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Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*

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ABSTRACT

We studied the effect of two probiotic *Bacillus subtilis* strains on the growth performance, digestive enzyme activity, immune gene expression and disease resistance of juvenile white shrimp (*Litopenaeus vannamei*). A mixture of two probiotic strains, L10 and G1 in equal proportions, was administered at two different doses 10^5 (BM5) and 10^8 (BM8) CFU g^{-1} feed to shrimp for eight weeks. In comparison to untreated control group, final weight, weight gain and digestive enzyme activity were significantly greater in shrimp fed BM5 and BM8 diets. Significant differences for specific growth rate (SGR) and survival were recorded in shrimp fed BM8 diet as compared with the control; however, no significant differences were recorded for food conversion ratio (FCR) among all the experimental groups. Eight weeks after the start of the feeding period, shrimp were challenged with *Vibrio harveyi*. Statistical analysis revealed significant differences in shrimp survival between probiotic and control groups. Cumulative mortality of the control group was 63.3%, whereas cumulative mortality of the shrimp that had been given probiotics was 20.0% with BM8 and 33.3% with BM5. Subsequently, real-time PCR was employed to determine the mRNA levels of prophenoloxidase (proPO), peroxinectin (PE), lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP) and serine protein (SP). The expression of all immune-related genes studied was significantly up-regulated ($P < 0.05$) in the shrimp fed BM5 and BM8 diets compared to the control group. These findings demonstrate that administration of *B. subtilis* strains, L10 and G1, can improve growth performance and disease resistance through an enhanced immune response in shrimp.

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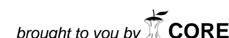

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1. Introduction

World aquaculture production has increased significantly in the last years [1]. Among them, shrimp farming is one of the fastest growing aquaculture sectors in many tropical countries; however, this development has been accompanied by adverse impacts due to the intensification, such as problems related to diseases and deterioration of environmental conditions [2]. The occurrence of infectious diseases in shrimp aquaculture is a serious problem due to the overuse or misuse of antibiotics and antibiotic resistance genes among opportunistic pathogens such as *Vibrio* species [3–5]. In addition, the invertebrate immune system is highly depends on the innate mechanisms and in theory, is incapable of responding to specific vaccines [6]. In fact, the development of vaccines for shrimp infectious diseases have had limited success and limited information has resulted from these studies [7]. Therefore, other alternatives such as probiotic bacteria have been examined in order to control the infectious diseases not only in shrimp aquaculture but also in other farmed aquatic species [8]. A recent study has demonstrated that dietary administration of an antagonistic bacterium, *Psychrobacter* sp., improved the feed utilization, enzyme activity and immune response of grouper, *Epinephelus coioides* [9]. Moreover, dietary administration of *Lactobacillus plantarum* resulted in an enhanced immune response in white shrimp, *Litopenaeus vannamei* [10] and rainbow trout, *Oncorhynchus mykiss* [11].

Application of *Bacillus subtilis* as probiotic has brought very promising results for shrimp aquaculture. This bacterium is a non-pathogenic Gram positive spore-forming which has been used to

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improve the growth performance and also shrimp health and disease management [12–15]. In addition it is well documented that *Bacillus* species are able to produce a wide range of extracellular substances and antimicrobial peptides against variety of microorganisms [16–19].

We have recently reported the isolation, identification and characterization of two *B. subtilis* strains, L10 and G1, with antagonistic ability against two shrimp pathogens, *Vibrio harveyi* and *Vibrio parahaemolyticus* [20]. The aims of the current study was thus to investigate whether a mixture containing two strains, L10 and G1, could improve the growth performance, digestive enzyme activity, immune gene expression and disease resistance of juvenile white shrimp (*L. vannamei*).

2. Materials and methods

2.1. Bacterial strains

B. subtilis strains L10 and G1, previously isolated and identified from fermented pickles [20], were used as potential probiotics. A virulent strain, *V. harveyi* ATCC 14126, was used for experimental infection in this study. All strains were preserved at −20 °C in Luria-Bertani broth (LB; Difco) with 15% sterile glycerol, prior to use.

2.2. Preparation of the feed

Probiotic *B. subtilis* strains, L10 and G1, were grown in LB broth using a shaking incubator at 30 °C for 48 h. The cultures were then centrifuged at 3000 *g* for 10 min at 4 °C and, after discarding the supernatant, the pelleted bacteria were re-suspended and washed three times in sterile Normal Saline Solution (NSS, 0.9% NaCl). The cell densities of the suspensions were calculated using spectrophotometer at 600 nm and also correlated to the colony-forming unit (CFU) using the spread-plate technique. These suspensions were kept at 4 °C until used.

