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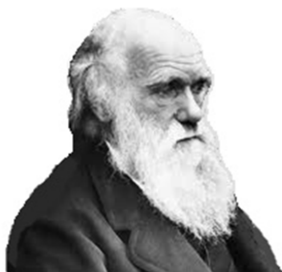

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Immune response of shrimp *Peneaus monodon* against *Vibrio parahaemolyticus*

Rajikkannu Muthaiyan[1], **S. Krishnan**[1], **Selvakumar Periyasamy**[2], **Zhievino Chetri**[3] and **R. Nandakumar**[3,*]

[1]Department of Zoology, Periyar E.V.R. College, Tiruchirappalli – 620 023, Tamil Nadu, India

[2]Center of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai - 608 502, Tamil Nadu, India

[3]Department of Zoology, St. Joseph University, Virgin Town, Ikishe, Model Village, Dimapur – 797 115 Nagaland, India

*E-mail address: drnkzoo@gmail.com

ABSTRACT

The study was carried out on impact of vibriosis by using *Vibrio parahaemolyticus* in *Peneaus monodon* Fabricius, 1798, collected from Nagapattinam. The impact was studied on heamatological and biochemical parameters. Feeds were prepared by coating probiotic strains of *B. firmus* and *B. coagulans*. Laboratory investigations were carried out concerning impact of pathogenic *Vibrio parahaemolyticus* of total count, differential count, Nitroblue tetrazolium activity, Prophenoloxidase activity and bacterial clearance in *P. monodon*. Total heamocyte was enumerated in vibriosis by induced shrimps, all results showing an increase in total haemocyte count after the time of infection. After feeding, the total heamocyte count was 1442 cells/cu·mm in control animals, in *B. coagulans* supplemented animals 2757 cells/cu.mm and in *B. firmus* was 2448 cell/cu·mm. After feed supplementation the shrimps were infected with *V. parahaemolyticus* and their impact on total haemocyte count was assessed in all three groups. In control, the total haemocyte count was increased with 2560 cells/cu·mm, whereas in *B. coagulans*is 5126 cells/cu·mm, and in *B. firmus*is 4697cells/cu·mm. Two-way Analysis of Variance for total haemocyte count in normal and infected haemolymph showed a significant variation. The control samples recorded the lowest, and after infection *B. coagulans* supplemented animals recorded maximum counts among three groups. Differential count was evaluated by studying the three types of cells. There was not much variation in the percentage of cells, but a slight decrease was observed in the hyaline cells after 24 hours of infection. The NBT activity was 1.4 (NBT activity) in control animals, 2.7 in *B. coagulans* and 2.4 in *B. firmus* supplemented animals. Maximum reduction was observed in control

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animals with 0.92, 1.7 in *B. coagulans* and 1.4 in *B. firmus* supplemented animals. There was an initial spurt of vibrios when cultured in selective medium TCBS in all three treatments and gradual decrease in the vibrios count during 24 hours observation.

Keywords: *Penaeus monodon*, *V. parahaemolyticus*, NBT activity and Pathogenesis

1. INTRODUCTION

Cellular defenses in crustaceans rely on haemocytes with several functions, such as coagulation, phagocytosis and encapsulation (1,2). Certain types of haemocytes have a molecular mechanism associating several proteins: the prophenoloxidase system (pro-po-system) (3).

In invertebrates, including crustaceans, haemocytes are known to participate in different types of cellular immune reactions, such as phagocytosis, encapsulation and intra, as well as extra cellular cytotoxicity against infectious agents. Phagocytosis, which represents the primary defense mechanism against invasion by pathogenic organisms, occurs due to the ability of the haemocytes to ingest and destroy microorganisms. Incidents of activation of the haemocyte cell membrane during phagocytosis has been reported (4-6).

Peneaid shrimp`s immunology has recently become a priority because of the dramatically increasing impact of infectious-diseases on the sustainability and economic durability of shrimp aquaculture. Crustacean immune responses are based on both, cellular and humoral components, which cooperate to eliminate potentially infectious microorganisms. The first immune process is the recognition of microorganisms, and it is mediated by the plasma and haemocytes (7). In invertebrates, haemocyte oxidative microbicidal system has been first demonstrated in gastropods (8,9). Then it was shown in several marine bivalves (10,11), with a special attention to the interaction of the oxidative metabolism with the specific intracellular parasites. Adaptations for avoiding or bypassing the haemocytic oxidative microbicidal system have been demonstrated in several pathogens, using in vitro host –pathogen models with isolated specific pathogens (12).

