

Immune response and disease resistance in the white shrimp, *Litopenaeus vannamei* induced by potential probiotic *Lactobacillus bulgaricus*

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Summary: In the present study, *Lactobacillus bulgaricus* E20 isolated from the gut contents of farm-reared white shrimp, *Litopenaeus vannamei*, after determination of its characteristics using biochemical, molecular and probiotal examinations, was used in the diet of white shrimp to evaluate the survival rate, immune status, and disease resistance. *L. bulgaricus* was administered at two different doses 1×10^7 (T1) and 1×10^9 (T2) CFU g⁻¹ feed to shrimp for 30 days. A control group was also included with normal feed. The survival rate, total hemocyte counts, phenoloxidase activity, respiratory burst, gut lactic acid bacteria and gut total bacteria were evaluated at the end of trial and after challenging shrimp with *Vibrio parahaemolyticus*. Higher survival rates were observed in shrimp fed probiotic diets as compared with the control ($P < 0.05$). The best immune performance in terms of hemocyte counts, phenoloxidase activity, and respiratory burst was seen in the probiotic-treated groups especially in T2. Also, an improvement was seen in the number of lactic acid bacterias in shrimp that had been given probiotics. There were no significant differences between groups (treated and control) after exposing with *V. parahemolyticus* ($P < 0.05$). Shrimp fed with probiotic diets revealed lower cumulate mortality than the control group. These findings demonstrated that administration of *L. bulgaricus* can improve survival rate and disease resistance through an enhanced immune response in shrimp.

Keywords: Immune responses, *Lactobacillus bulgaricus*, *Litopenaeus vannamei*, probiotic.

Introduction

It is well known that in the intensive culture, shrimp are exposed to different stressful conditions which usually led to the deterioration of immune response and outbreak some infectious diseases (10). As a result, such conditions increase mortality and led to great economic losses. At present, antibiotics and chemicals are used widely to eliminate bacterial diseases in aquaculture sector (26, 36). However, some bacterial strains show resistance to a range of antibiotics, can survive and transfer this resistance to other bacteria (38). Also, the accumulation of antibiotics in aquaculture products such as shrimp can cause some serious problems for consumers (19). Consumption of probiotics is a good alternative to eliminate multiple problems due to chemical use in the industry. Probiotics promote the natural immunity mainly by replacing pathogens with new microbial communities that in turn enhance the disease resistance of target animal (10). Nowadays, wide use of probiotics in aquaculture sector is well promising to replace antibiotics (10, 42) and is used in various species (7, 17, 18, 39, 44).

Lactobacillus is a non-pathogenic, Gram-positive, non-spore-forming and non-flagellated rods or coccobacilli that are used widely as probiotic in aquaculture (34). Like other lactic acid bacteria (LAB), it

can prevent colonization of some harmful bacteria by reducing the pH of the gastrointestinal tract and producing bacteriocins and organic acids which are well known inhibitors to some pathogens (25).

Crustaceans have a non-specific immune system that absolutely relies on hemocytes and perform functions like phagocytosis, encapsulation, nodule formation and mediation of cytotoxicity (29). Due to the primary and undeveloped immune system in shrimp, the prevalence of some infectious diseases such as vibriosis in shrimp ponds is unavoidable. Therefore, use of probiotics in shrimp farming is a feasible way to reduce the morbidity and mortality by bacterial diseases especially those of *Vibrio* genus (41).

The objective of this study was to evaluate the inhibitory role of *Lactobacillus bulgaricus* as a potential probiotic to *Vibrio parahaemolyticus* in *L. vannamei*.

Materials and Methods

Bacteria were recovered from the digestive tracts of *L. vannamei*, as described by Irianto and Austin (13). In brief, the intestines of reared shrimp in Khuzestan province, Iran, were removed, homogenized and diluted. Dilutions at 10^{-2} to 10^{-4} were prepared in fresh saline and 0.1 ml of volumes were spread over plates of de Man,

Rogosa, and Sharpe (MRS) agar and incubated at 30°C for about 72 h. Some colonies were selected and evaluated for inhibitory effects against pathogenic *V. parahaemolyticus*. The probiotic activities of colonies were examined in vitro using an agar diffusion method and inhibition zones measured (30). Finally, one isolate of LAB (*L. bulgaricus*) with the high inhibitory effects was selected for the experiment.

