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Effects of dietary *Bacillus subtilis* and *Shewanella algae* in expression profile of immune-related genes from hemolymph of *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus*

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ABSTRACT

B. subtilis and *S. algae* effects in growth, survival and innate immunity were assessed on *L. vannamei* juveniles. During 60 days, shrimp were reared in three treatments: Bs, fed with 10^6 CFU of *B. subtilis* per gram of commercial feed, Sa, fed with 10^6 CFU of *S. algae* per gram of commercial feed and Control (without bacterial addition). Then, the animals were subjected to a *V. parahaemolyticus* challenge. For this purpose, four treatments were established: Control (shrimp not submitted to probiotic treatments), Vibrio (*Vibrio* challenged shrimp), Vibrio + Bs (Bs challenged shrimp) and Vibrio + Sa (Sa challenged shrimp). Shrimp hemolymph was sampled 45-days after rearing and 24 h post-challenge for quantification of prophenoloxidase (proPO), lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) and hemocyanin (HEM) transcripts by qPCR. Moreover, shrimp final weight and survival were also verified. *B. subtilis* administration enhanced shrimp growth and improved proPO, LGBP and HEM expression levels before and after challenge. After 60-days of feeding, Sa final weight was higher than the Control, whereas Vibrio + Sa cumulative mortality after 48 h of *Vibrio* challenge was lower than Vibrio group. These results could be correlated with the proPO and LGBP up regulation in Vibrio + Sa compared to Vibrio group, protecting *L. vannamei* from the bacterial infection. Together, these results suggest the probiotic potential of *B. subtilis* e *S. algae* in the modulation of immune-related genes as a tool to control *V. parahaemolyticus* infection inside shrimp.

1. Introduction

Despite the remarkable growth in world production of cultivated shrimp, there are great mortalities due to diseases [1]. In this concern, infectious diseases are the most significant cause of economic losses in shrimp farms [2]. Disease outbreaks are particularly caused by the main shrimp pathogens: white spot syndrome virus (WSSV), yellow headed virus (YHV), infectious myonecrosis virus (IMNV), and *Vibrio* bacteria [3,4]. Some *Vibrio* species can cause vibriosis (e.g. *V. alginolyticus* and *V. harveyi*), as well as it was recently discovered early mortality syndrome (EMS), which is closely related to *V. parahaemolyticus* [5,6].

Once foreign particles enter the haemocoel, shrimp engage an innate immune response that includes cellular and humoral reactions [7]. The main immune reactions occur in hemolymph, which contain three different hemocytes types: hyaline, granular and semigranular [8]. Several immune molecules are produced and stocked in the granules of the hemocytes before they are released into the hemolymph after activation by cell wall components of bacteria and/or fungi, such as peptidoglycan, lipopolysaccharides and β-glucans [9].

Pattern recognition proteins recognize and bind microbial cell wall components and activate different immune response [10,11]. The innate immunity system of shrimp protects from pathogenic infections

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and mainly consists in prophenoloxidase (proPO) system, coagulation system, phagocytosis, encapsulation and nodule formation, antimicrobial peptides formation and cell agglutination [12,13].

Previous studies report *Bacillus* species as able to induce the innate immune system of crustaceans [14,15]. Indeed, *Bacillus* spp. is often used as a beneficial microorganism in aquaculture systems once can added to health-promoting functional foods as therapeutic, prophylactic, and growth supplements [16]. The oral administration of *Bacillus* PC465 enhanced the growth performance and survival of *L. vannamei*, improved digestion and nutrient absorption, microbial structures in the gut, immune status and response to viral infection [17]. The probiotic supplementation significantly increased the transcription of prophenoloxidase enzyme (proPO), penaeidin 3a (Pen-3a) an antimicrobial peptide, peroxinectin, a cell adhesion molecule, C-type lectin 3 (Lec-3), a pattern recognition receptor, and thioredoxin (Trx) antioxidant enzyme in *L. vannamei* hemocytes. Likewise, probiotic treatment increased the transcription of hemocyanin in *L. vannamei* hepatopancreas [17].

Shewanella spp., e.g. *Shewanella algae* [18], *Shewanella putrefaciens* e *Shewanella baltica* [19] has also been used as probiotics to increase nutritional and disease resistance [20]. According to Chabrillón et al. [21] and Shakibazadeh et al. [22], different species of the genus *Shewanella* have antagonistic abilities that affect *Vibrio* species and can further modulate immunological genes from animals e.g. fish from rearing [23], however, studies about stimulation of shrimp immune genes by *Shewanella* spp. are still scarce. Taking into account all these previous considerations, the aim of the current study was to compare the dietary effects of *B. subtilis* and *S. algae*, isolated from *L. vannamei* gut reared in Brazilian northeast, on *L. vannamei* juveniles growth, innate immune and *V. parahaemolyticus* challenge resistance.