The bacteria, *Vibrio alginolyticus*, *Vibrio harveyi* and *Photobacterium damselae* subsp. *damselae* (also known as *Vibrio damsela*) are considered to be secondary and opportunistic pathogens, and have been demonstrated to cause disease outbreaks of vibriosis associated with poor environmental conditions. These diseases have also been reported to be associated with increases of Vibrio population in the culture pond waters. Decapod crustaceans have three types of circulating haemocytes: hyaline cell (HC), semi-granular cell (SGC), and granular cell (GC).

They are involved not only in phagocytosis, an important process in eliminating microorganisms or foreign particles, but also in the production of melanin via the prophenoloxidase (proPO) system which is an important component of the cellular defense reaction (7). Phenoloxidase is the terminal enzyme in the proPO system and is activated by cell polysaccharides like a-1, 3-glucan, lipopolysaccharide or peptidoglycan from microorganisms through pattern recognition proteins (13).

In the peneaid shrimps, *Penaeus monodon*, (14) have used two different methods for the demonstration of oxygen metabolism, leading to different results. The authors were able to spectrophotometrically detect the generation of oxygen metabolites, based on nitro blue tetrazolium (NBT) reduction. However, using a chemiluminescence (CL) method, the same

elicitors used, failed to elicit haemocyte activation. CL activity has been successfully demonstrated in *P. japonicus* haemocytes upon PMA or Zymosan stimulation [11].

These different results probably underline the need of method standardization in research of crustacean haemocyte functioning in vitro.

Prophenoloxidase (proPO) from hemocytes is stimulated into phenoloxidase through limited proteolysis by a specific serine type protease, which is itself a latent enzyme, and is controlled by a cascade. This chain reaction is induced by a minute amount of cell wall components of microbes, so that invaders are killed by highly reactive quinones produced by activated phenoloxidase. The components involved in this activation are called the 'proPO System' of which the final reaction is the removal of the propeptide of prophenoloxidase, although all of the reactions and components involved in this have not yet been clarified. The endogenous conversion of hemocyanin into a phenoloxidase like enzyme, however, has not been investigated at all in crustaceans [7].

Disease outbreaks are recognized as a significant constraint to aquaculture production and trade, affecting both the economic development and socioeconomic revenue of the sector in many countries including India. The total potential area shrimp farming in the country is estimated to be 1.2 million ha. Prawn culture industry has encountered various disease problems including serious vibriosis [15]. Other works with a fin fish have shown that various Vibrio spp. produce toxins with haemocytic activity [16] which may be important as virulence factors.

Crustaceans have an innate immune system, characterized by lack of immunoglobulin and memory, but are efficient enough to protect and preserve themselves from all intruding pathogens. Crustacean hemocytes play a central role in the host immune response, performing functions, such as phagocytosis, encapsulation, nodule formation and mediation of cytotoxicity. In decapod crustaceans, based on the presence of cytoplasmic granules and relative size of granules, crustacean hemocytes are usually divided into three main types, hyaline (agranular), semigranular (small granular) and granular (large granular) hemocytes [17]. They are involved not only in phagocytosis, an important process of eliminating microorganisms or foreign particles, but also in the production of melanin by the prophenoloxidase (proPO) system. Prophenoloxidase system prevalent in crustacea makes them capable of resisting every possible foreign particle entering their body by promoting cell-to-cell communication and subsequently eliminating them. Phenoloxidase (PO) is the terminal enzyme in the proPO system and acts as both, recognition and effectors component of the arthropod defense system since it can be specifically activated by sugars of constitutive polysaccharides from the fungal or bacterial cell walls.

Circulating haemocytes in crustaceans also play an important physiological roles including hardening of the exoskeleton; wound repair; carbohydrate metabolism; lipoprotein or amino-acid transport and storage; prevention of blood loss (haemolymph coagulation). Disease fighting properties of heamocytes include confinement of invasive organisms by a clot formation, phagocytosis, and encapsulation [18]. It is therefore, important to extend knowledge on the interaction between crustaceans with pathogens, and their products.

In the present study a novel haemocytic assay using haemolymph of tiger prawn (*Penaeus monodon*) is dealt with an infected bacteria. As infected bacteria can be isolated from haemolymph of invertebrates [19] the haemocytolytic assay described here should be useful for furthering pathogenesis studies in invertebrates.

