Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 25(3): 241 – 261 (2021)
www.ejabf.journals.ekb.eg

The influence of continuous and intermittent *Bacillus subtilis* AQUA-GROW® application on the white leg shrimp, *Litopenaeus vannamei*, immune-related genes

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ARTICLE INFO

Article History:
Received: April 19, 2021
Accepted: May 26, 2021
Online: June 5, 2021

Keywords:
Bacillus subtilis,
Gene expression,
Immune-related genes,
Litopenaeus vannamei

ABSTRACT

In the framework of fact, prolonged and excessive use of probiotics has been implicated in immunosuppression. The realization of immuno-stimulants frequent application impacts is the groundwork for tactical protection of shrimp culture. Therefore, changes in the gene expression of five immune attributable genes (prophenoloxidase, serine proteinase, transglutaminase, lysozyme; and superoxide dismutase) of *Bacillus subtilis* supplemented shrimp, *Litopenaeus vannamei* over a period of time (12 weeks) were assessed before and after *Vibrio parahaemolyticus* bacterial challenge. *L vannamei* of weight measured 0.78±0.08 (SE) was randomly divided into three groups. Frist group (T1) was the normalizing group (control) fed plain diet, the second group (T2) fed *B. subtilis* supplemented diet daily, the third group provided *B. subtilis* supplemented diet followed by basal diet changeably week after week. The findings before bacterial challenge manifested that proPO of (T2) expressed significantly in 1st, 2nd, 4th, and 7th weeks. In (T2) SP expressed highly in the 2nd and 7th week. proPO, SP, TGase, and LYZ of (T3) presented significant up-regulation in the 7th week. TGase of (T2) and SOD of (T3) expression levels increased significantly in 4th and 7th weeks. LYZ and SOD of (T2) expression levels increased significantly in the 2nd, 4th, 7th weeks. In response to induced infection, proPO of (T2) and (T3) proPO, SP, and SOD expression levels were observed to respond to bacterial infection at early and late infection phases 3 and 24 (hpi). This work suggested that the transient application of *B. subtilis* for shrimp *L. vannamei* enhances the immune system, and their immune status is poised and all set against *V. parahaemolyticus* by rapid and early defensive responses.

INTRODUCTION

Aquaculture is one of the braces of the economic issues toward food demand achievement and unemployment combating. However, disease outbreak is considered a major obstacle in aquaculture attributed to the economic impoverishment of the aquaculture industry **(Chauhan and Singh, 2018)**. A wide range of antibiotics has

tremendously been used as a strategy for disease control in aquaculture. Antibiotics application in shrimp culture system concerned from human health attitude as human health has been fraught with danger from the antibiotic application, due to the evolution of antibiotic-resistant strains **(Ninawe and Selvin, 2009)**. So there is an increasing concern to detect an eco-friendly alternative to overcome the diseases. The employment of probiotics is a potential substitutional procedure for controlling infectious agents and treating diseases **(Chauhan and Singh, 2018)**. Probiotics show effective impacts through immunity enhancement and improved physiological responses of shrimp **(van Hai and Fotedar, 2010)**.

Implementation of *Bacillus subtilis* probiotic exhibited favorable impacts in shrimp aquaculture. *B. subtilis* has no pathogenic effect, but can form spore. It is a Gram-positive bacterium that has been improved growth parameters and the reluctance of disease **(Balcázar and Rojas-Luna, 2007; Shen e*t al.*, 2010; Zokaeifar *et al*., 2012)**. Besides, *Bacillus* species can produce variable substances of anti-microbial activity **(Perez *et al.*, 1993; Korenblum *et al.*, 2005; Zokaeifar *et al.*, 2012).**

Over dosage or prolonged administration of probiotics leads to immune fatigue and subsequent immunosuppression **(Bai *et al.*, 2010; van Hai and Fotedar, 2010).** The effective protection of shrimp culture systems against pathogens is achieved by appreciating the prominence of probable immuno-stimulant dose and the frequency **(Sajeevan *et al.*, 2009; Babu *et al.*, 2013*).***.**

The current study aimed to assess the continuous and intermittent *B. subtilis* supplementation effect on the innate immune response and subsequently disease overcome against *V. parahaemolyticus* in whiteleg shrimp, *Litopenaeus vannamei*.

MATERIALS AND METHODS

1. Experimentation with animals

A bevy of supposedly healthy cultured whiteleg shrimp, *Litopenaeus vannamei,* was purchased from a private shrimp hatchery in Damietta governorate in July 2020.

2. Acclimation procedures of shrimp to the culture system

Shrimps were accommodated to culture circumstances in 1 m^3 plastic tanks (200 shrimp per tank) containing 500 L of screened seawater (30 %) and continual aeration for 14 days with gradual salinity increasing until reach (35 %). Shrimp subsisted on ad libitum commercial diet two-times daily at 9:00 am and 9:00 pm (control diet; **Aller ® Egypt**, 38 % protein, Table1).

3. Preparation of AQUAGROW supplemented experimental diets

Commercial feed (**ALLER® Egypt**) was used as a basal medium for *B. subtilis* supplementation (**AQUA-GROW®** manufactured by **Canal AquaCure Company, Egypt**, **Table 2**). The feed was sprinkled with probiotic to obtain a definite concentration of around 2×10^9 CFU /Kg feed. Probiotic supplemented feed was desiccated at room ambient temperature and preserved at 4 °C until use.

Table 1. Nutrient composition of the commercial feed (control).

Parameter	%
Crude protein	38
Crude fat	7
NFE (Nitrogen-Free Extract)	41.1
Ash	10.7
Fiber	3.2

Table 2. AQUA-GROW® composition per 100 g

Bacillus subtilis	2×10^{11}
Vit[a]. A	12000000 IU
Vit. D_3	2500000 IU
Vit. E	25000 mg
Vit. C	50000 mg
Choline	50000 mg
Betaine	50000 mg
Lysine	75000 mg
L- methionine	50000 mg
L- threonine	10000 mg
Valine	25000 mg

[a] Vitamin

4. Experimental design

After the period of adaptability, a total of 315 whiteleg shrimp, *Litopenaeus vannamei*, were subjected to weight measuring and parted at a random manner in 9 rounded plastic tanks containing 25 l seawater for a 12-week feeding trial. Every 35 individuals, approximately weighed 0.78 g ± 0.08 (SE) was stocked in each tank to establish three experimental groups conducted in 3 replicates for each group. Frist group turned out to be a control and consumed a plain un-supplemented diet throughout the experimental time series (T1). The second group daily fed *B. subtilis* supplemented diet throughout the experiment (T2), while the third group fed *B. subtilis* supplemented diet intermittently week after week (T3). Shrimp were provided with their allotted diets two-time per day at 5% of the body weight.

5. Immune challenge with *Vibrio parahaemolyticus*

At the 12th week of the feeding trial, the pathogenic *vibrio parahaemolyticus* was cultivated in Brain Heart Infusion broth (BHI) at a temperature of 25 °C for 24 h followed by centrifugation (1000 ×g). The fluid (supernatant) was eliminated, and the

bacterial suspension was performed by the addition of sterile saline solution (SSS; 1.5% NaCl) to the bacterial pellet **(Vieira *et al.*, 2010)**. Bacterial concentration was estimated spectrophotometrically at optical density 600 nm (OD_{600}) **(Won *et al.*, 2020)**. The final bacterial concentration was customized to 10^7 CFU/ml. Challenge experiment was conducted by random selecting ten shrimp from each tank and stocked in new 25 l plastic tanks. Infection was induced by the injection of each individual in the 2nd abdominal segment with 25 µl SSS bacterial suspension of final concentration 10^7 CFU/ml.

