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Survival and immune-related gene expression in *Litopenaeus vannamei* co-infected with WSSV and *Vibrio parahaemolyticus*

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A B S T R A C T

In the present study, survival and immune-related gene expression were investigated in *Litopenaeus vannamei* co-infected with white spot syndrome virus (WSSV) and *Vibrio parahaemolyticus* by ingestion and immersion, respectively. The expression of five immune-related genes (translationally controlled tumor protein [TCTP], toll receptor [LvToll], penaeidin4, crustin, and lysozyme) in hemocytes of experimental shrimp was measured by real-time quantitative PCR. Shrimp infected first with *V. parahaemolyticus* and then with WSSV showed a mortality of $46.66 \pm 0.88\%$. Shrimp infected first with WSSV and then with *V. parahaemolyticus* showed a mortality of $45.00 \pm 1.53\%$. Shrimp infected with WSSV and *V. parahaemolyticus* at the same time showed a mortality of $55.00 \pm 2.08\%$. Gene expression of LvToll, crustin, and lysozyme was upregulated whereas gene expression of TCTP and penaeidin4 was downregulated. Under the present experimental conditions and infection routes, *V. parahaemolyticus* was the main responsible for the mortality of cultured white shrimp. Regarding immune response, significant changes were found in gene expression in hemocytes of *L. vannamei* during the time-course of a successful immune response to pathogens *V. parahaemolyticus* and WSSV.

Statement of relevance: Bacterial and viral co-infection in white shrimp culture.

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1. Introduction

Worldwide, viruses are problematic in shrimp farming (Lo et al., 1996a, b; Wang et al., 1999). However, *Vibrio* spp. can generate significant production losses too (Goarant et al., 2006; Tran et al., 2013). Under field conditions, animals are often infected with more than one pathogen (Phuoc et al., 2009) that can accelerate and enhance shrimp mortality (Nurhayati and Widarnani, 2015). Co-infections with viruses and *Vibrio* in shrimp have been studied, such as WSSV-*Vibrio campbelli* (Phuoc et al., 2008), WSSV-*Vibrio* spp. (Phuoc et al., 2009), and infectious myonecrosis virus-*Vibrio harveyi* (Nurhayati and Widarnani, 2015). Research findings have demonstrated that a primary WSSV infection may weaken shrimp, increasing their susceptibility to bacterial infections (secondary infection) (Selvin and Lipton, 2003). *Vibrio* spp. may act as opportunistic agents in secondary infections (Saulnier et al., 2000a) or be true pathogens (*Vibrio penaeicida*, *Vibrio parahaemolyticus*) (Nunan et al., 2014; Saulnier et al., 2000b; Tran et al., 2013).

Internationally, efforts have been made for preventing and controlling diseases to make aquaculture development sustainable. Therefore, a better understanding of the molecular responses and defense mechanisms of shrimp against invading pathogens is needed (Yeh et al., 2009).

The non-specific immune system of crustaceans is based on cellular and humoral effectors that ensure efficient defense responses to eliminate infectious pathogens. Hemocytes are cell fractions in the hemolymph that play important roles in the immune response, like recognition (Cheng et al., 2005; Sivakamavalli et al., 2014), phagocytosis, encapsulation, nodule formation, cytotoxicity (Sritunyalucksana et al., 1999), and cellular communication (Liu et al., 2005). The humoral response is composed of the hemolymph clotting mechanism, prophenoloxidase (proPO) system, melanization, lysosomal hydrolytic enzymes, antimicrobial peptides, nitric oxide, respiratory burst, opsonization, and agglutination (Bachère et al., 2000; Cerenius and Söderhäll, 2004; Jiravanichpaisal et al., 2006; Yeh et al., 2006).

The expression of immune-related genes is considered a potential marker of a shrimp's health status (Liu et al., 2005), as it provides relevant information about the activation and modulation of the immune system (Wang et al., 2007; Wang et al., 2010). Therefore, the aim of this work was to improve the knowledge of the expression of immune–related genes involved in interactions between WSSV and *V. parahaemolyticus* in shrimp.

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2. Materials and methods

2.1. Experimental animals

Pathogen-free shrimp, weighing 10–60 mg, were obtained from rearing facilities of the state of Sinaloa, Mexico (Proveedora de Larvas, S.A. de C.V. and Acuacultura Integral, S.A. de C.V.) and transported to the lab facilities of CIIDIR-IPN Sinaloa. The outdoor culture area was covered with a shadow mesh. Shrimp stock was cultured in 1000-L plastic tanks with 500 L of filtered (20 μm) seawater (30‰) and under continuous aeration. Shrimp 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 before feeding.