The present study aims were to clarify the relationship between the host and pathogen by describing the changes in total and differential haemocyte counts and immunomodulation by

probiotic supplemented animal. Median lethal dose for the pathogen *Vibrio parahaemolyticus* and the shift in the immune parameters in relation to Vibriotoxicity have also been analysed.

2. METHODS AND METERIALS

The experiment was carried out to assess the haematological and biochemical changes by the effect of *Vibrio parahaemolyticus* on *Penaeus monodon*, and bacterial clearance after challenging with *Vibrio parahaemolyticus*, in probiotic feed supplemented animal.

2. 1. Study animals

The juvenile shrimps were weighed accurately in digital electronic balance before the start of the experiment. Post larvae (Pl-20) of *Penaeus monodon* was collected from a private farm in Nagapattinam district, Tamil Nadu, India. Animals were introduced into plastic trough the 45 liter capacity, filled with 40 liter of salinity adjusted sea water. In each plastic trough, 25 animals were maintained. Continuous aeration was given by using compressor air pump to maintain dissolved oxygen at a level of more than 5 ppm in each trough. Water exchange was carried out daily at a rate of 25%. The animals were fed with a commercial compounded feed two times a day and acclimatized to continuously aerated sea water under the laboratory conditions in large plastic tubs.

2. 2. Bacterial strains

V. parahaemolyticus were isolated from sea water shrimps. Collected shrimps were surface - disinfected by wiping with 75% alcohol. Hepatopancreas were removed and tissue was homogenized with 20 mL of 0.85% NaCl solution. A series of 10 fold dilutions of each water and hepatopancreas sample were made using sterile saline (0.85% NaCl solution, w/v) as dilution blanks, and 0.1 mL from each dilution was plated on agar plates by a spread plate method. Total heterotrophic aerobic bacterial counts were made using Zobell's agar medium. *Vibrio* spp. were enumerated using thiosulfate-citrate-bilesalt-sucrose (TCBS) agar. For the enumeration of total bacteria and vibrios, the inoculated plates were incubated at 25 °C in the dark for 5 days and 48 h, respectively, and identification of *Vibrio* was carried out by biochemical tests.

2. 2. 1. Compounded feed

For the sustenance of prawn under laboratory conditionthe essential nutrients in adequate composition should be given. A pelleted feed based on the recommendations was compounded in the laboratory.

The composition of the feed is as given below:

- Fish meal -39 g
- Corn flour -15 g
- Groundnut oilcake -20 g
- Tapioca flour -20 g
- Calcium carbonate -0.5 g

- Calcium phosphate -3 g
- Vitamin and mineral mix -2.5 g.

2. 3. Feed additives

2. 3. 1. Selection of probiotics

The probiotic strains were isolated from the gut of *Penaeus monodon* and identified by biochemical test.

2. 3. 2. Probiotic Strains

Putative probiotic strains of *B. firmus* and *B. coagulans* were identified. Pure culture was isolated and mass cultured at 37 ºC for 24 hours in a temperature controlled shaker. Bacterial pellets were harvested every 24 hours and stored in a sterile container.

2. 3. 3. Feed Coating

Selected strains of probiotics *B. firmus* and *B. coagulans* were mass cultured and the concentration of colony forming units were determined by adjusting the culture to OD -1. Feed pellets were warmed to 60 °C and blended with the molten agar containing plant extract. The mixture was stirred well with sterile glass rods to have a uniform coating of the bacteria over the feed pellets. Similarly, the probiotic cells of *B. subtilis* and *B. coagulans* were coated on feed pellets, with molten agar.

2. 3. 4. Feeding

Shrimps were fed twice a day at 3-5% of their body weight. Feeding was done usually at morning and evening. Unutilized feed and excreta of shrimps, settled at the bottom of the tubs were siphoned out every day, prior to water replenishment. Feed rations were adjusted according to daily intake by the shrimps.

2. 4. *Vibrio parahaemolyticus* challenge test

To study the bacterial clearance, the shrimps were infected with *V. parahaemolyticus*. The shrimps were challenged with known sublethal concentration of *V. parahaemolyticus*. The challenge trials were conducted in duplicates.

2. 4. 1. Challenge test

To study the bacterial clearance of prawns, animals were stocked in 45 litre troughs with a stocking density of 8 prawns per trough. All the prawns were injected intramuscularly with an LD_{50} dose of 100 μL *V. parahaemolyticus* in saline adjusted to 1.0 OD animal between 5th and 6th abdominal segments. The infected animals showed signs of red discolouration, erratic swimming, and lethargy and swam near the water surface of trough before death.

