Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 4, No. 5, p. 273-284, 2014
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Effects of the mixed probiotic microalgae-bacteria, *Skeletonema costatum-Bacillus subtilis*, on Stress tolerance and innate immunity prophenoloxidase (proPO) activating in *Penaeus monodon* larvae

Gholamreza Rafiee[1*], Ahmad-ali Pourbabaee[2], Ali Agharokh[3], Mehran Avakhkeysami[4]

[1]*Department of Fisheries, University of Tehran, Iran*

[2]*Biotech,nology Laboratory, Department of Soil Science, University of Tehran, Iran*

[3]*Department of Fisheries, University of Tehran, Iran*

[4]*Research center of Fisheries, Ministry of Jahad-Keshavarzi. Gilan Province, Iran*

Article published on May 20, 2014

Key words: probiotic, *p. monodon*, *S. costatum,* prophenoloxidase, immune system.

Abstract

Shrimp farming like other industries, requires modern methods and techniques for increasing production yield. Novel technologies and other sciences such as biotechnology, nanotechnology and microbiology have important role in fishes aquaculture activities. Larvae of *P. monodon* were fed by conjugated *B. subtilis* and *S. costatum* with a high total protein rate. In this study tried to use a microalgea as a carrier for beneficial bacteria. These fed larvae by this carrier food showed responses to tolerance tests and prophenoloxidase I and II (proPO I & II) expression rates well in compare to the control samples. The analysis of immune-related gene expression of prophenoloxidase I and II of monodon post larvae were increased after being fed with 10^7 and 10^9 cellml^{-1} significantly. It is therefore said that 10^9 cellml^{-1} concentration of algae-bacteria can be very suitable for shrimp larva breeding. Because comparing the expression rates with control sample, proPO I and II genes of treated 10^9 cellml^{-1} sample were recorded 2.018 and 12.008 fold respectively.

***Corresponding Author:** Gholamreza Rafiee ✉ ghrafiee@ut.ac.ir

Introduction

The applying of probiotics as a food supplements dates for rearing animals back to the 1970s (Farzanfar, 2006). Based on statistics of the FAO[1] marine and brackish water shrimp rearing production in the world expanded from 8987 metric tons in 1970 to 3,146,918 metric tons in 2006. The considerable increase in cultured shrimp production has occurred despite relatively poor basic knowledge of the cultivated species and enormous mortality due to diseases like white spot syndrome virus (WSSV), estimated at approximately US $1 billion per year since the early 1990s (Flegel *et al.,* 2008). Moreover penaeid shrimps rearing, among the farm crustacean and oyster, is an economical and important activity which serves as a producing high quality seafood and generation of employment (Nayak *et al*., 2011).

Probiotics are alive cells that have spectacular effects on the health conditions of cultivated fishes by improving its intestinal balances by increasing feed value and enzymatic contribution for digestion, inhibition of pathogenic dangerous, antimutagenic and anticarcinogenic properties, growth-promoting factors, and an increased immune abilities (Verschuere *et al.,* 2000) The genus Bacillus contains some of the bacteria used as probiotics in fish and crustacean aquaculture and is able to secrete many exoenzymes to improve feed digestion and absorption, and also water quality (Maw-Sheng *et al.,* 2009). Most of the probiotic studies in shrimp focused on the phase of growth out, but little was related to shrimp larval development and culture (Ziaei-Nejad *et al.,* 2006).

Innate immunity has a high role in insects and crustaceans lives because of lack antibodies (Iwanaga *et al.,* 2005). Innate immunity involves phagocytosis, encapsulation, hemocyt coagulation and activation of the prophenoloxidase (proPO) or melanization cascade (Jiravanichpaisal *et al.,* 2006; Lemaitre *et al.,* 2007) that every of these, is a part of the overall invertebrate immune system (Maningas *et al.,* 2008). Activation of proPO generates phenoloxidase (PO), which catalyzes the oxygenation of monophenols to o-diphenols and the oxidation of o-diphenols to the corresponding o-quinones (Cerenius *et al.,* 2008).These are reactive intermediates for melanin synthesis and other physiological processes such as cuticle clerotization, wound healing and pathogen sequestration (Cerenius *et al.,* 2004). In most cases, the proPO cascade is triggered by a small amount of microbe-derived molecules such as lipopolysaccharides(LPSs), b-1, 3-glucans, or peptidoglycan. Pattern-recognition proteins bind these molecules and initiate the proPO system through a quick proteolytic cascade are proteolytic cascade, and many proteins involved in the serine proteinases (Cerenius *et al.,* 2010). The final serine proteinase that converts the inactive proPO form into its active one is called prophenoloxidase-activating enzyme (PPAE) (Cerenius *et al.,* 2004).

The aim of this research is study effects of the mixed probiotic microalgae-bacteria, *Skeletonema costatum-Bacillus subtilis*, on Stress tolerance and innate immunity prophenoloxidase (proPO) activating in *Penaeus monodon* larvae.

