

Effect of microbial immunostimulants on WSSV infection percentage and the expression of immune-related genes in white shrimp (*Litopenaeus vannamei*)

*Efecto de los inmunoestimulantes microbianos en el porcentaje de infección con WSSV y la expresión de genes relacionados con el sistema inmune del camarón blanco (*Litopenaeus vannamei*)*

*Efeito de imunoestimulantes microbianos sobre a porcentagem de infecção por WSSV e a expressão de genes relacionados ao sistema imune em camarões brancos (*Litopenaeus vannamei*)*

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Abstract

Background: The white spot syndrome virus (WSSV) causes high mortalities in aquaculture. The use of immunostimulants increases animal resistance. **Objective:** To evaluate the WSSV infection percentage and the immunostimulant effect of lactic acid bacteria and yeast (MI= microbial immunostimulants) on WSSV infected *Litopenaeus vannamei*. **Methods:** A bioassay was performed for 33 d, with treatments in triplicate. The MI was added to the feed at 8.5 mg/kg feed and offered to shrimp (9.9 ± 3.1 g) daily, every 2 days, or every 3 days. Shrimp were infected with WSSV at 9 and 19 days. The expression of four immune system-related genes was studied using qRT-PCR. **Results:** No significant differences were observed in growth and survival among treatments. At the end of the bioassay, WSSV infection percentage (low viral load) decreased 8.3 and 25% in treatments III and IV as compared to the control group. Treatments with MI showed significant differences in the relative expression of *LvToll*, transglutaminase, and prophenoloxidase genes when MI was offered daily as compared to the control group. The MI did not regulate the expression of the superoxide dismutase gene. The WSSV infection percentage decreased when feed with MI was offered every 3 d. **Conclusion:** The MI decrease WSSV infection percentage in *L. vannamei* infected with low viral load when it is offered every three days. The MI up-regulates *LvToll*, *Tgase*, and *proPO* genes when it is offered daily. Further research is needed regarding prophylactic treatment with microbial immunostimulants against WSSV in commercial shrimp farms.

Keywords: *Candida parapsilosis*; gene expression; immune system; *Litopenaeus vannamei*; *Pediococcus parvulus*; shrimp; white shrimp.

Resumen

Antecedentes: El virus del síndrome de la mancha blanca (WSSV) ocasiona alta mortalidad en acuicultura. El empleo de inmunoestimulantes incrementa la resistencia de los animales. **Objetivo:** Evaluar el porcentaje de infección de WSSV y el efecto inmunoestimulante de bacterias ácido lácticas y levadura (MI= inmunoestimulantes microbianos) en *Litopenaeus vannamei* infectado con WSSV. **Métodos:** Se realizó un bioensayo durante 33 días con tratamientos por triplicado. Se agregó MI al alimento (8,5 mg/kg de alimento), suministrándolo al camarón ($9,9 \pm 3,1$ g) diariamente, cada 2, o cada 3 días. Los camarones se infectaron con WSSV a los 9 y 19 días. Se estudió la expresión de cuatro genes relacionados con el sistema inmune utilizando qRT-PCR. **Resultados:** No hubo diferencias significativas en el crecimiento y la sobrevivencia entre los tratamientos. Al final del bioensayo, el porcentaje de infección de WSSV (baja carga viral) disminuyó un 8,3 y un 25% en los tratamientos III y IV en comparación con el grupo control. Los tratamientos con MI mostraron diferencias significativas en la expresión relativa de los genes *LvToll*, transglutaminasa, y profenoloxidasas cuando se suministró MI diariamente en comparación con el grupo control. Los MI no regularon la expresión del gen de la superóxido dismutasa. El porcentaje de infección de WSSV disminuyó cuando el alimento con MI se aplicó cada 3 d. **Conclusiones:** Los MI disminuyen el porcentaje de infección de WSSV en *L. vannamei* infectado con baja carga viral cuando se aplican cada tres días. Además, los MI causan una sobre-expresión de los genes *LvToll*, *Tgase* y *proPO* cuando se administran diariamente. Se necesita más investigación sobre los inmunoestimulantes microbianos como tratamiento profiláctico contra WSSV en granjas comerciales de camarón.