2. 4. 2. Bacterial clearance test

Shrimps were acclimatized in the laboratory condition for a period of 15 days. A bacterial suspension of 0.1 mL was injected into tail muscle of each shrimp. Then they were kept in

seawater aquaria equipped with aeration for three hours. Haemolymph was collected from each shrimp without anticoagulant and 30 μL of whole blood was dropped on TCBS agar. A two-fold dilution of the whole blood was made using sterile 2.6% NaCl solution. Haemolymph was withdrawn every three hours and plated for vibrios in TCBS medium. The colonies were enumerated after 48 h incubation.

2. 5. Haemolymph Collection

Hemolymph was withdrawn from the pericardial sinus of the prawn, by a 20 G needle and diluted fivefold with haemolymph anticoagulant media (HAM, pH 7.8) to prevent coagulation and aggregation of hemocytes.

2. 5. 1. Total haemocyte count

Total haemocyte count was made using an improved Neubauer hemocytometer, following the method as described for counting WBC.

2. 5. 2. Differential haemocyte count

Fixed haemolymph was smeared on a slide and stained with Giemsa solution (10%) for 10 minutes. The differential haemocytes were then characterized according to [(17)], and 200-300 cells from each smear were counted under light microscope at 1,000×.

2. 6. Biochemical analysis

Haemolymph was used for the estimation of prophenol oxidase, nitrobue tetrazolium analysis.

2. 6. 1. Quantitative assay of Nitroblue tetrazolium

0.1 mL of freshly drawn haemolymph was immediately mixed with anticoagulant solution and then centrifuged at 300 g for 10 minutes. After removing the supernatant, precipitate was washed with MCBHSS for three times, then stained with Nitro blue tetrazolium (NBT) (0.3%, 100 μL) for 30 minutes at 37 ºC. The staining reaction was terminated by removing the NBT solution and adding absolute methanol. After three washing with 70% methanol, the haemocytes were air-dried and coated with a solution of KOH (120 μL, 2 M) and DMSO (140 μL) to dissolve the cytoplasmic formazan; the optical densities of the dissolved cytoplasmic formazan at 630 nm (OD_{630}) were then measured with a spectrophotometer [(14)].

2. 6. 2. Determination of prophenol oxidase activity

Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxy phenylalanine (L-DOPA, Hi Media, Mumbai). The diluted haemolymph was centrifuged at 300 g at 4 °C for 10 min. The supernatant fluid was discarded and the pellet was rinsed and re-suspended gently in 1 mL cacodylate-citrate buffer (sodium cacodylate 0.01 M, sodium chloride 0.45 M, trisodium citrate 0.10 M, pH 7.0), and then centrifuged again. The pellet was then re-suspended in 200 μL cacodylate buffer. One hundred mL of the cell suspension was incubated with 50 mL of trypsin (1 mg mL^{-1}), which served as an elicitor, for 10 min at 25 °C. To this 50 mL of L-DOPA (3 mg mL^{-1}) was added,

followed by 800 µL of cacodylate buffer added 5 min later. The optical density was measured at 490 nm using a spectrophotometer. The control solution, which consisted of 100 mL of cell suspension, 50 mL cacodylate buffer (to replace the trypsin) and 50 µL of L-DOPA, was used for the background phenoloxidase activity in all test conditions. The background phenoloxidase activity optical density values were in the range of 0.03 to 0.09. The phenoloxidase activity optical density was expressed as dopachrome formation per 50 mL haemolymph.

2. 7. Statistical Analysis

For all the animals under study, the mean value of glycogen and protein content in each tissue, both in control and experimental fish, was estimated out and standard deviation was calculated by the following formula:

$$\sigma = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

Where:

$\sum x$ = sum of variables
$\sum x^2$ = sum of the squares of variables
n = number of observations.

Standard Error was calculated from the following formula:

$$SE = \frac{SD}{\sqrt{n}}$$

Where:

SD = Standard deviation of the sample
n = Sample size.

Two-way ANOVA of the results and post-hoc (SNK) test were carried out using a statistical package (SPSS version.20).