Identification of the bacteria was carried out based on colony, cell morphology, gram staining and biochemical testing (12). PCR analysis of ribosomal RNA (rRNA) gene was done in order to confirm probiotic bacteria isolated from the intestine of shrimp (2). Briefly, PCR was carried out after 2 min of initial denaturation at 92°C, and 35 cycles of 30s of denaturation at 95°C, 45s of the annealing at 57°C, 45s of primer extension at 72°C and 5 min of final extension. Amplification products were analyzed by electrophoresis in 1% (w/v) agarose gel containing ethidium bromide (1 mg ml⁻¹).

Shrimp weighing 1.3 ± 0.07 g were kept in the tanks of 200-L in 3 groups, each group containing 300 ± 20 shrimp in 3 replicates. Animals were first acclimated to laboratory conditions for one week with water quality consisting of pH 7.5-7.9, salinity 29-32 ppt, daily water exchange 40-50% and temperature 29 ± 1 °C. Preparation of probiotics was in accordance with Planas et al. (25). Bacteria were first cultured in broth agar under anaerobic conditions for 18-20 h at 37°C. Bacterial cells were harvested by centrifugation at 8000 g in 4° C for 5 min, washed 3 times with phosphate buffer saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH: 7.2) and re-suspended in the sterile PBS. Total count of the bacterial suspension was measured by serial dilutions using pour plate count. Required doses of probiotics (10^7 and 10^9 CFU/ml) were adjusted using McFarland standard tubes and then were sprayed on each grams of food (25). Shrimp were fed four times a day with a pellet (Havourash, Iran) at 10% of biomass. A control group was also included without probiotic treatment. The trail was run for 30 days.

Dead shrimp were removed daily and the survival rates were calculated at the end of the trial. Following immune parameters were measured at the end of the rearing period (day 30) and also 10 days after challenging the shrimps with *V. parahaemolyticus*.

Hemolymph was collected from the ventral sinus of 30 shrimp per treatment using a 26-gauge needle with anticoagulant solution (Trisodium citrate 30 mM, sodium chloride 0.34 M, EDTA 10 M, pH 7). An aliquot of haemolymph was used for total hemocyte count and the rest was centrifuged at $300 \times g$, 4°C for 15 min and the supernatant fluid was separated for immunological analysis (40).

Haemocytes were counted three times using haemocytometer under a light microscope at 400× magnification. The detection of phenoloxidase (PO) activity was carried out according to Smith and Soderhll (35) by measuring L-dihydroxyphenyl alanine (L-Dopa) transformation in dopachrome by the formation of the red pigment DOPA-chrome, after oxidation of the enzyme substrate L-DOPA. Briefly, 50 µL of hemolymph samples were added in a 96 well micro plate. Haemolymph was incubated with 50 µl of 0.1% trypsin in Cacodylate buffer (CAC) at 25°C for 10 min at room temperature, and 50µL of L-DOPA was then added. Absorbance was then measured at 490 nm using a microplate reader. One unit of enzyme activity was defined as an increase in absorbance of 0.001/min/mg protein. Protein content in serum was measured by the Bradford method (6).

Respiratory burst activity (RB) was assayed by using the reduction of Nitro Blue Tetrazolium (NBT) and measuring of superoxide anion (33). A volume of 50 µL of diluted hemolymph was placed into the bottom microplate, incubated at 37°C for 1 h, followed by centrifugation at 1000g for 20 min at 4°C. The pellet was incubated with 100 mL NBT for 2h at room temperature. The suspension was centrifuged at 1000g for 10 min, and then fixed with 100 µL of absolute methanol. Formazan pellet was washed with 70% methanol for three times and dried. Then, formazan was dissolved in KOH (2M) and 140mL dimethylsulfoxide (DMSO). Finally, optical density was calculated at 630 nm using a microplate reader.

To evaluate the antagonistic activity of Lactobacilli, bacteria were cultured in the center of MRS agar plates and were incubated for 48 h at 37° C. Then vibrio bacteria were cultured on the previous plates. After incubation, inhibition areas (between lactobacilli and vibrio) were measured (14).

