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Effect of mixed-*Bacillus* spp isolated from pustulose ark *Anadara tuberculosa* on growth, survival, viral prevalence and immune-related gene expression in shrimp *Litopenaeus vannamei*

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ABSTRACT

The widespread overuse of antibiotics in aquaculture has led to the emergence of antibiotic-resistance shrimp pathogens, the negative impact on shrimp gut microbiota, and the presence of antimicrobial residues in aquaculture products, with negative consequences on human health. Alternatively, probiotics have positive effects on immunological responses and productive performance of aquatic animals. In this study, three probiotic bacteria, (*Bacillus licheniformis* MAt32, *B. subtilis* MAt43 and *B. subtilis* subsp. *subtilis* GAtB1), isolated from the *Anadara tuberculosa* were included in diets for juvenile shrimp, *Litopenaeus vannamei*, to evaluate their effects on growth, survival, disease prevalence, and immune-related gene expression. Shrimp naturally infected with WSSV and IHHNV were fed with the basal diet (control, T1) and diets supplemented with four levels of bacilli probiotic mix (1:1:1) at final concentration of (T2) 1×10^6, (T3) 2×10^6, (T4) 4×10^6, and (T5) 6×10^6 CFU g^{-1} of feed. The specific growth rate of shrimp was significantly higher in T2 than in T1 (control) treatment, and the final growth as well as the survival were similar among treated groups. The prevalence of WSSV and IHHNV infected shrimp was reduced in T2 and T4 treatments, respectively, compared with control. The mRNA expression of proPO gene was higher in treatment T4 than control. The LvToll1 gene was significantly up-regulated in treatments T4 and T5 compared to control. The SOD gene was up-regulated in treatment T5 compared to control. In contrast, the mRNA expression of the Hsp70 gene was down-regulated in treatments T4 and T5 respect to control, and the TGase gene remained unaffected by the level of bacillus probiotic mix. As conclusion, the bacilli probiotic mix (*Bacillus* spp.) enhanced immune-related gene expression in WSSV and IHHNV naturally infected shrimp. This is the first report of probiotic potential of bacteria isolated from *A. tuberculosa* on the immune response and viral prevalence in shrimp *Litopenaeus vannamei*.

1. Introduction

The main role of aquaculture in the production of quality food worldwide has generated a wide field of study [1]. In Mexico, shrimp (*Litopenaeus vannamei*) is the main economically important aquaculture specie. The rapid expansion and intensification of shrimp farming has led to the occurrence of infectious diseases, causing considerable economic losses. The use of large amounts of antibiotics in shrimp aquaculture is a common strategy to control these microbial diseases; however its application has serious concerns due to the emergence of antibiotic-resistance pathogens, the negative effect on shrimp gut and environmental microbiota, and the presence of antibiotic residues in seafood [2]. Therefore, research is becoming increasingly focused on the use of preventive approaches to control diseases through improved immunity.

As a marine invertebrate, shrimp lack of an adaptive immune system and depends on humoral and cellular innate immune responses for detection and elimination of pathogens [3]. Because disease is one of the critical factors in shrimp aquaculture, several

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studies have focused on enhancing the immune response by different strategies, such as phage therapy [4], vaccines [5], prebiotics [6], probiotics [7], and synbiotics [8]. Probiotics are defined as live microorganisms which confer a health benefit to the host when administered in adequate amounts [9]. The use of probiotics in shrimp aquaculture has expanded because of positive results in the control of bacterial [10] and viral diseases [11] by enhancing the immune response [12] and antioxidant defenses [13] as well as promoting growth and survival [14].

Bacillus spp. have showed great potential as probiotics for shrimp [15–17]. Moreover, probiotic mix of several species or strains is gaining attention because they can result in enhanced inmunomodulatory responses and/or disease resistance [18]. However, studies of co-administration of several *Bacillus* species or strains in shrimp are limited [19–21]. Interestingly, in a recent study we found that several strains of *Bacillus* sp. isolated from the pustulose ark *Anadara tuberculosa* (Sowerby I, 1833) have probiotic potential for white shrimp *Litopenaeus vannamei* [22]. Therefore, the aim of this study was to determine the effect of potential probiotic bacteria, alone or in combination, isolated from the gut of *A. tuberculosa* on the growth performance, survival, viral prevalence and immune-related gene expression in juveniles of white shrimp (*L. vannamei*). This is the first report of probiotic potential of bacteria isolated from *A. tuberculosa* on the immune response and viral resistance in shrimp.

2. Materials and methods

2.1. Experimental shrimp

A batch of apparently healthy shrimp (1 ± 0.1 g) was collected from a commercial farm (Acuicola Cuate Machado, Guasave, Sinaloa, Mexico). It was transported to the laboratory facilities of Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR)-Sinaloa in plastic containers provided with seawater and aeration. Shrimp were screened by single or nested PCR to determine either WSSV or IHHNV prevalence (as explained later). The collected shrimp carried the WSSV but with low viral charge (nested PCR).