3. RESULTS

3. 1. Blood Cell count

3. 1. 1. Total Haemocyte Count

Total number of haemocytes present in test shrimps challenged with *Vibrio parahaemolyticus* with median lethal dose was found to be higher after feed supplementation and after infection increasing trend was observed in the haemocyte count. Total heamocyte count in after 30 days of probiotic supplementation was in control animals 1442 cells /cu·mm,

in *B. coagulans* supplemented animals 2757 cells /cu·mm and in *B. firmus* supplemented animals 2448 cell/cu·mm (**Table 1**).

After feed supplementation the prawns were infected with *V. parahaemolyticus* and its impact on total haemocyte count was assessed in all three groups. It showed an increasing trend in all three groups. The increase was slightly higher than the control in probiotic supplemented group, this may be due to disease combating ability and immunomodulatory effect of probiotic supplemented in the feed. In control the increase in the total haemocyte count was with 2560 cells/cu·mm, whereas in *B. coagulans* supplemented animals it was 5126 cells/cu·mm and in *B. firmus* supplemented animal's 4697 cells/cu·mm.

Table 1. Total haemocyte count in normal and *V. parahaemolyticus* challenged *P. monodon*, supplemented with live probiotic *B. coagulans*and *B. firmus*in (mean values ± Standard deviation)

S. No	Treatment	Control	*B. coagulans*	*B. firmus*
1	Normal	1442 ±114	2757 ±214	2448 ±233
2	Infected	2560 ±103	5126 ±237	4697 ±52

Two-way Analysis of Variance for total haemocyte count in normal haemolyph and diseased haemolymph showed a significant variation. Haemocyte count increased with after infection, whereas in different groups of animals a significant result was shown at 5% level interval. In the haemolymph of vibriosis induced animals, a marked variation was observed between treatments of animals. There was a remarkable variation between each treatment and total haemocyte count. The control samples recorded the lowest and *B. coagulans* supplemented animals after infection recorded maximum counts among three groups (**Table 2a** and **b**).

Table 2(a). F-test for Total haemocyte count in haemolymph of normal and *V. parahaemolyticus* challenged *Penaeus monodon*, supplemented with live probiotic *B. coagulans* and *B. firmus*

Source	Type III sum of squares	df	Mean squared	F
Corrected Model	30620944.444	5	6124188.889	200.944
Intercept	181121200.222	1	181121200.222	5942.860
Feed	12741825.444	2	6370912.722	209.039**
Treatment	16450848.000	1	16450848.000	539.777**

Feed * Treatment	1428271.000	2	714135.500	23.432**
Error	365725.333	12	30477.111	
Total	212107870.000	18		
Corrected Total	30986669.778	17		

a R Squared = .988 (Adjusted R Squared = .983)
** - P < 0.01

Table 2(b). SNK Test for total haemocyte countvariation in haemolymph
(Total haemocyte count against different feed supplementation)

	N	**Subset**		
Supplemented feeds		1	2	3
Control feed	6	2001.5000		
B. firmus supplemented	6		3572.8333	
B. coagulans supplemented	6			3942.0000
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares.
The error term is Mean Square (Error) = 30477.111.
a Uses Harmonic Mean Sample Size = 6.000.
b Alpha = .05.

3. 1. 2. Differential Count

Table 3. Differential count in normal and *V. parahaemolyticus* challenged *P. monodon*, supplemented with live probiotic *B. coagulans* and *B. firmus*

S. No	**Treatment**	**Control%**			***B. coagulans*%**			***B. firmus* %**		
		HG	**LG**	**SG**	**HG**	**LG**	**SG**	**HG**	**LG**	**SG**
1	Normal	70	21	9	75	15	10	70	18	12
2	Infected	52	35	13	67	12	21	66	31	3

HG-Hyaline cells

LG- Large granular cells
SG- Small granular cells

Differential count was enumerated by studying three different types of large granular cells, small granular cells and hyaline cells. Among all treatments hyaline cells recorded maximum count followed by large granular cells and small granular cells. There is a slight variation in the number of hyaline cells with the increase after infection (**Table 3**).

3. 2. Nitroblue tetrazolium activity (NBT)

The nitroblue tetrazolium activity was estimated in prawns from all the three feed groups. There was a significant variation in the NBT activity in all three groups; uniformly they recorded gain in the activity. The NBT activity was 1.4 (NBT activity Units/min/mg of protein) in control animals, 2.7 in *B. coagulans* supplemented animals, and 2.4 in *B. firmus* supplemented animals (**Table 4**).