Materials and methods

Materials

In this study we tried to culture *Penaeus monodon* shrimp (from zoa-1 to post larva-14) with conjugated *Skeletonema costatum-Bacillus subtilis* (microalgae-bacteria as a mixed food) and used the algae as an adhesive surface for siting bacterial place and measuring its effects on stress resistance and innate immune system.

Methods

The mixed algae-bacteria package food was prepared from Marine Research Center of Persian Gulf university-I.R.IRAN and the zoa from a private

[1]-Food and Agriculture Organization

shrimp propagation center in Bushehr Port[2]. This diet had been reared in different ecological circumstances in order to determine the highest total lipids, total proteins, total carbohydrates, total carotenoids, chlorophylls a and b in a previous study (unpublished documents).The highest total proteins had been obtained in salinity 40 ppt, temperature 25 ˚C, pH 8.0, light 1000 Lux/4 days and bacteria rate 10^7cllml^{-1}. The highest total proteins content of the diet was 5.01376 µg/ml.

Rearing water for culturing media was autoclaved firstly in 5 litters glass containers, at 121 °C and 1.055g mm-2 for 20 min (AuW)(12 kilograms, Reyhan Teb Co. I.R. IRAN made). Sterility of AuW was routinely confirmed by spreading aliquots of randomly selected samples on modified Sea Water Complex (SWC) agar (Merck Co., Germany) (Reichelt *et al.,* 1973). All rounded 5 litters glass flasks, air pipes, glass tubes and other tools were treated prior to use by autoclave set again. Flask heads were plugged with cotton together aluminum foil cover and air was supplied through 0.2 mm air filters and diffuser.

Zoa-1 stage of P. monodon were stocked at 100 larvae L–1 in these glass containers. Gentle aeration was supplied through an autoclaved plastic tube from the bottom of each flask at the rate of 1-2 bubbles s–1. Moreover by this increasing dissolved oxygen, the gas bubbles can keep the diet particles in suspension form. The mixed food (algae + bacteria) was fed in three replicates and in five levels (101,103, 105, 107 and 109 cellml-1).

• *Effects of algae-bacteria complex on larval growth and survival rates*

Circumstances for breading

After preparing this stock[3] (*S. costatum- B. subtilis*), with high total proteins, the *P. monodon* shrimps were fed from zoa-1 to post-larvae-14 stages. These trails had 3 replicates and one control. After pumping water from the Persian Gulf Sea and passed through sand filtering system, it was autoclaved in mentioned circumstances above for rearing water. The 5 liter-glass containers were stocked with 500 *P. monodon* zoa-1 stage. Moderate aeration was done through a transparency tube from the bottom of each container at the rate of 1–2 bubbles s^{-1}. The salinity and temperature of culture water in the glass flasks were 33 ppt and 30 ˚C respectively. Three 3 ml water samples from each glass tank were collected to evaluate the algae number once every 3 days before adding algae-bacteria food. Counting of algae was carried by spectrophotometer set PerkinElmer-Lambda 25 model out. Each of these water samples was analyzed in triplicate bacteriologically too. It was used Thiosulphate Citrate Bile Salt Agar (Merck Co. Germany) with 3 ppt salinity (as a supplement) and incubated at 27 ˚C during 24 hours for investigation of probably vibrio presence (Zou *et al.,* 2009).

In order to reduce stresses, chemical factors including ammonia and nitrate were measured during the feeding time, exception of pH (Metrohm 827-Swiss model) that measured daily (Bower *et al.,* 1987) (Table 1). After counting algae number in 3 samples (3 ml) of rearing water and knowing status of feeding, new algae-bacteria stock solution as a food was added to the larvae rearing water resulting in 10^1, 10^3, 10^5, 10^7 and 10^9 cellml^{-1} respectively, once every 2 days at 08:00 pm o'clock. The food for control treatment was prepared from privet shrimp propagation center[4]. The larvae shrimps were fed with live mixed diet (*S. costatum-B. subtilis*) during the experimental period for ten times totally.

Ten samples from each replicate were randomly sub-sampled once every 2 days to see the larval development under microscope (Ceti modle, made Belgium). When all zoa and Mysis had metamorphosed to the post larval stage, the resistant or survival rate was estimated in pl-14. At the end of this studying period (approximately 3 weeks),

2- Abzian-parvar busher, shrimp propagation private center
[2]- This stock was prepared from Persian Gulf university

3- Abzian-parvar-e Bushehr privet center (managed by Dr. Rasekhi)

survival percent (Table 2) and resistance rate to salinity (fresh water, 33 and 66 ppt) and nitrite (300 ppt) stresses for the post larvae were measured (table 3). All treatments were run in triplicate. During the experiments, larval development and water quality parameters including ammonia-N and nitrite-N, total bacterial count, and probably Vibrio sP. count were determined. The Bower-Bidwell and Bendschneider methods were used for ammonia and nitrate assessment respectively (Bower *et al.*, 1978; Bendschneider *et al.,* 1952). At the end of experiment, larval survival rate, and stress tolerance to water changes in salinity and nitrite-N status were evaluated. It must be mentioned that during the tests no food was added, water no changed and aeration carried gently and continuously out.

* *Salinity and nitrite-N stress assessments*

After breading *p. monodon* post-larvae with algae-bacteria, were tested for the tolerance to salinity and nitrite-N(concentrations mentioned above).