2.2. Shrimp acclimation to culture system

Shrimp were acclimated to culture conditions in 120-L outdoor plastic tanks (10 shrimp/tank) containing 80 L of filtered (20 μm) seawater (30‰) and constant aeration for 2 days. Shrimp were fed ad libitum twice daily at 0900 and 1700 h with commercial feed (Camaronina ® Purina, Vevey, Suiza, 35% protein).

2.3. Preparation of experimental diets with potential probiotic bacteria

A mixture (1:1:1) of potential probiotic bacteria (*Bacillus licheniformis* Mat32), *B. subtilis* MAt43, and *B. subtilis* GAtB1), isolated and characterized by Ref. [22]was used. Strains used were grown in tryptic soy broth (22092 Sigma-Aldrich) with 2.5% NaCl for 24 h at 35 °C. The number of viable bacteria for feed inoculation was determined based on the count of CFU per milliliter of a bacterial suspension (2.5 NaCl) with an optical density of 1 [22]. The bacterial mixture was sprayed on commercial feed at 1×10^6, 2×10^6, 4×10^6, and 6×10^6 CFU g feed^{-1}, using Dry Oil (DO, Innovaciones Acuícolas, Culiacán, Mexico) as an adhesive and feed attractant following the manufacturer's instructions. Feed was dried according to previous viability studies [23] at room temperature and stored at 4 °C until use.

2.4. Preparation of WSSV paste

A batch of shrimp was infected experimentally with WSSV by injection. After infection, symptomatic shrimp were collected and stored at −80 °C. Shrimp were thawed, and abdominal muscle and gills were dissected and cut into fine pieces with a scalpel. Tissues were chopped and then used to feed shrimp during the bioassay. Before supplying, the tissue was analyzed by single PCR to confirm the high viral load.

2.5. Experimental design

A 32-day bioassay was performed with the same shrimp (10 per tank, 1 ± 0.1 g) and conditions of the culture system from the acclimation period. Animals were fed twice a day at 0900 and 1700 h according to their weight (10–13%). Animals were selected at random and treatments were done in triplicate: (T1) control, commercial feed; (T2) commercial feed + bacterial mixture at 1×10^6 CFU g^{-1} feed; (T3) commercial feed + bacterial mixture at 2×10^6 CFU g^{-1} feed; (T4) commercial feed + bacterial mixture at 4×10^6 CFU g^{-1} feed; (T5) commercial feed + bacterial mixture at 6×10^6 CFU g^{-1} feed. Shrimp were infected three times, at day 12 (0.250 g of WSSV-shrimp chopped/tank, nested PCR). Reinfection was done at day 24 as in day 12. Uneaten food and waste were removed by siphoning every three days, and lost water was replaced. 50% water exchange was done every six days. Every three days, temperature, oxygen (oximeter YSI model 55, Yellow Springs Instruments, Yellow Springs, OH, USA), salinity (refractometer W/ATC 300011, Sper Scientific, Scottsdale, AZ, USA), and pH (pHep HI 98127, Hanna Instruments, Woonsocket, RI, USA) were monitored. Ammonia, nitrites, and nitrates were determined at the beginning and at the end of the bioassay.

2.6. Growth performance

Weight was measured at the beginning and at the end of the bioassay in order to obtain specific growth rate. Specific growth rate (SGR) was calculated as:

$$\mathrm{SGR}(\%/\mathrm{day}) = \left(\ln W_f - \ln W_i\right) \times 100/\mathrm{t} \qquad 1$$

Where: W_i = initial weight and W_f = final weight.

2.7. Viral prevalence

At the end of the experiment twelve shrimp per treatment (four per tank) were used to determine WSSV prevalence. DNA was extracted individually from a mix of gills and pleopods with DNAzol® reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Viral detection was performed by single and nested PCR, using the primers WSSV out-1/WSSV out-2 and WSSV in-1/WSSV in-2 [47], which amplified genome fragments of 982 and 570 bp, respectively. IHHNV detection was performed by single PCR, using the primers IHHNV392F and IHHNV392R [24], which amplified a genome fragment of 392 bp. Negative samples were tested with an internal control that amplified a 298 bp segment of shrimp GAPDH DNA using the primers GAPDH298F and GAPDH298R by one-step PCR [24].