6. Hemolymph and hepatopancreas sampling

Samples of hemolymph and hepatopancreas were collected from shrimp of each treatment replicates on days 0, 1, 3, and 7 of the first week, then on days 3 and 7 of the 2nd week, and day 3 of the 4th, 7th, and 12th weeks throughout the experiment. Hemolymph and hepatopancreas were collected during the challenge test, before infection and 3, 6, 12, 24 hr post-infection. Hemolymph was obtained from the ventral sinus cavity using a 26 G syringe. The extracted hemolymph and hepatopancreas were added immediately in Trizol (**QIAzol® Lysis Reagent: QIAGEN)** for total RNA isolation.

7. Total RNA extraction, cDNA synthesis, and gene expression analysis by quantitative real-time PCR

The operating protocol for this analysis was in running order according to the methods showed by **Aguilera-Rivera *et al.* (2019)**. Concisely, Total RNA was isolated from hepatopancreas and hemolymph (the volume of samples did not exceed 10 % of the used Trizol volume) from freshly collected shrimp following instructions of Trizol reagent (**QIAzol® Lysis Reagent**). The isolated RNA was measured at 260 and 280 nm with a spectrophotometer (UNICO- UV-VIS Spectrophotometer). Only RNA samples had (A260/A280) ratio higher than 1.8 were submitted to subsequent analysis **(Aguilera-Rivera *et al.*, 2019)**. **RevertAid First Strand cDNA Synthesis Kit®** (Thermo Scientific) was used to efficiently synthesize cDNA from RNA templates according to kit instructions.

Specific primers (**METBION®)** for immune-related genes were selected to perform quantitative real-time PCR (RT-qPCR), based on previous published *L. vannamei* primer sequences of the following genes; prophenoloxidase, transglutaminase, superoxide dismutase, lysozyme and serine proteinase (immune-related genes), and β-Actin gene (internal control) **(Han-Ching Wang *et al.*, 2010; Zokaeifar *et al.*, 2012; Fierro Coronado *et al.*, 2018; Jiao *et al.*, 2021)** (**Table 3**). The RT-qPCR was performed by **7500 Fast Real-Time PCR System®** Applied biosystem using SYBR Green Mastermix (**Top Real SYBR mix®, Biovision**). Sample duplication was operated for each sample. RT-qPCR cycle set consisted of 15 min of denaturation at 95℃ then 40 thermal cycles at 95℃ for 15 s followed by 60℃ for 30 s along with 1 min at 60℃. ΔC_t was calculated for each cDNA sample, from the indicated threshold cycle (Ct) of the required gene customized to the Ct of internal control gene *β-actin* in the same sample and subsequent

determination of $\Delta\Delta C_t$ value. Finally, the fold change relative to the basal expression of the control was represented as $2^{-\Delta\Delta ct}$.

Table 3. sequences of primers submitted for RT-qPCR to assess the immunological state of shrimp, *Litopenaeus vannamei*

Immune element/response	Gene	Abb. name [a]	PrimerF/R[b]	(5̀ -3̀) primer sequence
ProPO activating system	Prophenoloxidase	proPO	proPO-F	GAG ATC GCA AGG GAG AAC TG
			proPO-R	CGT CAG TGA AGT CGA GAC CA
	Serine proteinase	Sp	SP-F	CGT CGT TAG GTT AAG TGC GTT CT
			SP-R	TTT CAG CGC ATT AAG ACG TGT T
Clotting system	Transglutaminase	TGase	TGase-F	CCT CAG GAT CTC CTT CAC CA
			TGase-R	TTG GGA AAA CCT TCAT TTC G
Anti-microbial peptide system	Lysozyme	Lyz	Lyz-F	GAA GCG ACT ACG GCA AGA AC
			Lyz-R	AAC CGT GAG ACC AGC ACT CT
Antioxidant defense mechanism	Superoxidase dismutase	SOD	SOD-F	ATC CAC CAC ACA AAG CAT CA
			SOD-R	AGC TCT CGT CAA TGG CTT GT
Internal control	β-actin		β-actin-F	CCA CGA GAC CAC CTA CAA C
			β-actin-R	AGC GAG GGC AGT GAT TTC

[a] Abbreviation gene name [b] Forward/ reverse primers

8. Statistical analysis

The variables of the study were analyzed and described from a statistical point of view by using means and f-values to find differences between the control group and the experimental treatments by using (Univariate Analysis of Variance) and the significant variation ($P<0.05$) was detected by Tuckey's range test. All statistical analysis was figured by SPSS.

RESULTS

1. Immune-associated genes expression levels of *B. subtilis* supplemented shrimp during a serial course of time

Time series course RT-qPCR assessment was operated to explore the expression modulation of 5 immune-involved shrimp genes upon 12 weeks *B. subtilis* supplementation trial. Following the $2^{-\Delta\Delta ct}$ equation, the data are stated as the fold change

in expression levels of the target gene customized to an internal reference gene (β-actin) and in proportion to the control **(Livak and Schmittgen, 2001)**.

The expression levels of proPO gene from shrimp fed *B. subtilis* supplemented diet by continuous manner (T2) exhibited notable ($p<0.05$) up-regulation in 1st, 2nd, 4th, and 7th weeks relative to the control set, with fold difference 3.4, 3.2, 5.2, and 4.4, respectively. Nevertheless, the reduction ($p>0.05$) of proPO expression level of (T2) was observed in the 12th week with a relative fold change of 1.5. However, shrimp fed *B. subtilis* supplemented diet week after week (T3) showed significant up-regulation ($p<0.05$) of its proPO expression levels in the 1st week and reach its peak in the 7th week compared to the control group, with a fold difference of 3.6 and 11.1, respectively (**Fig. 1**).

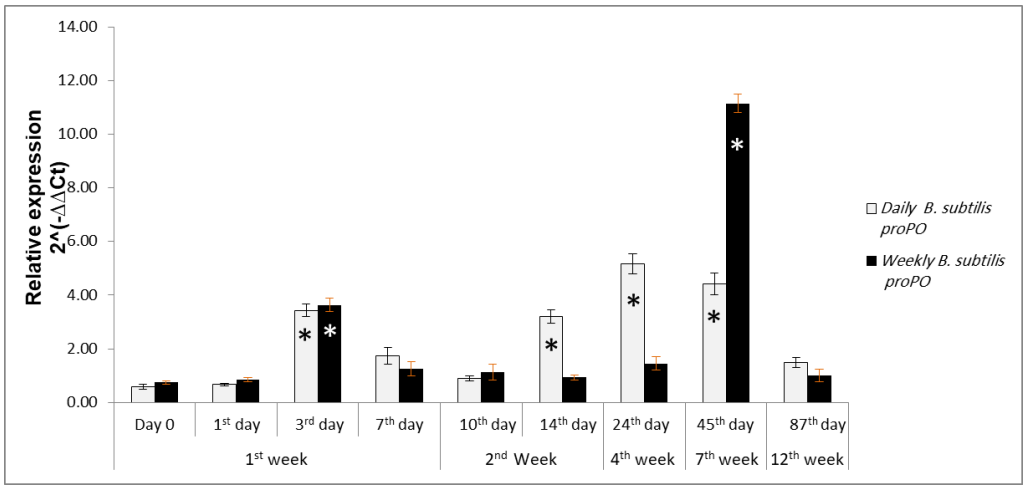

Fig. 1 Relative expression level means ± standard error (SE) of hemolymph and hepatopancreas prophenoloxidase (ProPO) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value (p<0.05) presented by (*)

Serine proteinase (SP) expressed in (T2) obviously ($p<0.05$) in 2nd week reached its peak in 7th week in comparison with control (4.6 and 7.6 relative fold change, respectively), but its expression level decreased slightly ($p>0.05$) in 4th and 12th weeks. On the other hand, SP expression reached its peak ($p<0.05$) in (T3) in the 7th week to be higher than that of (T2) in the same week, with a fold difference of 9.7 and 7.6, respectively (**Fig. 2**).