Commercial feed (BLANCA, Malaysia) was used as the basal diet for the supplementation of probiotic strains. *B. subtilis* strains L10 and G1 were sprayed into the feed to give a final concentration of approximately 10^5 CFU g^{-1} (L10, 5×10^4 and G1, 5×10^4 CFU g^{-1}), named BM5 and 10^8 CFU g^{-1} (L10, 5×10^7 and G1, 5×10^7 CFU g^{-1}), named BM8. The amount of the probiotic strains in the feed was determined using the spread-plate technique. Briefly 1 g of each feed type was randomly sampled and serially 10-fold diluted in phosphate-buffered saline solution (PBS; pH 7.2) and 100 μl of each dilution was then spread on mannitol-egg yolk-polymyxin agar (MYP agar, Difco, USA) in order to estimate the probiotic concentration (CFU g^{-1}).

2.3. Shrimp and experimental conditions

Healthy juvenile shrimps were provided by the Marine Science Research Station and Biology Field Station, UPM, Port Dickson, Malaysia and the experiment was conducted at the same place. Shrimp had not been exposed to shrimp diseases and were deemed pathogen-free. One hundred juvenile shrimp were weighted, randomly distributed in 6 tanks containing 500 l seawater, and acclimatized for 3 days prior to the experiment.

One group served as the control and was fed un-supplemented diet during the entire experimental period. The other two groups were fed *B. subtilis*-supplemented diets at two different doses 10^5 and 10^8 CFU g^{-1} feed until the end of the experiment. Experiment was conducted in duplicate for 8 weeks and the feed preparation procedure was done twice a week. Shrimps were fed their specific diets three times a day at 5% of the body weight.

2.4. Water supply and analysis

The water was directly supplied from the sea and it was treated before use. After filtration, the salinity was reduced to 20 ppt using fresh water. The tanks were maintained under constant aeration (DO, 5 ± 0.5 mg l^{-1}), with a 50% water change twice a week, ambient temperature of 28 ± 1 °C and pH of 7.3–8.2. Temperature, DO and pH were measured using an YSI (Yellow Spring Inc.). Water chemical parameters were measured spectrophotometrically once a week using the HACH kit (product no: 2107569 for Nitrite, 2605345 for Nitrate, and 2653299 for Ammonia). During the experiment the chemical parameters were recorded within the acceptable ranges of nitrite-N ($<0.01 \pm 0.001$ mg l^{-1}), nitrate-N ($<4 \pm 1$ mg l^{-1}), and ammonia-N ($<1.1 \pm 0.1$ mg l^{-1}).

2.5. Biometry and sampling for analysis

At the end of the experiment, the final weight, survival rate, weight gain, feed conversion ratio (FCR), and specific growth rate (SGR) of different treatments were calculated according to Robertson et al. (2000); Felix and Sudharsan (2004) and Venkat et al. (2004):

$$\text{Weight gain (g/shrimp)} = \text{Final weight (g)} - \text{Initial weight (g)}$$

$$\text{Feed conversion ratio (FCR)} = \text{Total Feed Given (g)}/\text{Weight Gain (g)}$$

$$\text{Specific growth rate (SGR; \%/day)} = ([\ln \text{final wt} - \ln \text{initial wt}]/\text{Days}) \times 100$$

$$\text{Survival rate (\%)}: (\text{Final numbers}/\text{Initial numbers}) \times 100$$

One shrimp from each replicate was randomly collected at week 0, 4, and 8 of the experiment to estimate the *Bacillus* sp. and *Vibrio* sp. levels in shrimp gastrointestinal tract (GIT). For enzyme activity assays, 5 shrimp from each replicate were randomly collected at the end of week 8 and the GIT of each shrimp was removed aseptically and then immediately packed and immersed in liquid nitrogen. Samples were kept in −80 °C until analysis. The same procedure with one shrimp from each replicate was followed for immune-related gene expression assays (see below).

2.6. Bacteriological analysis

In order to estimate the *Bacillus* sp. and *Vibrio* sp. levels in shrimp GIT, the spread-plate technique was followed as mentioned above. Shrimp were aseptically dissected using a sterile surgical scissor and the GIT was removed and homogenized in a sterile glass homogenizer with PBS. To ensure the colonization of *B. subtilis* in shrimp GIT, at the end of week 8, DNA of 10 random colonies from the plates within the acceptable range of colony counts (30–300 CFU $plate^{-1}$) were extracted and identified using the PCR amplification of 595-bp fragment corresponding to an internal portion of the '*B. subtilis* group' 16S rRNA [21]. The Primers Bsub5F (5′-AAG TCG AGC GGA CAG ATGG-3′) and Bsub3R (5′-CCA GTT TCC AAT GAC CCT CCCC-3′) were used for the PCR amplification. DNA extraction was followed by the boiling technique. Briefly, colonies were aseptically removed and homogenized in 1.5 ml Eppendorf tubes containing 1 ml sterile double distilled water (ddH_2O). The suspension was incubated in water bath at 95 °C for 15 min. Cellular debris were then pelleted using centrifugation on 13000 *g* for 10 min. 2 μl of the supernatant was applied to master mix kit,