Three replicate groups of 50 post-larvae from each algal-bacterial treatment were considered to salinity tests of fresh water, 33 (control) and 60 ppt. The stress salinity test was done in 1000-ml glass beakers with a salinity of 33 ppt and gentle aeration. After that post-larvae were transferred immediately to glass beakers with fresh water, 33 and 60 ppt salinity separately. In that time the mortality was recorded at 1-hour and 10 minutes (for nitrite test) intervals until all post-larvae had died in one treatment firstly.

Table 1. Chemical circumstances of rearing water (30 ppt salinity) tanks of post-larvae were fed with mixed algae-bacteria and comparing them with control sample.

Paramet.		pH changes				ammonia changes /ppm				nitrite changes / ppm				
	Algae-bacteria Fcu/ml	Post-larvae replicants			pH mean	Post-larvae replicants			Mean ammo. /ppm	Post-larvae replicants			Mean nitrite /ppm	Vibrio infection
Samples		3	2	1		3	2	1		3	2	1		
Control	Hatchery food	7.9	7.8	7.8	7.83	0.77	0.77	0.75	0.76	0.76	0.77	0.76	0.763	+
Treatment 1	10^1	7.9	7.8	8.0	7.9	0.75	0.70	0.60	0.68	0.75	0.75	0.7	0.73	+
Treatment 2	10^3	8.0	8.1	7.9	8.0	0.60	0.60	0.70	0.63	0.60	0.70	0.7	0.66	+
Treatment 3	10^5	7.9	7.8	7.9	7.8	0.70	0.60	0.70	0.66	0.65	0.65	0.65	0.63	+
Treatment 4	10^7	8.1	8.0	8.2	8.1	0.73	0.72	0.74	0.73	0.65	0.68	0.68	0.67	+
Treatment 5	10^9	8.2	8.0	8.1	8.1	0.78	0.77	0.76	0.77	0.76	0.69	0.70	0.71	+

Table 2. Survival percentage of *p. monodon* larvae in different growth stages with different level of conjugated algae-bacteria food in 33 ppt.

Algae-Bacteria	Zoa stage %				Mysis stage %				Post larvae %			
	Replicate				Replicate				replicate			
	1	2	3	Mean	1	2	3	mean	1	2	3	mean
10^1	88	89	89	88.6	82	84	84	83.3	40	46	47	46.3
10^3	94	93	89	92.0	87	81	84	84.0	52	44	46	47.33
10^5	93	94	89	92.0	88	83	83	84.66	52	45	47	48.0
10^7	93	92	94	93.0	88	86	86	86.0	54	48	48	50.0
10^9	96	94	95	95.0	90	89	92	90.3	59	51	57	55.66
control*	85	86	87	86.0	87	82	84	84.3	57	48	56	53.6

* The food for feeding control samples and all growing stages, had been from the private Propagation Shrimp Center(Abzian-Parvar-e Bushehr center).

Except 10^9 cell/ml algae-bacteria treatment that had less microbial contamination, the rest samples were infection to *Vibrio sp*. based on counting colonies. For nitrate trail, the post-larvae were introduced to nitrate-N in 300 ppm concentration suddenly (Li, *et al*., 2008). For all these trails, three replicates were mentioned. When the survival experiments were doing, the salinity was 33 ppt and aeration was done by diffuser gently and continuously. The dead post-larvae shrimps were recorded 1- hour (for salinity and fresh water) and 10 minutes (for nitrate) intervals. These trails had been continued to

All larvae had died in one of treatments as the first. When these experiences were doing, no live food was fed and no water changed. Dead larvae were considered as a shrimp that did not show any reaction to mechanical motions under light microscope (Kuan, *et al*., 2010) (Table 3).

Based on these data (table 3), the resistant of *P. monodon* to fresh water was very high so that mean rates of death percentage during 23 hours was 82.6% and 84% in fresh-water and 66 ppt salinity respectively. Also in nitrate stress test, all the post larvae in control 1 were dead during 138 minutes and had lesser resistance than the other stress tests. These experiments were done in three replicates for control and the treatment that fed with mixed algae-bacteria food with 10^9 cell/ml concentration. Because of this concentration had the best effect on *P. monodon* based on table 2 data. In addition, the distances between obtained results of treatments and control were down. The reason that can be said for these results is, the bought food from privet hatchery had been mixed with kind of probiotic.

Table 3. Resistance conditions of 50 *P. monodon* post larvae fed with 10^9 /cell ml^{-1} algae-bacteria complex in stress positions.

