, salicylic acids, carotenoids, steroids, terpenes, and phytosterols (Dagne *et al.*, 2000; Boudreau & Beland, 2006). Glucomannan and acemannan have immunostimulant, antiviral, and anti-

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bacterial properties (Waihenya *et al.*, 2002; Ferro *et al.*, 2003; Mahdavi *et al.*, 2013; Mojtaba & Esmail, 2013; Dotta *et al.*, 2014). Also, the anthraquinone, aloe-emodin, prevents virus adsorption and replication into the cell (Reynolds & Dweck, 1999; Hu *et al.*, 2003; Li *et al.*, 2014). The effect of plants on shrimp immune system can be analyzed using the expressions of immune-related genes as potential markers (Liu *et al.*, 2006) since the mRNA provides information about the potential synthesis of a particular gene product (Sánchez-Paz *et al.*, 2003).

In *Penaeus vannamei*, high survival was observed in animals fed *A. vera* and challenged with *Vibrio parahaemolyticus*, the causative agent of AHPND (Trejo-Flores *et al*., 2016). Therefore, the present study aimed to evaluate the effect of *A. vera*, added to the diet, on the expression of some immune-related genes in *P. vannamei*.

MATERIALS AND METHODS

Experimental animals

Shrimps were obtained from Proveedora de Larvas, S.A. de C.V. (Sinaloa, Mexico) and cultured in 1000 L plastic tanks with 500 L of filtered (salinity of 30) seawater and continuous aeration. Tanks were located in an outdoor culture area covered with a shadow mesh. Shrimps were fed twice daily at 09:00 and 17:00 h with commercial feed (Purina®, 35% protein) according to weight tables. Uneaten food and waste material were removed daily by siphoning. Shrimps were analyzed by PCR to verify that they were WSSV and IHHNV-free.

Shrimp acclimation to culture conditions

Shrimps were maintained for 3 days in 50-L glass aquaria containing 46 L of filtered (20 μm) seawater and under continuous aeration. Shrimps were fed as above. Uneaten food and waste material were removed daily by siphoning.

Preparation of the experimental diet with powdered *Aloe vera*

Whole leaves were sun-dried and ground in a Waring® blender. The powder obtained was then minced in a hammer mill (Thomas Scientific 3383-L60, GE Motors & Industrial Systems, USA) to obtain a fine powder. At the same time, commercial feed (Purina®, 35% protein) was pulverized in a coffee grinder.

A homogeneous paste with commercial feed, *A. vera* powder, grenetin, and distilled water was prepared. The concentration of the plant in the feed was based on the work of Trejo-Flores *et al*. (2016). The paste was prepared by adding 40 g of gelatin and 410 mL of distilled water to each kilogram of feed. Subsequently, the paste was pelletized in a meat grinder (Torrey®, model M-22 R, Monterrey, Nuevo León, Mexico) and then dried at room temperature with a fan for 24 h. The pelleted feed was stored at -20°C.

Bioassay (time series for gene expression)

A bioassay was conducted for 72 h in 50 L glass tanks with 50 shrimp (2.9 ± 0.8 g) and constant aeration. Six shrimps were removed at 0, 6, 12, 24, 48 and 72 h (Wang *et al.*, 2008). Samples of time zero (control) were removed just before feeding with aloe (1 g kg-feed^{-1}). After feeding with aloe during 24 h, uneaten food and waste material were removed by siphoning and then shrimp were fed only with the commercial feed. Hemolymph and hepatopancreas were sampled from each shrimp for the analysis of immune-related genes and fixed until their analyses. Shrimps were WSSV-free. Temperature ranged from 28.4 ± 1.2 to 28.9 ± 2.7°C; salinity was between 30.5 ± 1.4 and 30.9 ± 1.1, dissolved oxygen ranged from 5.6 ± 0.5 to 5.2 ± 0.3 mg mL^{-1}, and pH was between 8.0 ± 0.4 and 8.1 ± 0.3.