Palabras clave: Camarón; camarón blanco; *Candida parapsilosis*; expresión de genes; *Litopenaeus vannamei*; *Pediococcus parvulus*; sistema inmune.

Resumo

Antecedentes: O vírus da mancha branca (WSSV) causa alta mortalidade na aquicultura e o uso de inmunoestimulantes aumenta a resistência em animais. **Objetivo:** Este trabalho avaliou a prevalência do WSSV e o efeito inmunoestimulante de bactérias ácido lácticas e levadura (MI= inmunoestimulantes microbianos) em *Litopenaeus vannamei* infectado con WSSV. **Métodos:** Se realizou um bioensayo durante 33 días com tratamientos por triplicado. Se agregó MI al alimento (8,5 mg/kg feed) e se o fornecimento de camarão ($9,9 \pm 3,1$ g) diariamente, cada 2 e cada 3 d. Los camarones foram infectados con WSSV a los 9 y 19 d. Se estudió a expressão de quatro genes relacionados com o sistema imune, use qRT-PCR. **Resultados:** No hubo diferencias significativas no crecimiento e na supervisão entre os tratamientos. Al final del bioensayo, a prevalência do WSSV diminuiu um 8.3 e não 25% nos tratamientos III e IV em comparação com o grupo de controle. Os tratamentos com MI mostraram diferenças significativas na expressão relativa dos genes. *LvToll*, transglutaminasa e prophenoloxidase quando se administraron em diários de MI em comparação com o controle

de grupo. Los MI não regularam a expressão do genótipo da superóxido dismutasa. A prevalência do WSSV diminuiu quando o alimento com MI se aplicó cada 3 d. **Conclusões:** Los IM diminuir a prevalência de WSSV em *L. vannamei* infectado com baixa carga viral quando aplicável a cada três dias. Além disso, os IM causaram uma sobre-expressão dos genes *LvToll*, *Tgase* e *proPO* quando se administran diariamente. Há mais informações sobre imunoestimulantes microbianos como tratamento profilático contra WSSV en granjas comerciales de camarón.

Palavras-chave: Camarão; camarão branco; *Candida parapsilosis*; expressão de genes; *Litopenaeus vannamei*; *Pediococcus parvulus*; sistema immune.

Introduction

Worldwide aquaculture production exceeds 60 million tons per year and contributes with more than 40% to the production of aquatic organisms (FAO, 2016). However, diseases caused by viruses and bacteria are a primary concern for the development of this important activity (Lightner, 2011). White spot disease caused by white spot syndrome virus (WSSV) is a devastating disease for cultured penaeid shrimp (Joseph *et al.*, 2015) and can not be treated with antibiotics as it happens with diseases caused by bacteria (Gatlin, 2002).

Immunostimulants are an alternative to antibiotics to prevent diseases in cultured animals (Cheng *et al.*, 2014) since they increase animal resistance (Song and Hsieh, 1994). Regarding crustaceans, the immunostimulatory effect of molecules (sodium alginate, glucans, lipopolysaccharides, nucleotides, peptidoglycans, and chitosan) and of whole cells (yeasts and bacteria) have been studied (Luna González *et al.*, 2013a; Ringø *et al.*, 2012; Sajeevan *et al.*, 2009a, b). Regarding yeast, *Candida parapsilosis* possesses immunostimulatory activity against WSSV and has supported high post-challenge WSSV survival (Wilsa *et al.*, 2015).

Crustaceans, such as *Litopenaeus vannamei*, have two lines of defense. The first line is a physical barrier consisting of the cuticle, which prevents the entry of pathogens in the body. The second line is based on cellular and humoral effectors, which combine to combat pathogens, heal wounds and respond to biotic and abiotic stressors (Lemaitre and Hoffman, 2007). The cellular effectors in shrimp are hemocytes (granular, semigranular, and hyaline), which play a central role in phagocytosis, melanization, encapsulation, nodulation, cytotoxicity, apoptosis, interfering RNA,

autophagy, interferon-type system, and coagulation of the hemolymph (Goncalves *et al.*, 2014; Sun *et al.*, 2010). The humoral effectors are the second line of defense and include agglutinins, profenoloxidase system, coagulation proteins, hydrolytic enzymes, antimicrobial peptides, nitric oxide, respiratory burst, and opsonization (Morales and Cuéllar *et al.*, 2014; Cerenius and Söderhäll, 2004; Destoumieux *et al.*, 2000; Jiravanichpaisal *et al.*, 2006). The expression of immune-related genes in shrimp provides important information about the activation of the immune system and its modulation (Wang *et al.*, 2007).

