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Time course RT-qPCR study on immune, growth, and stress-related gene expression in *Bacillus subtilis* supplemented overstocked shrimp "*Litopeneaus vannmei*"

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ABSTRACT

The feasibility of *Bacillus subtilis* application on immune, growth, and stress responses in *Litopeneaus vannmei* has been assessed by evaluating changes over time of some immune, growth, and stress-related gene expression. A total of 315 *L. vannamei* weighted 0.78 g ± 0.08 (SE) divided to 3 experimental groups; frist group (T1) was considered as a control, second group (T2) fed *B. subtilis* supplemented daily throughout the experiment. In contrast, the third group (T3) fed *B. subtilis* supplemented diet for a week followed by a week of basal diet alternatively. The expression levels of Toll receptor (LvToll), Penaeidin4 (PEN4), Protein kinase C delta type (PKC-delta), Ras-related protein Rap-2a (PAP-2a), Heat shock protein 70 (HSP70), and Heat shock protein 90 (HSP90) were evaluated before and after *Vibrio parahaemolyticus* challenge. Results showed that down-regulation of LvToll, HSP70, and HSP90 expression levels was predominant in (T3) and transient up-regulation of PEN4, PKC, and RAP-2a levels expression in the same group in 1st and 7th week in which *B. subtilis* was supplemented. On the other hand, T2 showed up-regulation of lvToll and HSP70 in the 2nd and 12th weeks of trial, PEN4 in 1st, 2nd, and 7th weeks, HSP90 in the 7th and 12th weeks; up-regulation of PKC and PAP-2a was observed nearly upon the entire experimental period. Immune response against *V. parahaemolyticus* was on guard in (T3) than (T2). LvToll, PEN4, HSP70, HSP90, and PKC (T3) showed rapid upregulation in response to induced bacterial infection. RAP-2a responds to the bacterial challenge by significant down-regulation in both groups. This study suggests that the transient, weekly application of probiotic *B. subtilis* to shrimp may enhance the immune status, improve host stress tolerance, modulate pro-inflammatory responses, and trigger growth-associated responses of shrimp.

INTRODUCTION

The white-leg *Litopeneaus vannmei* shrimp is a prominent cultured shrimp species worldwide, and it is regarded as the cultivated species with the premier single yield production **(Yu *et al.*, 2019)**. Outbreaks of bacterial related-diseases are one of the main

obstacles impede sustainability of *l. vannamei* culture in many countries. Outbreaks have drawbacks on shrimp culture economic returns as they make culture practice financially not rewarding, annual deficit attaining about one billion USD through last decade **(Alfiansah *et al.*, 2020)**.

Increasing stocking densities in shrimp ponds influence growth negatively **(Yu *et al.*, 2009)**. Density with strings attached growth is a characteristic phenomenon in high stocking shrimp culture on account of many factors, which involve: deficiency of feed sources and living space, notable cannibalism, deterioration of water quality and gradual accumulation of unfavorable sediments **(Arnold *et al.*, 2006)**.

Many studies praised probiotic application in aquaculture owing to their capacity to keep bacterial population balance and decrease the density of pathogenic bacteria. Aquaculture probiotic application is an approach toward eco-friendly aquaculture practices **(Swapna *et al.*, 2015)**.

Upon the systemic level, probiotics have mostly been recorded to enhance hematological and immunological parameters. In contrast, upon the localized level, they can sustain barrier function and regulate gene expression pathways. Since aquatic animals are mainly dependent on their innate immune response, this system may contribute resistance against broad-spectrum multiple pathogens implemented in disease **(Irianto and Austin, 2002)**.

Administration of probiotics enhances food utilization and attains optimal growth **(Bachruddin *et al.*, 2018)**. Probiotics can release enzymes extracellularly like families of proteases, amylases, and lipases and supply the host with elements essential for growth like vitamins, fatty acids, and amino acids **(Bachruddin *et al.*, 2018)**. Therefore, probiotics are considered as practical growth promotor. Environmental stress is an essential factor promoting disease and mortality occurrence in cultured aquatic animals. Consequently, drastic environmental changes can be contributed to disease outbreaks **(Liu *et al.*, 2010)**. The capacity of stress resistance was improved upon probiotics application **(Rollo *et al.*, 2006; Taoka *et al.*, 2006; Liu *et al.*, 2010)**.

This study aimed to assess the feasibility of applying *Bacillus subtilis* by two different routines to overstocked *L. vannamei* by evaluating time course RT-qPCR of immune, growth, and stress-related gene expressions.

MATERIALS AND METHODS

Animals of experimentation

A group of apparently healthy cultured whiteleg shrimp, *Litopenaeus vannamei,* was obtained from a private shrimp hatchery in Damietta governorate in July 2020.

3.2. Acclimation procedures of shrimp to the culture system

Shrimp 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 an *ad*

libitum commercial diet twice per day at 9:00 am and 9:00 pm (control diet; **ALLER® Egypt, 38% protein, Table 1**).

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

Preparation of AQUAGROW® supplemented experimental diets

Commercial feed (**ALLER® Egypt**) was used as a vehicle for the *Bacillus subtilis* supplementation (**AQUA-GROW®** manufactured by Canal AquaCure Company, Egypt, **Table 2**). The probiotics were sprinkled on the feed to attain a final concentration of about 2×10^9 CFU/Kg feed. Probiotics supplemented feed was desiccated at ambient temperature then preserved at 4°C until use.

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

Experimental design

After the adaptability period, a total of 315 whiteleg shrimps, *Litopenaeus vannamei*, were submitted to weight measuring, divided by a random manner in 9 rounded plastic tanks containing 25 liters seawater for a 12-week feeding trial. Shrimp were stocked in high density. Every 35 individuals approximately weighed 0.78 g ± 0.08 (SE) were stocked in each tank to establish three experimental groups conducted in 3 replicates for each group. First group was considered as a normalizing (control) group and was fed basal diet upon course time of the experiment (T1). The second group fed *B. subtilis*

supplemented diet daily throughout the experiment (T2). Simultaneously, the third group fed *B. subtilis* supplemented diet intermittently week after week (T3). Shrimp fed their determined diets twice daily at 5% of the body weight.