Hemolymph and hepatopancreas extraction

Hemolymph (200 μL) was withdrawn from the pleopod base of the first abdominal segment with a sterile 1 mL syringe (27 G×13 mm needle) preloaded with 400 μL of a cooled anticoagulant solution (450 mM NaCl, 10 mM KCl, 10 mM Hepes, and 10 mM EDTA Na_2, pH 7.3) (Vargas-Albores *et al*., 1993). Hemolymph was centrifuged at 800 *g* for 15 min at 4°C. The plasma was discarded, and the cell pellet was rinsed with 250 μL of cold anticoagulant by centrifuging as above. The supernatant was removed, and the hemocytes were suspended in 250 μL of cooled Trizol Reagent® (Invitrogen, Carlsbad, CA, USA) and stored at -70°C until use. A hepatopancreas sample was placed in cold RNAlater (Invitrogen®).

Total RNA isolation and cDNA synthesis

Total RNA was extracted from hemocytes and hepatopancreas with Trizol Reagent according to manufacturer's instructions. The concentration and purity of RNA were checked in a nanophotometer (Implen, Inc., Westlake Village, CA, USA). The RNA was treated with DNAse I (1 U μL^{-1}, Sigma-Aldrich®, St. Louis, MO, USA). First-strand cDNA synthesis was performed from 500 ng of total RNA using reverse transcriptase (Improm II, Promega®, Madison, WI, USA) with the oligo dT20. The cDNA was diluted with 80 μL of ultrapure water and stored at -70°C until analysis. Five microliters of this cDNA dilution were used as template in each qRT-PCR reaction.

Expression analysis of immune-related genes by qPCR

The expression of immune-related genes in hemocytes (translationally controlled tumor protein [TCTP, GenBank EU305625], cytosolic manganese superoxide dismutase [cMnSOD, GenBank DQ298207.1], and penaeidin4 [GenBank DQ211701]) and hepatopancreas (heat shock protein 70 [HSP70, GenBank JQ736788]) was determined by quantitative real-time PCR using a CFX96 system and the CFX Manager version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA) (Table 1). Penaeidin4, TCTP, and SOD were determined in hemocytes because these molecules are crucial in the immune function of hemocytes against the pathogen. Hepatopancreas has been previously reported to be a sensitive tissue in crustaceans (Bhavan & Geraldine, 2001). Therefore, the impact of *A. vera* on the digestive gland during digestion was analyzed through the expression of HSP70 since this gene plays an important role in stress and the defense against microbial infections in shrimp (Rungrassamee *et al*., 2010).

To find the best normalization factor to evaluate the expression of target genes (Table 1), a stability analysis of four reference genes (40S-S24 [GenBank unpublished], β-actin [GenBank AF300705], EF1α [GenBank # GU136230], and ubiquitin [GenBank KJ831562]) was done with two algorithms: GeNorm (Vandesompele *et al*., 2002) and NormFinder (Andersen *et al*., 2004), using the RefFinder web application [http://150.216.56.64/referencegene.php]. Hence, hemocyte gene expression was normalized to the geometric mean of 40S-S24, β-actin, and ubiquitin expression. Hepatopancreas gene expression was normalized with β-actin, EF1α, and ubiquitin.

A qPCR master mix (2X) (1.5 µL of reaction buffer 10X, 0.75 µL of 50 mM $MgCl_2$, 0.3 µL of 10 mM dNTPs, 0.75 µL of EvaGreen® 20x [Biotium, Hayward, CA, USA]; 0.1 µL of 5 U μL^{-1} Biolase DNA Polymerase [Bioline, Taunton, MA, USA], and 4.1 µL of ultrapure water) was prepared for all reactions of the experiment, separated in aliquots (reactions per plate), and stored at -20°C until use. Amplification was performed in duplicate in a 96-well plate in a 15 µL reaction volume containing 7.5 µL of PCR Master Mix 2X, 0.35 µL of each primer (10 µM, Sigma-Aldrich®), 1.8 µL of ultrapure water, and 5 µL of cDNA. Amplification conditions were as follows: 95°C for 3 min followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, 72°C for 30 s, and 79°C for 5 s (to acquire fluorescence). After each reaction, a dissociation curve from 65 to 90°C was recorded at increments of 0.5°C and examined for unique and specific products.