At the end of trial, the shrimp were challenged with *V. parahaemolyticus* PS-017. The bacteria were grown for 48 h at 37°C in Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) containing 2% NaCl. Bacterial cells were harvested by centrifugation at 3500g for 10 min and washed 3 times. The suspension was diluted to 10^5 up to 10^8 and was added to water. LD50 was calculated using software Probit (1). 40 shrimp from each treatment were transferred to 10 L fiberglass tanks. Shrimp were exposed to *V. parahaemolyticus* PS-017 at 10^7 CFU ml⁻¹ by adding the bacteria to water without water exchanging for 24h. Dead shrimp were collected daily and the cause of mortality was confirmed by reisolating the bacteria of the shrimp haemolymph on TCBS. The challenge test was continued for 10 days.

Intestines of 10 shrimp collected from each treatment were dissected and homogenized liquid was serially diluted in sterile saline (from 10^{-2} to 10^{-5}) and then, spread

on plates of MRS agar and Tryptic Soy Agar (TSA) to determine lactic acid bacteria and total bacteria counts in triplicate after incubation at 30°C for 48h. Bacterial counts (BC) were calculated by the following formula:

BC (CFU per shrimp) = a number of bacterial colonies on plate × dilute multiple × volumes of the homogenized liquid/number of shrimp.

All statistics were performed using SPSS software version 21. Differences among the means were tested for statistical significance using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Since LABC was considered 0 in control group, T-test was used to count lactic acid bacteria. A significance level of P<0.05 was considered in the present study.

Results

The isolated LAB was gram-positive bacilli, non-motile, oxidase and catalase negative that produced acid from maltose, raffinose, ribose, and fructose, whereas they did not utilize rhamnose and xylose. They grew on MRS agar at 15°C and did not produce acid from cellobiose, galactose, lactose, melezitose, melibiose whereas they didn't grow at 45°C. Based on morphological and biochemical tests and PCR analysis of LAB rRNA genes, they were identified as *L. bulgaricus*. *L. bulgaricus* was examined for probiotic activity against pathogenic *V. parahaemolyticus* that revealed they had high inhibitory effects against *V. parahaemolyticus*. The inhibition zone of *L. bulgaricus* was 32.5 ± 3.5 mm and there was a significant difference observed among *L. bulgaricus* and the other LAB was obtained from the digestive tracts of *L. vannamei*.

Survival rates of shrimp were measured for 30 days before and 10 days after exposure to *V. parahaemolyticus* (Table 1). Before the beginning of the trial, mortality was low and survival rate had no significant differences (p>0.05) among groups. After probiotic feeding period, findings showed significant differences in terms of survival rates between the probiotic received shrimp and those in control group (p<0.05).

Table 1. The survival rates of *L. vannamei* reared with and without *L. bulgaricus* before and after treatment and after challenge by *V. parahaemolyticus*.

Treatment	Before probiotic feeding	After probiotic feeding	After challenge test
Control	98.20± 0.54 ^a	82.10± 1.90 ^a	33.34±3.20 ^a
T1	98.50± 0.40 ^a	92.40± 1.50 ^b	53.34± 4.10 ^b
T2	97.10± 0.62 ^a	93.20± 1.60 ^b	60.00± 3.20 ^c

Data in the same column having different letters are significantly different (P<0.05).

After the challenge test, there was also a significant difference between shrimp fed with *L. bulgaricus* supplementation and control, so that the probiotic treatments showed the higher survival rates than control at the end of challenge period (p<0.05).

Profile of hemocyte count was presented in Figure 1. The total hemocyte count (THC) showed no differences before feeding experiment among treatments (p>0.05). However, the THC increased after probiotic feeding period, especially in treatment T 2, although there were no significant differences between probiotic treatments. 3 days after challenge THC decreased in shrimps of control group, however the probiotic treatments showed less decline compared to control group and had no significant differences between them (p>0.05).

Figure 2 shows the respiratory burst activity (RB) of white shrimp during the experiment. RB activity showed no significant differences among treatments before probiotic supplementation. After 30 days of feeding with probiotics, RB activity increased which T2 group showed the highest RB activity compared to other experimental groups. After challenge with vibrio, RB activity in probiotic received shrimp had no significant differences with control. Furthermore, RB activity in control was less than the probiotic treatments (P<0.05).