2.8. Hemolymph extraction and hemocyte sampling

Hemolymph (100 μL) of each intermolt shrimp (6 per treatment) was extracted from the pleopod base of the second abdominal segment with 1-mL syringes (27G × 13 mm needle) loaded with

600 μL of a precooled (4 °C) anticoagulant solution (27 mM trisodium citrate $Na_3C_6H_5O_7$, 385 mM NaCl, 115 mM glucose, at pH 7.5). Hemolymph was centrifuged at 800 g for 10 min at 4 °C. Plasma was removed, and hemocyte pellets were rinsed twice with 250 μL of cold anticoagulant (4 °C) by centrifugation, as above. The supernatant was removed, and hemocytes were suspended in 300 μL of precooled TRIzol Reagent (MRC, Cincinnati, OH, USA) and stored at -70 °C until total RNA extraction.

2.9. Hepatopancreas sampling

A fragment of hepatopancreas from the shrimp were dissected and placed individually in 600 μl of precooled Trizol and stored at -70 °C until total RNA extraction.

2.10. Total RNA isolation and cDNA synthesis

Total RNA was extracted from hemocytes according to the protocol of TRI Reagent (MRC) and treated with DNAse I (Sigma St. Louis, MO, USA). Quantification and quality determination of total RNA was performed in a nanophotometer (IMPLEN Inc., Westlake Village, CA, USA) and RNA integrity was verified by electrophoresis on 1.2% agarose gel.

For cDNA synthesis, reverse transcriptase (Improm II, Promega, Madison, WI, USA) was used to synthesize the first strand (cDNA) with oligo dT20 primer using 500 ng of total RNA. cDNA was suspended in 130 μL of ultrapure water for a total volumen of 150 μl of cDNA and stored at -70 °C until use as template in the quantitative real-time PCR (RT-qPCR).

2.11. qRT-PCR gene expression

The relative expression of four immune-related genes (prophenoloxidase [proPO], Toll receptor [LvToll], transglutaminase [TGase], an antioxidant-related gene superoxide dismutase [SOD]), and four housekeeping genes (EF1α, L21, β-actin, and 40S-S24) were determined in hemocytes; and the stress-related heath shock protein 70 (Hsp70) gene was determined in hepatopancreas (Table 1).

Relative expression was performed in a CFX96 Touch Real-Time System (Bio-Rad®) with the software CFX Manager Bio-Rad® Versión 2.1. Amplifications were performed in triplicate in 96-well plates. The PCR reactions contained 7.5 μl of PCR Master Mix 2X (1.5 μl 10 × reaction buffer 0.75 μl $MgCl_2$ 50 mM 0.1 μl DNA Polymerase 5U/μl; 0.3 μl dNTPs 10 mM [Bioline™, Taunton, MA, USA] 0.75 μl EvaGreen® 20 × [Biotium, Hayward, CA, USA]), 0.35 μl each primer 10 μM (Sigma® Aldrich, St. Louis, MO, USA), 5 μl cDNA, and ultrapure water to achieve a final volume of 15 μL. Thermocycler conditions were as follows: 5 min at 95 °C, 40 cycles of 10 s at 95, 15 s at 60 °C, 30 s at 72 °C, and 5s at 79 °C (to acquire fluorescence). After each reaction, a dissociation curve from 65 °C to 90 °C was recorded at increments of 0.5 °C to confirm unique and specific products. Efficiency of the PCR reactions was determined by calculating a slope with five serial dilutions (dilution factor of 5 or 10), of a representative pool of cDNA of all samples [E = 10 (-1/slope) - 1].

To determine most stable reference genes, the relative expression of housekeeping genes were analyzed with web application RefFinder (http://www.leonxie.com/referencegene.php), using GeNorm and NormFinder algorithms. For the reference genes, only those with major stability were used for the gene expression analysis. EF1-α was the least stable gene for hemocyte and hepatopancreas samples, so it was discarded.

To calculate the expression of target genes, Cq values were transformed to relative quantities (RQ) using the equation:

$$RQij = E(Cq(mean) - Cq(ij)) \quad (2)$$

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

Table 1
Sequences of primers used for conventional PCR and quantitative real time PCR.

Conventional PCR			
Tissue	Target sequence gene	Sequence 5′-3′	Reference
Gill and pleopod	WSSV Out	F ATC ATG GCT GCT TCA CAG AC R GGC TGG AGA GGA CAA GAC AT	[47]
	WSSV In	F TCT TCA TCA GAT GCT ACT GC R TAA CGC TAT CCA GTA TCA CG	[47]
	IHHNV	F GGG CGA ACC AGA ATC ACT TA R ATC CGG AGG AAT CTG ATG TG	[48]
	GAPDH	F TCA CCG TCT TCA ACG AGA TG R ACC CTC CAG CAT CTC GAA CT	[50]
RT-qPCR			
Hemocytes	Superoxide dismutase	F ATC CAC CAC ACA AAG CAT CA R AGC TCT CGT CAA TGG CTT GT	[31]
	proPhenoloxidase	F GAG ATC GCA AGG GAG AAC TG R CGT CAG TGA AGT CGA GAC CA	[31]
	LvToll	F ATG TGC GTG CGG ATA CAT TA R GGG TGT TGG ATG TCG AGA GT	[31]
	Transglutaminase	F CCT CAG GAT CTC CTT CAC CA R TTG GGA AAA CCT TCA TTT CG	[31]
Hepatopancreas	HSP70	F CTC CTG CGT GGG TGT GTT R GCG GCG TCA CCA ATC AGA	[49]
Housekeeping	EF-α	F CTG TGG TCT GGT TGG TGT TG R TCG GAT GAG TTC TTG GGT TC	
	β-actina	F CCA CGA GAC CAC CTA CAA C R AGC GAG GGC AGT GAT TTC	[31]
	L21	F GTT GAC TTG AAG GGC AAT G R CTT CTT GGC TTC GAT TCT G	[49]
	40S-S24	F CAG GCC GAT CAA CTG TCC R CAA TGA GAG CTT GCC TTT CC	