HotStarTaq®*Plus* PCR (Qiagen, Germany) as template. A total of 8 μl of PCR amplification products was verified by 2.0% (w/v) agarose gel electrophoresis stained with ethidium bromide for 2 h with 85 V, and photographed under UV illumination. 100 bp ladders were used as markers.

2.7. Digestive enzyme analysis

The crude extract of the GIT was used to quantify the digestive enzyme activity of shrimp in different treatments. The whole of the GIT of one or two shrimp were dissected out, pooled, weighted and homogenized with cold deionized water (1:10). The homogenate was then centrifuged at 5000 *g* for 20 min at 4 °C. The supernatant was carefully separated and passed through 0.45 μm-pore-size filters (Sartorius, Germany). Aliquots were made in 1.5 ml Eppendorf tubes in triplicates and kept at −20 °C to analyze different enzymes.

The total protein activity was measured using bovine serum albumin as standard according to [22]. Total protease activity was assayed using casein as the substrate which reacts with Folin's reagent [23]. A calibration curve of absorbance at 440 nm was prepared using tyrosine as standard. One unit of protease activity was defined as the number of micromoles of tyrosine released per min per mg of protein at 37 °C. Total amylase activity was determined according to Rick and Stegbauer [24] using 1% soluble starch as substrate reacting with 3,5-dinitrosalicylic acid. A calibration curve of absorbance at 550 nm was prepared using a standard maltose solution. One unit of amylase activity was defined as the number of micromoles of maltose released per min per mg of protein at 37 °C.

2.8. Experimental infection

After eight weeks of the feeding period, an experimental infection was induced in shrimp with the pathogenic bacterium, *V. harveyi* ATCC 14126. *V. harveyi* was grown overnight in LB medium and the concentration was adjusted to 10^7 CFU ml^{-1} using NSS as previously mentioned. A total of 30 shrimp in the intermolt stage were collected from the treatment and control groups and injected with 20 μl of the bacterial suspension into the third abdominal segment resulting 10^6 CFU $shrimp^{-1}$. Immediately after injection, shrimp were transferred into the 20 l tanks with 10 shrimp each. The experiment was conducted in triplicates and the water was supplied from the previous tanks in order to minimize the stress. A group of untreated shrimp with *B. subtilis*-supplemented diet, which was injected with NNS, served as positive control (NB). During the experimental infection, shrimp were fed their specific diets as previously described. The mortality was monitored daily for up to 10 days.

2.9. Relative mRNA expression of immune-related genes

The expression of immune-related genes of shrimp following the challenge with *V. harveyi* was determined by real-time RT-PCR at 24 h post-injection. One shrimp from each replicate was randomly collected for RNA extraction. Since it was almost impossible to collect hemocytes from the shrimp (∼3–4 g), the whole body of shrimp was therefore freeze-dried using ample amounts of liquid nitrogen and homogenized using RNase free mortar and pestle. 100 mg of the homogenized was subjected for RNA extraction and purification using the guanidinium thiocyanate method [25]. The reverse transcription was used to synthesize the first-strand cDNA using QuantiTect Rev. Transcription Kit (Qiagen) containing the oligo-$(dT)_{18}$. The manufacturer's recommendations were followed to maximize cDNA synthesis.

Four specific primers [10], and β-actin as the housekeeping gene were used to determine immune-related gene expression. The primer sequences and product sizes are presented in Table 1.

For real-time-PCR, the QuantiTect® SYBR® Green PCR master mix kit (Qiagen) containing HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTP mix, Fluorescent dyes (SYBR Green I and ROX as the reference dye) and RNase-free water was mixed with synthesized cDNA. The real-time-PCR thermal profile for all immune-related genes was at 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, according to manufacturer's recommendation. Melting curves were generated by ramping the temperature from 70 to 90 °C in 2 s for each step afterward. In order to obtain the efficiency, separately one standard curve was generated for each gene using the same thermal profile as mentioned above. Amplification of cDNA was performed using the Rotor-Gene Q instrument (Qiagen). Each sample from the treated and untreated shrimp was analyzed with four replicate.