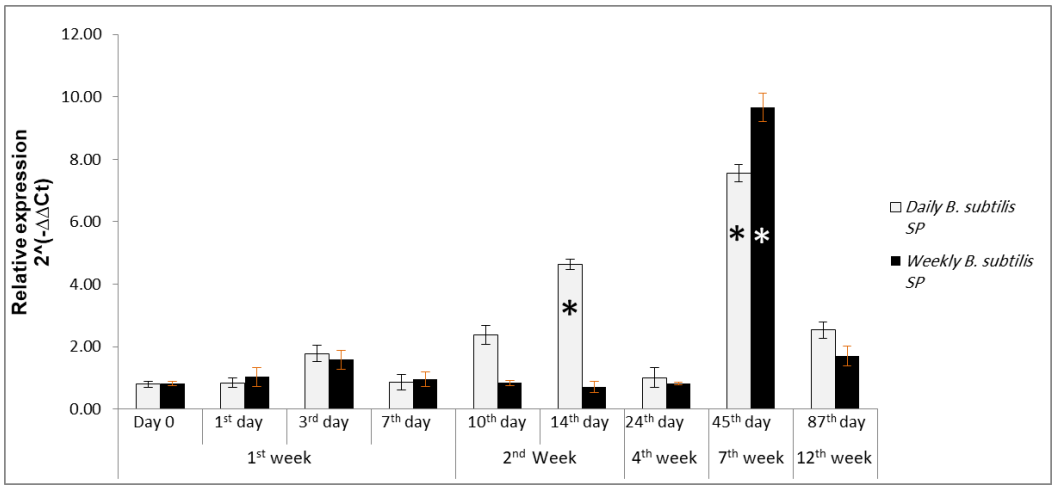

Fig. 2 Relative expression level means ± standard error (SE) of hemolymph and hepatopancreas serine proteinase (SP) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

Daily consumption of diet supplemented with *B. subtilis* up-regulated the transglutaminase (TGase) mRNA expression levels of (T2) in 2nd (statistically of no significant $p>0.05$), 4th weeks ($p<0.05$). It showed up its superior value ($p<0.05$) in 7th week relative to the control group (2.4, 3.6, and 9.2 fold change, respectively). Conversely, the level of TGase expression in the 12th week showed a decline in its value comparing to its 7th-week value (2.6 and 9.2 fold change relative to control, respectively). While (T3) TGase expression level increased in 4th ($p>0.05$) and 7th ($p<0.05$) week with proportional fold change (2.4 and 9.9) respectively (**Fig. 3**).

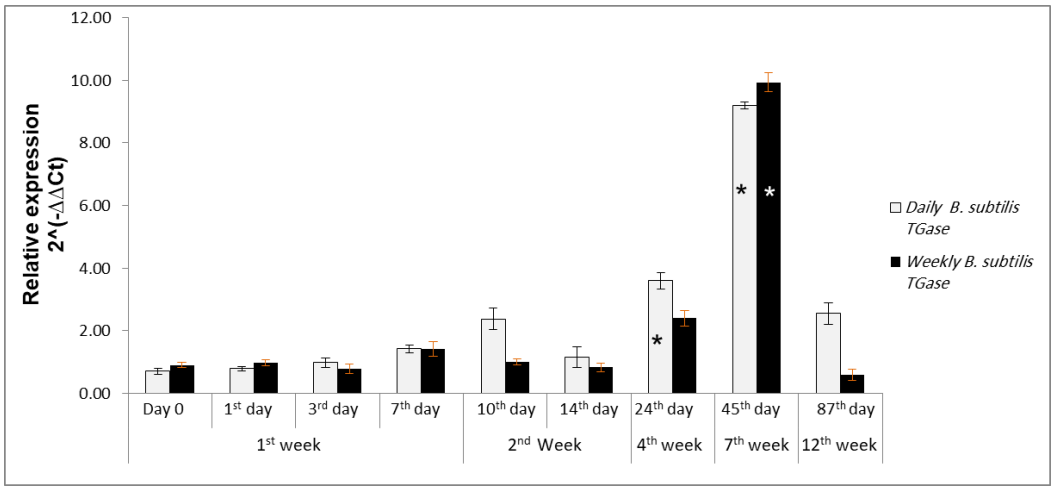

Fig. 3 Relative expression level means ± standard error (SE) of hemolymph and hepatopancreas transglutaminase (TGase) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

The expression levels with slight up-regulation ($p>0.05$) of (T2) Lysozyme (LYZ) were detected in the 1st week of the feeding trial, followed by its peak ($p<0.05$) in the 2nd and 4th week (7.8 and 6.9 relative fold change, respectively). The expression levels decreased afterward in the 7th and 12th weeks (5.4 and 3.9 relative fold change,

respectively), but still statistically of significant value ($p<0.05$). Otherwise, LYZ expression levels of (T3) up-regulated ($P>0.05$) in the 1st week and reached a superior level (P<0.05) in the 7th week (4 relative fold change) (**Fig.4**).

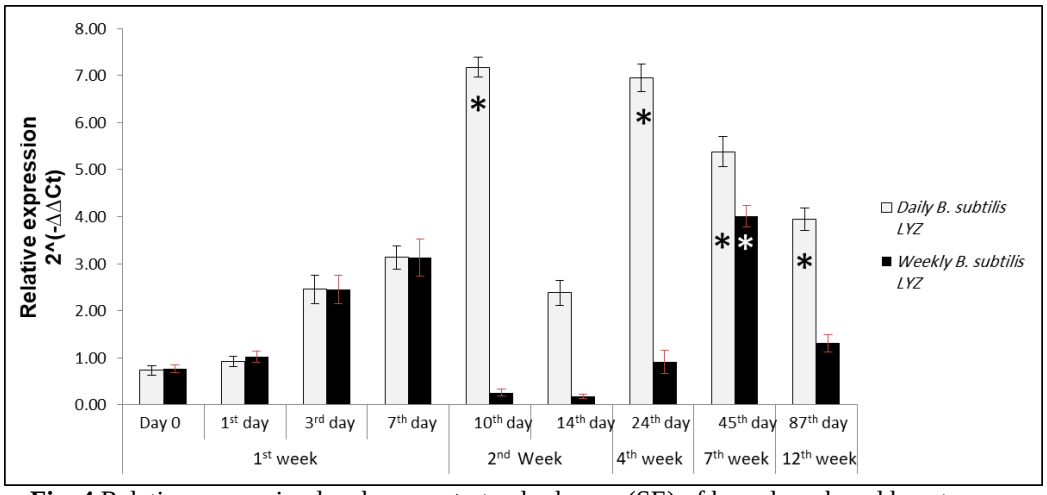

Fig. 4 Relative expression level means ± standard error (SE) of hemolymph and hepatopancreas lysozyme (LYZ) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

Sodium dismutase (SOD) mRNA expression levels of (T2) started to increase significantly in the 2nd week and attained their maximum ($p<0.05$) in the 4th week with 4.1 relative fold change. After that, SOD decreased expression levels were observed in the 7th and 12th weeks (2.9 ($p<0.05$) and 1.3 relative fold change, respectively). Furthermore, SOD expression levels of (T3) up-regulated significantly ($p<0.05$) (3.9 relative fold change) in the 4th week and reached its topmost value ($p<0.05$) (7.5 relative fold change) in the 7th week (**Fig. 5)**.