Shrimp challenge with *Vibrio parahaemolyticus*

At the end of the 12th week of the feeding trial, the pathogen *Vibrio parahaemolyticus* was propagated 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)**. The concentration of the bacterial solution was measured using a spectrophotometer at optical density 600 nm (OD_{600}) **(Won *et al.*, 2020)**. The final bacterial concentration was optimized to be 10^7 CFU/ml. The challenge experiment was preceded by a random selection of 10 shrimp from each tank and stocked in new 25 l plastic tanks. The infection was provoked by injection of each individual in the 2nd abdominal segment with 25 µl SSS bacterial suspension of final concentration 10^7 CFU/ml

Hemolymph and hepatopancreas extraction

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

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

The RNA isolation procedures were conducted following **Aguilera-Rivera *et al.* (2019)**. Shortly, total RNA was isolated from 100 mg of hepatopancreas and hemolymph from freshly collected shrimp following Trizol kit instructions. The extracted RNA absorbance ratio was measured at 260 and 280 nm with a spectrophotometer (**UNICO-UV-VIS Spectrophotometer**). Only (A260/A280) ratio of RNA samples maximal than 1.8 was subjected to the following the procedures suggested by **Aguilera-Rivera *et al.* (2019)**. **RevertAid First Strand cDNA Synthesis Kit®** (**Thermo Scientific**) was assorted to synthesize the first-strand cDNA from RNA templates with efficacy according to kit instructions.

Specific primers (**METBION®)** for immune-related genes were selected to perform quantitative real-time PCR (RT-qPCR), from previous published *L. vannamei* primer sequences of the following genes; Toll, Penaeidin4 (immuno-related genes), Heat shock protein 70 (Lvhsp70) and Heat shock protein 90 (Lvhsp90) (stress related genes), Protein kinase C delta type (PKC) and Ras-related protein Rap-2a (Rap-2a) (growth related genes) and β-Actin gene (endogenous control) **(Wang *et al.*, 2007; Wang *et al.*, 2010;**

del Carmen Flores-Miranda *et al.*, 2015; Trejo Flores *et al.*, 2018; Yu *et al.*, 2019) (**Table 3**). The RT-qPCR was conducted by **7500 Fast Real-Time PCR System®** Applied biosystem through the use of SYBR Green Mastermix (**Top Real SYBR mix®, Biovision**). A duplicate of each sample was performed. RT-qPCR cycle setting has consisted of 15 min of denaturation at 95°C then 40 thermal cycles at 95°C for 15 s followed by 60°C for 30 s along with 1 min at 60°C. ΔCt 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 ΔΔCt value. Finally, the fold change relative to the basal expression of the control was represented as 2-ΔΔct.

Table 3 Sequences of primers submitted for RT-qPCR to assessed immune, stress and growth responses of shrimp, *Litopenaeus vannamei*

Related system/response	Target gene	Abb. Name [a]	PrimerF/R[b]	(5 -3̀) primer sequence
Pattern recognition receptor	Toll receptor	LvToll	LvToll-F	ATG TGC GTG CGG ATA CAT TA
			LvToll-R	GGG TGT TGG ATG TCG AGA GT
Anti-microbial peptide system	penaiedin4	PEN4	PEN4-F	GCC CGT TAC CCA AAC CAT C
			PEN4-R	CCG TAT CTG AAG CAG CAA AGT C
Stress	Heat shock protein 70 (Lvhsp70)	HSP70	hsp70 -F	GGC AAG GAG CTG AAC AAG TC
			hsp70 -R	TCT CGA TAC CCA GGG ACA AG
	Heat shock protein 90 (Lvhsp90)	HSP90	hsp90 F	TGG GGC TTC TAC TCC GCC TAC C
			hsp90 R	ACG GTG AAA GAG CCT CCA GCA
Growth	Protein kinase C delta type	PKC-delta	PKC-F	GTG CTG AGC CTC GGA ACC A
			PKC-R	GCC GCA GTG TTG TAT GTG GA
	Ras-related protein Rap-2a	Rap-2a	RAP-2a-F	GCC GTG CGT GCT TGA GAT
			RAP-2a-R	TTG ATG TCC TGG AAG GTC TGG
Internal control	β-actin		β-actin-F	CCA CGA GA CCA CCT ACA AC
			β-actin-R	AGC GAG GGC AGT GAT TTC

[a] Gene name abbreviation

[b] Forward/ Reverse primers

Statistical analysis

The study variables 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**). Tuckey's range test was used to identify the significant variation ($P<0.05$). All statistical analysis was figured by SPSS.

RESULTS

Transcriptional expression of shrimp genes pre- and post-bacterial challenge during a time course trial

Immune-related genes expression

The transcriptional regulation of the immune, stress and growth-related shrimp genes was evaluated using time-course RT-qPCR assessment throughout 12 weeks *B .subtilis* feeding trial. Following the $2^{-\Delta\Delta ct}$ equation, the data have been presented as the fold change in expression levels of target gene customized to an internal reference gene (β-actin) and in proportion to the control **(Livak and Schmittgen, 2001)**.

RT-qPCR analysis revealed that Toll (LvToll) expression levels of the continuous (T2) *B. subtilis* supplemented groups fluctuated slightly (insignificant, $p>0.05$) up and down around that of the control group over the study accompanied with significant ($p<0.05$) up-regulation in the 2nd and 12th weeks. At the same time, down-regulation was the overmastering response of Toll expression of (T3) with a significant ($p<0.05$) decrease in the 2nd and 7th weeks **(Fig. 1)**. However, the response of LvToll expression after *V. parahaemolyticus* injection was insignificant down-regulation of its expression levels of (T2) and (T3) at early and mid-infection phase ($p>0.05$) followed by an obvious increase in its expression level of both treatment groups ($p<0.05$) at late infection phase (24 hpi) with relative fold change (7.7 and 9.8, respectively) **(Fig. 2)**.