the dataset for each gene. Relative expression was calculated with the next equation according to (RQ-target)/Geometric mean of the most stable reference genes (RQ-reference genes).

2.12. Statistical analysis

A one-way analysis of variance (ANOVA) using the *F* test was used to examine differences in AG, SGR, viral prevalence and relative gene expression. Values of $p < 0.05$ were considered as significantly different. Results given as percentages were arcsine transformed. When significant differences were found, the Tukey test was performed to identify the source of these differences ($p < 0.05$). All statistical analyses were performed using Statistica 7.0 software (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Growth performance

Shrimp treated with probiotics showed no significant differences in final growth ($p > 0.05$). In T2 group, the specific growth rate (SGR) of shrimp was significantly higher than in T1 group (control) ($p = 0.029$) and T4 group ($p = 0.0009$). A significant difference was found in SGR among group T4 with T2 ($p = 0.001$), T3 ($p = 0.006$), and T5 groups ($p = 0.008$) (Table 2).

3.2. Survival and viral prevalence

Shrimp survival was high (98.9 ± 1.92 to 100± 0.0%) in all treatments and no significant differences were found among treatments ($p > 0.05$). Initial stock showed 50% of shrimp infected with WSSV and 100% with IHHNV. After 32 days of culture, the WSSV prevalence in treatment T2 was significantly lower than all treatments. Similarly, the IHHNV prevalence in treatment T4 was significantly lower than all treatments (Table 3).

Table 2
Growing performance summary of *Litopenaeus vannamei* cultures with different concentrations of probiotic mix. Values represent mean ± SE. Wi = initial weight, Wf = final weight, SGR = specific growth rate. Different letters represent significant difference (p < 0.05). T1 = Control, T2 = 1×10^6 CFU gr-1, T3 = 2×10^6 CFU gr-1, T4 = 4×10^6 CFU gr-1, T5 = 6×10^6 CFU gr-1.

Treatment	Wi (g)	Wf (g)	SGR (% d^{-1})
T1	0.98 ± 0.06	4.02 ± 0.35	5.03 ± 0.03^{ab}
T2	0.98 ± 0.07	4.25 ± 0.40	5.25 ± 0.19^{c}
T3	0.96 ± 0.07	4.07 ± 0.40	5.14 ± 0.08^{bc}
T4	0.96 ± 0.06	3.75 ± 0.36	4.85 ± 0.07^{a}
T5	0.97 ± 0.06	4.08 ± 0.32	5.12 ± 0.09^{bc}

Table 3
Summary of survival and viral prevalence for *Litopenaeus vannamei* after 32 days of culture. Different letters represent significant difference (p < 0.05). T1 = Control, T2 = 1×10^6 CFU·gr-1, T3 = 2×10^6 CFU·gr-1, T4 = 4×10^6 CFU·gr-1, T5 = 6×10^6 CFU·gr-1.

Treatment	Survival (%)	Viral prevalence (%)[a]			
		$WSSV_i$	$WSSV_f$	$IHHNV_i$	$IHHNV_f$
T1	98.9 ± 0.00	50	42^{bc}	100	75^{b}
T2	98.9 ± 1.92	50	25^{a}	100	75^{b}
T3	100 ± 0.00	50	67^{c}	100	67^{b}
T4	98.9 ± 1.92	50	67^{c}	100	33^{a}
T5	98.9 ± 1.92	50	42^{bc}	100	75^{b}

[a] $WSSV_i$ = Initial WSSV prevalence; $WSSV_f$ = Final WSSV prevalence; $IHHNV_i$ = Initial IHHNV prevalence; $IHHNV_f$ = Final IHHNV prevalence.

3.3. Relative gene expression analysis

The SOD, proPO, LvToll, and Hsp70 gene expressions were modulated but not that of the TGase gene.