2. Materials and methods

2.1. Candidate probiotics

The probiotic candidate strains *Bacillus subtilis* (IPA-S.51) and *Shewanella algae* (IPA-S.252) were provided by Instituto Agronômico de Pernambuco (IPA). Both strains were previously isolated from the hepatopancreas/stomach of *L. vannamei* grown at 30 $g\,L^{-1}$ of salinity. Its 16S rRNA gene region was sequenced with fD1 (forward) (5′- AGAGT TTGATCCTGGCTCAG-3′) e rD1 (reverse) (5′- AAGGAGGTGATCCA GCC-3′) and then the sequences were compared with GenBank, National Center for Biotechnology Information (NCBI) through BLASTn program. *B. subtilis* and *S. algae* were grown in Tryptic Soy Broth (TSB) at 30 °C during 48 h. After that, bacterial cells were collected by centrifugation ($1800 \times g/20$ min) and then suspended in sterile sea water for further addition in shrimp feed.

B. subtilis (IPA-S.51) and *S. algae* (IPA-S.252) probiotic potential was previously confirmed by screening the *in vitro* inhibition against *Vibrio* spp. pathogens and analyzing the probiotic effects on *L. vannamei* juveniles fed with the strains [24].

2.2. L. vannamei feed

Experimental feed were prepared adding *B. subtilis* and *S. algae* separately to a commercial pelleted feed with 40% of crude protein (Camaronine 40CR2, Purina, Agribrands do Brasil SA, São Paulo, Brazil) (adapted from Jiang et al. [25]). At the end of feed preparation the bacteria concentration in the mixture was approximately 10^6 Colony Forming Units (CFU) g^{-1}. The bacteria CFU g^{-1} of feed was according to previous study with *B. subtlis* (IPA-S.51) and *S. algae* (IPA-S.252) offered to *L. vannamei* juvenile shrimps [24]. After feed preparation, the *B. subtlis* (IPA-S.51) and *S. algae* (IPA-S.252) concentration in the diet was confirmed by plating technique, in which a sample of each feed was serially diluted (1/10) in saline solution (2% NaCl) and grown on Tryptone Soy Agar (TSA supplemented with 2% NaCl) for 24 h at 30 °C. The feed was stored for a maximum period of 72 h at 4 °C, until use. After 72 h, the probiotics concentration in the feed was inferior to 10^6 CFU g^{-1}.

2.3. Shrimp and rearing trials

L. vannamei postlarvae (PL10) were obtained from a commercial larviculture (Aquatec®), located in Canguaretama, Rio Grande do Norte and transferred to LTA/UFRPE (Laboratório de Tecnologia em Aquicultura/Universidade Federal Rural de Pernambuco). The animals were acclimatized in a 1000 L tank, until reaching a weight of 1.05 ± 0.25 g. After, shrimp were stocked in (width x length x height = 0.48 m × 0.56 m x 0.89 m) polypropylene tanks containing 50 L of seawater (23 $g\,L^{-1}$) with constant aeration and maintained at 28 - 29 °C through heaters with thermostat. Shrimp were distributed in three treatments with four replicates each: Bs, fed with *B. subtilis*; Sa, fed with *S. algae* and Control, without bacterial addition. Each group included four replicates. The stocking density was 130 shrimp m^{-2} and 25% of the water was changed daily. During the whole experiment (60 days), shrimp were fed four times a day. The amount of feed supplied was equivalent to 10% of the biomass of each tank. The shrimp final weight was measured after 60 rearing days.

2.4. V. parahaemolyticus challenge

Vibrio parahaemolyticus (ATCC 17802) was grown in Tryptone Soy Broth (TSB supplemented with 2% NaCl, Difco) for 24 h at 30 °C before being centrifuged at 1800 *g* for 20 min. Fluid supernatants were removed and the pellets re-suspended in sterile saline (2% NaCl) in order to stabilize a stock solution for infection assays in a concentration between 3×10^7 and 1×10^7 CFU mL^{-1}.