The aim of this work was to determine the effect of microbial immunostimulants on WSSV infection percentage and on the expression of immune-related genes in white shrimp (*Litopenaeus vannamei*), cultured in the laboratory and challenged with WSSV.

Materials and methods

Ethical considerations

This study complies with the Mexican Official Standard NOM-062-ZOO-1999, technical specifications for the production, care and use of laboratory animals.

Animals and acclimatization

Shrimp were obtained from Cuate Machado farm (Guasave, Sinaloa, Mexico) and transported to the CIIDIR-IPN facilities in a plastic tank (250 L) with sea water and constant aeration. Animals were maintained in an outside culture system in 120 L plastic tanks with 80 L filtered (20 µm) sea water (30 ‰) and continuous aeration. Shrimp were fed with commercial feed (Purina®, Ciudad de Mexico, Mexico, 35% protein) twice daily at 09:00 and 16:00 h.

Preparation of viral inoculum and tissue paste

A viral inoculum (stored at $-70\text{ }^{\circ}\text{C}$) with high viral load (single PCR) was used to infect shrimp to obtain fresh muscle and gills with WSSV. Muscle and gill tissues of moribund shrimp were analyzed by polymerase chain reaction (PCR) to confirm the presence of the virus. Half of the tissues obtained were stored at $-70\text{ }^{\circ}\text{C}$ in Falcon tubes and the other half were placed in cold Eppendorf tubes to be macerated with a plastic pistil. After maceration, 1 g of tissue was placed in a 20 mL Falcon tube with 10 mL of cold saline solution (2%). Individual samples were centrifuged at 17,000 g, for 10 min, at $4\text{ }^{\circ}\text{C}$. The supernatants were filtered with syringe filters (0.45 and 0.25 μm), placed in cold Eppendorf tubes (1.5 mL) and stored at $-70\text{ }^{\circ}\text{C}$. The presence of WSSV in the inoculum was verified with simple (>1000 copies of viral DNA, Lo *et al.*, 1996a, b) and nested (10-50 copies of viral DNA, Lo *et al.*, 1996a, b) PCR.

Microbial immunostimulants (MI)

Lactic acid bacteria (*Pediococcus parvulus* Lta2) and yeast (*Candida parapsilosis* Lt6) were isolated and characterized according to Apún Molina *et al.* (2009) and Luna González *et al.* (2013b), and tested in *L. vannamei* challenged with *Vibrio sinaloensis* according to Flores Miranda *et al.* (2011).

Preparation of the experimental diet with powdered immunostimulants

Microorganisms were grown and washed as described in Apún Molina *et al.* (2009). Cells were centrifuged at 5,000 g for 10 min and dried in an oven (Felisa, Zapopan, Jalisco, Mexico) at $74\text{ }^{\circ}\text{C}$ for 4 h. The dried cell pellet was ground in a mortar and then included in the commercial feed pellet (35% protein) at 8.5 mg/kg feed (50% *P. parvulus* and 50% *C. parapsilosis*). The amount of microbial immunostimulants (MI) was based on the work by Flores Miranda *et al.* (2011).