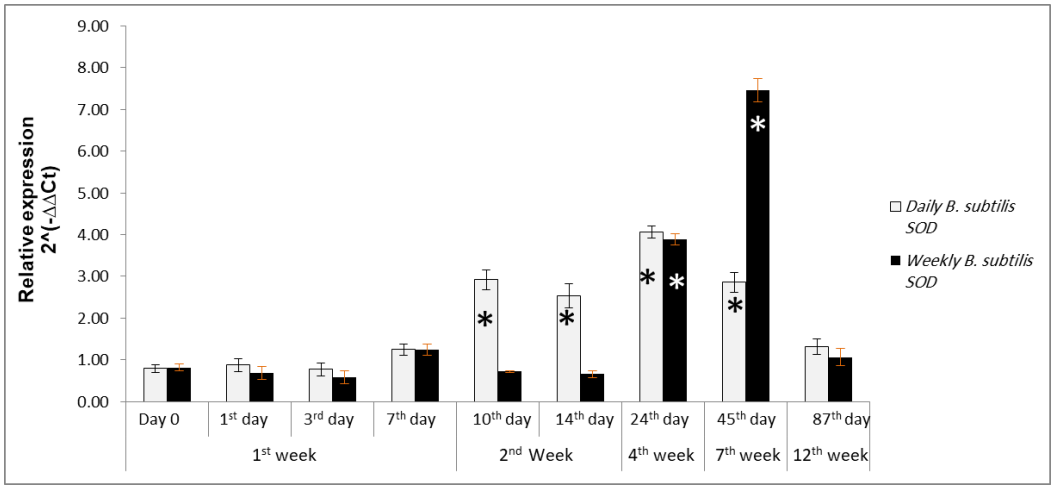

Fig. 5 Relative expression level means ± standard error (SE) of hemolymph and hepatopancreas superoxide dismutase (SOD) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

2. Immune-associated genes expression levels of *B. subtilis* supplemented shrimp in response to *Vibrio parahaemolyticus* challenge

The expression levels of proPO in the continuous and pulsed *B. subtilis* probiotic feeding regime (T2 and T3 respectively) showed up-regulation at 3 hours post-infection (hpi) ($p<0.05$) with relative fold differences of (4.3 and 3.7, respectively) then the expression levels of both groups showed a decline in proPO expression levels followed by up-regulation of proPO expression levels of significant value ($p<0.05$) in both groups at 24 hpi with relative fold change 3.6 and 9.9 in (T2) and (T3), respectively (**Fig. 6**).

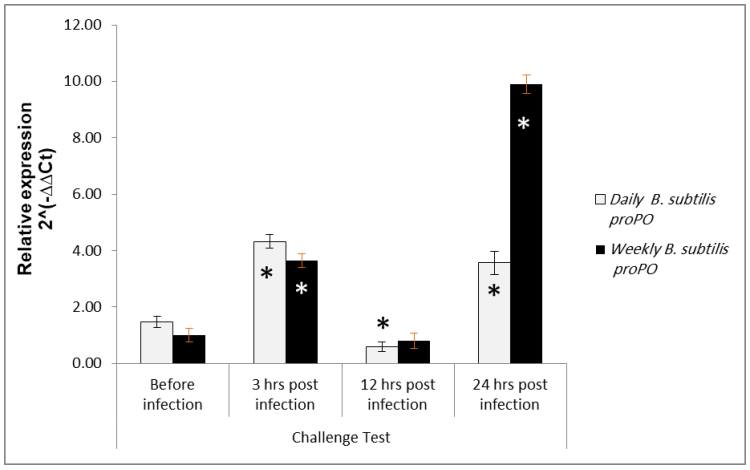

Fig. 6 Relative expression levels (mean±SE) of hemolymph and hepatopancreas prophenoloxidase (proPO) in response to *Vibrio parahaemolyticus* challenge after 12-week *Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

SP expression of (T2) down-regulated ($p<0.05$) in the first 12 hpi followed by non-significant up-regulation with relative fold change (4.4) at 24 hpi. However, SP expression of (T3) peaked ($p<0.05$) at 3 hpi followed by down-regulation at 12 hpi then showed a significant up-regulation ($p<0.05$) of SP expression level at 24 hpi (22, 0.54, and 8.6 fold change comparing with the control set, respectively) (**Fig. 7**).

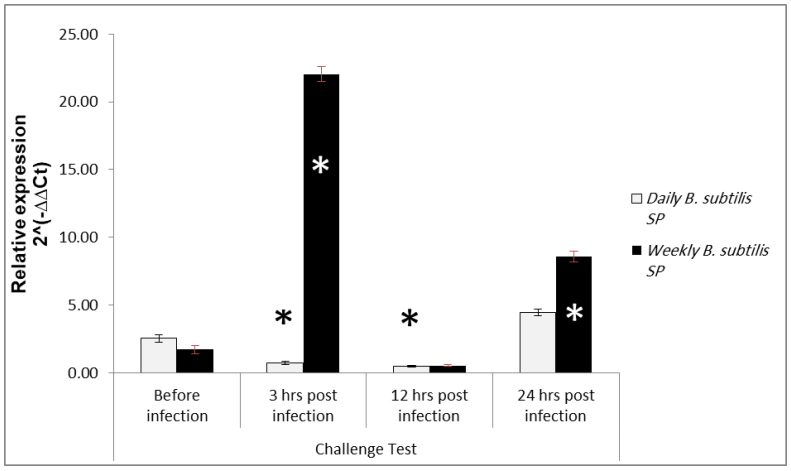

Fig. 7 Relative expression levels (mean±SE) of hemolymph and hepatopancreas serine proteinase (SP) in response to *Vibrio parahaemolyticus* challenge after 12-week *Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

Expression response of (T2) TGase after *V. parahaemolyticus* injection showed non-significant (*p>0.05*) differences with that of control, whereas (T3) TGase levels of expression insignificantly down-regulated (*p>0.05*) at early and mid-infection phase (0.7 and 0.8 relative fold change at 3 hpi and 12 hpi, respectively) followed by an obvious (*p<0.05*) up-regulation of the expression level at 24 hpi (13.3 relative fold change) (**Fig. 8)**.

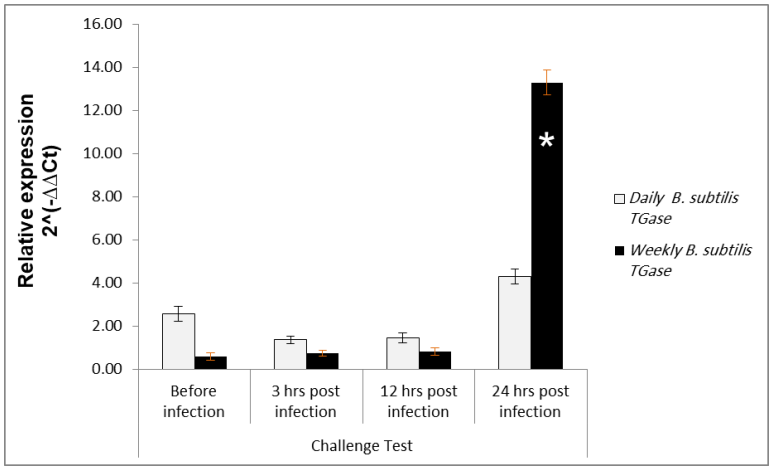

Fig. 8 Relative expression levels (mean±SE) of hemolymph and hepatopancreas transglutaminase (TGase) in response to *Vibrio parahaemolyticus* challenge after 12-week *Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value (*p<0.05*) presented by (*)

Lysozyme (LYZ) expression respond to *V. parahaemolyticus* challenge at early and mid-infection phase by slight (*p>0.05*) reduction of the expression levels of (T2 andT3) followed by notable (*p<0.05*) up-regulation in (T3) at late infection phase (24 hpi) with fold change 19.7 relative to control set. LYZ expression of (T2) in response to induced infection showed insignificant (*p>0.05*) differences with that of the control group (**Fig. 9**).