The mRNA expression of SOD gene was up-regulated in treatment T5 ($p = 0.018$) than in treatments T1 (control), T2, T3, and T4 (Fig. 1). The mRNA expression of proPO gene was higher in treatment T4 than in treatments T1 (control) ($p = 0.016$) and T2 ($p = 0.008$) (Fig. 2a). Similarly, the LvToll gene was significantly up-regulated in treatments T4 ($p = 0.003$) and T5 ($p = 0.001$) compared to treatments T1 (control), T2, and T3 (Fig. 2b). Although slight modifications were observed, there was no significant influence of the level of bacillus probiotic mix on the expression of TGase gene (Fig. 3).

In contrast, the mRNA expression of the Hsp70 gene was down-regulated in treatments T4 ($p = 0.037$) and T5 ($p = 0.037$) compared to treatments T1, T2, and T3 (Fig. 4).

4. Discussion

In this work, the effect of combined bacillus species (*B. subtilis* and *B. licheniformis*) on survival, growth, and immune response of white shrimp infected with WSSV and IHHNV was studied since the above mentioned species have been studied as probiotics for shrimp farming [19].

Substances or probiotics applied to shrimp culture systems or feed should not affect their productive performance. In our study, bacillus probiotic mix did not affect shrimp survival. Moreover, the growth was positively affected by the administration of bacilli at 1×10^6 UFC g^{-1} feed. Previously, we observed a higher specific growth rate in shrimp treated with bacteria mix isolated from pustulose ark *A. tuberculosa* [22]. Similar results on growth performance in *Penaeus latisulcatus* fed mixed probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) have been reported [25]. In addition, in *L. vannamei* a significant increase in the final weight and the average daily gain have been obtained using 1×10^7 UFC g^{-1} of live *Bacillus coagulans* (a similar dose used in our study), unlike the control group fed with the basal diet or dead bacteria [26]. More recently, a mechanism of action of probiotic bacteria that showed increased growth of *L. vannamei* was an associated effect of high production of lytic enzymes and digestibility of the diet [27]. Therefore, our results and previous reports support the use of

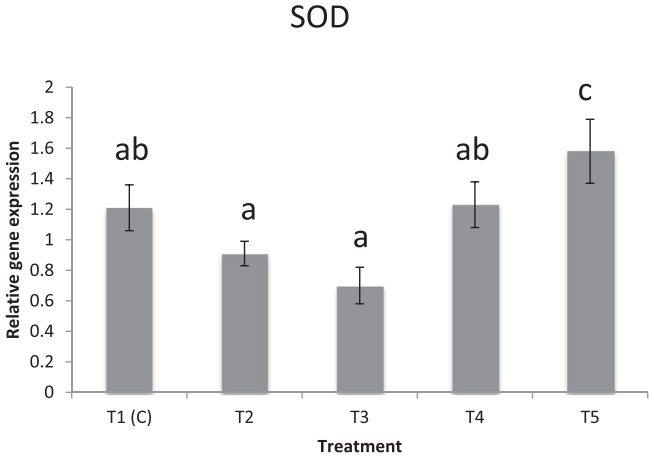

Fig. 1. Relative gene expression for (a) prophenoloxidase (proPO) and (b) receptor toll-like (LvToll) in hemocytes. T1 = Control, T2 = 1×10^6 CFU gr-1, T3 = 2×10^6 CFU gr-1, T4 = 4×10^6 CFU gr-1, T5 = 6×10^6 CFU gr-1. Data represents average relative gene expression ± SE. Uppercase letters represent significant differences (p < 0.05).