Experimental design

The bioassay was conducted for 33 d with shrimp weighing 9.9 ± 3.1 g. Ten shrimp were placed per tank. Treatments in triplicate were as follows: I) Control

group, shrimp fed commercial feed + WSSV; II) shrimp fed commercial feed + MI (8.5 mg/kg feed) daily + WSSV; III) shrimp fed commercial feed + MI (8.5 mg/kg feed) every 2 d + WSSV; IV) shrimp fed commercial feed + MI (8.5 mg/kg feed) every 3 d + WSSV. Shrimp were fed feed plus MI, except for the days (9 and 19) when animals were fed 250 mg per tank of shrimp paste (muscle and gills) with a high WSSV load (single PCR, > 1000 viral particles). Healthy shrimp were fed on dead shrimp with WSSV to simulate a natural infection. Initially, animals were fed 5% of the mean body weight and adjusted thereafter according to the feeding response in each tank. Uneaten food and waste material were removed every 3 d before feeding, and 50% of the water was exchanged every 5 d.

Physicochemical parameters like pH (HI 98127 pH, Hanna Instruments, Woonsocket, RI, USA), salinity (refractometer W/ATC 300011, Sper Scientific, Scottsdale, AZ, USA), dissolved oxygen, and temperature (YSI model 55 oxygen meter, Yellow Spring Instruments, Yellow Springs, OH, USA) were monitored every 2 d. At the beginning and the end of the bioassay, ammonium, nitrites, and nitrates were determined by the method of Strickland and Parsons (1972).

At the end of the bioassay, weight and survival of shrimp were determined. Shrimp were analyzed separately for WSSV by single or nested PCR. In addition, hemolymph was extracted for the immune system analysis. During the bioassay, the physicochemical parameters (temperature, salinity, pH, dissolved oxygen, ammonium, nitrites, and nitrates) were kept within the optimal intervals for shrimp culture according to Brock and Main (1994) and Boyd and Tucker (1988). Temperature ranged from 29.0 ± 0.2 to $29.5 \pm 0.3\text{ }^{\circ}\text{C}$; salinity was between 31.5 ± 0.6 and $32.2 \pm 0.6\text{‰}$; dissolved oxygen ranged from 5.6 ± 0.06 to 5.8 ± 0.05 mg/L; pH was between 8.4 ± 0.02 and 8.5 ± 0.02 ; ammonium ranged from 0.2 ± 0.2 to 0.6 ± 0.20 mg/L; nitrite was between 0.012 ± 0.001 and 0.014 ± 0.002 mg/L; and nitrate ranged from 0.05 ± 0.05 to 0.7 ± 0.01 mg/L.

DNA extraction and WSSV infection percentage

Genomic DNA was extracted from abdominal muscle and gill lamellas (1:1 w/w) (100 mg) with

DNAzol reagent (MRC[®], Cincinnati, OH, USA) following manufacturer's instructions. Quantification and quality assessment of DNA were performed in a Pearl nanophotometer (Implen, Inc. Westlake Village, CA, USA). DNA was stored at -20 °C, until use. Twelve shrimp per treatment (four per tank) were sampled for WSSV infection percentage by single and nested PCR, using the primers WSSV out-1/WSSV out-2 (5'-ATC ATG GCT GCT TCA CAG AC-3' [forward]/5'-GGC TGG AGA GGA CAA GAC AT-3' [reverse]) and WSSV in-1/WSSV in-2 (5'-TCT TCA TCA GAT GCT ACT GC-3' [forward]/5'-TAA CGC TAT CCA GTA TCA CG-3' [reverse]) (Kimura *et al.*, 1996), which amplified genome fragments of 982 and 570 bp, respectively. Negative samples were analyzed by one-step PCR with an internal control that amplified a 298 bp segment of shrimp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA, using the primers GAPDH298 and GAPDH298 (5'-TCA CCG TCT TCA ACG AGA TG-3' [forward]/5'-ACC CTC CAG CAT CTC GAA CT-3' [reverse]) (Tang and Lightner, 2001).

Hemolymph extraction

Two-hundred microliters of hemolymph were sampled from 3 intermolt shrimp per tank, 9 per treatment, 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 (SIC-EDTA, Na₂) (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 placed in cold Eppendorf tubes (1.5 mL) and then centrifuged at 800 *g* for 6 min at 4 °C. Plasma was discharged and the cellular pellet was rinsed with 250 µL of cold anticoagulant solution and resuspended in 300 µL of Trizol reagent (Invitrogen, Carlsbad, CA, USA). Samples were stored at a -70 °C until used.