2.2. V. parahaemolyticus growth

V. parahaemolyticus was isolated from shrimp with AHPND (López-León et al., 2016). Bacteria were grown in tryptic soy broth (TSB) supplemented with 2.5% NaCl and incubated at 30 °C for 18 h. Samples were centrifuged at 2379*g* (Sigma 2-6E) for 20 min and the cell pellet was resuspended in 1 mL of sterile saline solution (2.5% NaCl). The bacterial solution was adjusted spectrophotometrically to an optical density of 1 at 580 nm.

2.3. Preparation of WSSV-positive shrimp tissue

The viral inoculum (50 μL) was injected to juvenile shrimp (5–8 g) in the second abdominal segment with an insulin syringe. After 24 h, the moribund shrimp were killed and the presence of WSSV was determined by single or nested PCR with primers of Kimura et al. (1996). Then, the organisms were stored at −80 °C until used for tissue preparation. Shrimp were thawed and abdominal muscle, gills, and pleopods were dissected and cut into fine pieces with a scalpel. The resulting WSSV tissue was used to feed shrimp during the bioassay. Shrimp tissues were positive to WSSV by single PCR, which means a relatively high viral load. Sensitivity of single PCR is about 1000 viral particles whereas sensitivity of nested PCR is 10–50 viral particles (Lo et al., 1996a; Lo et al., 1996b; Takahashi et al., 1996).

2.4. Shrimp acclimation to culture conditions

Shrimp maintained in an outside culture system were acclimated to culture conditions for 3 days in 120-L plastic tanks containing 40 L of filtered (20 μm) sea water (30‰) and under continuous aeration in groups of 30 organisms per tank, selected at random. Shrimp were fed as above. Uneaten food and waste material were removed daily before feeding.

2.5. Experimental design

2.5.1. Bioassay 1 (Vibrio LC_{50})

A bioassay was conducted to determine the median lethal concentration (LC_{50}) of *V. parahaemolyticus* in animals from the acclimation culture. Bacteria were put in the water of the culture system. Each treatment had three replicates (60 shrimp, 20 $tank^{-1}$). The bioassay was conducted for 4 d with shrimp weighing 2.15 ± 0.21 g. The bioassay consisted of five treatments: I) Control without *Vibrio*; II) *Vibrio* (1×10^3 CFU mL^{-1}); III) *Vibrio* (1×10^4 CFU mL^{-1}); IV) *Vibrio* (15×10^4 CFU mL^{-1}); V) *Vibrio* (35×10^5 CFU mL^{-1}). Shrimp were fed as above. Water physicochemical parameters (pH, temperature, salinity, and dissolved oxygen) were determined daily (Boyd and Tucker, 1998). Animals were cultured under natural photoperiod. During the bioassay, mortality was recorded three times a day. Shrimp were grown in optimal conditions; however, no cleaning of the tanks was made during the challenging period and temperature was maintained between 28 and 30 °C to favor shrimp infection.

Mortality results from the bioassay were used to calculate the LC_{50} by using Probit analysis (Finney, 1952) with StatPlus® 2009 professional 5.8.4.

2.5.2. Bioassay 2 (co-infections with WSSV and V. parahaemolyticus)

The bioassay was conducted for 5 d days with shrimp weighing 2.15 ± 0.21 g. Bacteria ($LC_{50} = 96.5 \times 10^3$ CFU mL^{-1}) from the first bioassay and shrimp paste with WSSV (100 mg $shrimp^{-1}$) were added to the water of each tank. Twenty shrimps were placed in each tank. The bioassay consisted of four treatments, each one in triplicate: I) Control group without pathogens; II) *Vibrio* LC_{50} (inoculated at first day) + WSSV (inoculated at 48 h); III) WSSV (inoculated at first day) + *Vibrio* LC_{50} (inoculated at 48 h); IV) *Vibrio* LC_{50} + WSSV (both pathogens inoculated at first day). Water physicochemical parameters (pH, temperature, salinity, and dissolved oxygen) were determined daily (Boyd and Tucker, 1998). Animals were cultured under natural photoperiod. During the bioassay, mortality was recorded two times a day. Shrimp were grown in optimal conditions; however, no cleaning of the tanks was made during the challenging period and temperature was maintained between 28 and 30 °C to favor shrimp infection. Survival was determined and hemolymph was extracted from live animals by taking two batches of samples, the first batch was taken 24 h after the first infection and the second one 24 h after the second infection (72 h). Three shrimp per tank (9 shrimp $treatment^{-1}$) were sampled.