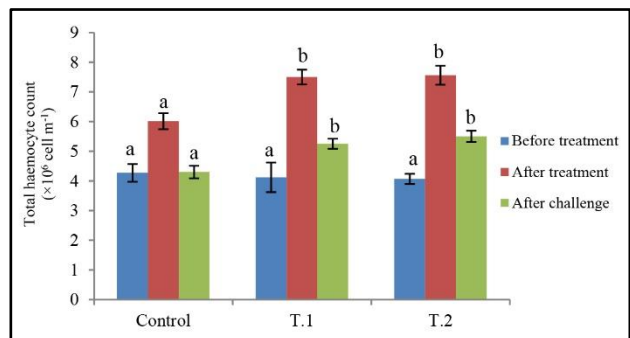


Figure 1. Total haemocyte count (×10⁶ cell m⁻¹) of *L. vannamei* reared with and without *L. bulgaricus* before and after treatment and after challenge by *V. parahaemolyticus*. Error bars represent mean± SE. Dissimilar superscripts are significantly different (P<0.05).

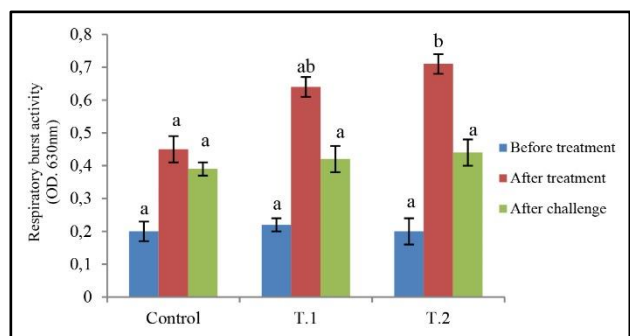


Figure 2. Respiratory burst activity (OD. 630nm) reared with and without *L. bulgaricus* before and after treatment and after challenge by *V. parahaemolyticus*. Error bars represent mean± SE. Dissimilar superscripts are significantly different (P<0.05).

Table 2. Total Bacterial Count (TBC) and Lactic Acid Bacteria Count (LABC) in gastrointestinal tract of *L. vannamei* reared with and without *L. bulgaricus* before and after treatment and after challenge by *V. parahaemolyticus*.

	Treatment	TBC (10 ⁶ CFU/shrimp)	LABC (10 ⁴ CFU/shrimp)	Ratio (%) (LAB /TBC)
Before probiotic feeding	Control	2.62± 0.38 ^a	ND	ND
	T1	2.49± 0.62 ^a	ND	ND
	T2	2.55± 0.30 ^a	ND	ND
After probiotic feeding	Control	7.93± 0.12 ^b		
	T1	7.12± 0.41 ^a	8.90±0.10 ^b	1.25
	T2	7.30±0.09 ^a	9.20±0.10 ^b	1.26
After challenge test	Control	9.10±0.71 ^b		
	T1	8.04±0.59 ^a	5.79±0.20 ^b	0.72
	T2	8.51±0.27 ^a	5.93±0.10 ^b	0.69

Data in the same column having different letters are significantly different (P<0.05).

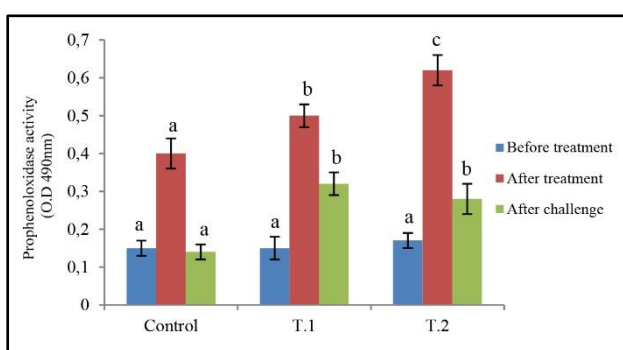


Figure 3. Pro phenoloxidase (proPO) activity (O.D. 490nm) of *L. vannamei* reared with and without *L. bulgaricus* before and after treatment and after challenge by *V. parahaemolyticus*. Error bars represent mean± SE. Dissimilar superscripts are significantly different (P<0.05).