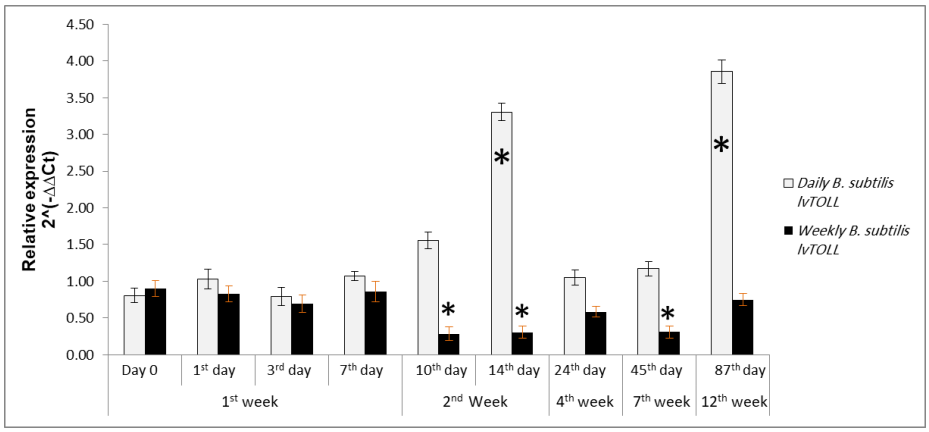

Fig. 1 Relative expression levels (mean± standard error SE) of hemolymph and hepatopancreas Toll receptor (LvToll) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

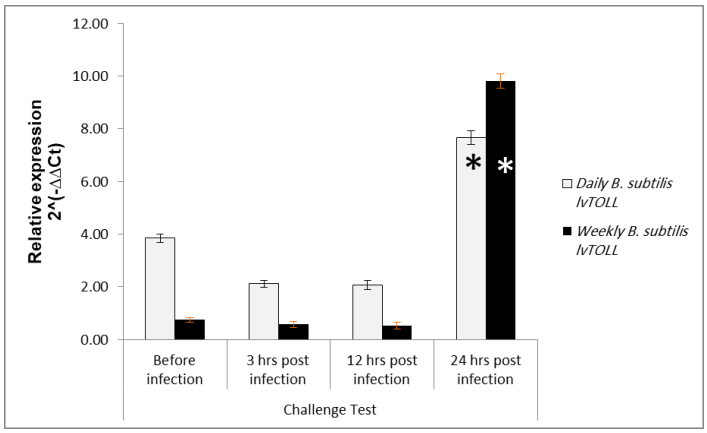

Fig. 2 Relative expression levels (mean±SE) of hemolymph and hepatopancreas Toll receptor (LvToll) in response to *Vibrio parahaemolyticus* challenge after 12-week *Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

Penaiedin4 (PEN4) levels of expression were upregulated in two *B. subtilis* supplementation regime before *V. parahaemolyticus* challenge. PEN4 expression levels of (T2) upregulated significantly ($p<0.05$) on 3rd day of the 1st week and peaked on 3rd day of the 2nd week (2.6 and 3.1 fold change relative to the control group) then decreased near to the control level at the end of the 2nd week and extended toward the 12th week. In contrast, PEN4 of (T3) increased obviously ($p<0.05$) on the 3rd day of the 1st week and reach its peak in the 7th week (2.9 and 4.3 fold change compared with the control) **(Fig. 3)**.

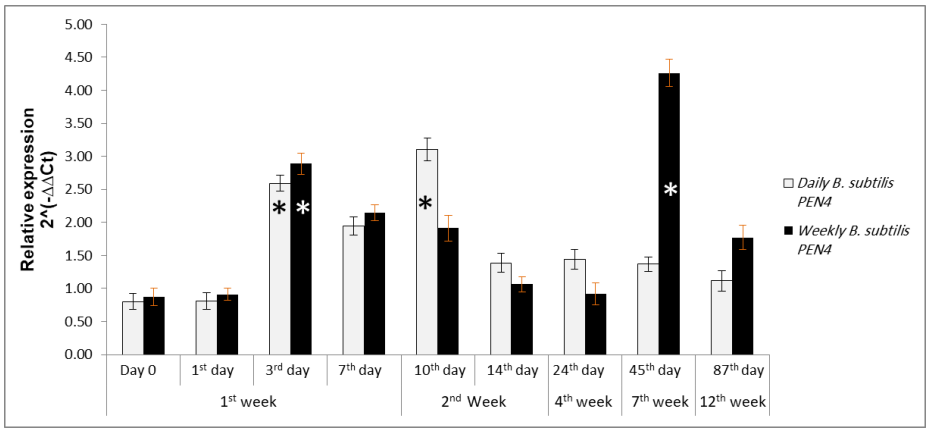

Fig. 3 Relative expression levels (mean± standard error SE) of hemolymph and hepatopancreas penaiedin4 (PEN4) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

The early dominant response of penaieden4 (PEN4) levels of expression after *V. parahaemolyticus* challenge was a meager down-regulation of PEN4 expression levels of (T2 and T3) at the first 12 hpi ($p>0.05$). There was a significant up-regulation of PEN4 expression levels of (T3) ($p<0.05$) at 24 hpi with a relative fold change of 8.7 by the time, there were insignificant differences in the expression responses of PEN4 between the control group and (T2) in response to challenge with *V. parahaemolyticus* ($p>0.05$) **(Fig. 4)**.

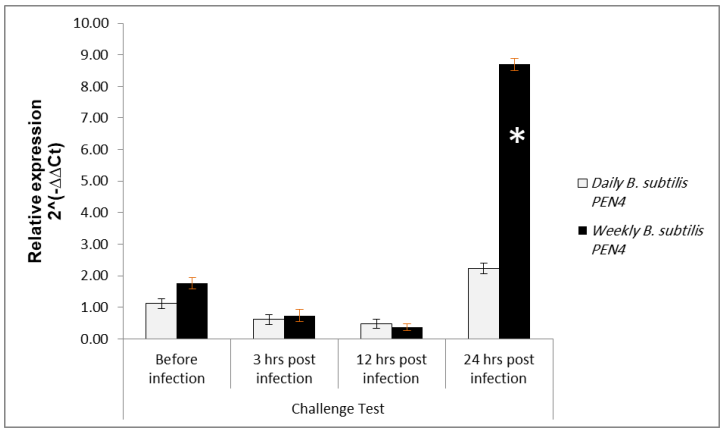

Figure 4: Relative expression levels (mean±SE) of hemolymph and hepatopancreas penaiedin4 (PEN4) in response *to Vibrio parahaemolyticus* challenge after 12-week *Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

Stress-related genes expression

Over the 12-week feeding trial, the down-regulation was the predominant effect of *B. subtilis* administration on (T3) transcriptional levels of heat shock protein 70 (HSP70), which was statistically significant ($p<0.05$). While (T2) exhibited a trivial

decrease ($p>0.05$) of HSP70 levels of expression accompanied with significant up-regulation ($p<0.05$) in the 2nd and 12th weeks in the same group **(Fig. 5)**. On the other hand, HSP90 levels of (T2) showed significant differences with that of the control ($p<0.05$), T2 HSP90 levels of expression fluctuated around the control level with a significant up-regulation was observed in the 7th and 12th week. However, throughout the 12-week feeding trial, the down-regulation ($p<0.05$) was the obvious effect of *B. subtilis* administration on HSP90 levels of (T3) **(Fig. 6)**.