2.5.3. Hemolymph extraction

Hemolymph (200 μL) of individual shrimp was withdrawn from the pleopod base of the first abdominal segment with a sterile 1-mL syringe (27 G × 13 mm needle). Before hemolymph extraction, the syringe was loaded with 400 μL of a precooled (4 °C) solution (450 mM NaCl, 10 mM KCl, 10 mM Hepes, and 10 mM EDTA-Na2, pH 7.3) used as an anticoagulant (Vargas-Albores et al., 1993). Hemolymph was centrifuged at 800*g* for 10 min at 4 °C. The plasma was removed, and the hemocyte pellet was rinsed with 250 μL of cold anticoagulant (4 °C) by centrifuging as above. The supernatant was removed, and the hemocytes were suspended in 250 μL of precooled Trizol Reagent® (Invitrogen, Carlsbad, CA, USA) and stored at −70 °C until use.

2.6. Total RNA isolation and cDNA synthesis

Total RNA of hemocytes was extracted with Trizol Reagent according to manufacturer's protocol. A nanophotometer (Implen, Inc., Westlake Village, CA, USA) was employed to analyze the RNA concentration and purity. The RNA was treated with DNAseI (1 U μL^{-1}, Sigma-Aldrich®, St. Louis, MO, USA). Reverse transcription was used to synthesize the first strand of cDNA using reverse transcriptase (Improm II, Promega®, Madison, WI, USA) with the oligo dT20, using 500 ng of total RNA. The cDNA was diluted with 80 μL of ultrapure water and stored at −70 °C until analysis. Five microliters of this cDNA dilution was used as template in each qRT-PCR reaction.

2.7. Expression analysis of immune-related genes by qPCR

The expression of five immune-related genes (translationally controlled tumor protein [TCTP], toll receptor [LvToll], penaeidin4, crustin, and lysozyme) in hemocytes (Table 1) of experimental shrimp was measured by quantitative real-time PCR using a CFX96 system and the CFX Manager version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA).

To find the best reference to normalize the expression, a stability analysis of four reference genes (40S–S24, β-actin, EF1a, and L21) (Table 1) was done with two algorithms: GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004), using the RefFinder web application (http://fulxie.0fees.us/?type=reference). The expression of target genes was normalized to the geometric mean of the most stable reference genes (EF1α, β-actin, and L21).

Table 1
Specific primers used for qPCR amplifications of white shrimp genes.

Genes	Primers	Sequence (5′–3′)	References
Immune-related			
Lysozyme	Lys-F	GAAGCGACTACGGCAAGAAC	Wang et al. (2010)
	Lys-R	AACCGTGAGACCAGCACTCT	
Toll receptor	LvToll-F	ATGTGCGTGCGGATACATTA	Wang et al. (2010)
	LvToll-R	GGGTGTTGGATGTCGAGAGT	
Crustin	Crus-F	GAGGGTCAAGCCTACTGCTG	Wang et al. (2010)
	Crus-R	ACTTATCGAGGCCAGCACAC	
TCTP	TCTP-F	CAATGGACCCTGATGGC	Wu et al. (2013)
	TCTP-R	GCTTCTCCTCTGTTAGACCGTAT	
Penaeidin4	Pen4-F	GCCCGTTACCCAAACCATC	Wang et al. (2010)
	Pen4-R	CCGTATCTGAAGCAGCAAAGTC	
Housekeeping			
40S-S24	Lv40S_S24-F	CAGGCCGATCAACTGTCC	Álvarez-Ruiz et al. (2015)
	Lv40S_S24-R	CAATGAGAGCTTGCCTTTCC	
L21	L21Lv-F	GTTGACTTGAAGGGCAATG	Stephens et al. (2012)
	L21Lv-R	CTTCTTGGCTTCGATTCTG	
EF1α	LvEf-F	CTGTGGTCTGGTTGGTGTTG	Álvarez-Ruiz et al. (2015)
	LvEf-R	TCAGATGGGTTCTTGGGTTC	
β-Actin	Actin-F	CCACGAGACCACCTACAAC	Wang et al. (2007)
	Actin-R	AGCGAGGGCAGTGATTTC	

A qPCR master mix (2×) was carefully prepared for all reactions of the experiment, separated in aliquots (reactions per plate) and stored at −20 °C until use. Amplifications were performed in duplicate in a 96-well plate in a 15-μL reaction volume containing 7.5 μL of PCR Master Mix 2× (1.5 μL of reaction buffer 10×, 0.75 μL of 50 mM MgCl2, 0.3 μL of 10 mM dNTPs, 0.75 μL of EvaGreen® 20× [Biotium, Hayward, CA, USA]; 0.1 μL of 5 U μL^{-1} Biolase DNA Polymerase [BiolineTM, Taunton, MA,USA], and 4.1 μL of ultrapure water), 0.35 μL of each primer (10 μM, Sigma-Aldrich®, St. Louis, MO, USA), 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]. 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, 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).