The comparative quantitation analysis was performed by REST© software package version 2.0.13, 2009 [26] for group-wise comparison and statistical analysis of real-time PCR data. REST© applies the efficiency-corrected comparative CP method and performs randomization tests to estimate a sample's expression ratio and the likelihood of up or down-regulation, taking into account the reference genes and the individual amplification efficiency of each gene [27]. The relative expression ratios were calculated by a mathematical model, which included an efficiency correction for real-time PCR efficiency of the individual transcripts, as follows:

$$\text{Ratio} = (E_{\text{target}})^{\Delta CP}\text{target}^{(\text{control}-\text{sample})} \Big/ \left(E_{\text{ref}}\right)^{\Delta CP} \text{ref}^{(\text{control}-\text{sample})}.$$

The relative expression ratio of a target gene was computed based on its real-time PCR efficiencies (E) and the crossing point

Table 1
Specific primers used to evaluate immune status of shrimp, *Litopenaeus vannamei*.

Gene	Primer	Sequence (5′–3′)	Product size (bp)	References
Prophenoloxidase	ProPO-F	GCCTTGGCAACGCTTTCA	68	[44]
	ProPO-R	CGCGCATCAGTTCAGTTTGT		
Peroxinectin	PE-F	TGGACCTCGCGGGAGAT	56	[45]
	PE-R	GACCGATAGCCACCATGCTT		
Lipopolysaccharide and β-1,3-glucan-binding protein	LGBP-F	CATGTCCAACTTCGCTTTCAGA	64	[43]
	LGBP-R	ATCACCGCGTGGCATCTT		
Serine protein	SP-F	CGTCGTTAGGTTAAGTGCGTTCT	61	[46]
	SP-R	TTTCAGCGCATTAAGACGTGTT		
Housekeeping	β-actin-F	GAGCAACACGGAGTTCGTTGT	68	GenBank accession
	β-actin-R	CATCACCAACTGGGACGACATGGA		no: AF300705

difference (ΔCP) for an unknown sample versus a control. For each gene, cDNA dilution curves were generated and used to calculate the individual real-time PCR efficiencies ($E = 10^{[-1/\text{slope}]}$) [26,28]. The hypothesis test performed 2000 random reallocations of samples and control between the groups. Statistical differences were significant when $P < 0.05$.

Box plot graphs were generated using the REST© software to present the relative gene expression or up/down regulation of the shrimp immune genes of treatments in comparison to control.

2.10. Statistical analysis

Data on growth parameters, enzyme activity, and bacteriological analysis in digestive tract among treatments were analyzed by using ANOVA and Duncan's multiple range test was used to determine the significant variation ($P < 0.05$). All statistical analysis was performed using SPSS, version 15 (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Growth performance

The effect of diets containing *B. subtilis* strains, L10 and G1, on growth performance were established in this study. Analyzed data on the growth performance of shrimp in different treatments and control, including initial weight, final weight, weight gain, SGR, FCR, and survival rate are showed in Table 2. There were no significant differences for initial weight between treatments and control at the start of the experiment. At the end of the experiment, statistical analysis showed that shrimp fed BM8 and BM5 diets grew significantly faster than the control group. Final weight was recorded significantly different ($P < 0.05$) for BM8 (3.46 ± 0.06 g) and BM5 (3.39 ± 0.11 g) compared to the control (2.49 ± 0.13 g). In addition significant differences ($P < 0.05$) were recorded in weight gain for BM8 (2.79 ± 0.007 g) and BM5 (2.65 ± 0.02 g) in comparison with control (1.82 ± 0.13 g). No significant differences ($P > 0.05$) were recorded in survival rate and SGR neither between shrimp fed BM8 and BM5 diets nor between shrimp fed BM5 diet and control. However, the survival rate and SGR in the shrimp fed BM8 diet were significantly different ($P < 0.05$) from those of the control group. There was no significant difference ($P > 0.05$) in FCR among all the experimental groups (Table 2).