For the *V. parahaemolyticus* challenge, ten shrimp from each treatment reared for 60 days were distributed in three experimental tanks of polypropylene (the same used for the entire experiment), the water aeration and temperature were maintained as described for the rearing. The challenge was performed by injecting 100 µl of a *V. parahaemolyticus* suspension in shrimp third abdominal segment. The final concentration of the injected solution was between 3×10^6 and 1×10^6 CFU.mL^{-1}. The *V. parahaemolyticus* (ATCC 17802) concentration for the challenge was according to Peña-Navarro and Varela-Mejías [26].

Four treatments were established: animals fed without bacterial addition and challenged with *V. parahaemolyticus* (Vibrio), animals fed with *B. subtilis* and challenged with *V. parahaemolyticus* (Vibrio + Bs), shrimp fed with *S. algae* and challenged with *V. parahaemolyticus* (Vibrio + Sa) and Control, where animals not submitted to treatments with the experimental diets were injected with 100 µl of sterile saline solution to evaluate a possible mortality due to the injection procedure. Shrimp cumulative mortality was recorded after 24, 48, 72 and 96 h of *V. parahaemolyticus* challenge.

2.5. Quantitative RT-PCR (qRT-PCR)

The hemolymph of eight animals were sampled at the end of the 45 and 60 rearing days and 24 h after *V. parahaemolyticus* challenge via injection for determination of immune related genes expression. The hemolymph samples were collected by sterile syringes of 1 ml.

Quantitative RT-PCR (qRT-PCR) was performed using three biological samples for each group (Controls, with probiotics and infected with *V. parahaemolyticus*), each obtained from a pool of eight shrimp. The amplifications were performed in a 96-well plate and each individual sample was run in duplicate wells. A negative control with ultrapure water were included for all PCR screens.

Total RNA was extracted from the hemolymph using TRIzol reagent (Invitrogen) and purified using the RNeasy mini kit (Qiagen, Germany). After that, it was quantified using NanoVue equipment (GE Healthcare). The RNA concentration used was 200 ng cDNA synthesis was performed

using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's guidelines. Quantitative RT-PCR was performed following the methods described by Livak and Schimittgen [27] for delta delta Ct calculations to conduct relative quantification of the transcripts. The calibrator was the control. Hemolymph cDNAs from *L. vannamei* were quantified using SYBR Green PCR Master Mix (Applied Biosystems) in a Step One Plus PCR System (Applied Biosystems). The qRT-PCR reaction consisted of 1 μL of 10-fold diluted cDNA (5 ng), 12.5 μL of SYBR Green and 0.2 μM of each primer in a 25 μL total volume. β-actin was used as the internal control. The PCR program comprised 40 cycles at 94 °C (15 s) and 60 °C (1 min), followed by melt curve generation. Melt curves were analyzed to check the specificity of amplification.

Gene-specific primers were designed as: prophenoloxidase (proPO), forward primer, 5′-CGGTGACAAAGTTCCTCTTCG-3′ and reverse primer, 5′-TGCAGGTCGCCGTAGTAAG-3' [28]; lipopolysaccharide and β-1,3-glucan-binding protein (LGBP), forward primer, 5′-CATGTCCAA CTTCGCTTTCAGA-3′ and reverse primer, 5′- GCTCCGTAGGGCCAGT TAC-3' [29], β-actin (B-ACT), forward primer, 5′- CCACGAGACCACC TACAAC-3′ and reverse primer, 5′-TCCTTCTGCATCCTGTCGG-3' (GenBank no.: AF300705) and hemocyanin (HEM), forward primer, 5′-CTTAGTGGTTCTTGGGCTTGTC-3′ and reverse primer, 5′-GGTCTCC GTCCTGAATGTC-3' (GenBank no.: X82502).

2.6. Statistical analysis

Significant differences between Bs, Sa and Control final weight gain and survival data after *V. parahaemolyticus* challenge (means ± Standard Error) were performed by One-way (ANOVA) followed by Fisher post hoc test. Differences in data collected from qRT-PCR experiments after 45-day rearing and *V. parahaemolyticus* challenge were also assessed by One-way (ANOVA) followed by Fisher post hoc test. Two sample T test was used to verify significant differences in immune genes expressions before and after *V. parahaemolyticus* challenge. The statistical analyses were performed with Origin 8.0 software.

3. Results

3.1. Immune status analysis

Fig. 1 shows the proPO, LGBP and HEM mRNA expression levels in different shrimp treatments reared for 45 days.