Parameter / Treatment	Resistance rate to different salinities			Resistance to nitrite 300 ppm
	Fresh water (death %)	33 ppt	66 ppt	
Replicate 1	78%	100%	86%	82%
Replicate 2	84%	100%	82%	86%
Replicate 3	86%	100%	84%	80%
Replicate mean rates	82.66%	100%	84%	82.66%
Control 1	100% Death – 23 h.	100%	96%	100% Death- 138min.
Control 2	92%	100%	92%	90%
Control 3	94%	100%	100% Death- 8 h.	94%
Control mean rates	95.33 %	100%	96.0 %	94.66 %

Immune circumstances analysis

After getting outcome from the 101, 103,105, 107 and 109 cell/ml algae-bacteria treatment and control in resistance rate estimation, 10 post-larvae from each of them were taken randomly and immediately used for total RNA isolation and immune gene expression measurements of prophenoloxidase I (proPO I) (Lai *et al.,* 2005), and prophenoloxidase II (proPO II) (Yeh *et al.,* 2009) using real-time reverse transcription polymerase chain reaction (RT-PCR). Each of these treatments was carried out with three replicates. In this study, the β-Actin (GB: AF300705) that was used as a positive control and the needed primers are brought in table four (table 4).

Table 4. The sequences of primers used in this study (prophenoloxidase I, II and glutathione).

Genes	Primer sequence	Cod of bank gene	Source
ProPo I-F	5`- GCCTTGGCAACGCTTTCA-3`	GB:AY723296	(Liu, *et al*., 2007)
ProPo I-R	5`- CGCGCATCAGTTCAGTTTGT-3`		
ProPo II-F	5`- GAGAGGCTGAACCGAGACTGA-3`	GB:EU373096	(Sotelo Sotelo-Mundo, *et al*., 2003)
ProPo II-R	5`- AAGAAAACGGCCCCCAATT-3`		

β-actin-F	5`-GAGCAACACGGAGTTCGTTGT-3`	
β-actin-R	5`-CATCACCAACTGGGACGACATGGA-3`	GB: AF300705

Few post larvae from each treatment with total approximately 20 mg weights were taken and frozen in liquid nitrogen immediately. They were made powder by sterile pounder to homogenize. One ml RNX was added to this homogenized powder and mixed well and leaved it in room temperature for 5 minutes. Then 200 µl chloroform was added to it and after leaving in 4 °C temperature (on ice small flakes) for 5 minutes, centrifuged with 12000 g by refrigerator- centrifuge set (Eppendorf 5418-Germany model) for 15 minute. Liquid phase (upper liquid) was transferred to a new tube and added isopropanol as much as it's volume. It was mixed gently and after leaving in 4 °C temperature (on ice powder) for 15 minutes, centerifuged again with 12000 g for 15 minutes. The precipitated material (or RNA) was washed with one ml ethanol 75%. After that centrifuged again with 7500 g in 4 °C. This precipitated RNA was dried in room temperature for 15-30 minutes and mixed with enough distilled water (relation to precipitation amount, 20-100 µl).

The RNA of Penaeus monodon post-larvae was used as a model by fermentas special kite for preparing cDNA. This experiment was performed according to the manufacturer's recipe. The cDNA was made by transcription M-MuLV enzyme and Oligo (dt) or random hexamer as a primer based on standard method and frequent two stages.

Random Hexamer method

Firstly, random hexamer 2 µl, double distilled water (without any RNA and DNA) 10 µl and RNA 4 µl were mixed in a sterile micro-tube and put it on ice (component 1). After centrifuging this component (1) in a few minutes and 70 °C, was put on ice pieces. Then 5x-buffer 8 µl, RNAase inhibitor 1 µl, double distilled water(without any RNA and DNA) 11 µl, mM dNTP 10 mix 2 µl and M-MuLV 2 µl were added to it (component 2). This sample was centrifuged in 6 minutes and kept in temperature 37° C. The reaction was stopped in ten minutes by sample treatment in 70 °C. The prepared cDNA was used in PCR standard reactions. The different materials (from Bioneer Co.-South Korea) were used for performance of RT-PCR (table 5).

Table 5. needed materials for RT-PCR

Need materials	XSYBR Mix 2	PrimerF qPCR (10/pmol)	PrimerR qPCR (10/ pmol(	Tag Polymerase	cDNA Template	Water DNase
Volume (µl)	6.25	0.5	0.5	0.1	0.5	4.65

The reagent RocheKit for total RNA isolation and FermentasKit for preparing cDNA were used in this study. The expression of proPO II in post-larvae was increased as the Algae-bacteria concentration went in the culture media up. ΔCt of proPO II was the lowest in the 109 cellml-1 algae-bacteria treatment, which significantly differed from the control and 107 ml-1 treatment. ProPO II ΔCt of post-larvae in the 107 cellml-1 treatment was significantly lower than that ones in the control treatment. The ΔΔCt of proPO II between the control and 107 and 109 ml-1 treatments were 3.323 and 3.523, respectively. The relative expressions of proPO II in the 107 and 109 ml-1 treatments were 10.012 and 11.5213 fold, respectively, higher compared to the control (table 6)(Fig 1).