3.2. Bacteriological analysis

The levels of *Bacillus* spp. and *Vibrio* spp. counted at week 0, 4, and 8 were analyzed and are shown in Table 3. There were significant differences ($P < 0.05$) for *Bacillus* spp. and *Vibrio* spp. counts between treatment and control groups. The highest ($P < 0.05$) *Bacillus* spp. counts on MYP agar at week 4 and 8 were recorded for BM8 followed by BM5 and control. The population of *Bacillus* spp. in the GIT of shrimp fed BM8 and BM5 diets increased through 8 weeks of culture from 3.92 ± 0.02 to 6.47 ± 0.03 and 5.86 ± 0.03 log CFU g^{-1}, respectively. In contrast, no increase of *Bacillus* spp. concentration was observed in the control (Table 3). In order to confirm at the genus level, the yellow colonies counted on MYP agar as *Bacillus* spp. [21] were randomly picked ($n = 10$) and subjected for DNA amplification using specific primers. Out of the 10 colonies, 9 and 7 of them showed specific amplification at 595 bp, which were isolated from shrimp fed BM8 and BM5 diets, respectively (Fig. 1), whereas only 2 colonies were recorded to be *Bacillus* spp. in the control (Fig. 1). The bacteriological analysis and the molecular detection assay clearly showed that *B. subtilis* strains L10 and G1 were able to colonize in the GIT after shrimp had received the diet containing both strains for 8 weeks. In addition, there were considerable reductions ($P < 0.05$) of *Vibrio* spp. in the GIT of shrimp fed BM8 and BM5 diets at week 4 and 8. Compared to the control, significant differences ($P < 0.05$) in the reduction of *Vibrio* spp. levels were observed through 8 weeks of experiment, ranging from 5.53 ± 0.01 to 4.85 ± 0.07 and 5.18 ± 0.1 log CFU g^{-1} in the shrimp fed BM8 and BM5 diets, respectively (Table 3).

Table 2
Data of growth performance and survival of *Litopenaeus vannamei* cultured with probiotics *Bacillus subtilis*, strains L10 and G1.

Treatments	BM8: 10^8 cfu g^{-1}	BM5: 10^5 cfu g^{-1}	Control
Initial weight (g)	0.67 ± 0.06^a	0.74 ± 0.1^a	0.67 ± 0^a
Final weight (g)	3.46 ± 0.06^a	3.39 ± 0.11^a	2.49 ± 0.13^b
Weight gain (g)	2.79 ± 0.007^a	2.65 ± 0.02^a	1.82 ± 0.13^b
SGR (%)	2.97 ± 0.14^a	2.77 ± 0.27^{ab}	2.38 ± 0.09^b
FCR	1.86 ± 0.03^a	1.85 ± 0.02^a	1.92 ± 0.02^a
Survival (%)	100 ± 00^a	95.5 ± 6.36^{ab}	86.5 ± 2.12^b

Values (means ± SD) with different superscript in a row show significant differences ($P < 0.05$).

Table 3
Log mean of *Bacillus* spp. and *Vibrio* spp. count (cfu g^{-1}) in gastrointestinal tract of shrimp *Litopenaeus vannamei* in different treatments and control during 55 days of culture with *Bacillus subtilis*, strain L10 and G1.

	Time (week)	BM8: 10^8 cfu g^{-1}	BM5: 10^5 cfu g^{-1}	Control
Log *Bacillus* spp. count	0	3.92 ± 0.02^a	3.92 ± 0.02^a	3.92 ± 0.02^a
	4	5.19 ± 0.05^a	4.51 ± 0.09^b	3.89 ± 0.02^c
	8	6.47 ± 0.03^a	5.86 ± 0.03^b	3.93 ± 0.05^c
Log *Vibrio* spp. count	0	5.53 ± 0.01^a	5.53 ± 0.01^a	5.53 ± 0.01^a
	4	5.17 ± 0.06^b	5.1 ± 0.21^b	5.92 ± 0.2^a
	8	4.85 ± 0.07^b	5.18 ± 0.1^b	5.92 ± 0.2^a

Values (means ± SD) with different superscript in a row show significant differences ($P < 0.05$).

3.3. Total protein and enzymes activity

Total protein, protease and amylase activity showed highly significant differences ($P < 0.05$) among all the experimental groups after 8 weeks of culture as summarized in Table 4. The highest protein, protease and amylase activity ($P < 0.05$) was recorded for the GIT of shrimp fed BM8 followed by BM5 and control, respectively. Clearly *B. subtilis* strains L10 and G1 showed their ability to increase digestive enzyme activities and induce a higher total protein content in the GIT of shrimp fed BM8 and BM5 diets.

3.4. Mortality and immune-related gene expression

After 8 weeks of the feeding period, shrimp fed BM8 and BM5 diets and the control were infected with *V. harveyi*. Cumulative

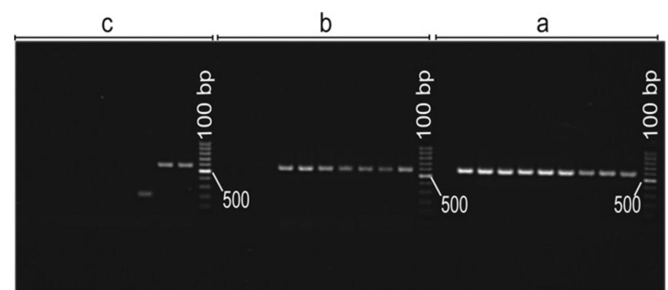

Fig. 1. *Bacillus* spp. detection in gastrointestinal tract (GIT) of shrimp *Litopenaeus vannamei* after 8 weeks of culture with or without probiotics *B. subtilis*, strains L10 and G1. DNA amplification of random colonies from shrimp GTI of; BM8: 10^8 cfu g^{-1} (a), BM5: 10^5 cfu g^{-1} (b) NB: no addition of probiotic (c).