Table 4. Nitroblue tetrazolium activity in normal and *V. parahaemolyticus* challenged *Penaeus monodon*, supplemented with live probiotic *B. coagulans*and *B. firmus*in (mean values ± Standard deviation) (NBTactivity Units/min/mg of protein)

S. No	Treatment	Control	*B. coagulans*	*B. firmus*
1	Normal	1.4 ±0.1	2.7 ±0.2	2.4 ±0.2
2	Infected	0.92 ±0.1	1.7 ±0.1	1.4 ±0.1

Similar to that of differential counting, there was a gradual decrease in NBT activity after infection. A maximum reduction was observed in control animals with 0.92, 1.7 in *B. coagulans* supplemented animals, and 1.4 in *B. firmus* supplemented animals. Thus, the probiotic supplementation has diseased the combating ability. Two way analysis of variance showed a significant variation among the feed supplementation and treatment. Student Newman's Keuls post hoc analysis revealed that *B. coagulans* supplemented group recorded maximum NBT activity followed by *B. firmus* and control groups (**Table 5a** and **b**).

Table 5(a). F-test for Nitroblue tetrazolium activity inhaemolymph ofnormal and *V. parahaemolyticus* challenged *Penaeus monodon*, supplemented with live probiotic B. coagulansand B. firmus

Source	Type III sum of squares	df	Mean squared	F
Corrected Model	7.301	5	1.460	58.349

Intercept	55.658	1	55.658	2224.127
Feed	3.525	2	1.763	70.439**
Treatment	3.430	1	3.430	137.082**
Feed * Treatment	.345	2	.172	6.892**
Error	.300	12	2.502E-02	
Total	63.259	18		
Corrected Total	7.601	17		

a R Squared = .960 (Adjusted R Squared = .944)
** - P < 0.01

Table 5b. SNK Test for Nitroblue tetrazolium acitivity inhaemolymph (NBT activity against different feed supplementation)

Supplemented feeds	**N**	**Subset**		
		1	2	3
Control feed	6	1.1610		
B. firmus supplemented	6		1.8957	
B. coagulans supplemented	6			2.2187
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares. The error term is Mean Square (Error) = 2.502E-02.
a Uses Harmonic Mean Sample Size = 6.000.
b Alpha = .05.

3. 3. Prophenoloxidase activity

The prophenol oxidase activity was estimated in prawns from all the three feed groups. There was a significant variation in the prophenol oxidase activity in all three groups, uniformly they recorded gain in the activity. The prophenol oxidase activity was 1.2 (prophenol oxidase activity Units/min/mg of protein) in control animals, 1.5 in *B. coagulans* supplemented animals, and 1.4 in *B. firmus* supplemented animals (**Table 6**).

Similar to that of differential counting, also there was a gradual decrease in prophenol oxidase activity after infection. A maximum reduction was observed in control animals with 0.74, 1.9 in *B. coagulans* supplemented animals, and 1.3 in *B. firmus* supplemented animals. Thus, the probiotic supplementation has diseased the combating ability.

Two way analysis of variance showed a significant variation among the feed supplementation and treatment. Student Newman's Keuls post hoc analysis revealed that *B.*

coagulans supplemented group recorded maximum prophenol oxidase activity followed by *B. firmus* and control groups (**Table 7a** and **b**).

Table 6. Prophenol oxidase activity in normal and *V. parahaemolyticus* challenged *Penaeus monodon*, supplemented with live probiotic *B. coagulans*and *B. firmus*in (mean values ± Standard deviation) (Phenoloxidase activity Units/min/mg of protein)

S. No	Treatment	Control	*B. coagulans*	*B. firmus*
1	Normal	1.2 ±0.1	1.5 ±0.1	1.4 ±0.1
2	Infected	0.74 ±0.2	1.9 ±0.1	1.3 ±0.1

Table 7(a). F-test for Prophenol oxidase activity inhaemolymph ofnormal and *V. parahaemolyticus* challenged *Penaeus monodon*, supplemented with live probiotic *B. coagulans* and *B. firmus*

Source	Type III sum of squares	df	Mean squared	F
Corrected Model	2.065	5	.413	28.245
Intercept	32.214	1	32.214	2203.064
Feed	1.586	2	.793	54.243**
Treatment	4.109E-02	1	4.109E-02	2.810**
Feed * Treatment	.438	2	.219	14.965**
Error	.175	12	1.462E-02	
Total	34.454	18		
Corrected Total	2.241	17		

a R Squared = .922 (Adjusted R Squared = .899)
** - P < 0.01

Table 7(b). SNK Test for Prophenol oxidase activity inhaemolymph (Prophenol oxidase activity against different feed supplementation)

Supplemented feeds	N	Subset		
		1	2	3
Control feed	6	.9667		

B. firmus supplemented	6		1.3533	
B. coagulans supplemented	6			1.6933
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares.
The error term is Mean Square (Error) = 1.462E-02.
a Uses Harmonic Mean Sample Size = 6.000.
b Alpha = .05.