The dietary inclusion of *B. subtilis* IPA-S.51 significantly improved the transcription of proPO and HEM in *L. vannamei* juveniles hemolymph compared to Control and Sa. After 45-day rearing the proPO, LGBP and HEM expressions of Bs were 1.71, 2.73 and 3.06-fold, respectively. Shrimp fed with *S. algae* IPA-S.252 and Control had a similar proPO and HEM expression trend. The proPO and HEM levels of Sa were 0.93 and 1.26-fold, whereas the proPO and HEM levels for Control were 1 and 1-fold, respectively. The LGBP gene was significantly up-regulated in all shrimp fed with *B. subtilis* (2.73-fold) and *S. algae* (3.28-fold) compared to the Control (1.01-fold).

The mRNA expression levels of proPO, LGBP and HEM in different treatments challenged with *V. parahaemolyticus* via injection are shown in Fig. 2.

After 24 h of *V. parahaemolyticus* challenge, the expression levels of proPO in Vibrio + Bs (4.15-fold) was higher than Vibrio group (2.28-fold), but similar to Vibrio + Sa (3.18-fold). Vibrio and Vibrio + Sa groups did not show significant differences in proPO expression.

The LGBP levels were up regulated in Vibrio + Bs (40.40-fold) and Vibrio + Sa (104.90-fold), but Vibrio + Sa showed a higher mRNA expression level compared to Vibrio + Bs and Vibrio (10.91-fold). LGBP expression of Vibrio + Bs was also higher than Vibrio group. The HEM expression was superior for Vibrio + Bs (60.36-fold) compared to the other groups: Vibrio (16.02-fold) and Vibrio + Sa (16.81-fold). Both Vibrio and Vibrio + Sa had similar LGBP levels.

Significant differences in proPO, LGBP and HEM expressions were found in shrimp groups after and before *V. parahaemolyticus* challenge. Bs group had all the immune genes up regulated after challenge. Regarding to Sa group, LGBP and HEM levels after *V. parahaemolyticus* injection were higher, however the proPO levels were similar before and after the challenge. The groups fed with feed without candidate probiotics supplementation (Control and Vibrio) only had a LGBP expression up regulated after challenge, proPO and HEM levels did not have significantly differences before and after *Vibrio* challenge.

3.2. Growth and survival measurements

Bacillus subtilis supplementation was associated with a significant ($p < 0.05$) increase in *L. vannamei* final weight after 45 days of rearing [24]. The Bs final weight (6.2 ± 0.04 g) was significantly higher than Control (5.58 ± 0.08 g) and Sa (5.83 ± 0.1 g), whereas both Control and Sa final weight were significantly similar. According to Interaminense et al. [24], shrimp survival after the 45-day feeding period was not significantly different among shrimp groups. Control and Sa survival was 86.8 ± 8 and 75 ± 15% respectively and Bs had an 100% of survival.

Sa had the higher final weight after 60-day feeding period, (9.35 ± 0.1 g) and was significant different of Control (7.68 ± 0.1 g) and Bs (8.4 ± 0.1 g). Bs also had a significantly superior final weight compared to the Control. Shrimp cumulative mortality after 24 h of *V. parahaemolyticus* challenge did not have significant differences: Sa (65 ± 5%), Bs (75 ± 5%) and Vibrio (85 ± 5%) (Fig. 3). At 48 h of challenge significant differences between Sa (85 ± 5%) and Vibrio (100%) were observed. Bs (92.5 ± 2,5%) cumulative mortality was similar to the other groups and 100% of mortality was observed at 72 and 96 h of challenge (Fig. 3). For shrimp Control group that just received a saline solution injection, there was not observed mortality.

4. Discussion

The effective performance of a probiotic depends on their adhesion of the gut cells, which in turn improves the host immune system [30]. Once the probiotic adheres to the cell, various biological activities take place, which primarily include the release of cytokines and chemokines, strictly linked to the stimulation of the immune system [31].

In the present study, *B. subtilis* was reisolated from *L. vannamei* hepatopancreas and intestine during the experimental rearing. Moreover, Bs group had a higher level of the proPO mRNA expression than Sa and Control after 45 days of rearing. Shrimp proPO system could be activated by several microbial polysaccharides and specific pattern recognition proteins (PRPs), such as LPS- and β-1, 3-glucan-binding protein (LGBP) and peptidoglycan-binding proteins (PGBP) [32]. In this regard, our study found that at the 45-day rearing, Bs group had a significantly higher LGBP level than the control group, which may be correlated with the up regulating of proPO gene.