Table 4
Digestive enzyme activity of *Litopenaeus vannamei* in treatments and control after 55 days fed with or without *Bacillus subtilis*, strains L10 and G1.

Activity (U g^{-1} protein)	Treatments		
	BM8: 10^8 cfu g^{-1}	BM5: 10^5 cfu g^{-1}	Control
Total protein	3.45 ± 0.11^a	2.99 ± 0.19^b	2.43 ± 0.1^c
Protease	1.43 ± 0.09^a	1.31 ± 0.03^b	0.93 ± 0.03^c
Amylase	1.48 ± 0.14^a	1.19 ± 0.13^b	0.68 ± 0.12^c

Values (means ± SD) with different superscript in a row show significant differences ($P < 0.05$).

mortality of shrimp after being injected with a high dose (10^6 CFU $shrimp^{-1}$) of *V. harveyi* showed significant differences ($P < 0.05$) between BM8 (20 ± 10%) and BM5 (33.3 ± 5.7%) compared to the control group (63.3 ± 15.3%) (Fig. 2). No mortality was observed in shrimp from the positive control which had been injected with NSS.

The proPO gene was significantly up-regulated ($P < 0.05$) in all shrimp fed *B. subtilis*-supplemented diets compared to the control group, with mean expression ratios of 48.77, 23.37 and 5.31 for BM8, BM5 and NB, respectively. PE showed significant up-regulation ($P < 0.05$) ratios including 784.4 for BM8, 11.3 for BM5 and 6.63 for NB compared to the control group. Expression of LGBP found to be significantly up-regulated ($P < 0.05$) in comparison to control with mean ratios of 24.83, 11.08 and 15.26 for BM8, BM5 and NB, respectively. In respond to pathogen *V. harveyi*, SP gene expression of shrimp in BM8, BM5 and NB were recorded in comparison to control, with significant up-regulations ($P < 0.05$) with mean ratios of 61.55, 28.34 and 11.33, respectively (Fig. 3).

4. Discussion

Manipulation of microbiota using probiotics have been reported as a worthy practice for aquaculture in order to control or inhibit the pathogen bacteria, improve the growth performances and digestive enzymes, and enhance the immune responses of the host against pathogens or physical stress [3,8,29]. We have recently reported the identification, characterization and safety of *B. subtilis* strains L10 and G1 as potential probiotics for shrimp culture [20]. In this study we determined the growth performance and digestive enzyme activity of shrimp fed diets containing *B. subtilis* strains, L10 and G1, at two different doses of 10^5 (BM5) and 10^8 (BM8) CFU g^{-1} feed. Survival and immune status of treated shrimp have also been investigated through an experimental infection with *V. harveyi*.

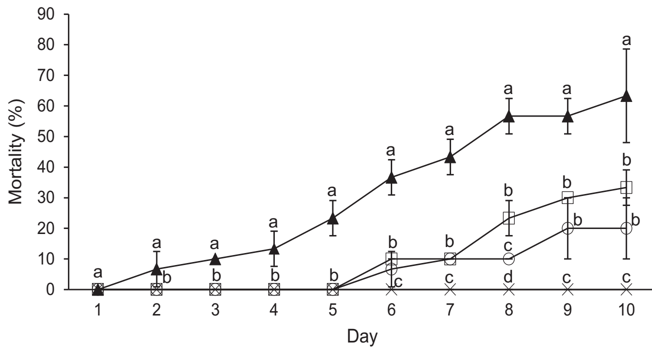

Fig. 2. Cumulated mortality (%) of white shrimp, *Litopenaeus vannamei*, for 10 days after being injected with *Vibrio harveyi* (10^6 cfu $shrimp^{-1}$) for BM8, BM5, and control or injected with NSS for NB statistical analysis showed the highest mortality ($P < 0.05$) of shrimp during 10 days of the challenge test for shrimp in control group compared to those shrimp in BM8, BM5 and NB. Different letters at the point of each day reperesent significant differences ($P < 0.05$) among treatments. Bars reperesent the SD for each point. (○) BM8: 10^8 cfu g^{-1} (□) BM5: 10^5 cfu g^{-1} (×) NB: no addition of probiotics but injected with NSS (▲) control: no addition of probiotics but injected with *V. harvyi*.