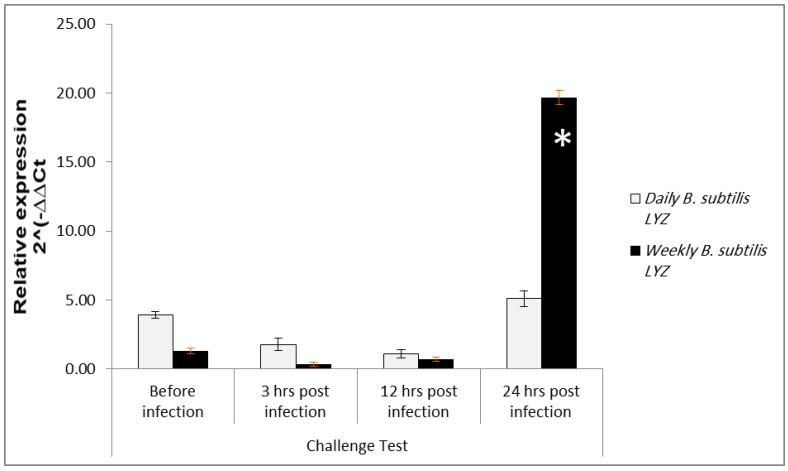

Fig. 9 Relative expression levels (mean±SE) of hemolymph and hepatopancreas lysozyme (LYZ) in response to *Vibrio parahaemolyticus* challenge after 12-week *Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value (*p<0.05*) presented by (*)

Results manifested non-significant differences (*p>0.05*) between (T2) SOD expression levels and that of control in response to challenge with *V. parahaemolyticus*. On the other hand, (T3) SOD expression obvious (*p<0.05*) up-regulation was observed throughout the challenge period (6.7and 7.7 relative fold change at 3 hpi and 24 hpi, respectively) (**Fig. 10**).

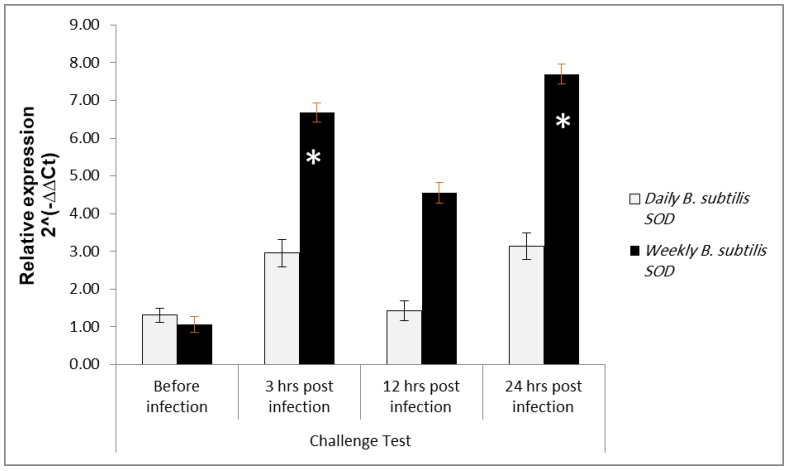

Fig. 10 Relative expression levels (mean±SE) of hemolymph and hepatopancreas superoxide dismutase (SOD) in response *to Vibrio parahaemolyticus* challenge after 12-week *Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value (*p<0.05*) presented by (*)

DISCUSSION

Species of genus *Bacillus* can boost innate and acquired immune responses of the host owing to their immunostimulatory capacity and improvement of favorable intestinal microflora **(Kuebutornye *et al.*, 2019)**. *B. subtilis* probiotic supplementation induces expression of proteins (pathogen recognition protein, PRPs) in the host, which involved in recognition of pathogen molecular pattern (lipopolysaccharide-b-1,3-glucan binding protein, LGBP) and subsequently increasing expression of the proPO system **(Zokaeifar *et al.*, 2012)**.

The proPO system triggered by the recognition of the specific structure of pathogen by mean of proteins known as pattern-recognition proteins (PRPs), boost a serine proteinase pathway, eventually resulting in the splitting of the inert proPO to the active PO that involved in melanin and toxic reactive intermediates synthesis against microbes **(Amparyup *et al.*, 2013).**

Shrimp proPOs have been reported to be restricted only to the hemocytes **(Ai *et al.*, 2009; Masuda *et al.*, 2012)**. Accordingly, proPO RNA from all shrimp belonged to the *Penaeidae* family, except for one from *M. japonicus* **(Masuda *et al.*, 2012)**, are recorded to be expressed entirely in hemocytes **(Sritunyalucksana *et al.*, 1999; Amparyup *et al.*, 2009; Gao *et al.*, 2009)**. Contrariwise, The peak value of proPO expression was observed in hepatopancreas at 6 and 24 hours, respectively, followed by the injection of LPS (lipopolysaccharide) and a notable decrease of proPO expression in hemocytes from 3 h

to 48 h was noticed following injection with laminarin, LPS and poly I:C (mimicking viral infection) suggesting that the hepatopancreas was also implicated in immune factors synthesis **(Ji *et al.*, 2009)**.

Continuous administration of AQUA-GROW® *B. subtilis* supplement associated with a remarkable up-regulation of proPO gene expression levels through the first seven weeks peaked at the 7th week. A simple correspondence was reported that the prophenoloxidase expression in shrimp *Litopenaeus vannamei* fed the *B. subtilis* (at 10^8 CFU/g diet) improved significantly in comparison with those on the control group after eight weeks feeding trial **(Won *et al.*, 2020)**.

As previously mentioned, the awareness of immunostimulants application issues, including the concentration and frequency protocols, is an effective approach to control diseases in shrimp **(Sajeevan *et al.*, 2009; Babu *et al.*, 2013)**. Shrimp *L. vannamei* immune fatigue was recorded after continuous immuno-stimulants β-glucan or glycyrrhizin administration **(Bai *et al.*, 2010)**. The above findings interpret the decreased result of proPO expression level in the 12^{th} week of the recent study. Also, *L. vannamei* PO activity was significantly low after 12 weeks feeding regime with multispecies probiotic bacteria **(Kesselring *et al.*, 2019)**. However, any decline of proPO system efficiency might contribute to incompetence phagocytic activity; superabundance levels, or extended period of prophenoloxidase subsequent cytotoxic medium can also involve in the destruction of host tissue and cell death **(Cerenius *et al.*, 2008; Amparyup *et al.*, 2013)**. The proPO expression level dropped near the control level on the 7^{th} day of the first week, and the 3^{rd} day of the second week. It was suggested that the early decrease of proPO level might be a protective immune response.

ProPO expression levels of intermittently *B. subtilis* supplemented group showed up-regulation only in weeks of *B. subtilis* supplemented diet (2^{nd} and 7^{th} week) followed by reducing its value after shifting supplemented diet to control (basal) diet. This present finding was in agreement with previous findings that assessed probiotic *Lactobacillus plantarum* action accordance with time in *L. vannamei*, indicating that the time-related action of *L. plantarum* is short as a meager difference in hemolymph proPO activity in 6^{th} day after alternating supplemented diet to basal diet **(Vieira *et al.*, 2008)**. The above finding was supported by **Duc le *et al.*, (2004)** study, which stated that *B. subtilis* cleared from the gut rapidly after 6 days.

Boosting the proPO system is regulated by a multistep signaling pathway. The pathogen recognition depending on their PAMPs (pathogen-associated molecular pattern) by PRPs (pathogen recognition protein), provokes serine proteinases (SPs) cascade, which ended by the proteolytic splitting of the proPO proenzyme to its active form PO enzyme. PAMPs include lipopolysaccharide (LPS) peptidoglycan (PGN). **Chiu *et al.* (2007)** stated that a shrimp that fed *L. plantarum*-supplemented diet showed increasing proPO generating PO activity improvement, which promoted the shrimp's ability to resist *Vibrio alginolyticus* infection. Similarly, up-regulation of proPO was observed against *V.*

harveyi after a feeding trial of two probiotic *B. subtilis* strains **(Zokaeifar *et al*., 2012)**. Likewise, in the present study, proPO levels of expression up-regulated in both treatments at 3 hpi and 24 hpi after challenge with *V. parahaemolyticus*.