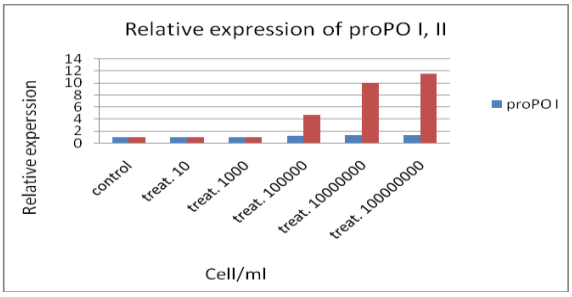

Fig 1. Relative expression of two genes, Prophenol Oxidases I and II

Table 6. Expression rates of proPo I and II with their standard deviations in different mixed algae-bacteria food concentrations and comparing control sample

Gen / Treatment	Replicate number	proPo I			proPo II		
		ΔΔct mean	Expression rate mean	Standard deviation	ΔΔct	Experssion rate mean	Standard deviation
Control	3	0.00	1.000	0.000	0.00	1.000	0.000
Treat. 10^1	3	0.09	1.0643	0.009	0.07	1.049	0.011
Treat. 10^3	3	0.0833	1.05953	0.020	0.06	1.04258	0.038
Treat. 10^5	3	0.3133	1.2429	0.020	2.25	4.7571	0.168
Treat. 10^7	3	0.4866	1.40148	0.027	3.323	10.012	0.191
Treat. 10^9	3	0.47733	1.3883	0.032	3.523	11.5213	0.266

The number cycle was considered 40 cycles for all the genes. Reaction phases included, 1- initial single strand production, 94 ˚C in 2 minutes 2- single strand, 94 ˚C in 15 seconds 3- connecting primer and linear, 60 ˚C in 30 seconds.

Estimation of comparing effectiveness in propagation of control innate gene and prophenoloxidases I and II

Because of impossible to collect post larval hemocytes for analyzing the immune responses, expressions related genes proPO I and II were used as indicators for estimating the immune status improvement of post larvae by an SYBR green real-time PCR.

Based on comparing gene expression method that was used in this study, it's needed considered effectiveness of propagation of control innate gene and prophenoloxidases I and II. Because propagation effectiveness of target gene and innate control gene must be a same approximately. Therefore, standard input logarithmic curves for each genes, (prophenoloxidase I , II and also β-Actine gene as an innate control) against all Ct rates were drawn. Based on RT-PCR reaction, for each genes and mean Ct rates, two replicates performed and also proportion of prophenoloxidases I and II rates to β-Actine rates calculated. Using Excel statistic software, input log rates were considered as X-axis and gene rates as Y-axis. After drawing XY-scatter, y=ax+b linear formula was gotten. When the propagation effectiveness of target gene and innate control gene rates are equal, absolute slope must be zero approximately (Pfaffi *et al*., 2001).

The $2^{-\Delta\Delta C_t}$ equation was used for quantification of RT-PCR data. It's clear that the initial concentration of gene increases, the cycle number or CT decreases. Therefor:

$2^{-\Delta\Delta C_t}$ = (CTPro – CTβActin) in treated group-(CTPro – CTβActin) in control group

For making equilibrium data in experimental groups and for normalizing them based on control innate gene expression rates, it was calculated the portion of gene expression rate to control group ones (Livak and Schmittgen, 2001).

The obtained data were investigated for normality of variance before further analysis. Where these conditions were satisfied, parametric test such as T-

tests and analysis of variance (ANOVA) were used. The conditions of homogeneity were violated, the non-parametric alternatives such as the Kruskal–Wallis tests and Mann–Whitney Rank sum tests were used. Significant levels of difference was set at $P< 0.05$ (Sokal *et al.,* 1995).

Discussion and results

There are not significant differences in pH, ammonia-N or nitrite-N among post larvae treatments during this experiment. The change ranges of pH, nitrite-N and ammonia-N in treatment sample were recorded 7.8-8.1, 0.63-0.73 and 0.63-0.77 ppm, respectively. Based on table 1, nitrite and ammonia in control samples are higher than the other treatments totally (Table 1).

There were more Vibrio grew on control TCBS media than the other treatments particular post-larvae stages. It was shown that the bought food from the private hatchery had more microbial pollution in comparing to our algae-bacteria complex food particularly 101 and 103 treatments in zoa and mysis stages.

The survival percentages of *P. monodon* larvae, after being bred with the algae-bacteria at the concentrations of commercial hatchery food (control), 10^1, 10^3, 10^7 and 10^9 ml^{-1} is shown in table 2. Results showed that larval survival percentage was increased after feeding with the algae-bacteria at the 10^9 concentration. There were more larvae at the zoea 3 stage with algae-bacteria at 10^9 ml^{-1} concentration in compared of control sample on the 3rd day. In this investigation, all larvae approximately had metamorphosed into post-larvae after 6-7 days. In all stages, algae-bacteria complex at 10^9 ml^{-1} concentration treatment had a better development than not only control samples but also than the others treatments. The best means were estimated for all the three growth stages in zoa , mysis and post-larvae survival percentages aspects after feeding with algae-bacteria (Table 2). For zoa is 86.0 % and 95.0% , mysis is 84.3% and 90.3 % and post-larvae is 53.6 % and 55.66% for control and 10^9 ml^{-1} treatments respectively.