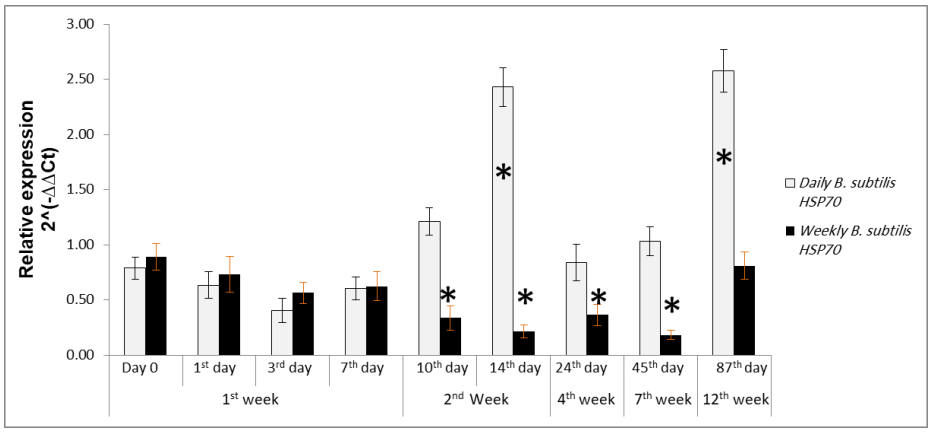

Fig. 5 Relative expression levels (mean± standard error SE) of hemolymph and hepatopancreas Heat shock protein 70 (HSP70) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

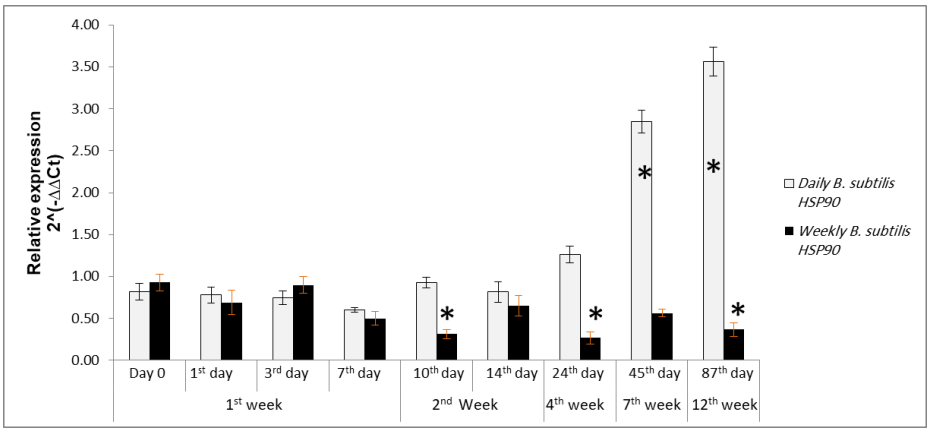

Fig. 6 Relative expression levels (mean± standard error SE) of hemolymph and hepatopancreas Heat shock protein 90 (HSP90) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

HSP70 levels of (T2) increased significantly ($p<0.05$) as a response to *V. parahaemolyticus* injection at 12 hpi (4.1 relative fold change). In contrast, HSP70 of (T3) respond to the bacterial challenge by a notable increase of its levels of expression at 12 hpi and 24 hpi with 2.7 and 3.6 fold change comparing with the control level, respectively ($p<0.05$) **(Fig. 7)**.

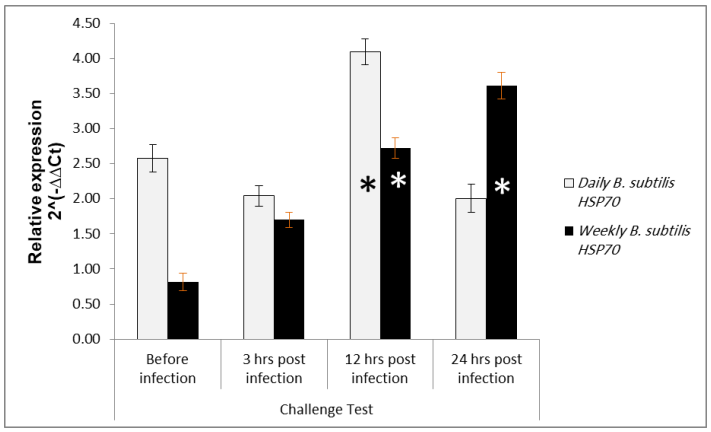

Fig. 7 Relative expression levels (mean±SE) of hemolymph and hepatopancreas Heat shock protein 70 (HSP70) in response to *Vibrio parahaemolyticus* challenge after 12-week *Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

When (T3) was challenged, HSP90 levels increased obviously ($p<0.05$) at 12 and 24 (hpi) presented a 3.4 and 3.9 relative fold change, respectively. There were no significant differences between the responses of (T2) HSP90 and that of the control against *V. parahaemolyticus* **(Fig. 8)**.