Total RNA isolation and cDNA synthesis

Total RNA of hemocytes was extracted with Trizol reagent according to manufacturer's protocol. The RNA concentration and purity was analyzed in a Pearl nanophotometer. The RNA was treated with DNase I (1 U/µL) (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, 500 ng of total RNA and an incubation temperature of 42 °C for 60 min. 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 qPCR reaction.

Expression analysis of immune-related genes by qPCR

The expression of four immune-related genes (toll receptor [*LvToll*], superoxide dismutase [*SOD*], prophenoloxidase [*proPO*], and transglutaminase [*TGase*]) 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 genes to normalize the expression of target genes, a stability analysis of *ribosomal protein (40S-S24)*, *actin (β-actin)*, *elongation factor 2 (EF2)*, and *ribosomal protein (L21)* genes (Table 1) was done with the algorithms GeNorm (Vandesompele *et al.*, 2002) and NormFinder (Andersen *et al.*, 2004), using the RefFinder web application (<http://150.216.56.64/referencegene.php>). The expression of target genes was normalized to the geometric mean of the most stable reference genes (*EF1α*, *β-actin*, and *L21*).

The expression of target and reference genes was determined using the primers found in Table 1. Amplifications were performed in triplicate in a 96-well plate with a final volume of 15 µL, containing 7.5 µL of PCR Master Mix 2x (1.5 µL of 10x reaction buffer, 0.75 µL of 50 mM MgCl₂, 0.3 µL of 10 mM dNTPs, 0.75 µL of EvaGreen 20x [Biotium, Hayward, CA, USA], 0.1 µL of 5 U/µL Biolase DNA polymerase [Bioline[™], Tauton, MA, USA], and 4.1 µL ultrapure water), 0.5 µL of each primer (10 µM, Sigma-Aldrich, St. Louis, MO, USA), 3.5 µL of ultrapure water, and 3 µL of cDNA. The thermocycler conditions were as follows: 95 °C for 3 min followed by 40 cycles at 95 °C for 10 s, 60 °C for 15 s, 72 °C for 30 s, and 79 °C for 5 s. 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.

Table 1. Specific primer sequences used for quantitative real time PCR.

Genes	Primers	Sequence (5' - 3')	References
Immune-related			
<i>Transglutaminase</i>	TGase-F	CCTCAGGATCTCCTTCACCA	Wang <i>et al.</i> (2010)
	TGase-R	TTGGGAAAACCTTCATTTTCG	
<i>Superoxide dismutase</i>	SOD-F	ATCCACCACACAAAGCATCA	Wang <i>et al.</i> (2010)
	SOD-R	AGCTCTCGTCAATGGCTTGT	
<i>Toll receptor</i>	LvToll-F	ATGTGCGTGCGGATACATTA	Wang <i>et al.</i> (2010)
	LvToll-R	GGGTGTTGGATGTCGAGAGT	
<i>Prophenoloxidase</i>	proPO-F	GAGATCGCAAGGGAGAAGT	Wang <i>et al.</i> (2010)
	proPO-R	CGTCAGTGAAGTCGAGACCA	
Housekeeping			
40S-S24	Lv40S_S24-F	CAGGCCGATCAACTGTCC	Álvarez-Ruiz <i>et al.</i> (2015)
	Lv40S_S24-R	CAATGAGAGCTTGCCTTTCC	
L21	L21Lv-F	GTTGACTTGAAGGGCAATG	Stephens <i>et al.</i> (2012)
	L21Lv-R	CTTCTTGGCTTCGATTCTG	
EF2	LvEf-F	CTGTGGTCTGGTTGGTGTG	Álvarez-Ruiz <i>et al.</i> (2015)
	LvEf-R	TCAGATGGGTTCTTGGGTTC	
β -actin	Actin-F	CCACGAGACCACCTACAAC	Wang <i>et al.</i> (2007)
	Actin-R	AGCGAGGGCAGTGATTC	

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 [$E = 10(-1/\text{slope}) - 1$]. The expression of target genes was calculated by transforming the Cq values to relative quantities (RQ), using the equation $RQ_{ij} = E^{[(Cq \text{ mean}) - Cq(ij)]}$, where E is the gene-specific efficiency, and $[(Cq \text{ mean}) - Cq(ij)]$ 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 $RQ^{\text{target}}/\text{Geometric mean of } RQ^{\text{reference genes}}$ (Vandesompele *et al.*, 2002).