The proPO gene expression peaked at the late phase of infection, which was detected in freshwater crayfish, shrimp *P. vannamei*, and mud crab following *Aeromonas hydrophilla* challenge 24 (hpi), *Vibrio harveyi* at 36 (hpi) and *Vibrio parahaemolyticus* at 72 (hpi), respectively **(Han-Ching Wang *et al.*, 2010; Liu *et al.*, 2013; Zhang *et al*., 2019; Deris *et al.*, 2020)**. The previous finding suggests that *B. subtilis* supplementation triggers an early immune response against bacterial infection *V. parahaemolyticus* at 3 (hpi). The drop observed at 12 (hpi) in proPO level after *V .parahemolyticus* challenge may be attributed to modulate massive melanin production **(Amparyup *et al.*, 2013; Deris *et al.*, 2020)**. Also, the previous report detected that *L. vannamei*, LvPPAE1 (prophenoloxidase-activating enzyme) expression levels declined in hemocytes and increased in the gill following *V. harveyi* injection **(Jang *et al.*, 2011)**.

The SP cascade activated by the interaction between pathogen-related PAMPs and PRP of the host ended by PO boosting, which mediated melanin synthesis and its precipitation in every respect to the infection site or surrounded the surface of the foreign pathogen **(Vieira *et al.*, 2008**; **Amparyup *et al.*, 2013)**. In the present study, serine proteinase expression levels of (T2) increased 2nd and 7th week, then followed by a decline in its level at 12th week was in agreement with the finding described by **Won *et al.* (2020)**. The increased proPO gene expression in (T2 and T3) was not positively related to SP expression, suggesting the proteinase activity modulated by proteinase inhibitors **(Amparyup *et al.*, 2013),** as a negative modulator of the proPO regulation cascade. However, it was reported that proPO gene expression was accompanied by the up-regulation of the SP gene in shrimp received *B. subtilis* supplementation **(Zokaeifar *et al.*, 2012)**. Conversely, proPO gene expression was not correlated with SP expression **(Liu *et al.*, 2010)**.

SP expression levels of Chinese white shrimp increased at 6 h post- *Vibrio* challenge in hepatopancreas, hemocytes then showed a decrease in its value at 12 hpi in both hemocytes and hepatopancreas then at 24 hpi SP level increased in hepatopancreas with decline hemocytes SP expression **(Ren *et al.*, 2009)**. SP transcriptional levels of Vibrio challenged treatment (T3) has improved than control with obvious up-regulation ($p<0.05$) at 3 and 24 hpi, suggesting that pulsed *B. subtilis* supplementation improves the immune response of shrimp through a rapid increase of SP expression to quench infection. While (T2) SP expression response was insignificant ($p>0.05$) suggesting that the continuous application of *B. subtilis* for a long time may interfere with the immune capacity of shrimp.

Transglutaminase (TGase) is reported to play a key role in the blood coagulation mechanism, which is a conserved process implicated in the defense mechanism in invertebrates. TGase levels of expression are expressed extensively in the hemocytes and

hematopoietic tissue, and the presence of LPS and β-1,3-glucans trigger TGase release as a rapid and active response of hemocytes **(Fagutao *et al.*, 2012; Fierro Coronado *et al.*, 2018)**.

It was previously stated, TGase expression of shrimp-post-larvae significantly increased after15-day administration of *Bacillus* strain containing amorphous poly-beta-hydroxybutyrate **(Laranja *et al.*, 2017)**. It was in agreement with the present transglutaminase expression finding in this study; the expression levels of TGase elevated in both (T2) and (T3).

TGase expression of (T2) showed statistically insignificant difference with that of control ($p>0.05$) in response to the bacterial challenge by the time, its expression levels of (T3) was significantly up-regulated following *V. parahaemolyticus* infection suggesting the inducement effect of intermittent supplementation regime of *B. subtilis* on the disease resistance by somewhat a rapid immune response comparing with previous studies. When *Macrobrachium rosenbergii* hemocytes TGase activity was assessed following *V. harveyi* challenge, the TGase activity started to decrease after 0 (hpi). It reached the significant lowest level at (24 hpi) then a slight increase (statistically of no significance) was recorded until 48 (hpi) **(Arockiaraj *et al.*, 2013)**. In this work, the up-regulation of TGase accompanied by increasing the LYZ expression level supporting the fact that TGase is a substantial constituent involved in the shrimp immune response and is implicated in the modulation of anti-microbial peptides as lysozyme **(Fagutao *et al.*, 2012)**.

Lysozyme is produced mainly by shrimp hemocytes, a prominent AMP that directly began an assault against pervading pathogens by enhancing the hydrolysis of the cell wall of invading bacteria **(Mai and Hu, 2009)**. In a previous study, a 60-day extended experiment, lysozyme showed increased levels in the hemolymph of *L. vannamei* consumed a yeast supplemented diet **(Dengi *et al.*, 2013)**. This result, coupling with the finding of this study, whereas LYZ expression levels exhibited up-regulation in response to *B. subtilis* administration, indicates that lysozyme is triggered by various immunostimulants. In this work, a slight decrease ($p>0.05$) in LYZ levels of expression during the first 12 (hpi) may attribute to reduction of LYZ signals of transcription in circulating hemocytes and peripheral tissues, with its signal increasing in the muscular tissue at injection site suggesting that hemocytes infiltrated in the injection site early during the course of the immune response **(Burge *et al.*, 2007)**. Statistically, there were insignificant differences between (T2) and that of the control group (T1) in their response to the challenge ($p>0.05$). Moreover, the LYZ expression of (T3) responded to Vibrio challenge by significant up-regulation ($p<0.05$) at 24 (hpi). These concatenated results may elucidate the effect of frequent *B. subtilis* administration on disease resistance.

Hemocytes' respiratory burst has been extensively resorted to assess shrimp infection clearance activity **(Campa-Cordova *et al.*, 2002; Cheng *et al.*, 2005)**. They are produced by phagocytes, whereas many reactive oxygen intermediates (ROIs) are

released **(Muñoz *et al.*, 2000)**. Although reactive oxygen intermediates (ROIs) are essential for host defense, the overexpression of ROIs has a deleterious effect on the host cell **(Ji *et al.*, 2011)**. Superoxide dismutase (SOD) keeps the lowest attainable levels of ROIs intracellular **(Chiu *et al.*, 2007)**.

Many research glorified the influence of probiotics on shrimp respiratory burst enhancement **(Mujeeb Rahiman *et al.*, 2010; NavinChandran *et al.*, 2014)**. In the recent study, SOD levels expression up-regulated in both treatments (T2 and T3). This result coincides with the finding of many studies that evaluated shrimp, *Penaeus vannamei*, immune response by different immuno-stimulants **(Liu *et al.*, 2011; Subramanian *et al.*, 2013; Trejo Flores *et al.*, 2018)**. These facts indicated that the up-regulated SOD expression levels of shrimp administered *B. subtilis*-contained diet were a response to the reproducible respiratory burst. In other words, results found in this work suggest that *B. subtilis*, administered orally, may trigger the ROIs release in hemocytes by increasing phagocytosis.

In this study, an increased SOD level of transcription was observed post-injection with *V. parahaemolyticus* in (T3), suggesting that the (T3) reactive oxygen species increased in response to bacterial challenge **(Ji *et al.*, 2011)**. Likewise, the involvement of the increased SOD expression levels in defenses against diseases and phagocytic activity improvement in *Bacillus spp* supplemented shrimp was investigated **(Sánchez-Ortiz *et al.*, 2016)**. There were insignificant differences ($p>0.05$) between SOD expression responses in the control group (T1) and (T2) when challenged with *V. parahaemolyticus*. The immune system showed defiance for pathogen Fig.ht when the *B. subtilis* was provided to shrimp by intermittent manner.

CONCLUSION

The global shrimp culture is defiant by many critical disease-concerning issues attributed primarily for pathogenic bacteria and viruses. Awareness of the host immune system and its regulation pathways in response to pathogens is a major step toward achieving effective and eco-friendly strategies against pathogen-associated problems. Probiotics are considered to be the master solution to this issue. However, getting the preferable effects of probiotics will only be obtained by realizing their effect on the immune system. The present study represented that the intermittent application of *B. subtilis* for *L. vannamei* shrimp enhances the immune system, and their immune response is poised and all set against *V. parahaemolyticus*.