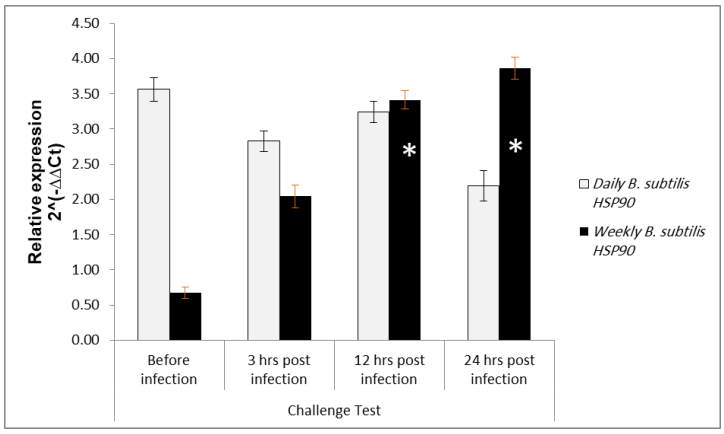

Fig. 8 Relative expression levels (mean±SE) of hemolymph and hepatopancreas Heat shock protein 90 (HSP90) in response *to Vibrio parahaemolyticus* challenge after 12-week *Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

Growth-associated genes expression

Protein kinase C delta type (PCK) expression levels showed significant up-regulation entire the course time *B. subtilis* administration in (T2), but the up-regulation ($p<0.05$) was observed in the 1st and 7th week of the trial in (T3) **(Fig. 9)**.

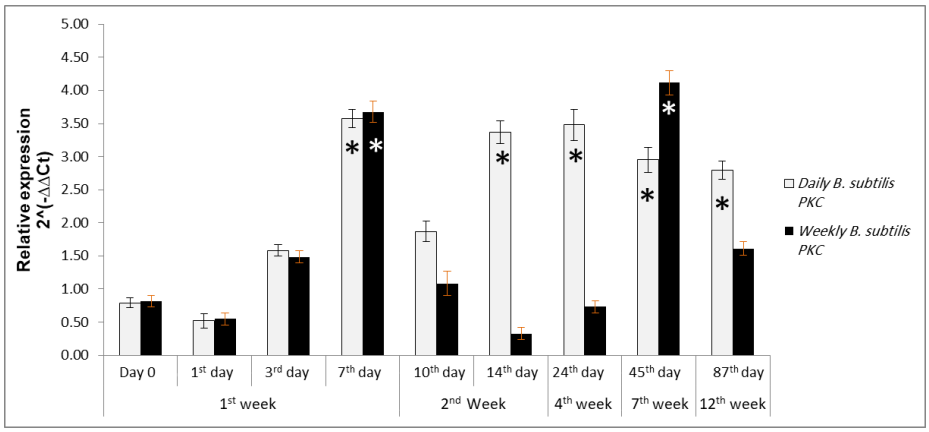

Fig. 9 Relative expression levels (mean± standard error SE) of hemolymph and hepatopancreas Protein kinase C delta type (PKC) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

PKC-delta levels of expression of (T2) exhibited a nominal increase ($p>0.05$) in response to the bacterial challenge at 24 hpi because there were no meaningful differences statistically between pkc of T2 and that of the control group. At the same time (T3) showed a significant increase ($p<0.05$) in PCK-delta expression levels at 24 hpi by a 5.7 fold change in proportion to that of control **(Fig. 10)**.

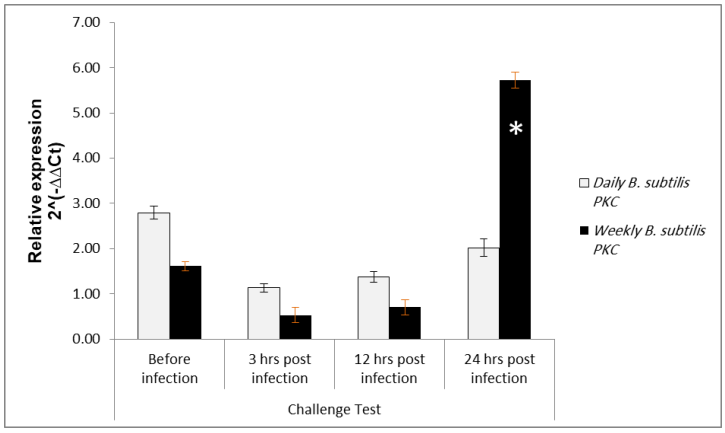

Fig. 10 Relative expression levels (mean±SE) of hemolymph and hepatopancreas Protein kinase C delta type (PKC) in response *to Vibrio parahaemolyticus* challenge after 12-*week Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

Meantime, *B. subtilis* administration presented an up-regulation of ras-related protein (Rap-2a) expression levels. Rap-2a expression levels of (T2) upregulated ($p<0.05$) 1st, 2nd, and 4th week reaching its peak at the end of the 1st week measuring 7 fold change relative to the control level then dropped around the control levels in

7th and 12th week. However, the expression level of (T3) Rap-2a exhibited expressive up-regulation ($p<0.05$) in the 1st and 7th week (7.5 and 4.2 fold change with control comparing respectively) **(Fig 11)**.

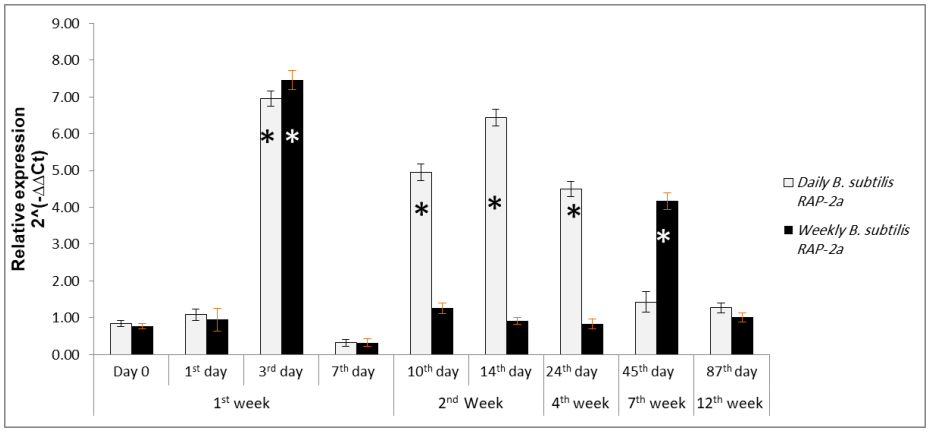

Fig. 11 Relative expression levels (mean± standard error SE) of hemolymph and hepatopancreas Ras-related protein Rap-2a (RAP-2a) of *Bacillus subtilis* supplemented *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

Statistically, the RAP-2 managed the bacterial challenge through an obvious down-regulation of its expression in both treatment groups (T2 and T3) ($p<0.05$) **(Fig. 12)**.