2.8. Statistical analysis

One-way variance (ANOVA) analysis was applied to examine the differences in survival (%) and expression of immune-related genes among treatments. Survival data were arcsine transformed according to Daniel (2002). Where significant ANOVA differences were found, a Tukey's HSD test was used to identify these differences at $P < 0.05$.

3. Results

3.1. Bioassay 1 (LC_{50})

Results of bioassay 1 showed that the *Vibrio*'s LC_{50} was 96.5×10^3 CFU mL^{-1}. This bacterial concentration was used to infect shrimp in the second bioassay.

3.2. Bioassay 2

3.2.1. Mortality

Results showed that 100% shrimp from treatment I (uninfected control) were alive. In treatment II, shrimp infected first with *V. parahaemolyticus* and then with WSSV (48 h), showed a cumulative mortality of 46.66 ± 0.88%. Shrimp died between 13 and 72 h post infection (43.33% died between 13 and 24 h and 3.33% died between 64 and 72 h). Most shrimp died very quickly after infection with *V. parahaemolyticus* but only two shrimp died after infection with WSSV. In treatment III, shrimp infected first with WSSV and then with *V. parahaemolyticus* (48 h) showed a cumulative mortality of 45.00 ± 1.53%. Shrimp died between 40 and 96 h post-infection (8.33% died between 40 and 48 h, 20% died between 64 and 72 h, and 16.66% died between 88 and 96 h). Only five shrimp died after infection with WSSV and mortality began at 40 h post-infection. Twenty two shrimp died after infection with *V. parahaemolyticus*, but mortality was slow as compared to treatment II as animals died at 48 h after infection. In treatment IV, shrimp infected with both WSSV and *V. parahaemolyticus* at the same time showed a cumulative mortality of 55.00 ± 2.08%. Shrimp died between 12 and 72 h post-infection (53.33% died between 12 and 24 h and 1.66% died between 64 and 72 h) (Fig. 1). Most shrimp died very quickly after infection with *V. parahaemolyticus* and WSSV at the same time.

3.3. Shrimp immune response to WSSV and V. parahaemolyticus

At 24 h, TCTP gene expression was significantly higher ($P = 0.03$) in healthy shrimp (treatment I) as compared to animals infected with both pathogens at the same time (treatment IV). At 72 h, no significant differences were found among treatments (Fig. 2).

At 24 h, LvToll gene expression was significantly higher in shrimp infected with both pathogens at the same time (treatment IV) as compared to healthy animals (treatment I; $P = 0.002$) and those infected first with *V. parahaemolyticus* and then with WSSV (treatment II; $P = 0.001$). At 72 h, LvToll gene expression was significantly higher in shrimp infected with both pathogens at the same time (treatment IV) as compared to healthy animals (treatment I; $P = 0.0001$), shrimp infected first with *V. parahaemolyticus* and then with WSSV (treatment II; $P = 0.022$), and shrimp infected first with WSSV and then with *V. parahaemolyticus* (treatment III; $P = 0.009$). Likewise, LvToll gene expression was significantly higher in shrimp infected with both pathogens at different times (treatment II [$P = 0.0006$] and treatment III [$P = 0.002$]) as compared to healthy animals (Fig. 3).

At 24 h, penaeidin4 gene expression was significantly higher in healthy shrimp (treatment I) as compared to shrimp infected first with *V. parahaemolyticus* and then with WSSV (treatment II; $P = 0.007$). At 72 h, no significant differences were found among treatments (Fig. 4).

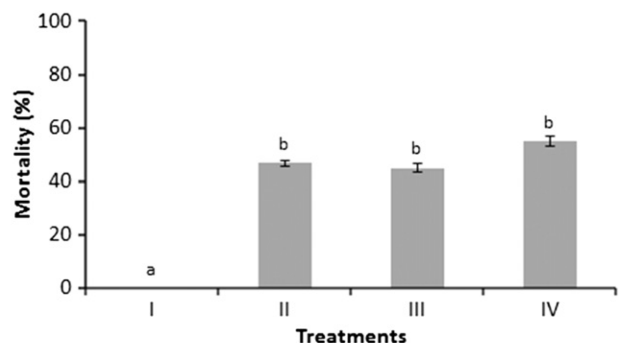

Fig. 1. Cumulative mortality of *L. vannamei* infected with *V. parahaemolyticus* IPNGS16 and WSSV. Treatments: I) Control group without pathogens; II) *Vibrio* LC_{50} (inoculated at time zero) + WSSV (inoculated at 48 h); III) WSSV (inoculated at time zero) + *Vibrio* LC_{50} (inoculated at 48 h); IV) *Vibrio* LC_{50} + WSSV (both pathogens inoculated at time zero). Columns and bars are mean ± SD.