Final weight of Bs group was significantly improved after 45 days of rearing compared to Control and Sa and there was not observed mortality in the group plots [24]. An increase in shrimp final weight of Bs group was also observed after 60 days of rearing compared to Control. Chai et al. [17], reported during a *Bacillus* PC465 feeding trial a significantly up-regulated transcriptions of immune-related genes as peroxinectin and hemocyanin and a “dose effect” was observed in shrimp infected by White Spot Syndrome Virus (WSSV) where in a much higher survival was observed for shrimp fed with 10^9 cells g^{-1} dose compared with 10^7 cells g^{-1} dose. According to the authors, growth may be promoted by an increase in digestive enzyme activities (protease, amilase and lipase) recorded in the study. In our previous work, we found that *B. subtilis* and *S. algae* had the ability of producing protease (unpublished results).

After *V. parahaemolyticus* challenge, proPO and LGBP genes were significantly higher to Bs group than the Control. These findings

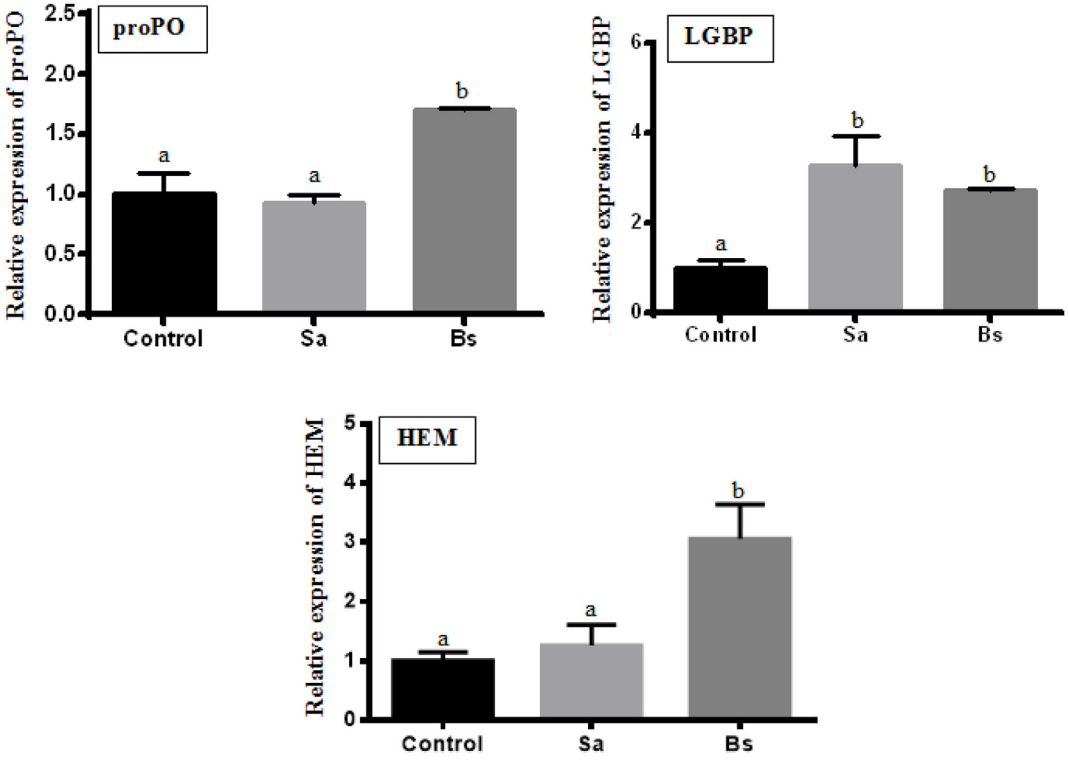

Fig. 1. Relative expressions through the real-time PCR analysis of immune-related genes, including prophenoloxidase (proPO), lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) and Hemocyanin (HEM) of hemolymph from *L. vannamei* fed with *S. algae* (Sa), *B. subtilis* (Bs) and without bacteria (Control) for 45 days. Each bar represents the mean fold change relative to the control ± S.D. Significant ($p < 0.05$) differences are indicated by different letters (a and b).

demonstrate that administration of *B. subtilis* (IPA-S.51) can improve growth performance and immune resistance in shrimp through an enhanced immune genes expression before and after a *V. parahaemolyticus* challenge. The shrimp proPO and LGBP expression in Bs were significantly up-regulated after *V. parahaemolyticus* challenge and these levels were higher compared to the levels observed after a 45-day rearing. This pattern of immune defense was suggested by Hao et al. [33]. The authors report that the probiotic application might result in an enhancement or prolongation of the desirable effects on the host immune response, growth performance and disease resistance.