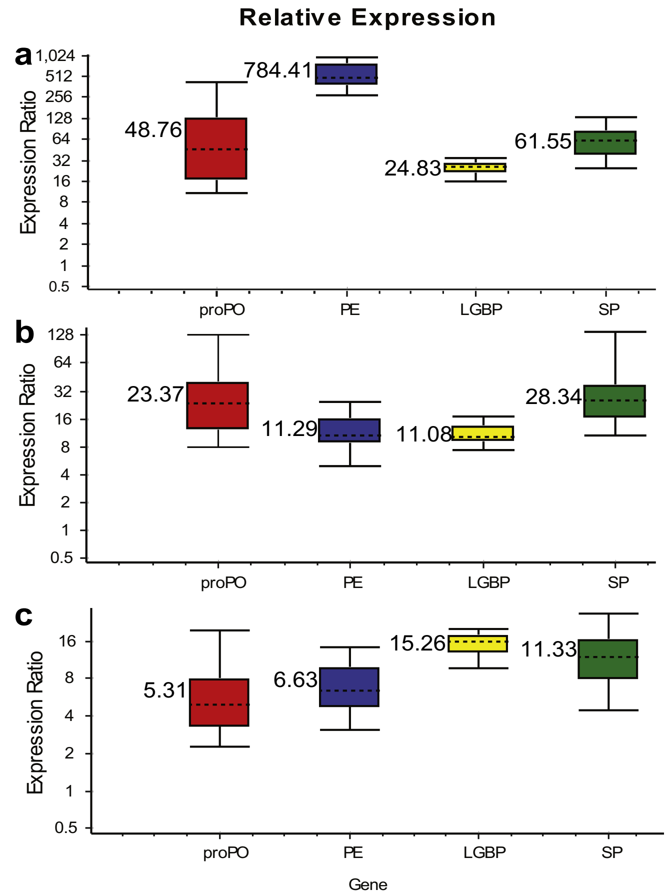

Fig. 3. The mRNA expression of four immune genes of *Litopenaeus vannamei* fed with *Bacillus subtilis* for 55 days and challenged with *Vibrio harveyi*. Gene expression profile of (a) shrimp fed *B. subtilis* L10 and G1 (10^8 cfu g^{-1}): proPo (P(H1) = 0.019), PE (P(H1) = 0.011), LGBP (P(H1) = 0.013), and SP (P(H1) = 0.025) are UP-regulated ($P < 0.05$) in sample group in comparison to control group, (b) shrimp fed *B. subtilis* L10 and G1 (10^5 cfu g^{-1}): proPo (P(H1) = 0.018), PE (P(H1) = 0.019), LGBP (P(H1) = 0.011), and SP (P(H1) = 0.021) are UP-regulated ($P < 0.05$) in sample group in comparison to control group, (c) shrimp fed without probiotics but injected with Normal Saline Solution: propo (P(H1) = 0.024), PE (P(H1) = 0.025), LGBP(P(H1) = 0.016), and SP (P(H1) = 0.031) are UP-regulated ($P < 0.05$) in sample group in comparison to control group. Control group was fed without probiotics but injected with *V. harveyi*. Data on the Boxplots represent the mean factors of gene expresions compared to control group. P(H1) represent the hypothesis test ($P < 0.05$).

Dietary administration of *B. subtilis* strains L10 and G1 significantly improved final weight, weight gain, SGR and survival of shrimp fed BM5 and BM8 diets (Table 2). Although several studies have demonstrated the beneficial effects of probiotics on the growth performance in shrimp [13,30,31], the exact mechanism of action is not well understood. The first explanation could be related to the action of competitive exclusion, by which probiotics may create a hostile environment for pathogen colonization. This mechanism of action has been determined in this study because of the GIT of treated shrimp was dominated by successful colonization of *B. subtilis* (Table 3). In addition, molecular detection of *B. subtilis* from MYP agar plates confirmed the presence of strains L10 and G1 in the GIT of the treated shrimp. On the other hand, the considerable reduction of *Vibrio* spp. populations in the GIT of shrimp fed BM5 and BM8 diets (Table 3) clearly showed the successful competitive exclusion of *B. subtilis* strains L10 and G1.

Another possible explanation for the improvement of the shrimp growth factors by *B. subtilis* may be due to the induction of digestive enzymes, including protease and amylase, which consequently stimulate the natural digestive enzyme activity of the host

[30,31]. In this study, the higher level of total digestive enzyme activity was recorded in shrimp fed *B. subtilis*-supplemented diets where the better growth performances were observed compared to control. Similar results have been reported by Ziaei-Nejad et al. [32] who observed a higher digestive enzyme activity in shrimp (*Fenneropenaeus indicus*) treated with *Bacillus* spp. than the controls.