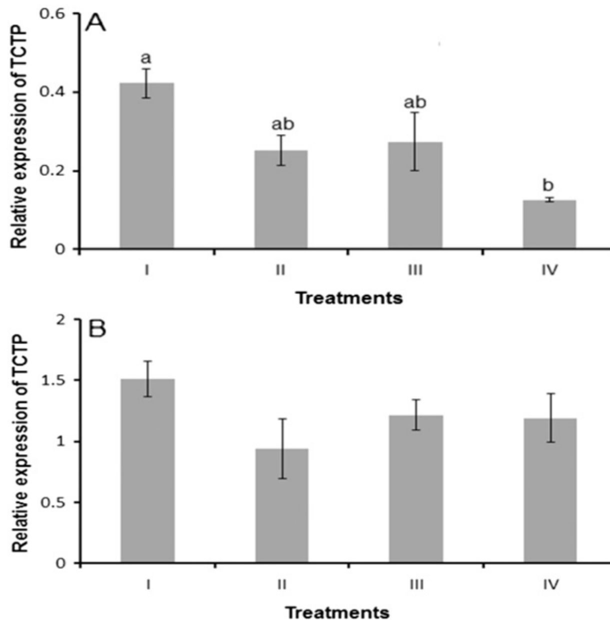

Fig. 2. Relative expression of TCTP gene in hemocytes of *L. vannamei* at 24 h (A) and 72 h (B) post-infection. Treatments: I) Control group without pathogens; II) *Vibrio* LC_{50} (inoculated at time zero) + WSSV (inoculated at 48 h); III) WSSV (inoculated at time zero) + *Vibrio* LC_{50} (inoculated at 48 h); IV) *Vibrio* LC_{50} + WSSV (both pathogens inoculated at time zero). Columns and bars are mean ± SE. Different letters indicate significant differences ($P < 0.05$).

At 24 h, crustin gene expression was significantly higher in shrimp infected with both pathogens at the same time (treatment IV) as compared to healthy shrimp (treatment I; $P = 0.006$), shrimp infected first with *V. parahaemolyticus* and then with WSSV (treatment II; $P = 0.0001$), and shrimp infected first with WSSV and then with *V. parahaemolyticus* (treatment III; $P = 0.04$). In treatment III, crustin gene expression in shrimp, infected first with WSSV and then with *V. parahaemolyticus*, was significantly higher as compared to shrimp infected first with *V. parahaemolyticus* and then with WSSV (treatment II; $P = 0.02$). At 72 h, no significant differences ($P > 0.05$) were found among treatments (Fig. 5).

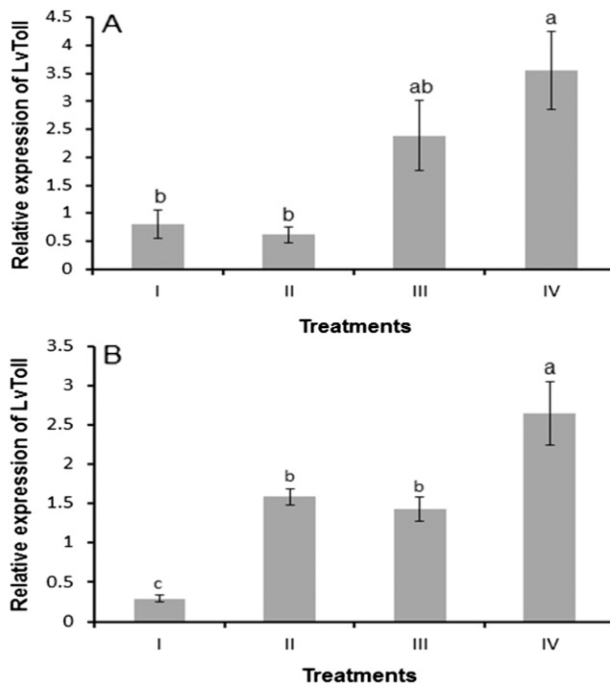

Fig. 3. Relative expression of LvToll gene in hemocytes of *L. vannamei* at 24 h (A) and 72 h (B) post-infection. Treatments: I) Control group without pathogens; II) *Vibrio* LC_{50} (inoculated at time zero) + WSSV (inoculated at 48 h); III) WSSV (inoculated at time zero) + *Vibrio* LC_{50} (inoculated at 48 h); IV) *Vibrio* LC_{50} + WSSV (both pathogens inoculated at time zero). Columns and bars are mean ± SE. Different letters indicate significant differences ($P < 0.05$).