Specific growth rate

At the end of the bioassays, the specific growth rate (SGR) was determined using the following formula:

$$\text{SGR (\%/d)} = 100 (\ln W_t - \ln W_0)/t$$

Where t is the culture period in days, W_0 is the natural logarithm of the weight of the shrimp at the beginning of the bioassay, and $\ln W_t$ is the natural

logarithm of the weight of the shrimp at day t (W_0 and W_t are in grams).

Statistical analysis

One-way variance analysis (ANOVA) using the F test was performed among treatments to examine the differences in growth performance and gene expression. Where significant ANOVA differences were found, a Tukey's HSD test was used to determine the nature of these differences at ($p < 0.05$) (Zar, 1996).

Results

Survival and growth

Survival in the bioassay was 100% in all treatments (Table 2). Initial weight ranged from 0.72 ± 0.01 to 0.75 ± 0.02 g. Final weight was between 4.04 ± 0.22 and 4.14 ± 0.18 g. Regarding SGR, values ranged between 5.82 ± 0.1 and $5.34 \pm 0.33\%/d$. No significant differences among treatments were found in growth (Table 2).

Table 2. Effect of MI on the survival, growth, and WSSV infection percentage in *L. vannamei* cultured in the laboratory.

Parameters	Treatments			
	I	II	III	IV
Survival (%)	100	100	100	100
Initial weight (g)	0.75±0.02	0.74±0.01	0.72±0.01	0.74±0.01
Final weight (g)	4.08±0.2	4.04±0.22	4.14±0.18	4.12±0.18
SGR (%/d)	24.2±0.5	24.3± 0.9	24.9±0.4	24.5±0.5
WSSV infection (%)	91.6±0.3	100±0.0	83.3±0.6	66.6±0.5

Treatments: I) Control group, shrimp fed commercial feed + WSSV; II) shrimp fed commercial feed + MI (8.5 mg/kg feed) daily + WSSV; III) shrimp fed commercial feed + MI (mg/kg feed) every 2 d + WSSV; IV) shrimp fed commercial feed + MI (mg/kg feed) every 3 d + WSSV. Data are mean±SE.

WSSV infection percentage

Nested PCR analysis showed that 58.3% of the organisms from the stock were infected with WSSV

(control without WSSV added). At the end of the bioassay, the WSSV infection percentage increased 8.4% in treatment II, where shrimp were fed daily with MI, as compared with control group. However, in treatments III and IV, where shrimp were fed every two and three days with the MI, WSSV infection percentage decreased by 8.3 and 25%, respectively, as compared with control group (Table 2).

Immune-related gene expression

Figure 1 summarizes the results of gene expression in white shrimp fed MI. The additive modulated the expression of the *LvToll*, *TGase*, and *proPO* genes. In *TGase* and *proPO*, significant differences ($p < 0.05$) were found among treatments I, II, and III, with higher gene expression in treatment II (shrimp fed daily with MI). In *LvToll*, significant differences ($p < 0.05$) were found in treatment II as compared with treatments I, III, and IV. The mRNA expression of *SOD* gene remains unchanged ($p > 0.05$).