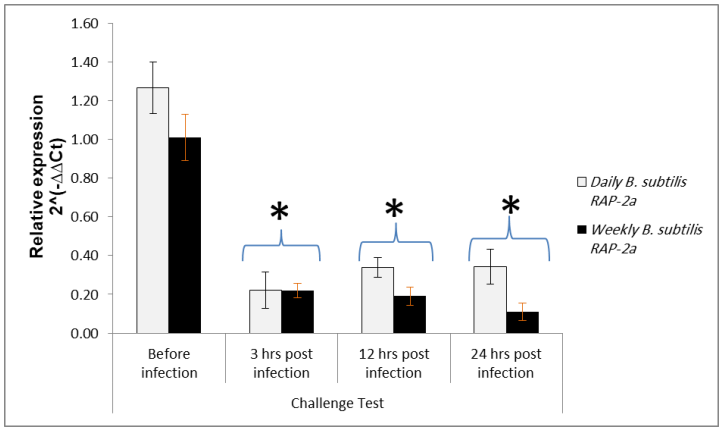

Fig. 12 Relative expression levels (mean±SE) of hemolymph and hepatopancreas Ras-related protein Rap-2a (RAP-2a) in response to *Vibrio parahaemolyticus* challenge after 12-week *Bacillus subtilis* supplementation trial, *Litopenaeus vannamei*. Significant value ($p<0.05$) presented by (*)

DISCUSSION

Probiotics application in aquaculture has acquired consideration as a microbial recommendation to sustain the health of many aquatic animals in captivity. Amongst the

many microbial immune-stimulants, probiotics from genus *Bacillus* can form spore that supports them to fight for their life against cruel environmental ambiance, is not virulent or disease-causing bacteria when was provided to aquatic animals, and can release anti-microbial elements that allow them to be a more suitable option in comparison with other probiotics. The application of Bacillus probiotics was used as a sustainable antibiotic alternative in aquaculture to optimize feed assimilation, modulates and relieve stress response, induces the immune response trigger, and enhances aquatic animals' ability to withstand diseases **(Kuebutornye *et al.*, 2019)**.

When the balance between the host and pathogen is interrupted, the disease will be the subsequent outcome. Therefore, shrimp is unprovided with adaptive immunity, placing reliance on innate immune responses to resist infection. Immune deficiency (IMD) and the Toll pathways are nuclear factor-κB (NF-κB) pathways involved in the immune response regulation of shrimp. Toll and IMD pathways are responsive to bacterial infection, which stimulates their associated NF-κB transcription factors, resulting in various anti-microbial peptides (AMPs) expression **(Li *et al.*, 2019a)**.

Toll-like receptors (TLRs) are identified as quite preserved proteins across the cell membrane of immune cells. TLRs respond quickly to PMAPs (pathogen-associated molecular patterns) of microorganisms (lipopolysaccharides, peptidoglycans, βglycans) **(Fierro-Coronado *et al.*, 2019)**. TLRs are attributed to pro-inflammatory cytokines, and chemokine production boosts anti-microbial responses **(Akira *et al.*, 2006; Asgari *et al.*, 2018)**

RT-PCR previous analysis reported that Toll was mainly expressed in hemocyte, gill, heart, brain, stomach, intestine, pyloric caecum, muscle, nerve, and spermary, with a lower-levelin eyestalk and hepatopancreas **(Yang *et al.*, 2007)**.

Dissimilar to pathogenic bacteria, which spark pro-inflammatory response following Toll receptors stimulation, probiotics impede the inflammation cascade by stimulating gut homeostasis by controlling NFκB induction **(Asgari *et al.*, 2018)**. Similarly, it was reported that *Lactobacillus reuteri* inhibited signals involved in the nuclear translocation of NFκB in intestinal epithelial cells result in regulating its anti-inflammatory effects **(Iyer *et al.*, 2008).**

Toll expression was detected to be regulated in various tissues (hemocytes, lymphoid organ, gill, hepatopancreas, stomach, midgut, and hindgut.) upon bacterial infection. Markedly Toll level increase was detected in hemocytes and lymphoid organs relative to control, at 72 and 48 (hpi), respectively. Nevertheless, contrary to other tissues, the hepatopancreas presented a notable decrease compared to the control through the challenge test **(Deepika *et al.*, 2020)**. In this study, the expression analysis of the Toll gene exhibited notable up-regulation ($p<0.05$) in both (T2 and T3) at 24 hpi in response to *V. parahaemolyticus*. Therefore, it can be assumed that *B. subtilis* induced a rapid LvToll gene expression of *L. vannamei,* triggered by *V. parahaemolyticus* through

signaling pathway activation leading to defense molecules expression against *V. parahaemolyticus* **(Rubio-Castro *et al.*, 2016)**.

The penaeidins are expressed in all shrimp of the family *Penaeidae*. It is a group of anti-microbial peptides which are fundamentally transcripted in hemocytes (Hu 2014). Penaeidins are impressionable to pathogenic agents and provide a line of defense to battle against infection **(Bachère *et al.*, 2000)**.

Furthermore, penaeidins contributed to opsonization by tagging the bacterial surfaces leading to immune reactivity enhancement, eliminating the marked antigen by phagocytosis **(Muñoz *et al.*, 2002)**.

A previous study demonstrated that the whole of the AMP groups' expression levels exhibited up-regulation before and after white spot virus challenge when bacterial probiotic was fed to shrimp **(Antony *et al.*, 2011)**. It agreed with the recent study's findings, as the PEN4 levels were upregulated in (T3) group compared with the control before and after the bacterial challenge. At the same time, (T2) expression levels of PEN4 presented insignificant differences ($p>0.05$) with that of the control levels after bacterial challenge. Toll and IMD pathways trigger the AMP penaeidin 4 (PEN4) expression in *Litopenaeus vannamei* **(Tassanakajon *et al.*, 2018)**. Although, before *V. parahaemolyticus* infection, Toll gene expression displayed down-regulation and non-significant differences in T3 and T2 compared with control, PEN4 manifested an upregulated notably ($p<0.05$) in both treatment groups, suggesting that PEN4 expression was mediated by IMD pathway. Data from a previous study signified that shrimp "*Litopenaeus vannamei*" immune boost generated by prompt increased IMD expressions in response after the administration of bio-encapsulated sulfated galactans **(Rudtanatip *et al.*, 2019)**.