Although the high mortality registered to Bs, these rates are in accordance with Tirasophon et al. [34] and Xia et al. [35]. The authors report that survival curves from different infection routes indicate that infection via injection causes more extensive mortality within a short period and is clearly a more eficiente route to ensure *Vibrio* infection in shrimps, but such infections may not necessarily result in a sustainable infection. The high mortality rates in a short time period (48 h) were also observed by Luis-Villaseñor et al. [36].

Within our experimental conditions, the shrimp treat with *B. subtilis* (IPA-S.51) and *S. algae* (IPA-S.252) reached approximately 10% and 20% less mortality, after 24 and 48 h of challenge, respectively, compared to infected shrimps that not received probiotics in diet.

The HEM expression was also higher to Bs before and after *V. parahaemolyticus* challenge in comparison with the other groups. Hemocyanin has been reported as a novel and important defense molecule in mollusks and arthropods [37]. Besides its primary function as a respiratory protein, it has been suggested that hemocyanin could be functionally converted into phenoloxidase like enzyme, antiviral agent, antimicrobial protein [38–40]. In fact, the phenoloxidase expression was higher to Bs group in comparison to Control and Sa and this level maybe could be influenced by the hemocyanin convertion during the offer of *B. subtilis* supplemented feed.

Huang et al. [41] reported that hemocyanin concentration decrease to the lowest level in *Vibrio*-resistant and normal shrimp about 3 and 12 h after pathogenic infection respectively. According to the authors, the hemocyanin concentration in resistant and normal shrimp decreased about 42.1% and 44.4%, and recovered at 12 h and 48 h after a *Vibrio harveyi* injection, respectively. In the present study, although the HEM level was not detected before 12 h of infection, the Control group kept the HEM level similar at 24 h post *Vibrio* injection. Vibrio + Bs presented a higher HEM level at 24 h post *Vibrio* injection compared to the HEM level of Control group reared for 45 days. Consequently, although the cumulative mortalities after 24 h of challenge did not present significant differences, Vibrio group cumulative mortality after 24 h of challenge were 10% superior to the rate verified to Vibrio + Bs. In fact, Vibrio and Control groups did not have differences between immune genes expression after *V. parahaemolyticus* challenge.

Positive results were also reported by Luis-Villaseñor et al. [36] after a *L. vannamei* challenge with *V. parahaemolyticus* injection (10^5 CFU g^{-1}). The authors observed a survival increase in shrimp treated with *Bacillus* mix (33%) compared to the control (9%). They concluded, after a molecular analysis, that *Bacillus* mix administration induced modulation of the intestinal microbiota of *L. vannamei* and increased its resistance to *V. parahaemolyticus*.

Hao et al. [33] also observed promising results in shrimp growth, i.e. final weight, weight gain, percent weight gain and specific growth rate were improved after 28-days of feeding regime with *Shewanella haliotis*, *Bacillus cereus* and *Aeromonas bivalvium*. In modern aquaculture, the ability of *Bacillus* spp. to sporulate, grow fast and tolerate a wide range of physiological conditions has been reported to improve the quality of sea water, reduce the load of harmful bacteria and maximize the host's response without antibiotics [42].

Regarding to immune expressions of shrimp fed with *S. algae*, only the LGBP gene was up regulate compared to Control after 45-day feeding regime. According to Amparyup et al. [42], the differences in LGBP transcript expression could be due to variations between the response to live and dead microorganisms cells or to different microorganisms and so cell wall components, in addition to differences