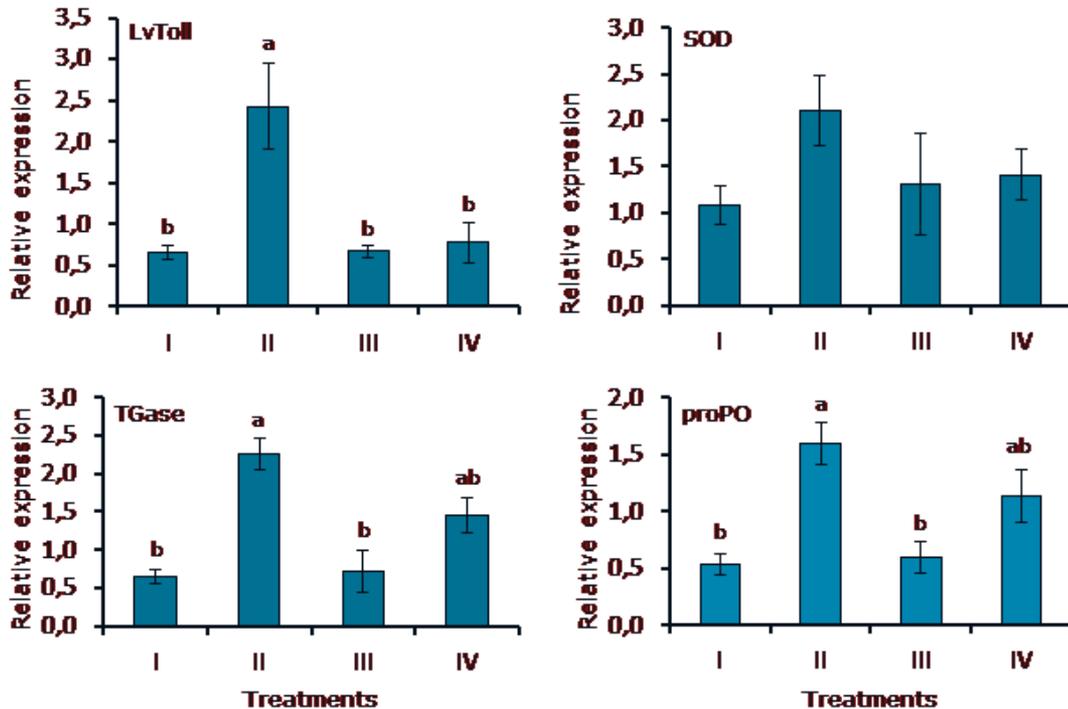


Figure 1. Immune-related gene expression in *L. vannamei* fed MI and challenged with WSSV. Treatments: I) Control group, shrimp fed commercial feed + WSSV; II) shrimp fed commercial feed + MI (mg/kg feed) daily + WSSV; III) shrimp fed commercial feed + MI (mg/kg feed) every 2 d + WSSV; IV) shrimp fed commercial feed + MI (mg/kg feed) every 3 d + WSSV. Relative expression of each gene was calculated with the equation $RQ_{target}/\text{Geometric mean of } RQ_{reference}$ genes. Data are mean±SE. Different letters indicate significant differences.

Discussion

The negative impact of white spot disease has been found in shrimp cultured worldwide. Because of the above, substances that strengthen the immune system of *L. vannamei* are now being investigated and to make it more resistant to diseases caused by pathogenic microorganisms; however, there is few scientific support for its function and dose/frequency of application (Andrews *et al.*, 2011; Sajeevan *et al.*, 2009a). Therefore, in this work, the immunostimulating effect of a mixture of microorganisms (lactic acid bacteria and yeast) on WSSV challenged shrimp was evaluated.

The growth of organisms is influenced by environmental conditions such as temperature, pH, salinity, and food availability (Oduleye, 1982). However, in the present work, growth was not negatively affected by the additives in the feed and because physicochemical parameters and water quality were kept within the optimal intervals for shrimp cultivation (Boyd and Tucker, 1988; Brock and Main, 1994). With respect to the survival of WSSV-infected shrimp, it was 100% in all treatments; results that agree with those reported by Partida Arangure *et al.* (2013), who worked with WSSV-infected shrimp using a mixture of lactic acid bacteria and bacilli in feed at a concentration of 1×10^5 CFU/g feed, as well as inulin at 0.4 to 0.8 g/kg feed. These findings corroborate that culture conditions in the laboratory are more stable than those found in the commercial shrimp farms.