3. 4. Bacterial Clearance

Clearance of *Vibrio* cells from the haemolymph of tiger shrimps *Penaeus monodon* was studied by calculating the total colonies in TCBS agar. Haemolymph was withdrawn after five minutes, ten minutes, two hours, and 12 hours of infection and pour plate was prepared using TCBS. The number of viable colonies was high in the five, ten minutes, two hours, and decreased after 12 hours (**Table 8**).

The same pattern was observed in shrimps from all the three treatments. However, the bacterial clearance could not be studied further due to the practical constraints.

Table. 8. *Vibrio parahaemolyticus* clearance after injection

S. No	Time after infection	Control	*B. coagulans*	*B. firmus*
1	Five minutes	1072	1372	1248
2	Ten minutes	736	988	1144
3	2 hours	768	1104	928
4	12 hours	600	872	-

4. DISCUSSION

The haemocytes in peneaid shrimp were divided into hyaline cells, semi-granular cells and granular cells. It is also otherwise classified into large granular, small granular, and agranular cells. Invertebrates, including crustaceans, appear to rely heavily on phagocytic blood cells as a mean of defense against infection agent's infection agents. Phagocytosis is a multiphase, cellaular process, comprising chemotaxis, attachments, ingestion, and killing. Invertebrates possess molecules belonging to the immunoglobulin super family (e.g hemolin, lachesim) they lack specific antibodies and true lymphocyte-like sub-population have yet to be demonstrated incontrovertibly. Despite of this, their immune defense is extremely efficient and, like vertebrates, is mediated by the complex cellular and humoral interactions. The haemocytes, particularly phagocytic and haemostatic cell types, are the most important in the immune functions of invertebrates. It is well known that the life cycle, food intake, disease

outbreak, pollutants, and environmental stresses affect both, the quantity and quality of circulating hemocyte counts of crustaceans [20-23]. Circulating hemocytes are also affected by extrinsic factors, such as temperature, pH, salinity, and dissolved oxygen, which have been reported to affect THCs in several species of decapod crustaceans. It has been reported that 1 ppm of copper salts did not suppress or stimulate haemocyte production at the same concentration [24] A. Significant reduction in the number of haemocytes was observed in the bivalve, *Villorita cyprinoid* exposed to Cu [25]. The total haemocytes population was found to be elevated in test prawn exposed to mercury. This is due to activation of defence mechanism to neutralize the toxic substances, Hg and Cu.

Viral and bacterial materials have been detected in the lymphoid organ of shrimp [26-28]. Therefore it was suggested that the lymphoid organ in the penaeid shrimp is responsible for the removal of foreign material from the haemolymph, before it goes from the arterial system into the open circulatory system. The sudden expansion of the humeral lumen could provide multiple contacts between large amounts of haemolymph and the cells in the tubule walls [29]. In this way, the lymphoid organ creates an effective trap by rapidly immobilizing foreign material from the haemolymph. Several factors like salinity and rise in temperature demonstrated to change the THC in *Crassostreavirginica* in nature. An increase in haemocyte count was also observed, following experimental infection in two bivalve species.

Most crustaceans, like crab, lobster and crayfish, do not possess a lymphoid organ, and fixed phagocytes in the outer wall of haemolymph vessels ion the digestive gland (hepatopancreas) are involved in the uptake of foreign material [30,31]. The lymphoid organ in penaeid shrimp was first described by [32], followed by [33], and [34]. Although the later authors attributed a haematopoietic function to the lymphoid organ, the limited number of mitotic figures do not support this supposition [35]. Since many of the settled phagocytes are destroyed during the course of some diseases [36], our findings also argue for replacement of the phagocytes by circulating haemocytes in the lymphoid organ, where possibly a haemocyte maturation occurs.