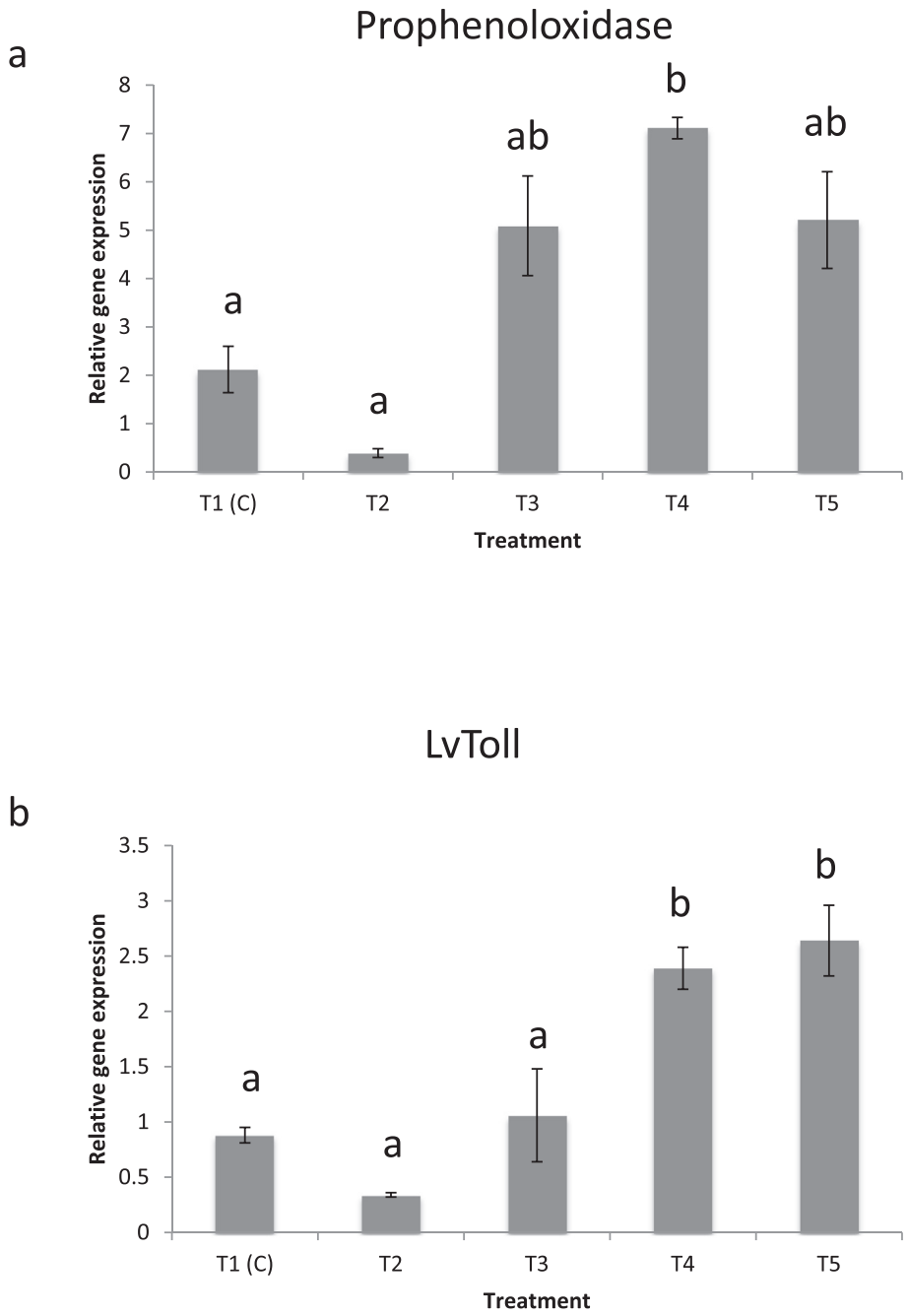

Fig. 2. Relative gene expression for transglutaminase (TG) in hemocytes. T1 = Control, T2 = 1×10^6 CFU·gr-1, T3 = 2×10^6 CFU gr-1, T4 = 4×10^6 CFU gr-1, T5 = 6×10^6 CFU gr-1. Data represents average relative gene expression ± SE. Uppercase letters represent significant differences ($p < 0.05$).

Bacillus species as potent growth promoters for shrimp [16].

Among the diseases that infect shrimp, viral infections with white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) are the most widespread and prevalent [28]. Despite no clinical signs are observed, a dual infection is often present. For example, Otta et al. [29] reported high percentage (60%) of co-infection with WSSV-IHHNV in *L. vannamei*. In our study, treatment T2 and T4 of bacillus probiotic mix reduced WSSV and IHHNV prevalence, respectively, compared to control. Our results agree with the findings of Leyva-Madrigal et al. [23] in *L. vannamei* since they found a decrease in the prevalence of WSSV in shrimp fed with probiotic lactic acid bacteria. Moreover, lobsters (*Panulirus ornatus*) fed with bacilli probiotic mix, which bacteria were isolated from different marine animals, were able to increase the survival of experimental infected animals [18]. In the above study, the mix probiotic formulation (composed of *Bacillus pumilus*, *B. cereus,* and *Lactobacillus plantarum*) improved the growth rate and feed conversion ratio of *P. ornatus* juveniles and resulted in higher survival after exposure to the pathogen *Vibrio owensii* compared with a single-strain probiotic preparation (*Bacillus pumilus*) [18]. Interestingly, Rengpipat et al. [30] found that the *Bacillus* S11 strain can protect *P. monodon* from microbial infection by the activation of immune defenses, suggesting that bacterial cell wall components (peptidoglycans and lipopolysaccharides) could be the main responsible for the immunostimulatory effect. In addition, Chai et al. [17] found higher survival of naturally or