Stress experiments

There were no any dead post-larvae had fed with *Skeletonema costatum-Bacillus subtilis* complex after being put to 33 ppt salinity suddenly . Based on table 3, the mortality of post-larvae after being suddenly transferred to freshwater media had a longest period time. All dead post-larval bodies were recorded after 23 hours of sudden exposure to fresh water in control replicate 1. So the mean percentages of death bodies for treatment and control replicates were 82.66 and 95.33% respectively. The cumulative mortalities of post-larvae in 66 ppt salinity was seen at treatment replicate 3 after passing 8 hours and the death mean treatment and control replicates at end of the trial touched points of 84% and 96% respectively. In nitrate stress, the control replicate 1 was the first replicate that all shrimp larvae were died during 138 minutes. Based on table 3, the death mean percentages for treatment and control replicates recorded 82.66 and 94.66 . From these data can find that *P. monodon* is sensitive to high salinities more than freshwater.

Todays, many of studies show the important effects positively like B. subtilis introduction to L. vannamei larvae caused accelerated larval development and survival rate at the concentration of 108 cful-1 (Liu *et al.,* 2010).Also, it is cleared that when L. vannamei juveniles was fed by B. subtilis – containing diet, their growth increased. Because the protease activity in their digestive tracts were promoted (Liu, *et al.*, 2009). This can lead us that the increasing of L. vannamei larval survival rate may can due to improve the nutritional circumstances caused by elevating digestive enzyme activities and nutrient absorption. In recent decades proved the positive effects of probiotics on shrimp or other fishes Larvie culture like Macrobrachium rosenbergii, zoea stage had a much better survival and a faster rate of metamorphosis when fed by B. subtilis treated (Keysami *et al.,* 2007). In another investigation was

found that shrimp larvae , Fenneropenaeus indicus, had a better survival and growth rates when they fed by 5 species of Bacillus including *B. subtilis, B. licheniformis*, *B. polymyxa*, *B. laterosporus*, and *B. circulans*. In this study the feeding operation from the zoea to the postlarval stages was done in containing water or with probiotic-enriched Artemia (Ziaei-nejad *et al.,* 2006). In addition, it was shown the probiotics including some of Bacillus strains able to inhibit many of pathogens cause diseases like *Vibrio harveyi* (Decamp *et al.,* 2008) (Vaseeharan *et al.,* 2003). But in present investigation we tried to make a complex of algae-bacteria as a food for getting better results than only bacteria. For this reason, the genetic analyzing is necessary to prove our goal.

Expression of the proPO I and II genes of post-larvae after being fed with algae-bacteria complex is shown in table 6. Comparing to the control, the mean ΔΔCt values of proPO I of post larvae in 10^7 and 10^9 ml^{-1} algae-bacteria treatments were 0.4866 and 0.47733 equivalent to 1.40148 and 1.388 fold higher expression respectively. Also ΔΔCt values of the proPO II gene in 10^7 and 10^9 ml^{-1} 3.323 and 3.523 equivalent to 10.012 and 11.5213 fold higher expression in comparing to the control respectively.

Discussion

Although some reports are regarded the advantages of using probiotics in shrimp aquaculture (Gatesoupe, 1999; Farzanfar, 2006) but very too less any report about bacteria and microalgae as a mixed diet. It's clear today that positive effects of probiotics in aquaculture industries for biological control of disease through improved shrimp immunity (Rengpipat *et al.,* 2000), pathogen inhibition(Decampo,2008) and shrimp growth performance (Wang 2007; Tseng *et al.,* 2009) are spectacular. Among the probiotics, like Bacillus sp. we tried to use the microalgae Skeletonema costatum as a surface for adhesion of this bacteria to increase nutrition effects. Also we believed that not only the own algae can be considered as food, but also nutritional extractions of this algae can help to better survival of bacteria.

The results showed that mixed algae-bacteria was able to protect larvae shrimp against of Vibrio and also improve their survival rate. There are many results in previous studies that shown the inhibition of Vibrio harveyi, Vibrio anguillarum, and Vibrio damsela by B. subtilis (Tseng , 2009)(Vaseeharan , 2003). We think that results could be better but It's guessed that the food was bought from the private shrimp propagation center for feeding control group, had been treated by bacteria.

In addition, the use of mixed B. subtilis-S. costatum in shrimp larval food may had delayed the primary growth of harmful bacteria like Vibrio sp. or may acted as a inhibitory factor against it. Also environmental stresses can be the factors to establish this delaying.

It is seen that the stress resistance of fish can be treated by probiotic agents (Taoka *et al.,* 2009). In many studies two probiotics, *Lactobacillus fructivorans* and *Lac. plantarum*, were investigated on rotifer *Brachionus plicatilis*, and/or *Artemia salina* for sea bream feeding or improving of food quality for *Sparus aurata,*fish. In these investigations, had been used these two microorganisms as the carrier caused changes in two stresses, in lower and higher cortisol level and Hsp70 gene expression respectively, and resulting decreased mortality in probiotic-treated sea bream fish when subjected to an acute pH stress (from 6.3 to 8.6) significantly (Rollo *et al.,* 2006).

In this study, tried to performance the stress tolerance in different ware salinity (fresh water and 60 ppt salinity) and 300 mgl^{-1} nitrite-N after *Penaeus monodon* postlarvae feeding with the algae-bacteria (*S. costatum-B. subtilis*) complex. It can be said that the physiological reactions of post-larvae to this integrated food, could adapted them to acute environmental stresses.