For each gene, efficiency (E) of the PCR reaction was determined by calculating a slope with five serial dilutions (dilution factor of 5 or 10) of a representative pool of cDNA [E = 10(-1/slope)-1]. The efficiency of the genes was between 1.91 and 1.98. To calculate expression of target genes, Cq values were transformed to relative quantities (RQ) using the equation $RQij = E^{[(Cq\ mean)-Cq(ij)]}$, where E is the gene-specific efficiency in decimal plus one, and [(Cq mean – Cq(ij)] is the absolute difference for each Cq sample against the mean Cq in the dataset for each gene. Relative expression of each gene was calculated with the equation RQ^{target}/geometric mean of $RQ^{reference\ genes}$ (Vandesompele *et al*., 2002).

Statistical analysis

One-way variance analysis (ANOVA) was applied to examine the differences in the expression of immune-related genes among treatments. Where significant ANOVA differences were found, a Tukey's HSD test was used with $P < 0.05$ as the criterion for significance.

RESULTS

Bioassay (shrimp immune response to *Aloe vera*)

To determine the transcriptional response of shrimp to *A. vera* added to feed, we evaluated mRNA expression of four immune-related genes in a serial course of time. The TCTP, cMnSOD, penaeidin4, and HSP70 gene expressions were modulated revealing the influence of *A. vera*.

Dietary *A. vera* significantly down-regulated the expression of TCTP in hemocytes. Conversely, the mRNA expression of cMnSOD and penaeidin4 genes was significantly up-regulated. The mRNA expression of TCTP gene was significantly down-regulated ($P < 0.05$) at 12, 24, 48 and 72 h after feeding with *A. vera* (Fig. 1). The mRNA expression of the cMnSOD gene was significantly up-regulated ($P < 0.05$) at 24, 48 and 72 h, but the reduction of its expression to the control level (0 h) was not observed in the time studied (Fig. 2). The mRNA expression of penaeidin4 was significantly up-regulated ($P < 0.05$) at 6 h and 12 h and the reduction of its expression to the control level (0 h) was observed from 24 h on (Fig. 3).

The dietary treatment significantly ($P < 0.05$) up-regulated the expression of HSP70 in the hepatopancreas at 48 h and 72 h, but the reduction of its expression to the control level (0 h) was not observed in the time studied (Fig. 4).

DISCUSSION

In the last years, medicinal plants have been used for the control and prevention of diseases of aquatic organisms (Citarasu *et al.,* 2006; Huynh *et al.,* 2011;

Table 1. Specific primers used for qPCR amplification of housekeeping and immune-related genes of *P. vannamei*.

Genes	Primers	Sequence (5´-3´)	References
Immune-related			
SOD	SOD-F	ATCCACCACACAAAGCATCA	Wang *et al.* (2010)
	SOD-R	AGCTCTCGTCAATGGCTTGT	
TCTP	TCTP-F	CAATGGACCCTGATGGC	Wu *et al.* (2013)
	TCTP-R	GCTTCTCCTCTGTTAGACCGTAT	
Penaeidin4	Pen4-F	GCCCGTTACCCAAACCATC	Wang *et al.* (2010)
	Pen4-R	CCGTATCTGAAGCAGCAAAGTC	
HSP70	hsp70 F	GGCAAGGAGCTGAACAAGTC	Flores-Miranda *et al.* (2014)
	hsp70 R	TCTCGATACCCAGGGACAAG	
Housekeeping			
40S-S24	Lv40S_S24-F	CAGGCCGATCAACTGTCC	Álvarez-Ruiz *et al.* (2015)
	Lv40S_S24-R	CAATGAGAGCTTGCCTTTCC	
Ubiquitin	Ubi-F	GGGAAGACCATCACCCTTG	
	Ubi-R	TCAGACAGAGTGCGACCATC	
EF1α	LvEf-F	CTGTGGTCTGGTTGGTGTTG	
	LvEf-R	TCAGATGGGTTCTTGGGTTC	
β-actin	Actin-F	CCACGAGACCACCTACAAC	Wang *et al.* (2008)
	Actin-R	AGCGAGGGCAGTGATTTC	