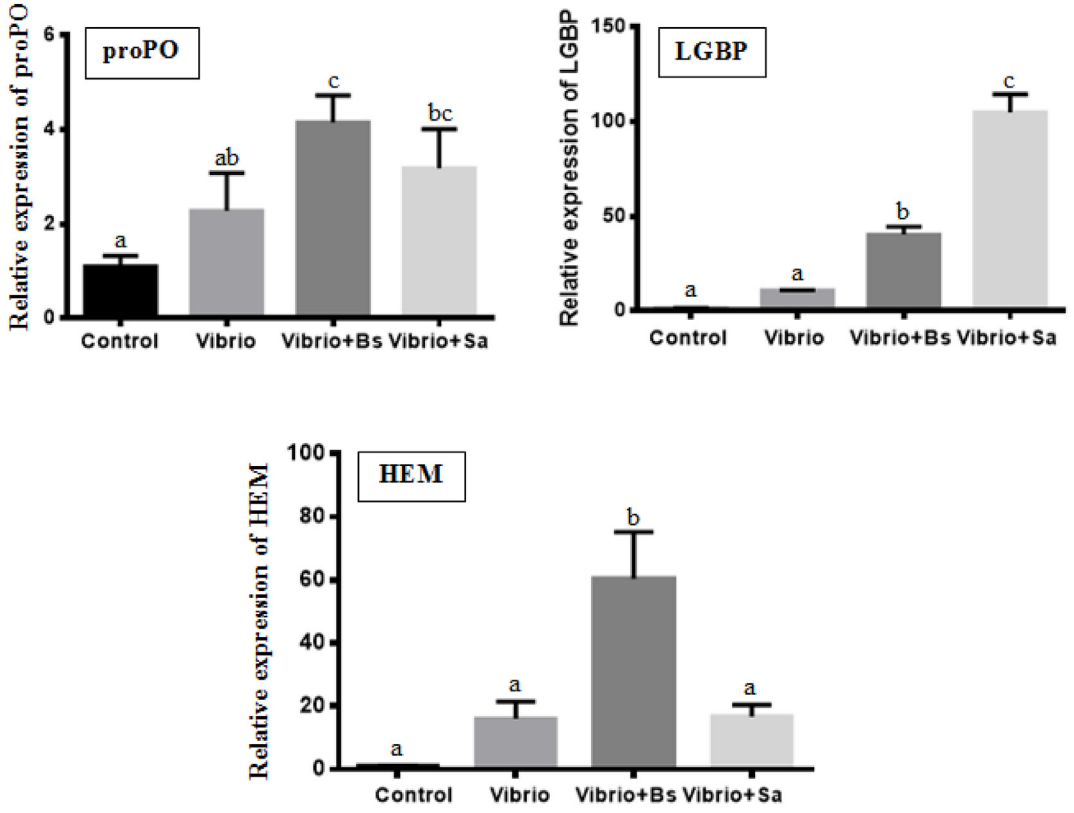

Fig. 2. Relative expressions through the real-time PCR analysis of immune-related genes, including prophenoloxidase (proPO), lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) and Hemocyanin (HEM) of hemolymph from *L. vannamei* reared for 60 days distributed in different treatments: animals fed without bacterial addition and challenged with *V. parahaemolyticus* (Vibrio), animals fed with *B. subtilis* and challenged with *V. parahaemolyticus* (Vibrio + Bs), animals fed with *S. algae* and challenged with *V. parahaemolyticus* (Vibrio + Sa) and Control, animals not submitted to treatments with experimental diets and injected with sterile saline solution. Each bar represents the mean fold change relative to the control ± S.D. Significant ($p < 0.05$) differences are indicated by different letters (a, b and c).

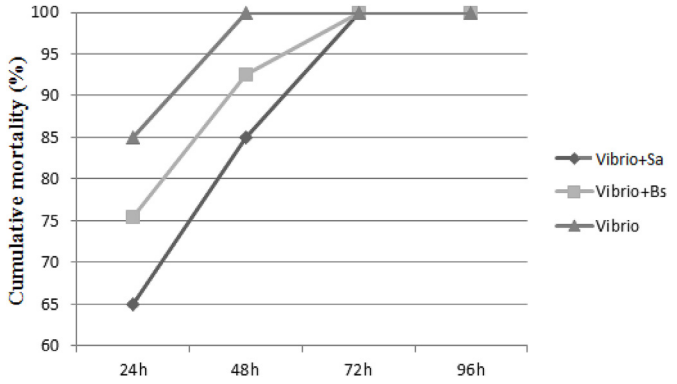

Fig. 3. Cumulative mortality (%) during 96 h of *L. vannamei* reared for 60 days distributed in different treatments: animals fed without bacterial addition and challenged with *V. parahaemolyticus* (Vibrio), animals fed with *B. subtilis* and challenged with *V. parahaemolyticus* (Vibrio + Bs), animals fed with *S. algae* and challenged with *V. parahaemolyticus* (Vibrio + Sa).

between host species or strains.

Some authors [11,43–45] indicate that the transcript level of LGBP is increased in response to *Vibrio* and LPS challenges after a variable time period of 3–24 h, thus, in the present work it is suggested that the proPO zymogen has not been converted to active PO (phenoloxidase) once that the LGBP could be not induced the activation cascade of serine proteinase (SPs) that leads to a final serine proteinase formation designated as a proPO-activating enzyme (PPAE). PPAE converts the inactive proPO zymogen in active phenoloxidase (PO) to produce the quinones, which can cross-link neighboring molecules to form melanin around invading microorganisms [44].