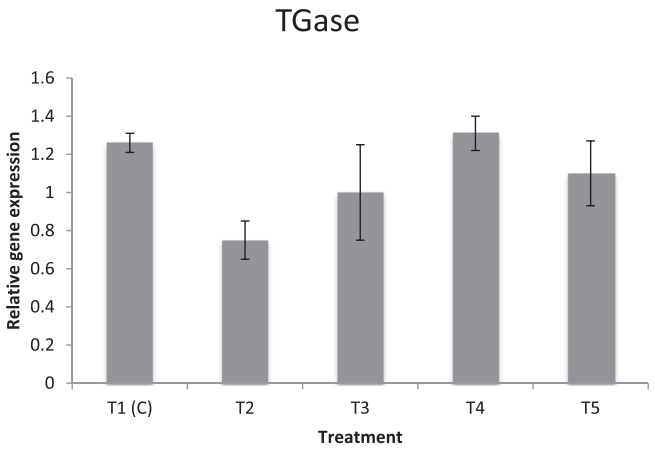

Fig. 3. Relative gene expression for super oxide dismutase (SOD) in hemocytes. T1 = Control, T2 = 1 × 10^6 CFU gr-1, T3 = 2 × 10^6 CFU gr-1, T4 = 4 × 10^6 CFU gr-1, T5 = 6 × 10^6 CFU gr-1. Data represents average relative gene expression ± SE. Uppercase letters represent significant differences ($p < 0.05$).

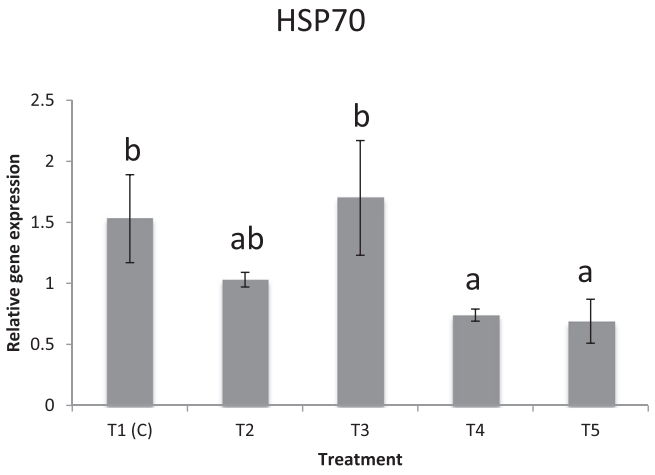

Fig. 4. Relative gene expression for heat shock protein 70 (Hsp70) in hepatopancreas. T1 = Control, T2 = 1 × 10^6 CFU gr^{-1}, T3 = 2 × 10^6 CFU gr^{-1}, T4 = 4 × 10^6 CFU gr-1, T5 = 6 × 10^6 CFU gr-1. Data represents average relative gene expression ± SE. Uppercase letters represent significant differences ($p < 0.05$).

experimentally WSSV infected shrimp fed with *Bacillus* sp. PC465 strain compared to control shrimp. Therefore, a likely response may have occurred in our study by the combination of *Bacillus licheniformis* Mat32, *B. subtilis* MAt43, and *B. subtilis* GAtB1 strains in fed shrimp. However, we can observe that this effect was not dose-dependent. Apparently, increasing dosage does not necessarily improve protection. Modulatory effects could be related with the dose of probiotic in shrimp which may vary with the shrimp genetic background, age, farming system, environmental conditions, feeding time, among others. For example, Liu et al. [34] found that the optimal dose of the single probiotic *B. subtilis* strain S12 as an effective immunopotentiator was 5 × 10^{10} CFU kg^{-1} instead of 5 × 10^9 CFU kg^{-1} or 5 × 10^{11} CFU kg^{-1} diet in *L. vannamei* infected with *Vibrio harveyi*. Similarly, two *B. subtilis* strains (L10 and G1 in equal proportions) added into the rearing water (1 × 10^5 UFC ml^{-1} and 1 × 10^8 UFC ml^{-1}) showed decreased *Vibrio* spp. count in culture water (1 × 10^5 UFC ml^{-1}) and reduced mortality after experimental infection with *V. harveyi* (1 × 10^8 UFC ml^{-1}) in *L. vannamei* [20], suggesting the different effects of the dose and route of administration of probiotic mix in shrimp. In comparison, in a previous study, we found that the combination of probiotics containing a *B. subtilis* strain promoted the growth than single probiotic strains; being *B. subtilis* the responsible of the enhanced hemocyte counts in *L. vannamei* [22]. Similar positive results have been found after oral administration of combined (1 × 10^8 UFC g^{-1}) probiotics, *B. licheniformis and B. subtilis*, in *Penaeus japonicus* shrimp [19]. Overall, and considering that the final WSSV viral prevalence in the treatment T3 and T4 was higher than that in the control, the optimal dose of *Bacillus* probiotic strains must be established and more studies should be carrying out to elucidate the effect of combined probiotics on viral disease prevalence in shrimp.