Based on bacteriological analysis in present study, however it's showed that using of mixed algae-bacteria did not produce any significant bacterial growth in the water despite the probability vibrio. But comparing control treatments, presence of vibrio bacteria decreased significantly in the algae-bacteria treatments of 10^7 and 10^9 ml^{-1} on the 14th day. Also vibrio bacteria were seen in all these treatments at the end of tests. But it's thought that algal-bacterial extractions may be the reason of keeping low vibrio.

Environmental stress can be one of the main contributing factors to elevate bacterial presence and mortality in aquaculture activities. Therefore, a sudden or severe change in environmental conditions can create bad mortality or disease outbreaks in aquaculture farms. Todays, the stress resistance of shrimp was shown to be improved by many reports (Taoka *et al.,* 2009).

1-Statistic analyses

In this study was used two softwares, Microsoft Office Excel and Spss16.0 for statistic analyzing. The kolmogorov-smirnov test was applied for data normalizing rate and one-way Anova was used for variations normality and used Duncan, s multiple range test in 0.05 level for comparing variations and with or no significant differences between means. Also used Duncan, s two-way test in 0.05 level for two factors including pasting time on gene expression and treatment dosage.

Acknowledgments

The authors acknowledge the Persian Gulf university assistantship. We are grateful to the Director of Persian Gulf Marine Research Center (PGMRC), DR. Hosseini, for providing the best facilities required and we also appreciate Dr. Abdi, Dr. Ghasemi (chief of genetic lab) and Jamali, Jafari, Ms Cheraghi (head of microbiology lab), Ms Hosseini, MsMoheymani , Namju, Kamyab, Ms Karimi, Jamhiri and Avijgan, the engineers of the center, for their kind helps. Thanks are due to all involved other specialists.

References

Bendschneider K , Robinson RJ. 1952. A new spectrometric method for the determination of nitrite in the seawater **11,** 87-96.

Bower CE, Bidwell JP. 1978. Ionization of ammonia in seawater: effects of temperature, pH and salinity **35,** 1012-1016.

Cerenius L, Söderhäll K. 2004. The prophenoloxidase-activating system in invertebrates. **198,** 116-126.

Cerenius L, 2010. Proteolytic cascades and their involvement in invertebrate immunity. **35,** 575-583. HYPERLINK "http://dx.doi.org/10.1016/j.tibs.2010.04.006" http://dx.doi.org/10.1016/j.tibs.2010.04.006

Cerenius L, Lee BL, Soderhall K. 2008. The proPO-system: pros and cons for its role in invertebrate immunity **29,** 263-271. HYPERLINK "http://dx.doi.org/10.1016/j.it.2008.02.009" http://dx.doi.org/10.1016/j.it.2008.02.009

Decamp O, Moriarty DJW, Lavens P. 2008. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquac Res **39,** 334-338. 10.1111/j.1365-2109.2007.01664.x

farzanfar A. 2006. The use of probiotics in shrimp aquaculture. 2006, Vol. 297.

Flegel TW. 2008. Shrimp disease control: past,present and future. In: Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M.,Subasinghe, R.P. (Eds.), 355-378.

Gatesoupe FJ. 1999. The use of probiotics in aquaculture **180,** 147-165. HYPERLINK "http://dx.doi.org/10.1016/S0044-8486(99)00187-8" \t "doilink" http://dx.doi.org/10.1016/S0044-8486(99)00187-8

Iwanaga Si, Bok LL. 2005. Recent advances in the innate immunity of invertebrate animals **38,** 128-150.

Jiravanichpaisal P, Lee BL, Söderhäll K. 2006. Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization **211,** 213-236.
HYPERLINK "http://dx.doi.org/10.1016/j.imbio.2005.10.015" \t "doilink" http://dx.doi.org/10.1016/j.imbio.2005.10.015

Keysami MA, Saad CR, Sijam K, Daud HM, Alimon AR. 2007. Effect of Bacillus subtilis on growth development and survival of postlarvae Macrobrachium rosenbergii (de Man) **13,** 131-136.
10.1111/j.1365-2095.2007.00463.x

Kuan FU, Liu CH, Winton C. 2010. Effects of the probiotic B. Subtilis E20, on the survival, development stress tolerance and immune status of white shrimp L. Vannamei **28,** 837-844.
HYPERLINK "http://dx.doi.org/10.1016/j.fsi.2010.01.012" \t "doilink" http://dx.doi.org/10.1016/j.fsi.2010.01.012

Lai CY, Cheng W, Kuo CM. 2005. Molecular cloning and characterisation of prophenoloxidase from haemocytes of the white shrimp, Litopenaeus vannamei **18,** 417-430.
HYPERLINK "http://dx.doi.org/10.1016/j.fsi.2004.10.004" \t "doilink" http://dx.doi.org/10.1016/j.fsi.2004.10.004

Lemaitre B, Hoffmann J. 2007. The host defense of Drosophila melanogaster **25,** 697-743.