The analysis showed that before the bioassay, 58.3% of the stock shrimp were infected with the virus (low viral load). Therefore, the infection in experimental shrimp was reinforced with shrimp paste. In this sense, it was observed that reinfection worked since 96.1% of the shrimp from the control group were WSSV positive. WSSV infection percentage decreased 8.3% in treatment III (shrimp fed MI every 2 d) and 25% in treatment IV (shrimp fed MI every 3 d) as compared with the control group (shrimp reinfected with WSSV). On the other hand, WSSV infection percentage in treatment II (shrimp fed MI daily) was increased 8.4% as compared with control group. The decrease in the WSSV infection percentage when the MI was administered

discontinuously agree with the results obtained by Itami *et al.* (1998) in *Penaeus monodon* challenged with *V. penaeicida* and fed peptidoglycans (0.2% of the diet) administered discontinuously. Similarly, Sajeevan *et al.* (2009a) reported a higher survival of *Fenneropenaeus indicus* challenged with WSSV orally and fed a glucan diet (0.2%) every seven days. In the same way, Flores Miranda *et al.* (2011) found a higher survival of *L. vannamei* challenged with strains of *V. sinaloensis* and fed every three days with the same MI of this study.

In all living organisms, cells regulate their activities by modulating the expression of their genes. Gene expression is generally proportional to the number of copies of messenger RNA (mRNA) of a given gene. This is a crucial fact when it comes to identifying the presence of specific cellular products since the mRNA is translated into the ribosomes to form proteins. Thus, it is possible to obtain data concerning the production of biological elements if the expression of the genes of a cell is known (McPherson *et al.*, 2008).

Toll receptors are highly conserved transmembrane proteins that are found in immune cells and recognize PAMPs (pathogen-associated molecular patterns) of microorganisms (lipopolysaccharides, peptidoglycans, betaglycans, flagellins, and others) (Akira and Hemmi, 2003). Transglutaminase (*TGase*) is involved in the polymerization of the coagulation protein in the plasma of decapod crustaceans (Liu *et al.*, 2007) and is released as an immediate response of hemocytes to the presence of LPS and β -1,3-glucans (Montaño Pérez *et al.*, 1999). Phenoloxidase can be found in hemocytes as the inactive form of phenoloxidase and plays an important role in the innate immune response because it allows the rapid response to bacteria and fungi (Amparyup *et al.*, 2013). On the other hand, the *SOD* enzyme converts the superoxide anion to hydrogen peroxide (Wang *et al.*, 2010) and thus maintains the state of health and survival of organisms (Campa Córdova *et al.*, 2005). In the present study, we found that the mRNA expression of *LvToll*, *TGase*, and *proPO* genes was significantly increased in treatment II, where the shrimp were fed daily with the MI as compared with control group and treatments III and IV, where shrimp were fed every two and three days with feed additives. However, in *SOD* gene expression, the trend was similar, but there

were no significant differences. It is possible that the increased gene expression of treatment II was due to the peptidoglycans and β -glucans of *P. parvulus* Lta2 and *C. parapsilosis* since WSSV load in all treatments was low (10-50 viral particles). Moreover, it is known that the expression of *LvToll*, *TGase*, *proPO*, and *SOD* genes can be suppressed or remain unchanged when shrimp are infected with WSSV (Ai *et al.*, 2008; Arts *et al.*, 2007; Wang *et al.*, 2010; Yeh *et al.*, 2009). Although genes were up-regulated in treatment II, the lower WSSV infection percentage occurred in treatment IV, where shrimp were fed every 3 days with MI. It is possible that continuous administration of immunostimulants causes immune fatigue in shrimp as reported by Bai *et al.* (2010) in *L. vannamei* fed β -glucan or glycyrrhizin. The above findings point out the importance of concentration and the frequency of application of immunostimulants in shrimp culture systems for effective protection against pathogens (Babu *et al.*, 2013; Sajeevan *et al.*, 2009a). Further research is needed about the effect of higher MI concentrations, added to the feed, in shrimp immune response and WSSV infection percentage.

In conclusion, despite the short period of time (14 days) from the last infection and the end of the bioassay, the tested MI decreased WSSV infection percentage in *L. vannamei* infected with low viral load when it was applied every three days. Also, results showed that MI up-regulates *LvToll*, *TGase*, and *proPO* genes, when it is given daily. Further research is needed about microbial immunostimulants as prophylactic treatment in commercial shrimp farms against WSSV.

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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