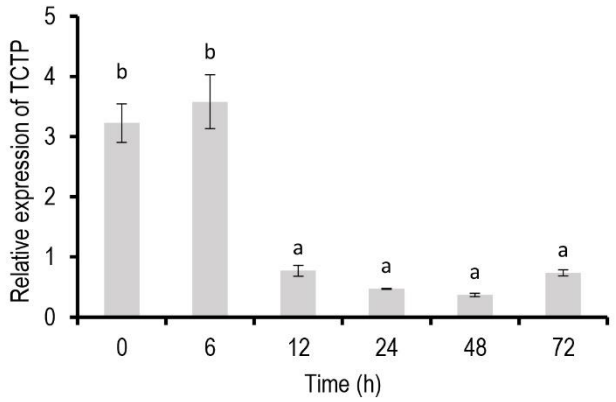

Figure 1. Relative expression of TCTP gene in hemocytes of *P. vannamei* fed with *A. vera*. Sampling (n = 6) times at 0, 6, 12, 24, 48 and 72 h. Relative expression was calculated with the equation RQ^{target}/geometric mean of $RQ^{reference\ genes}$. Reference genes: 40S-S24, β-actin, and ubiquitin. Results are mean ± SE. Different letters indicate significant differences ($P < 0.05$).

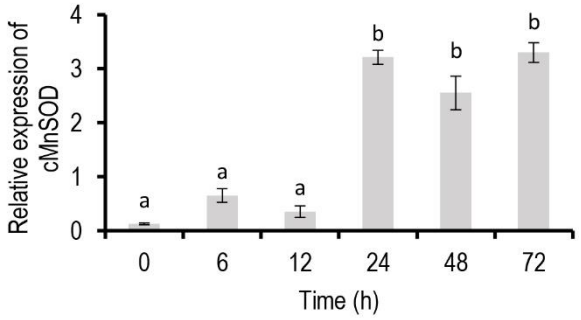

Figure 2. Relative expression of the cMnSOD gene in hemocytes of *P. vannamei* fed with *A. vera*. Sampling (n = 6) times at 0, 6, 12, 24, 48 and 72 h. Relative expression was calculated with the equation RQ^{target}/geometric mean of $RQ^{reference\ genes}$. Reference genes: 40S-S24, β-actin, and ubiquitin. Results are mean ± SE. Different letters indicate significant differences ($P < 0.05$).

Peraza-Gómez *et al.*, 2011; Medina-Beltrán, 2012) as with *P. vannamei*, where *A. vera* protects animals from the infection with WSSV and *V. parahaemolyticus* (Trejo-Flores *et al.*, 2016). Plant phytochemicals could be an alternative to the chemotherapeutic molecules due to antimicrobial and immunostimulant properties (Siravam *et al.*, 2004; Kirubakaran *et al.*, 2010). Although modulation of the immune response by plants has become the focus of scientific investigation (Galina *et al.*, 2009), there are no reports on the effect of *A. vera* on the immune system of *P. vannamei*. Therefore, this paper aims to address this issue.