In the case of shrimps untreated with IPA-S.51 and IPA-S.252, the proPO and HEM expression were not upregulated, however, the Control cumulative mortality was higher than the observed to IPA-S.252 at 48 h of *V. parahaemolyticus* challenge. Its known that PO is an important molecule of host defense system with functions to detect and kill invading pathogens as well as to synthesize melanin for wound healing and pathogen encapsulation [45].

Regarding to the melanization, is a process that is induced by activation of the proPO system and is essential for the immune defense. However, the melanin reaction is tightly controlled at multiple levels, including by inhibition of the serine protease (SP) cascade by proteinase inhibitors, the PO activity by PO inhibitors and the melanin reaction products by melanization-inhibiting protein (MIP). This tight control is required since excess melanin reaction products (highly reactive and toxic quinone intermediates) can cause damage and death to the host cells.

Thus, the absence of the PO convertion does not compromise the effectiveness of probiotic once that LGBP is able to binding to LPS and b-1,3-glucan leads to the expression of Antimicrobial Proteins (AMPs) and immune proteins to defense against infection and proPO.

Similar to prophenoloxidase, according to Shi et al. [46], the hemocyanin can be directly activated by microbial protease and enhanced by pathogen associated molecular patterns and thus, be recognized by LGBP. However, the HEM levels did not be improved in Sa group. Hao et al. [33] report that most probiotics could not affect only one pathway or immune mechanism (a probiotic may play the role of immunosuppressant or immunostimulant on different targets within the immune system). These immune genes expression results did not influenced the Sa final weight, after 45 days of rearing.

Although proPO and HEM genes have not been activated by *S. algae* supplemented feed after 45 days of rearing, an improvement in final weight after 60 days of rearing were observed. According to Zokaeifar et al. [15], beyond the expression of immune genes and a higher survival rate in treated shrimp may be also due to the competitive exclusion, higher digestive enzyme activity and better growth performance as observed in the present study.

However, a decrease in cumulative mortality in Sa group after 48 h of *V. parahaemolyticus* challenge were observed compared to the other shrimp groups. This could be correlated with the proPO and LGBP up regulation compared to Vibrio group and indicates a requirement for a longer period of time to the effective action of *S. algae* probiotic properties in comparison to *B. subtilis*.

Similar results were reported by Díaz-Rosales et al. [20]. The authors studied the effects of the dietary administration of *Shewanella putrefaciens* and *Shewanella baltica*, on the immunological responses of Senegalese sole (*Solea senegalensis*) and its survival after *Photobacterium damselae* sub sp. Piscicida challenge. The cumulative mortality percentage after the challenge was 100% in the groups fed with control diet and mortality rates observed in the groups fed with diets supplemented with *S. putrefaciens* and *S. baltica* ranged from 75 to100 and 65–80%, respectively. The authors also observed the improvement of growth by *Shewanella* supplemented feed offered to fishes compared to those fed with control diet.

Studies about *Shewanella* probiotic application and its effects are still scarce. The utilization of *Shewanella* genus is more widely studied in fish as Senegalesse sole, *Solea senegalensis* [20,23,47,48], Abalone, *Haliotis discus* [25], Gilthead sea bream, *Sparus aurata* L. [48,49], than in shrimp [18,32,50]. However, the *S. algae* safety to shrimp rearing was confirmed by Interaminense et al. [24] and to both shrimp rearing and human consumption was confirmed by Shakibazadeh et al. [51].

In conclusion, *B. subtilis* administration enhanced *L. vannamei* growth performance and improved proPO, LGBP and HEM genes levels before and after *V. parahaemolyticus* challenge in comparison to Control. *S. algae* feed supplementation also conferred greater growth performance after 60-day feeding regime and cumulative mortality decrease after 48 h of *V. parahaemolyticus* challenge, once that a quality shrimp feed offer and immune genes up regulation post-challenge consequently leads to higher digestive enzyme activity and stimulate antibacterial responses, respectively. In general, the supplementation of *L.vannamei* juveniles feed with *B. subtilis* and *S. algae* results in positive results to shrimp immune status, growth performance and decrease mortality after *V. parahaemolyticus* challenge. The present study confirms the strains probiotic potential, but pilot scale *in vivo* experiment is necessary to the production of a commercial probiotic product.

Conflicts of interest

None.

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