Li E. 2008. Acute toxicity of boron to juvenile white shrimp, Litopenaeus vannamei, at two salinities **278,** 175-178.
HYPERLINK "http://dx.doi.org/10.1016/j.aquaculture.2008.03.022" \t "doilink" http://dx.doi.org/10.1016/j.aquaculture.2008.03.022

Liu CH, Chiu CS, Lin PL, Wang SW. 2009. Improvement in the growth performance of white shrimp, Litopenaeus vannamei, by a protease producing probiotic Bacillus subtilis E20 from natto **107,** 1031-1041.
HYPERLINK "http://dx.doi.org/10.1016/j.fsi.2010.01.012" \t "doilink" http://dx.doi.org/10.1016/j.fsi.2010.01.012

Liu K. 2010. Effects of the probiotic, B. subtilis E20 on the survival, development, stress tolerance and immune status of white shrimp L. vannamei larvae **28,** 837-844.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Jou. Methods **25(4),** 402-408.

Maningas MB. 2008. Essential function of transglutaminase and clotting protein in shrimp immunity **45,** 1269–1275.

Maw-Sheng Y. 2009. A second proPO present in white shrimp Litopenaeus vannamei and expression of the proPOs during a Vibrio alginolyticus injection, molt stage, and oral sodium alginate ingestionFish. 49-55.

Nayak S, Ajay K, Meena A, Sreepada N. 2011. Profiling of a few immune responsive genes expressed in postlarvae of Fenneropenaeus indicus challenged with Vibrio harveyi D3. **107(2),** 168-172.
HYPERLINK "http://dx.doi.org/10.1016/j.jip.2011.04.001" \t "doilink" http://dx.doi.org/10.1016/j.jip.2011.04.001

Reichelt JL, Baumann L. 1973. Taxonomy of the marine luminous bacteria **94,** 283–330.
10.1007/BF00769027

Rengpipat S. 2000. immunity enhancement in black tiger shrimp (Penaeus monodon) by a probiont bacterium B. subtilis **191,** 271-288.
HYPERLINK"http://dx.doi.org/10.1016/j. aquaculture. 2005.07.021" \t "doilink" http://dx.doi.org/ 10.1016/j.aquaculture.2005.07.021

Rengpipat S, Phianphak W, Piyatiratitivorakul S, Menasveta P. 1998. Effects of a probiotic bacterium on black tiger shrimp Penaeus monodon survival and growth **167,** 301-313.
HYPERLINK "http://dx.doi.org/10.1016/S0044-8486(98)00305-6" \t "doilink" http://dx.doi.org/10.1016/S0044-8486(98)00305-6

Rollo A. 2006. live microbial feed supplement in aquaculture for improvement of stress tolerance **32,** 167-177.
10.1007/s10695-006-0009-2

Sokal RR, Rohlf FJ. 1995. The principles and practice of statistics in biological research. 1995, p. 887.

Taoka Y. 2009. Growth, stress tolerance and non-specific immune response of Japanese flounder Paralichthys olivaceus to probiotics in a closed recirculating system 72, 310-321.
10.1111/j.1365-2095.2011.00863.x

Tseng DY. 2009. Enhancement of immunity and disease resistance in the white shrimp, Litopenaeus vannamei by the probiotic, Bacillus subtilis E20 **26,** 339-344.
HYPERLINK "http://dx.doi.org/10.1016/j.fsi.2008.12.003" \t "doilink" http://dx.doi.org/10.1016/j.fsi.2008.12.003

Vaseeharan B, Ramasamy P. 2003. Control of pathogenic Vibrio spp. By Bacillus subtilis BT23, a possible probiotic treatment for black tiger shrimp Penaeus monodon. Lett Appl Microbiol **36,** 83-87.

Verschuere L, Heang H, Criel G, Dafnis S, Sorgeloos P, Verstraete W. 2000. Protection of Artemia against the pathogenic effects of Vibrio proteolyticus CW8T2 by selected bacterial strains **66,** 1139-1146.

Wang YB. 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp Penaeus vannamei **269,** 259-264.
HYPERLINK "http://dx.doi.org/10.1016/j.aquaculture.2007.05.035" \t "doilink" http://dx.doi.org/10.1016/j.aquaculture.2007.05.035

Yeh MS. 2009. A second ProPO present in white shrimp *L. vannamei* and expression of the ProPOs during a *Vibrio alginolyticus* injection, molt stage and oral sodium alginate ingestion. **26,** 49-55
HYPERLINK "http://dx.doi.org/10.1016/j.aquaculture.2005.07.021" \t "doilink" http://dx.doi.org/10.1016/j.aquaculture.2005.07.021

Ziaei-Nejad S. 2006. The effect of Bacillus spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the India white shrimp Fenneropenaeus indicus **252,** 516-524.
HYPERLINK "http://dx.doi.org/10.1016/j.fsi.2008.10.003" \t "doilink" http://dx.doi.org/10.1016/j.fsi.2008.10.003

Zou X, Wang Y, Li F. 2009. Effects of probiotic on larvae shrimp (p. vannamei) based on water quality survival rate and digestive enzyme activities **287,** 353-349.