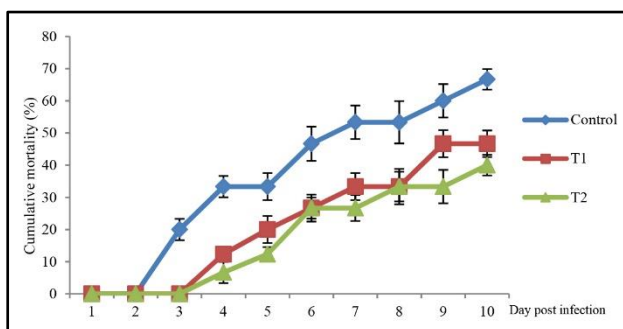


Figure 4. Accumulated mortalities of *L. vannamei* supplemented with or without probiotic infected with 10⁷ CFU ml⁻¹ with pathogenic *V. parahaemolyticus*. Different superscript letter at each interval indicated significant difference (P<0.05).

The proPO activities are presented in Figure 3 that showed no differences among all treatments before supplementation with probiotic. However, after 30 days feeding with diet supplemented probiotic, proPO increased significantly. At the end of the treatment, the highest values of proPO activity was found in T2

compared to other treatments and then after *Vibrio* challenge, proPO activity in T2 and T1 treatments were higher than in control (P<0.05).

Table 2 shows the total bacterial count (TBC) and lactic acid bacteria count (LABC) in the digestive tract of white shrimp. TBC showed no differences before supplementation with probiotic among treatments. At the end of probiotic feeding experiment (day 30), TBC in control group was higher compared to T1 and T2. After vibrio challenge test, the highest TBC was observed in control group, whereas there were no significant differences between probiotic receiving shrimp. After supplementation of shrimp with probiotic, LABC values showed no significant differences between treatments. Also, LABC was not observed in control group. After the challenge test, the amount of LABC was decreased in probiotic receiving groups. As well as, the LABC rate in control group was found to be 0.

In this study, lactobacilli had antagonistic effects against pathogens (*V. parahaemolyticus*). Mortality was observed 3 days after infection with *V. parahaemolyticus* PS-017. Vibriosis was observed with some symptoms in shrimp such as reduction in movement, decreased feed intake, expansion of chromatophores especially on the walking and swimming legs, the corrosion of tail and the reddish coloration on the appendages. The accumulated mortality of infected shrimp in probiotic receiving treatments was 40% (T1) to 46% (T2); whereas mortality was 66% in control. The survival rates in probiotic receiving treatments were significantly different from the control at the end of 10 days challenge with *Vibrio* (Figure 4).

Discussion and Conclusion

Several methods have been used in aquaculture to ameliorate the health condition of farmed animals such as reproducing specific disease-resistant shrimp (5), specific pathogen-free shrimp (24) and the use of probiotics (8, 27,

44). Probiotics can enhance the survival, immunity and disease resistance of aquatic animals (8, 27). Probiotic acts through several mechanisms including production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites and enhancement of the immune response (20). In the present study *L. bulgaricus* was isolated from the digestive tract of *L. vannamei* and characterized by morphological, biochemical and molecular analysis which showed inhibitory effects in vitro against *V. parahaemolyticus*. Our study demonstrated that *L. bulgaricus* can enhance resistance to vibriosis through activating immune defenses, as well as probably providing competitive exclusion in the digestive tract of shrimp.

Probiotics can elevate the survival rate by producing some compounds such as bacteriocins, lysozyme, and proteases, prevention of colonization of harmful bacteria in the gut, competition for consumption of nutrients and promote the immune system by stimulating the non-specific immune system (32). Data from this study showed that probiotics have enhancing effects on shrimp survival, confirming the results of previous studies (19, 42, 44).