The immune system of shrimp mainly consists of hemocytes that are activated by probiotics or infectious agents to regulate and generate effector immune responses. In our study, differences in the transcript level of immune-related genes in hemocytes were found in shrimp as a response of bacillus probiotic mix and the viral infections. The immunomodulatory gene expressions in shrimp fed with probiotics have been related with physiological functions that help in control of infectious diseases [31]. One of such responses is phagocytosis that produces reactive oxygen species (ROS) during microbial invasion, such as superoxide anion (O_2^-) that is converted in H_2O_2 by superoxide dismutase (SOD). In L. *vannamei*, the SOD has been used as a key parameter to evaluate the antioxidant and immune responses [32,33]. Here, the SOD gene in shrimp was significantly up-regulated with the highest dose of bacilli probiotic mix (treatment T5). Similarly, the up-regulation of SOD gene expression has been associated with disease resistance and increased phagocytosis activity in shrimp fed with *Bacillus* spp [34]. Since all treatments in our study were WSSV-IHHNV infected, our results suggest that SOD mRNA upregulation could be related to respiratory burst activity as a consequence of bacillus probiotic mix added to feed.

The prophenoloxidase (proPO) system plays an important role in shrimp immune response and defence against microbial pathogens, involving an enzymatic cascade that is regulated at transcriptional and translational levels [35]. In our study, the proPO gene expression was higher in treatments T3, T4, and T5 compared to control and T2 treatment. It is known that the up-regulation of proPO in *L. vannamei* fed with microbial immunostimulants is an induced response [36]. Similar to our results, the proPO gene was up-regulated and correlated with protection in *Bacillus PC465*-fed shrimp naturally and experimentally WSSV infected [17]. Interestingly, a probiotic bacilli mix composed of two *B. subtilis* strains (L10 and G1) into the rearing water also upregulated ProPO gene expression, and the immune resistance against *Vibrio harveyi* infection in *L. vannamei* [20]. In other study, shrimp fed with the probiotic *B. subtilis* E20-containing diets enhanced ProPO activity and resistance to *V. harveyi* infection [15]. Moreover, the mRNA proPO expression was also upregulated in shrimp when a probiotic mix composed of *Bacillus* sp. and *Lactobacillus* sp. was added into a bio-flock system [21]. Altogether, the results suggest that different delivery methods can be employed using bacilli probiotic mix, especially those composed of *Bacillus* species; and enhanced proPO gene expresion and disease resistance may be observed, which is relevant since the WSSV can down-regulate the proPO gene expression in shrimp as a strategy of immune evasion [37].

Toll-like receptor signaling is essential in mediating immune response and is activated according to bacterial or viral stimulation. It is knwon that Toll receptors play a significant role in shrimp host-response to WSSV [38]; however, despite that LvToll1 play an important role in phagocytosis, it is not fully clear its role during WSSV infection in shrimp [39,40]. In our work, the mRNA expression of LvToll1 was upregulated in treatments with the higher concentration of bacilli probiotic mix (T4 and T5). Similarly, the

TLR1 was up-regulated in the intestine and hepatopancreas of *Lactococcus lactis* probiotic strain D1813-fed kuruma shrimp [41]. Therefore, probiotics seems activate or enhance the systemic innate immunity of shrimp by LvToll1, and other TLRs, to respond against microbial invaders. In contrast, TGase and HSP70 gene expressions were not affected or down-regulated, respectively, by bacilli probiotic mix. Our results are similar to those found in brine shrimp (*Artemia franciscana*) fed with *Bacillus* sp. LT3 and challenged with *Vibrio campbellii* [42], in which TGase and HSP70 gene expressions remained unafected and downregulated, respectively. More studies are needed to elucidate the biological implications of probiotics on TGase and HSP70 expression responses. Moreover, studies of early and long-lasting elicited immune responses by combined probiotics, including local and systemic responses are necessary [18]. Remarkably, combined probiotic strains are promising for shrimp aquaculture; and recently Miandare et al. [43] demonstrated that a commercial multi-strain probiotic (PrimaLac®) enhanced the expression of immune related genes and improved the performance of *Litopenaeus vannamei*. Interestingly, *B. subtilis* has been used as both probiotic for human and animals; being able to stimulate proliferation of immune cells within the gut-associated tissue and promote potent immune responses [44]. Recently, *Bacillus subtilis* spores expressing Vp26 or Vp28 recombinant antigens on its surface demonstrated the great capacity of those antigens to induce immune protection against WSSV in orally vaccinated *L. vannamei* [45,46], leading a potential new biotechnological avenue for oral vaccine development using probiotic mix in shrimp aquaculture.

5. Conclusions

The bacilli probiotic mix (*Bacillus* spp.) induced immune responses able to affect the prevalence of WSSV and IHHNV naturally infected shrimp. Bacilli probiotic mix in viral infected *L. vannamei* induced the proPO, SOD, and LvToll immune-related genes. Therefore, future studies on feeding time and optimum doses could enable bacilli combined probiotics to improve immunity of *L. vannamei* to fight against naturally occurring viral diseases.

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