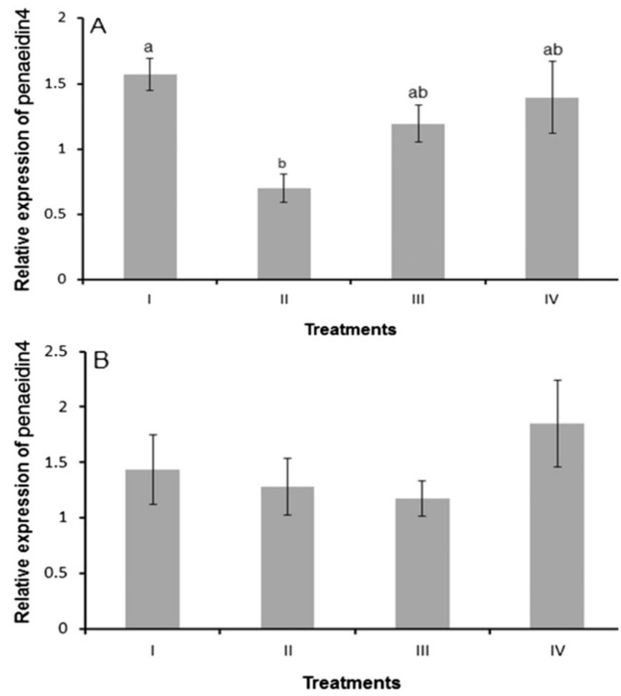

Fig. 4. Relative expression of penaeidin4 gene in hemocytes of *L. vannamei* at 24 h (A) and 72 h (B) post-infection. Treatments: I) Control group without pathogens; II) *Vibrio* LC_{50} (inoculated at time zero) + WSSV (inoculated at 48 h); III) WSSV (inoculated at time zero) + *Vibrio* LC_{50} (inoculated at 48 h); IV) *Vibrio* LC_{50} + WSSV (both pathogens inoculated at time zero). Columns and bars are mean ± SE. Different letters indicate significant differences ($P < 0.05$).

At 24 h, lysozyme gene expression in healthy shrimp (treatment I; $P = 0.0003$) and shrimp infected first with WSSV and then with *V. parahaemolyticus* (treatment III; $P = 0.02$) was significantly higher as compared to shrimp infected first with *V. parahaemolyticus* and then with WSSV (treatment II). At 72 h, lysozyme gene expression in shrimp infected first with *V. parahaemolyticus* and then with WSSV (treatment II) was significantly higher as compared to healthy shrimp (treatment I; $P = 0.003$) (Fig. 6).

4. Discussion

Worldwide, infectious diseases caused by viral and bacterial pathogens have become a serious concern in shrimp farming (Primavera, 1998). WSSV entry into the host cell directly or through endocytosis for their replication (22–24 h), assembly, and release (Chang et al., 1996; Chen et al., 2011; Escobedo-Bonilla et al., 2008; Mercer et al., 2010; Verbruggen et al., 2016). Regarding *V. parahaemolyticus*, this bacteria becomes virulent by acquiring a plasmid that expresses toxins (PirA and PirB) that induce cell death by membrane pore forming (Lee et al., 2015). Also, *V. parahaemolyticus* has the secretion systems type III (T3SS1) and VI (T6SS1 and T6SS2) that are important virulence factors (Cascales, 2008; Kongrueng et al., 2014).

Co-infections with virus and *Vibrio* in shrimp have been studied, such as WSSV-*V. campbelli* (Phuoc et al., 2008), WSSV-*Vibrio* spp. (Phuoc et al., 2009), and infectious myonecrosis virus-*V. harveyi* (Nurhayati and Widarnani, 2015). In this paper, a natural co-infection was performed with two primary pathogens, *V. parahaemolyticus* (waterborne route) and WSSV (per os). Results showed that, in a short