PEN4 expression stimulation by microbial infection is supposed as an interwoven response, interpreted in many previous studies. The recognition of pathogenic microbes sparks Toll and IMD transduction pathways and subsequent the NF-κB signaling, inducing gene expression associated with host immune responses **(Tassanakajon *et al.*, 2018)**. The infectious agents are revealed by Toll and/or IMD pathways prompted the expression of compatible AMPs in shrimp hemocytes **(Chen *et al.*, 2014)**. Furthermore, Toll-interacting protein (LvTollip) expressed in *Litopenaeus vannamei* induced PEN4 downregulation by interfering with the promoter of the NF-κB pathway associated with the expression of AMP penaeidin4 (PEN4). In like manner, it was reported that LvTollip expression was quick to respond to microbial infectious agents. In LvTollip-knockdown shrimp, the expression level of the AMP PEN4 was upgraded **(Wang *et al.*, 2013)**. These previous study facts validate the present study finding of the PEN4 up-regulation response to the bacterial challenge in (T3) at 24 hpi. Moreover, the 24 hpi PEN4 up-regulation of (T3) was accompanied by toll up-regulation at the same time, which supported the previous suggestions. The slight decrease ($p>0.05$) in the (T3) PEN4 levels at the first 12 hpi may be recommended to vast infiltration of granular hemocyte at the

site of bacterial injection. Penaeidin mRNA and protein are limited to granulocytes, and their expression and allocation are modified through profound alteration in haemocyte populations, as well as circulating and infiltrating shrimp tissues. There were two evident stages in the immune response, hemocytes migrated then infiltrated in the site of the induced infection within the early and med-infection phases (first 12 h) following the injection of the microbial agent, with a local and massive release of PEN4; followed by the manifestation of hemocytes into the hemolymph circulation displaying upregulated penaeidin-expression activity **(Muñoz *et al.,* 2002)**. Although Toll expression levels of (T2) showed significant up-regulation in response to the induced bacterial infection, PEN4 after a continuous 12-week time course *B. subtilis* administration trial exhibited non-significant up-regulation that time. This finding may be attributed to immune exhaustion followed by the continuous stimulation of immune factors contributing to PEN4 expression.

Many factors such as handling, vaccine application, physical, chemical, and biological characteristics of water, transportation and high stocking densities, as well as ammonia and nitrite in aquaculture, are stressors that affect the physiology and health of aquatic animals **(Kuebutornye *et al.,* 2019).** The members of heat-shocked proteins (HSPs) with a molecular chaperones activity are divided based on their molecular mass in kilodaltons (kDa) such as HSP110, HSP90, HSP70, HSP60, HSP21, and HSP10. In pathological conditions, such as necrotic cell death, the HSPs can be extracellularly liberated to induce an auto-immune response by stimulating receptors involved in innate immune system activation **(Routsias and Tzioufas, 2006)**. According to the challenge test of LvHSP70 and LvHSP90-silenced shrimp, shrimp did not have resistance capacity to acute hepatopancreatic necrosis disease (VPAHPND) infection **(Junprung *et al.,* 2017)**. Moreover, Hsps are theorized to be key mediators of stress tolerance. Cells can resist environmental stress by inducing Hsps stress - a phenomenon called "stress tolerance". Heat shock protein 70 (Hsp70) was recorded to reform partially denatured proteins, shared in the breakdown of irreversibly denatured proteins, and control protein aggregation; consequently, cells are preserved from harsh environmental stresses **(Laranja *et al.,* 2017)**.

T3 HSP70 expression was down-regulated in the presence of high density (stress), suggesting that intermittent administration of *B. subtilis* is the effective stress-relieving regime. It is permissible that *B. subtilis* given by pulsed regime plays an important role in shrimp to cope with environmental stress via HSP70 down-regulation. Novel studies are conducted to diminish stress and improve immune status in aquatic animals, applying variable feed additives in feed, including vitamins, immuno-stimulants, prebiotics and probiotics, revealing significantly improved tolerance against stress **(Dawood *et al.,* 2015; Dawood *et al.,* 2017)**. A mixture of *Bacillus* species was proved to minimize the intensity of cellular stress in sea bream larvae by decreasing the levels of HSP70 expression resulting in tolerance enhancement of the fish toward culture conditions

(Kuebutornye *et al.*, 2019). On the other hand, T2 HSP70 showed up-regulation ($p<0.05$) in the 2nd and 12th weeks, suggesting that the continuous *B. subtilis* administration may interfere with the host tolerance capacity to stress.

Similarly, HSP70 increased in hepatopancreas with increasing stocking density of juvenile shrimp *Litopenaeus vannamei* for 60 days resulting in decreased stress resistance capacity **(Gao *et al.*, 2017)**. Besides, the up-regulation of HSP70 expression in *Clostridium butyricum* supplemented shrimp at the end of the 56 days and before stress induction **(Duan *et al.*, 2017)**, in which the author attributed the improvement of the intestinal health condition to the up-regulation of HSP70 induced by *C. butyricum* supplemented diet. The suggestion of **Duan *et al.* (2017)** opposite the finding and interpretation of **Sánchez-Ortiz *et al.* (2016),** as HSP70 expression was down-regulated in shrimp administrated mixed-*Bacillus sp.*

The downregulation was an obvious response of Toll gene expression of (T3) accompanied by the downregulation of HSP70. This finding parallels the finding of **Gárate *et al.* (2013**) and **Mondal *et al.* (2021)**, TLRs expression is regulated in response to environmental stressors. Considering that, *B. subtilis* may regulate Toll expression and subsequent its pro-inflammatory factor through HSP70 mediation.

HSPs are fundamentally expressed as a consequence of infection and can be thought out as key biomarkers against viral and bacterial infections in *Crustacea*. Many previous studies have reported that HSP70 was in quick response against microbial challenge in aquatic animals **(Li *et al.*, 2018)**.