One of the studied immune effectors was TCTP, also known as fortilin, a multifunctional protein highly conserved in eukaryotes (Nayak *et al.*, 2010) that plays essential roles in cell growth (Gachet *et al.*, 1999), cell cycle progression (Cans *et al.*, 2003), and anti-apoptotic activity (Liu *et al.*, 2005). TCTP gene expression decreases in *P. monodon* infected with a high WSSV burden (Bangrak *et al.*, 2004). Furthermore,

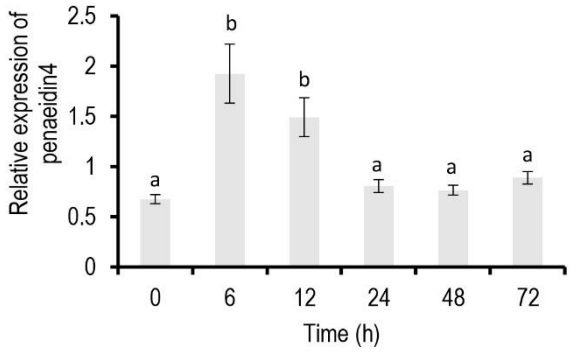

Figure 3. Relative expression of penaeidin4 gene in hemocytes of *P. vannamei* fed with *A. vera*. Sampling (n = 6) times at 0, 6, 12, 24, 48 and 72 h. Relative expression was calculated with the equation RQ^{target}/geometric mean of $RQ^{reference\ genes}$. Reference genes: 40S-S24, β-actin, and ubiquitin. Results are mean ± SE. Different letters indicate significant differences ($P < 0.05$).

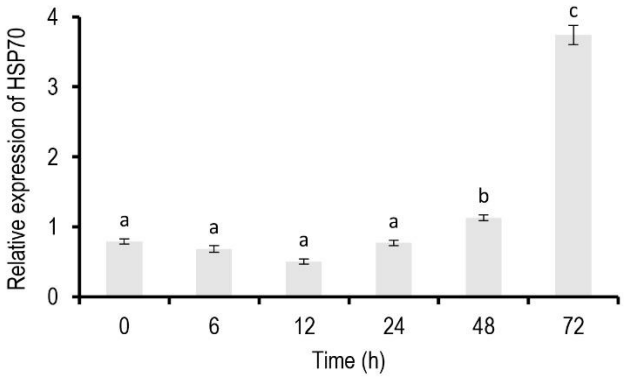

Figure 4. Relative expression of HSP70 gene in the hepatopancreas of *P. vannamei* fed with *A. vera*. Sampling (n = 6) times at 0, 6, 12, 24, 48 and 72 h. Relative expression was calculated with the equation RQ^{target}/geometric mean of $RQ^{reference\ genes}$. Reference genes: β-actin, EF1α, and ubiquitin. Results are mean ± SE. Different letters indicate significant differences ($P < 0.05$).

fortilin plays an essential role in response to *Vibrio harveyi* in *P. monodon* through its participation in the mechanisms of cell death (apoptosis) (Nayak *et al.*, 2010). In addition, TCTP plays an important role in the immune response to WSSV in gills of *P. vannamei* (Wu *et al.*, 2013). In this work, the expression of TCTP in hemocytes of shrimp fed with *A. vera* decreased significantly after 6 h. Therefore, it is possible that *A. vera* affects cellular processes in shrimp hemocytes that are very important in WSSV (Bangrak *et al.*, 2004) and *Vibrio* (Nayak *et al*., 2010) infections, such as cell growth (Gnanasekar *et al*., 2009) and/or cell death pathway (Bangrak *et al.*, 2004).