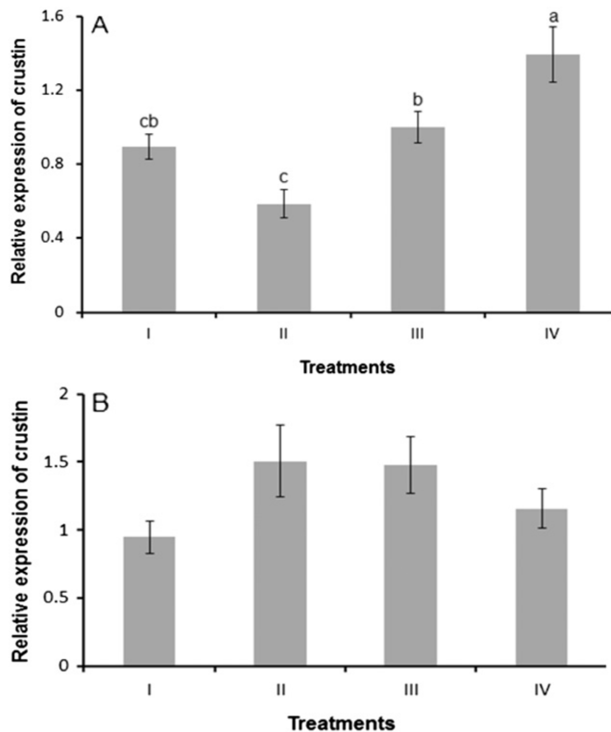

Fig. 5. Relative expression of crustin gene in hemocytes of *L. vannamei* at 24 h (A) and 72 h (B) post-infection. Treatments: I) Control group without pathogens; II) *Vibrio* LC_{50} (inoculated at time zero) + WSSV (inoculated at 48 h); III) WSSV (inoculated at time zero) + *Vibrio* LC_{50} (inoculated at 48 h); IV) *Vibrio* LC_{50} + WSSV (both pathogens inoculated at time zero). Columns and bars are mean ± SE. Different letters indicate significant differences ($P < 0.05$).

period of time (5 d), mortality (45–46.6%) of shrimp infected with both pathogens inoculated at different times was lower than in shrimp infected with both pathogens at the same time (55%). On the other hand, it is important to note that most shrimp (43.3%) infected first with *V. parahaemolyticus* and then with WSSV at 48 h died between 13 and 24 h, whereas most shrimp (36.6%) infected first with WSSV and then with *V. parahaemolyticus* at 48 h died between 64 and 96 h. It is important to mention that only five shrimp died after infection with WSSV alone and mortality began at 40 h post-infection. Most shrimp (53.3%) infected with both pathogens at the same time died between 12 and 24 h. Conversely, and taking into account the different infection routes, Phuoc et al. (2008) found that injection of *V. campbellii* causes mortality only if *L. vannamei* was injected previously with WSSV, considering that the tested strain is not a primary pathogen. In the same way, Phuoc et al. (2009) did not find any accelerated mortality in *L. vannamei* challenged with WSSV by injection and then infected with either *V. campbellii* (immersion) or *V. harveyi* BB120 (injection). The results showed that under the experimental conditions and infection routes, *V. parahaemolyticus* was the main responsible for the shrimp mortality. Regarding this assertion, it is widely assumed that environmental factors affect immune response and resistance in crustaceans (Gunalan et al., 2010; Le Moullac, 2000), especially in shrimp farms where environmental factors are not controlled as in the laboratory. In this sense, it is important to mention that, besides pathogenic mechanisms of each pathogen, the culture tanks were not cleaned during the challenging period, therefore, heterotrophic bacteria as *V. parahaemolyticus* can take advantage over the virus by increasing bacteria number that favor quorum sensing.

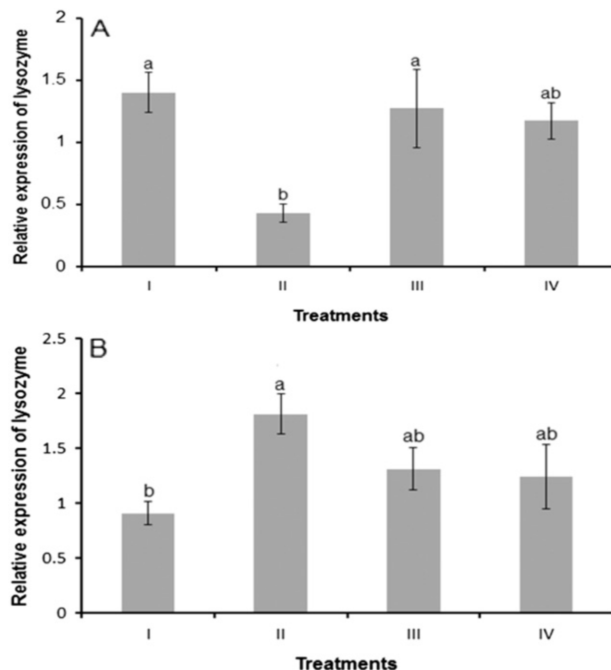

Fig. 6. Relative expression of lysozyme gene in hemocytes of *L. vannamei* at 24 h (A) and 72 h (B) post-infection. Treatments: I) Control group without pathogens; II) *Vibrio* LC_{50} (inoculated at time zero) + WSSV (inoculated at 48 h); III) WSSV (inoculated at time zero) + *Vibrio* LC_{50} (inoculated at 48 h); IV) *Vibrio* LC_{50} + WSSV (both pathogens inoculated at time zero). Columns and bars are mean ± SE. Different letters indicate significant differences ($P < 0.05$).

To understand the immune response of *L. vannamei* co-infected with WSSV and *V. parahaemolyticus*, the expression levels of five immune-related genes was determined. TCTP expression was studied in hemocytes because it plays important roles in cell growth (Gachet et al., 1999), cell cycle progression (Cans et al., 2003), anti-apoptotic activity (Liu et al., 2005), and immune response against WSSV (Wu et al., 2013). In this work, at 24 h, TCTP expression was down-regulated in hemocytes of shrimp infected with both pathogens at the same time (treatment IV). Results disagree with those found by Wu et al. (2013) in gills of *L. vannamei* challenged with WSSV in which TCTP gene expression increased. According to results, it is possible that combined pathogenic mechanisms of both pathogens potentiate the negative effect on the TCTP gene expression, affecting cellular process in shrimp hemocytes that are very important to destroy pathogens.