Shrimp relies more on non-specific immunity for the resistance to infections (29). Hemocytes are responsible for humoral and cellular defense against pathogens and they are used as an indicator to recognize health condition of shrimp in relation to infections and changes in environmental conditions (29). The results showed the dietary probiotic (*L. bulgaricus*) effects on immune system by increasing THC, as a reaction of shrimp immune system to a strange stimulator i.e. probiotic. In this regard, the highest and the lowest THC were observed in the shrimp fed with probiotic and probiotic free diet, respectively. Increase in total hemocytes was also found in *L. vannamei* fed with *Arthrobacter* XE-7 diet (16). A study presented by Chiu et al. (7) showed that the probiotics were capable to increase the THC value as well as enhancing the immune response during the period of challenge with pathogen infection. In our study, the elevations in hemocytes after challenge with *V. parahaemolyticus* are probably a defense as reported by Anderson and Siwicki (4).

Respiratory burst is a sequence of activities that lead to phagocytosis of foreign particles and thus production of reactive oxygen intermediates or oxygen radicals as the final product (29). In the present study, elevation of RB activity in probiotic receiving shrimp could demonstrate the immune stimulatory function of *L. bulgaricus* E20 as reported previously by Febrianti et al. (9), and Li et al. (16). However, after challenge with *V. parahaemolyticus*, RB activity decreased sharply in all treatments. Improvements in RB after treatment with probiotics such as *V. vulnificus* (37), *L. rhamnosus*, *Enterococcus faecium* and *B. subtilis* (21, 23) were also reported.

The proPO system is the most important component of crustacean immune system. Active phenoloxidase (PO) oxidizes phenols to quinones that leads to formation of melanine, which it can trap and barricade pathogens (3). The proPO activity after supplementation of probiotic showed an increasing tendency as reported previously by Li et al. (18) and Nurhayati et al. (22). T2 treatment (10^9 cfu g⁻¹) showed higher increase of PO value after supplementation with probiotic and then declined after infection, indicating an enhancement in immunity of shrimp. This suggests that *L. bulgaricus* possibly enhances the shrimp humoral immune responses through modification of the prophenoloxidase system. Perhaps, the supplementation of probiotics have raised the β -glucan-binding protein amount in the intestine, as it was reported by Hao et al. (11). Some studies have reported changes in the PO enzyme activity in shrimp treated with probiotics, for example in *L. vannamei* (39) and *P. monodon* (27).

Probiotic dietary increased the population of bacteria in shrimp digestive tract. Similar results were reported by Li et al. (15). In the present study LAB constitutes a small portion of the intestinal bacteria (less than 1.5 percent), although they could show its beneficial effects. This suggests that *L. bulgaricus* can adhere on shrimp intestine. The existence of beneficial intestinal bacteria suppresses potentially pathogenic bacteria (28). These findings amplify that *L. bulgaricus* dietary could restrain the growth of *V. parahaemolyticus* in shrimp intestine. In the present study, probiotic cells (LABC) were recognized at the end of experiment and after challenge with vibrio in shrimp. This indicates that probiotics have been able to settle and survive in the digestive tract of shrimp. Total bacteria declined after supplementation with probiotics, so that it reached to half of its amount before challenge. The alternation of microbial community of gut by probiotics may lead to immune responses (15).

After the 30-day supplementation with probiotics, shrimp were challenged with pathogenic bacterium *V. parahaemolyticus*. A higher resistance was found in shrimp fed with diets containing *L. bulgaricus*, especially in T2. The results showed that dietary supplementation of probiotic could improve the immune responses of white shrimp against vibriosis. THC declined after challenge in all probiotic treatments, so that there were no differences between probiotic supplemented groups and control that is consistent with the result of Li et al. (17). Hemocyte cell reduction could be a defense mechanism, probably due to the migration of hemocytes from circulation system to target tissues where many cells are infected (43). Reduction in mortality rates of probiotic supplemented shrimp after challenge may be related to the colonization of probiotics in the digestive tract, generation of various inhibitory substances such as bacteriocins, lysozymes and proteases and reduction of pH by secretion of organic

acids and some substances which had negative effects on *V. parahaemolyticus* (31).

In conclusion the probiotic, *L. bulgaricus*, provided both cellular and humoral immune defense responses in *L. vannamei* in terms of enhancing hemocyte counts, PO activity, and RB activity and rendered shrimp more resistant to *V. parahaemolyticus*. The best results were obtained in 10^9 CFU g⁻¹ dietary probiotic.

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