REFERENCES

Aguilera-Rivera, D.; Escalante-Herrera, K.; Gaxiola, G.; Prieto-Davó, A.; Rodríguez-Fuentes, G.; Guerra-Castro, E.; Hernández-López, J.; Chávez-Sánchez,

M. C. and Rodríguez-Canul, R. (2019). Immune response of the Pacific white shrimp, *Litopenaeus vannamei*, previously reared in biofloc and after an infection assay with *Vibrio harveyi*. Journal of the World Aquaculture Society., 50: 119-136.

Ai, H. S.; Liao, J. X.; Huang, X. D.; Yin, Z. X.; Weng, S. P.; Zhao, Z. Y.; Li, S. D.; Yu, X. Q. and He, J. G. (2009). A novel prophenoloxidase 2 exists in shrimp hemocytes. Dev Comp Immunol., 33(1): 59-68.

Amparyup, P.; Charoensapsri, W. and Tassanakajon, A. (2009). Two prophenoloxidases are important for the survival of *Vibrio harveyi* challenged shrimp *Penaeus monodon*. Dev Comp Immunol., 33: 247-256.

Amparyup, P.; Charoensapsri, W. and Tassanakajon, A. (2013). Prophenoloxidase system and its role in shrimp immune responses against major pathogens. Fish Shellfish Immunol., 34: 990-1001.

Arockiaraj, J.; Gnanam, A. J.; Palanisamy, R.; Kumaresan, V.; Bhatt, P.; Thirumalai, M. K.; Roy, A.; Pasupuleti, M.; Kasi. M.; Sathyamoorthi, A and Arasu, **A.** (2013). A prawn transglutaminase: molecular characterization and biochemical properties. Biochimie., 95: 2354-2364.

Babu, D. T.; Antony, S. P.; Joseph, S. P.; Bright, A. R. and Philip, R. (2013). Marine yeast Candida aquaetextoris S527 as a potential immunostimulant in black tiger shrimp *Penaeus monodon*. Journal of invertebrate pathology., 112: 243-252.

Bai, N.; Zhang, W.; Mai, K.; Wang, X.; Xu, W. and Ma, H. (2010). Effects of discontinuous administration of β-glucan and glycyrrhizin on the growth and immunity of white shrimp *Litopenaeus vannamei*. Aquaculture., 306: 218-224.

Balcázar, J. L. and Rojas-Luna, T. (2007). Inhibitory activity of *probiotic Bacillus* subtilis UTM 126 against Vibrio species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). Current microbiology., 55: 409-412.

Burge, E. J.; Madigan, D. J.; Burnett, L. E. and Burnett, K. G. (2007). Lysozyme gene expression by hemocytes of Pacific white shrimp, *Litopenaeus vannamei*, after injection with Vibrio. Fish Shellfish Immunol., 22: 327-339.

Campa-Cordova, A. I.; Hernandez-Saavedra, N. Y.; De Philippis, R. and Ascencio, F. (2002). Generation of superoxide anion and SOD activity in haemocytes and muscle of American white shrimp (*Litopenaeus vannamei*) as a response to beta-glucan and sulphated polysaccharide. Fish Shellfish Immunol 12: 353-366.

Cerenius, L.; Lee, B. L. and Soderhall, K. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol., 29: 263-271.

Chauhan, A. and Singh, R. (2018). Probiotics in aquaculture: a promising emerging alternative approach. Symbiosis., 77: 99-113.

Cheng, W.; Liu, C. H.; Kuo, C. M. and Chen, J. C. (2005). Dietary administration of sodium alginate enhances the immune ability of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. Fish Shellfish Immunol., 18: 1-12.

Chiu, C. H.; Guu, Y. K.; Liu, C. H.; Pan, T. M. and Cheng, W. (2007). Immune responses and gene expression in white shrimp, *Litopenaeus vannamei*, induced by *Lactobacillus plantarum*., 23: 364-377.

Deng, D.; Mei, C.; Mai, K.; Tan, B. P.; Ai, Q. and Ma, H. (2013). Effects of a yeast-based additive on growth and immune responses of white shrimp, *Litopenaeus vannamei* (Boone, 1931), and aquaculture environment. Aquaculture Research., 44: 1348-1357.

Deris, Z. M; Iehata, S.; Ikhwanuddin, M.; Sahimi, M. B. M. K.; Do, T. D.; Sorgeloos, P.; Sung, Y. Y. and Wong, L. L. (2020). Immune and bacterial toxin genes expression in different giant tiger prawn, *penaeus monodon* post-larvae stages following AHPND-causing strain of *Vibrio parahaemolyticus* challenge. Aquaculture Reports., 16: 100248.

Duc le, H.; Hong, H. A.; Barbosa, T. M.; Henriques, A. O. and Cutting, S. M. (2004). Characterization of Bacillus probiotics available for human use. Appl Environ Microbiol., 70: 2161-2171.

Fagutao, F. F.; Maningas, M. B.; Kondo, H.; Aoki, T. and Hirono, I. (2012). Transglutaminase regulates immune-related genes in shrimp. Fish Shellfish Immunol., 32: 711-715.

Fierro Coronado, J. A.; Luna González, A.; Caceres Martínez, C. J.; Álvarez Ruiz, P.; Escamilla Montes, R.; González Ocampo, H. A. and Peraza Gómez, V. (2018). Effect of microbial immunostimulants on WSSV infection percentage and the expression of immune-related genes in white shrimp (*Litopenaeus vannamei*). Revista Colombiana de Ciencias Pecuarias., 32: 221-231.

Gao, H.; Li, F.; Dong, B.; Zhang, Q. and Xiang, J. (2009). Molecular cloning and characterisation of prophenoloxidase (ProPO) cDNA from Fenneropenaeus chinensis and its transcription injected by *Vibrio anguillarum*. Mol Biol Rep., 36: 1159-1166.

Han-Ching Wang, K.; Tseng, C. W.; Lin, H. Y.; Chen, I. T.; Chen, Y. H.; Chen, Y. M.; Chen T. Y. and Yang, H. L. (2010). RNAi knock-down of the *Litopenaeus vannamei* Toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with *Vibrio harveyi*. Dev Comp Immunol., 34: 49-58.

Jang, I. K.; Pang, Z.; Yu, J.; Kim, S. K.; Seo, H. C. and Cho, Y. R. (2011). Selectively enhanced expression of prophenoloxidase activating enzyme 1 (PPAE1) at a bacteria clearance site in the white shrimp, *Litopenaeus vannamei*. BMC Immunol., 12: 70.

Ji, P.F.; Yao, C.L. and Wang, **Z. Y.** (2009). Immune response and gene expression in shrimp (*Litopenaeus vannamei*) hemocytes and hepatopancreas against some pathogen-associated molecular patterns. Fish Shellfish Immunology., 27: 563-570.

Ji, P.F.; Yao, C.L. and Wang, Z. Y. (2011). Reactive oxygen system plays an important role in shrimp *Litopenaeus vannamei* defense against *Vibrio parahaemolyticus* and WSSV infection. Dis Aquat Organ., 96: 9-20.

Jiao, L.; Dai, T.; Zhong, S.; Jin, M.; Sun, P. and Zhou, Q. (2021). *Vibrio parahaemolyticus* infection influenced trace element homeostasis, impaired antioxidant function, and induced inflammation response in *Litopenaeus vannamei*. Biol Trace Elem Res., 199: 329-337.

Kesselring, J.; Gruber, C., C.; Standen, B. and Wein, S. (2019). Continuous and pulse- feeding application of multispecies probiotic bacteria in whiteleg shrimp, *Litopenaeus vannamei*. Journal of the World Aquaculture Society., 50: 1123-1132.