Toll-like receptors (TLRs) have been recognized as a bridge between innate and acquired immunity (Goldstein, 2004). Pattern-recognition receptors detect pathogens and initiate inflammatory responses (Moreno and Sánchez-Ibarrola, 2003). In arthropods, the Toll-pathway is involved in responses against fungi, Gram-positive, and Gram-negative bacteria by activating signaling pathways that lead to expression of defense molecules (Aggarwal and Silverma, 2008; Brennan and Anderson, 2004). In *L. vannamei*, the LvToll receptors are involved in the defense response against pathogens, such as *V. harveyi* (Wang et al., 2010) and WSSV (Wang et al., 2012). In this study, compared to the control group, the expression level of LvToll at 24 and 72 h was significantly increased after infection with both pathogens, especially when shrimp were infected at the same time. It is important to note that the LvToll studied was one that recognizes *V. harveyi* but not WSSV (Wang et al., 2010). Therefore, it can be assumed that *V. parahaemolyticus* increases LvToll gene expression activating signaling pathways that lead to expression of defense molecules against *V. parahaemolyticus*.

Antimicrobial peptides have been found in all living organisms (Wang et al., 2010). Penaeidins are a family of antimicrobial peptides that have been found only in penaeid shrimp (de Lorgeril et al., 2008). These peptides not only seem to be induced by microbial infections (Destoumieux et al., 2000) but also act as a pro-inflammatory cytokine in the response induced by shrimp injuries (Li et al., 2010). Penaeidins are effective against fungi and Gram-positive bacteria but not Gram-negative bacteria (Destoumieux et al., 1997; Destoumieux et al., 1999). However, Wang et al. (2010) found that in *L. vannamei*

challenged with *V. harveyi*, penaeidin4 was significantly up-regulated at 18, 24, 36, and 48 h post-injection. Similarly, Vaseeharan et al. (2012) found that fein-penaeidin gene expression in *Fenneropenaeus indicus* was significantly increased at 6, 12, 24, 36, and 48 h after infection with *V. parahaemolyticus* injected at a concentration of 6×10^6 CFU. In this work, at 24 h, penaeidin4 mRNA expression was down-regulated in shrimp infected first with *V. parahaemolyticus* and then with WSSV. Down-regulation of penaeidin4 expression in hemocytes by *V. parahaemolyticus* infection might lead to decreased shrimp resistance to this pathogen.

Crustins are cationic cysteine-rich antibacterial peptides (Smith et al., 2008) found within granular hemocytes and plasma of crustaceans and their release is induced by Gram positive bacterial infection (Vargas-Albores et al., 2004). However, in *P. monodon* a five-fold up-regulation of crustin transcripts has been reported after challenging with *V. harveyii* (Amparyup et al., 2008). On the contrary, an unexpected down-regulation of crustin transcripts has been reported after challenging *L. vannamei* with LPS (Okumura, 2007) and *V. alginolyticus* (Jiménez-Vega et al., 2004; Vargas-Albores et al., 2004). In this work it was observed that crustin was expressed higher at 24 h in shrimp infected with both pathogens at the same time as compared to treatments I, II, and III (healthy animals and animals infected with pathogens at different times). Results disagree with the abovementioned works because it seems that both pathogens upregulated crustin mRNA expression.

Lysozyme is a bacteriolytic enzyme that is found in both prokaryotes and eukaryotes. It mainly hydrolyzes the β-1, 4-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycans leading to bacterial lysis (Jollès and Jollès, 1984). In shrimp, lysozyme displays antimicrobial activity against Gram-negative and Gram-positive bacteria including *Vibrio* species that are pathogenic to shrimp (Hikima et al., 2003; Supungul et al., 2010; Tyagi et al., 2007; Xing et al., 2009). In this work, lysozyme showed an active participation against *V. parahaemolyticus* infection because gene expression was downregulated and upregulated in treatment II at 24 and 72 h, respectively, as compared with the control group.

5. Conclusion

Results of this study showed that, under the used experimental conditions and infection routes, *V. parahaemolyticus* was the main responsible for the mortality of cultured white shrimp. Regarding immune response, changes were found in gene expression of TCTP, LvToll, penaeidin4, crustin, and lysozyme in hemocytes of *L. vannamei* during the time-course of a successful immune response to pathogens *V. parahaemolyticus* and WSSV.

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