Bacterial clearance from circulation in the present study, a lower THC was observed in the bacterial-compared to PBS-injected group. THC in crustaceans rapidly drops following injection of foreign material, while THC often increases after PBS injection. The decrease in THC is attributed to different defense activities of haemocyte for a reduced cell concentration in the haemolymoph. In addition, haemocytes aggregated into non-circulating clumps after an acute bacterial infection [36] and injection of foreign material [37,38]. Moreover, phagocytic haemocytes may leave the circulation after phagocytosis and enter the heart, connective tissue, gills or other haemal sinuses [39]. Strikingly in the present study, haemocytes seem to settle first, mainly in the lymphoid organ, before they phagocytose which agrees with the theory of [30] that fixed phagocytes in the most crustaceans are derived from circulating (hyaline) cells.

There was an increased bacterial clearance in the feed supplemented animals higher than the normal animals. There was a two-fold increase in bacterial clearance in first three hours after injection of *Vibrio parahaemolyticus*. Four-fold and five-fold increase could be observed after six and twelve hours of infection. Similar results in clearing viable cells in haemolymph were observed in crustaceans. An increased bacterial clearance in the shore crabs, *Carcinusmaenas*, and lobsters was also reported [40].

The benefits of growth enhancement was seen in immune stimulated shrimp larvae and juveniles. Circulating hemocytes have been reported to serve a variety of functions, including

carbohydrate metabolism, lipoprotein or amino-acid transport and storage, wound repair, hemolymph coagulation, and defense against invading microorganisms or parasites [41,42].

Nitroblue tetrazolium (NBT) staining has been used for both, qualitative and quantitative analyses of O_2-generated by hemocytes which is the first product of respiratory bursts [43]. The production of superoxide radicals, which decreased in hypoxic *L. stylirostris*, was due to a decrease in the THC, suggesting that NADPH oxidase which is responsible for the production of superoxide and was not affected by hypoxic conditions [44]. In the present study, the fact that no significant differences in respiratory bursts or increases in THC were observed among the prawns exposed to 1.2 mg L^{-1} saponin indicates that the activity of NADPH oxidase might have been depressed under saponin stress. THC and respiratory bursts of *L. stylirostris*, following 24 h of hypoxia exposure at 1 mg L^{-1} DO decreased, but its phenoloxidase activity and susceptibility to Vibrio infection increased. They indicated that the increase in phenoloxidase activity was related to a lower amount of plasma inhibitors, regulating the prophenoloxidase system. The THC, hyaline cells, phenoloxidase activity, and superoxide anions of *M. rosenbergii* decreased, following 120 h of exposure to low DO (2.75 mg L^{-1}), accompanied by an increase the susceptibility to *Lactococcusgarvieae* infection [45].

They also indicated that the resistance of prawn to *L. garvieaewas* correlated with phenoloxidase activity and clearance efficiency, which were more relevant than THC, DHC, phagocytosis, or NBT reduction. The increased susceptibility of *M. rosenbergii*, following exposure to ammonia-N was considered to be related to the decrease in the phenoloxidase activity, which was more relevant than the hemocyte count or respiratory burst products to the resistance of prawns infected by *L. garvieae* [46]. The above by-products could not be removed completely even after secondary physical/chemical treatments. These toxicants showed a high damage to Labeo rohita's biochemical, enzyme, immunological and haematological parameters [47]. A spectrophotometric nitroblue tetrazolium (NBT) reduction assay was used to demonstrate the production of superoxide anions (O_2^-) by haemocytes of the white shrimp *Penaeus vannamei*. It was found that haemocytes, without receiving an experimental stimulant, showed a rather high background activity [48].

In the present study, THC increased, whereas phenoloxidase activity decreased for prawns after 168 h of exposure to 0.9 and 1.2 mg L^{-1} saponin. This fact indicates that the decrease in phenoloxidase activity under saponin stress was not a consequence of the increase in THC, but may have possibly increased the susceptibility of prawns to the pathogen and increased hemolymph inhibitors regulating the prophenoloxidase system. In the present study, the total haemocyte count has decreased in the infected animal, and increased in the hepatopancreatic enzymes when compared with that of control. Changes in the biochemical alteration of the vibrio challenged prawns lead to structural manifestations of disruption in the absorptive, storage, and secretory functions of hepatopancreas, and in the physiological mechanisms of gills. Also exposure of prawns to even low level of bacterial infection can result in such deleterious changes.

5. CONCLUSION

Thus, the control samples recorded the lowest counts, whereas *B. coagulans* supplemented animals after infection recorded the maximum counts among the three groups.

Therefore, the probiotic bacteria, such as *B. firmus* and *B. coagulans*, have been considered to be a useful bacteria against the pathogen *V. parahaemolyticus*.

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