Korenblum, E.; Von Der Weid, I.; Santos, A.; Rosado, L.; Sebastián, G.; Coutinho, C.; Magalhaes, F.; De Paiva, M. and Seldin, L. (2005). Production of antimicrobial substances by *Bacillus subtilis* LFE- 1, B. firmus H2O- 1 and *B. licheniformis* T6- 5 isolated from an oil reservoir in Brazil. Journal of Applied Microbiology., 98: 667-675.

Kuebutornye, F. K.; Abarike, E. D. and Lu, Y. (2019). A review on the application of Bacillus as probiotics in aquaculture. Fish shellfish immunology., 87: 820-828.

Laranja, J. L. Q.; Amar, E. C.; Ludevese-Pascual, G. L.; Niu, Y.; Geaga, M. J.; De Schryver, P. and Bossier, P. (2017). A probiotic Bacillus strain containing amorphous poly-beta-hydroxybutyrate (PHB) stimulates the innate immune response of *Penaeus monodon* postlarvae. Fish Shellfish Immunol., 68: 202-210.

Liu, K. F.; Chiu, C. H.; Shiu, Y. L.; Cheng, W. and Liu, C. H. (2010). Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. Fish Shellfish Immunol., 28: 837-844.

Liu, X. L.; Xi, , Q. Y.; Yang, L.; Li, H. Y.; Jiang, Q. Y.; Shu, G.; Wang, S. B.; Gao, P.; Zhu, X. T. and Zhang, Y. L. (2011). The effect of dietary Panax ginseng polysaccharide extract on the immune responses in white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol., 30: 495-500.

Liu, Y. T.; Chang, C. I.; Hseu, J. R.; Liu, K. F. and Tsai, J. M. (2013). Immune responses of prophenoloxidase and cytosolic manganese superoxide dismutase in the freshwater crayfish *Cherax quadricarinatus* against a virus and bacterium. Mol Immunol., 56: 72-80.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods., 25: 402-408.

Mai, W. J. and Hu, C. Q. (2009). Molecular cloning, characterization, expression and antibacterial analysis of a lysozyme homologue from *Fenneropenaeus merguiensis*. Mol Biol Rep., 36: 1587-1595.

Masuda, T.; Otomo, R.; Kuyama, H.; Momoji, K.; Tonomoto, M.; Sakai, S.; Nishimura, O.; Sugawara, T. and Hirata, T. (2012). A novel type of prophenoloxidase from the kuruma prawn *Marsupenaeus japonicus* contributes to the melanization of plasma in crustaceans. Fish Shellfish Immunol., 32: 61-68.

Mujeeb Rahiman, K. M.; Jesmi, Y.; Thomas, A. P. and Mohamed Hatha, A. A. (2010). Probiotic effect of Bacillus NL110 and Vibrio NE17 on the survival, growth performance and immune response of *Macrobrachium rosenbergii* (de Man). Aquaculture Research., 41: e120-e134.

Muñoz, M.; Cedeño, R.; Rodríguez, J.; van der Knaap, W. P.W.; Mialhe, E. and Bachère, E. (2000). Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*. Aquaculture., 191: 89-107.

NavinChandran, M.; Iyapparaj, P.; Moovendhan, S.; Ramasubburayan, R.; Prakash, S.; Immanuel, G. and Palavesam, A. (2014). Influence of probiotic bacterium *Bacillus cereus* isolated from the gut of wild shrimp *Penaeus monodon* in turn as a potent growth promoter and immune enhancer in *P. monodon*. Fish shellfish immunology., 36: 38-45.

Ninawe, A. S. and Selvin, J. (2009). Probiotics in shrimp aquaculture: avenues and challenges. Crit Rev Microbiol., 35: 43-66.

Perez, C.; Suarez, C. and Castro, G. R. (1993). Antimicrobial activity determined in strains of *Bacillus circulans* cluster. Folia Microbiol (Praha)., 38: 25-28.

Ren, Q.; Xu, Z. L.; Wang, X. W.; Zhao, X. F. and Wang, J. X. (2009). Clip domain serine protease and its homolog respond to Vibrio challenge in Chinese white shrimp, *Fenneropenaeus chinensis*. Fish Shellfish Immunol., 26: 787-798.

Sajeevan, T. P.; Philip, R. and Bright Singh, I. S. (2009). Dose/frequency: A critical factor in the administration of glucan as immunostimulant to Indian white shrimp *Fenneropenaeus indicus*. Aquaculture., 287: 248-252.

Sánchez-Ortiz, A. C.; Angulo, C.; Luna-González, A.; Álvarez-Ruiz, P.; Mazón-Suástegui, J. M. and Campa-Córdova, Á. I. (2016). 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*. Fish shellfish immunology., 59: 95-102.

Shen, W. Y.; Fu, L. L.; Li, W. F. and Zhu, Y. R. (2010). Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*). Aquaculture Research., 41: 1691-1698.

Sritunyalucksana, K.; Cerenius, L. and Soderhall, K. (1999). Molecular cloning and characterization of prophenoloxidase in the black tiger shrimp, *Penaeus monodon*. Dev Comp Immunol., 23: 179-186.

Subramanian, D.; Jang, Y. H.; Kim, D. H.; Kang, B. J. and Heo, M. S. (2013). Dietary effect of Rubus coreanus ethanolic extract on immune gene expression in white leg shrimp, *Penaeus vannamei*. Fish Shellfish Immunol., 35: 808-814.

Trejo Flores, J.; Luna Gonzalez, A.; Alvarez Ruiz, P.; Escamilla Montes, R.; Fierro Coronado, J.; Peraza Gomez, V.; Flores Miranda, M.; G. Diarte Plata and Rubio Castro, A. (2018). Immune related gene expression expression in *Penaeus vannamei* fed Aloe vera. Latin American Journal of Aquatic Research., 46: 756-764.

Van Hai, N. and Fotedar, R. (2010). A Review of Probiotics in Shrimp Aquaculture. Journal of Applied Aquaculture., 22: 251-266.

Vieira, F. d. N.; Buglione Neto, C. C.; Mouriño, J. L. P.; Jatobá, A.; Ramirez, C.; Martins, M. L.; Barracco, M. A. A. M. and Vinatea, L. A. (2008). Time-related action of *Lactobacillus plantarum* in the bacterial microbiota of shrimp digestive tract and its action as immunostimulant. Pesquisa Agropecuária Brasileira., 43: 763-769.

Vieira, F. d. N.; Buglione Neto, C. C.; Mouriño, J. L. P.; Jatobá, A.; Ramirez, C.; Martins, M. L.; Schleder, D. D.; Andreatta, E. R.; Barraco, M. A. and Vinatea L. A. (2010). Effect of probiotic supplemented diet on marine shrimp survival after challenge with *Vibrio harveyi*. Arquivo Brasileiro de Medicina Veterinária e Zootecnia., 62: 631-638.

Won, S.; Hamidoghli, A.; Choi, W.; Bae, J.; Jang, W. J.; Lee, S. and Bai, S. C. (2020). Evaluation of Potential Probiotics *Bacillus subtilis* WB60, *Pediococcus*

pentosaceus, and *Lactococcus lactis* on Growth Performance, Immune Response, Gut Histology and Immune-Related Genes in Whiteleg Shrimp, *Litopenaeus vannamei*. Microorganisms., 8: 281.

Zhang, X.; Tang, X.; Tran, N. T.; Huang, Y.; Gong, Y.; Zhang, Y.; Zheng, H.; Ma, H. and Li, S. (2019). Innate immune responses and metabolic alterations of mud crab (*Scylla paramamosain*) in response to *Vibrio parahaemolyticus* infection. Fish Shellfish Immunol., 87: 166-177.

Zokaeifar, H.; Balcazar., J. L.; Saad, C. R.; Kamarudin, M. S.; Sijam, K.; Arshad, A. and Nejat, **N.** (2012). Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol., 33: 683-689.