In addition, it is important to mention that a better appetite was observed in shrimp fed diets containing *B. subtilis* L10 and G1 than the control group during the feeding period because of undigested feed residues were not found. A better feed digestion may be related to an increase of the digestive enzyme activity and subsequently increased the appetite in treated shrimp. Therefore by taking into account of the enzyme activity and appetite stimulation, together with the colonization of *B. subtilis* in the GIT of shrimp fed BM8 and BM5 diets, healthier shrimp and higher survival rate of 100 and 95.5% were resulted, respectively, compared to control with 86.5% (Table 2).

Eight weeks after the start of the feeding period, shrimp were challenged with pathogenic bacterium *V. harveyi*. As Fig. 2 shows, a higher resistance was observed in shrimp fed diets containing *B. subtilis* strains L10 and G1. A significantly lower mortality was recorded in shrimp fed BM8 and BM5 diets compared to the control group.

It is well known that hemocytes play important roles in the host immune responses. They are responsible for cellular defense mechanisms and also releasing humoral defense molecules in order to protect the body toward microbial intruders [33]. For the present study, it was almost impossible to collect hemocytes from juvenile shrimp ($\sim$3–4 g) to analyze the immune responses and biological activities such as pro-phenoloxidase activity (PO), phagocytosis activity, respiratory burst, cell adhesion activity, clearance efficiency, and superoxidase dismutase activity. Therefore, the expression of four immune-related genes was investigated using the real-time PCR in order to evaluate the immune status of shrimp after being injected with *V. harveyi* or NSS.

Activation of the prophenoloxidase-activating system is through recognition molecules in the hemolymph of invertebrates [34]. The prophenoloxidase activating system is a non-self-recognition system in invertebrates that is able to recognize and respond to intruders via lipopolysaccharides or peptidoglycan from bacteria and β-1,3-glucans from fungi [34]. Chiu et al. [10] reported that up-regulation of proPO resulted in increased PO activity in shrimp fed *L. plantarum*-supplemented diet, which enhanced the resistance against the pathogen, *Vibrio alginolyticus*. Similar results were found in our study, where up-regulation of proPO was recorded in shrimp fed BM8 and BM5 diets compared to the control group after challenge with *V. harveyi*. Chiu et al. [10] also suggested that the expression of PE gene can increase the biological activity of cell adhesion [35,36], opsonin [37], degranulation [38], peroxidase [39], and encapsulation [40] of shrimp. These biological activities might be achieved in the present study with the administration of *B. subtilis* strains L10 and G1, as PE gene up-regulation was enhanced in treated shrimp compared to the control.

Serine protein (SP) is responsible for converting proPO to PO [34]. The proPO gene expression in shrimp fed diets containing *B. subtilis* was accompanied with the up-regulation of SP gene for the same group of shrimp, suggesting that the up-regulation of proPO gene was a consequence of the gene expression of SP. Different result for SP regulation was observed by Liu et al. [41], although the gene expression of proPO was enhanced in shrimp treated with *B. subtilis* E20. Compared to the present study, it could be due to the period of their experiment which had been 2 weeks according to their study.

Lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP) is well known as another key of the immune response which recognizes and responds to microbial intruders and results in the activation of proPO system [42]. Cheng et al. [43] revealed that LGBP has a crucial role in shrimp defense during the early stage. They found the up-regulation of LGBP gene in the hepatopancreas of *L. vannamei*, 24 h post-injection with *V. alginolyticus*. These findings are comparable with the up-regulation of LGBP gene extracted at 24 h post-injection from shrimp fed diets containing *B. subtilis* strains L10 and G1. These facts suggest that the up-regulation and gene expression of proPO might be a consequence of LGBP up-regulation for treated shrimp with *B. subtilis* L10 and G1 as probiotics.

The manipulation of shrimp intestinal microbiota using *B. subtilis* strains, L10 and G1, induced the expression of immune-related genes and a better protection against *V. harveyi*. The four immune-related genes tested in this study including prophenoloxidase (proPO), the cell adhesive protein, peroxinectin (PE), the recognition protein, lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP), and serine protein (SP) which are relative to the proPO system showed up-regulation of these genes in shrimp fed diets containing *B. subtilis* strains L10 and G1. These findings are in agreement with the increase of the biological activities in treated shrimp, resulting in a higher survival rate after challenge with *V. harveyi*. It can be kept in mind that the expression of immune-related 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.

In conclusion, administration of *B. subtilis* strains L10 and G1 improved the growth performances, digestive enzyme activity and immune response against the pathogenic bacterium, *V. harveyi*. In addition, a better survival rate was obtained in shrimp fed probiotic diets after challenge with *V. harveyi*.

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