In *L. vannamei*, LvHSP70 enhanced the signaling cascade involved in the immune response to trigger the synthesis of immune-related proteins, subsequently keeping disease outbreaks from happening **(Sung *et al.*, 2011; Li *et al.*, 2019)**. Numerous stressors stimulate cells to provoke HSPs production like toxins, hypoxia, and bacterial diseases **(Georgopoulos and Welch, 1993; Li *et al.*, 2019)**. In parallel with the recent study, (T3) HSP70 expression responds to *V. parahaemolyticus* challenge by a significant up-regulation ($p<0.05$) at 12 and 24 hpi, suggesting that *B. subtilis* can improve the immunity and enhance stress tolerance by modulating HSP70 expression rapidly. While (T2) HSP70 upregulated ($p<0.05$) at 12 hpi then failed to keep its higher level, suggesting that the continuous application of *B. subtilis* may hinder the HSP70 ability to induce the immune pathway.

Heat shock protein 90 (HSP90) is one of the most copious eukaryotic cytosolic proteins, approximately equal to 1% of total soluble protein in some cells even if the stress is absent **(Buchner, 1999)**.

Upon stress absence status, HSP90 has played a major role in different cellular processes. It targeted specific intracellular signal transducers, which act as molecular switches through the modulation of their conformation. While, during stressful conditions, HSP90 put a stop to irretrievable protein aggregations. HSP90 is involved in the heat shock (stress) response of the cell, mainly a major defense implement for cell

preservation against and repairing from physical and chemical environmental stressors **(Jiang *et al.*, 2009)**.

A previous study reported that high stocking density is considered environmental stress for the juveniles of Macrobrachium nipponense. Modification of HSP70 and HSP90 expression levels was a response of a high stocking density (160 prawns/m^2) **(Sun *et al.*, 2016)**. The present work showed that (T2) HSP90 levels of expression fluctuated around the control level showing a significant up-regulation in the 7^{th} and 12^{th} week while, throughout the 12-week trial, a notable down-regulation ($p<0.05$) of its levels were in (T3). All findings were considered that transient application of *B. subtilis* reduces the response against stocking density stress. Additionally, HSP90 was reported to take part in the immune response against various microbial agents **(Zhu *et al.*, 2001; Tsan and Gao, 2009)**. In response to *V. parahaemolyticus* challenge, HSP90 expression levels of (T3) exhibited rapid, significant up-regulation ($p<0.05$) at 12 and 24 hpi, while its expression levels of (T2) showed insignificant differences comparing with the control. Recent and previous findings pointed out the importance of the probiotics application regime's frequency to obtain favorable disease resistance and optimal stress tolerance.

With the increased stocking density, individuals compete for feed and space, increasing their growth unconformity. The elevated culturing density is followed approach to improve productivity and boost economic feasibility in the aquaculture industry. It was nevertheless found that raising at an increased density can involve stress via the impairment of water quality or an injurious diverse communal behavior leading to decreased metabolic rate, weak immunity capacity, and reduced growth rate of individuals **(Sun *et al.*, 2016)**. It was found that the cultivation density of 175 tails/m^3 is the most appropriate stocking density of *L. vannamei* **(Marlina and Panjaitan, 2020)**.

The PKC-delta is a gene involved in metabolism, and it has been proved to participate in cell death (**Zhao *et al.*, 2012)**, and it also showed a controlling function during the molting process **(Chen *et al.*, 2017)**. The PKC-delta may modulate the molting process and promote growth subsequently **(Shyamal *et al.*, 2018)**. The PKC-delta was mainly expressed in muscle, heart, and stomach. The expression was of low levels in the other tissues **(Yu *et al.*, 2019)**.

The expression of PKC was upregulated obviously ($p<0.05$) in (T2 and T3) in response to *B. subtilis* administration, proposing that *B. subtilis* is an effectual growth promotor for *L. vannamei*. However, the increase of PCK in T2 and T3 was significant ($p<0.05$), the values of relative expression fold change were not very high attributed to the type of the samples (hemolymph and hepatopancreas) **(Yu *et al.*, 2019)**.

In mollusks, PKCs are also considered to be associated with the innate immune response of hemocytes, including phagocytosis regulation **(Plows *et al.*, 2006; Ramos MartíNez *et al.*, 2012)**.

A potential PKC isotype was cloned from *L. vannamei* denominated LvnPKC, sharing similarity with novel PKCs in vertebrates and invertebrates. LvnPKC expression

was increased significantly in hemocytes of shrimp challenged with *Vibrio alginolyticus*. Likewise, in the recent study, the PCK levels of expression in (T3) upregulated in response to *V. parahaemolyticus* challenge (24 hpi). Still, there were insignificant differences between its expression levels in (T2) and control. In *L. vannamei*, RACK1 (A receptor for activated PKC) was characterized and reported to be implicated in immune response and signal transduction in hemocytes in response to *V. alginolyticus* infection was detected **(Chang *et al.*, 2015)**.

Rap-2a is identified as a member of the Ras-associated protein family. It is an element of many signaling cascades, cytoskeletal re-arrangement regulation, migration of cells **(Taira *et al.*, 2004)**. The Rap-2a was particularly expressed in lymphoid organs and the digestive organs, including hepatopancreas, intestine, and stomach **(Yu *et al.*, 2019)**.

Rap-2a expression levels elevated significantly in (T2) during the first four weeks of the trial, followed by a drop to the control level at the 7^{th} and 12^{th} weeks. In the context of this finding, the drop in (T2) Rap-2a may be considered as a body response to avoid an unbalanced immune response as overexpression of Rap-2a was reported to lead to severe inhibition of NF-κB activation and subsequent TLR signaling molecules **(Carvalho *et al.*, 2019)**.

The obvious post-challenge response of Rap-2a was the down-regulation in (T2 and T3), suggesting that Rap-2a was implicated in TLR-mediated responses by contributing to balanced NF-κB activity status **(Carvalho *et al.*, 2019)**.

CONCLUSION

Enhancement of aquatic animal immunity, improvement of their stress tolerance capacity and high productivity achievement are pivotal approaches toward sustainable aquaculture implementation. The results showed that *B. subtilis* was involved in triggering the immune cascade, disease resistance against *V. parahaemolyticus*, improving the host's stress tolerance capability and promoting shrimp growth. *B. subtilis* can improve shrimp health status on immune, stress, and growth-related levels, as well as improve aquaculture environmental condition, but only on the understanding that is paying attention to probiotics application regime concerning the frequency of administration (week after week) to obtain the preferable results.

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