During the phagocytosis process carried out by hemocytes, reactive oxygen species (ROS) are produced, such as superoxide anion (O_2^-), which are microbicides (Campa-Córdova *et al.*, 2005; Medina-Beltrán *et al.*, 2012; Peraza-Gómez *et al.*, 2014). SOD production in crustaceans responds to pathogen infection, as observed in *P. monodon* challenged with *V. harveyi*, where the MnSOD gene is up-regulated in hemocytes. The authors found that the modulation of SOD gene expression is related to the presence of pathogens and defense processes (Nayak *et al.*, 2010). Regarding *A. vera*'s effect, in this work, SOD gene expression was significantly higher at 24, 48, and 72 h as compared to the initial hours (0, 6, and 12 h). Similar results in SOD gene expression were found in *P. vannamei*, treated with the polysaccharide extract of the root of *Panax ginseng* (0.4 g kg $diet^{-1}$) (Liu *et al.*, 2011), *P. monodon*, treated with sodium alginate (2.0 g kg^{-1}) (Liu *et al.*, 2006), and *P. vannamei*, fed with the extract of *Rubus coreanus* (0.25 and 0.5%) (Subramanian *et al.*, 2013). Results found in this work suggest that *A. vera*, administered orally, can enhance the release of the superoxide anion free radical in hemocytes by increasing phagocytosis due to immunostimulant substances (acemannans, glucomannans, aloctin A, and aloe emodin) (Hu *et al.*, 2003; Akev *et al.*, 2015).

Antimicrobial peptides (AMP) have a crucial role in innate immunity, which is evolutionarily conserved from invertebrates to vertebrates. Gene expression of these proteins appears to be induced only by microbial infections (Destoumieux *et al.*, 2000; Gueguen *et al.*, 2006); however, in the response induced by shrimp injury, AMP act as a pro-inflammatory cytokine, resulting in rapid gene expression (Li & Song, 2010). Penaeidins are effective against Gram-positive bacteria but not against Gram-negative (Destoumieux *et al.*, 1997, 1999). However, in *P. vannamei* challenged with *V. harveyi*, penaeidin4 was significantly up-regulated at 18, 24, 36, and 48 h postinfection (Wang *et al.*, 2010). Similarly, the expression of the gene fein-penaeidin in *Fenneropenaeus indicus* increased at 6, 12, 24, 36, and 48 h after infection with *V. parahaemolyticus* injected at a concentration of 6×10^6 CFU (Vaseeharan *et al.*, 2012). In this work, penaeidin4 gene expression increased significantly at 6 h, which proves its early expression in hemocytes due to immunostimulant substances of *A. vera* such as acemannans, glucomannans, aloctin A, and aloe emodin (Hu *et al.*, 2003; Akev *et al.*, 2015).

Heat shock proteins, such as Hsp70, are accessory proteins that play an important role in the immune response as chaperones and as inducers of pro-inflammatory cytokines secretion (Spagnolo *et al.*, 2007). Chaperones help in defense against microbial infections by sending signals to the immune system in the phagocytosis process and by stimulating the

production of antibodies and T lymphocytes in mammals (Valpuesta *et al.*, 2002). In penaeid shrimp (invertebrates), Hsp70 and Hsp10 have been reported in *P. vannamei* and Hsp82, Hsp29, and Hsp28 in *P. setiferus* (Gross *et al.*, 2001). Hsp70 is present at low levels in many cells and is induced by stress, regardless of their cell cycle stage. Furthermore, Hsp70 can be highly induced in infections caused by bacteria or viruses (Eisenhut, 2008; Espigares *et al.*, 2006) like in *P. vannamei* challenged with TSV (Taura syndrome virus) where Hsp70 gene expression was associated with resistance against TSV (Zeng *et al.*, 2008). In this work, the expression of the Hsp70 gene in the hepatopancreas was significantly increased after 48 h. It is possible that the increase in the gene expression was due to some adverse effects (stress) of plant molecules such as tannins (antinutritional factors) or aloin (laxative in humans) (Domínguez-Fernández *et al.*, 2012; Gilani *et al.*, 2012), which affect the hepatopancreas function when these molecules are in excess in the diet.

In previous work, our research group demonstrated the increase in survival of *P. vannamei* fed with *A. vera* and challenged with *V. parahaemolyticus*. However, this is the first report showing the effect of *A. vera* on the modulation of shrimp immune response. Further research is needed about the use of *A. vera* as a prophylactic treatment against *V. parahaemolyticus* in shrimp farms.

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