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Superoxide dismutase activity and catalase activity in *Vibrio Parahaemolyticus* infected marine prawn *Penaeus monodon*, subjected to probiotic feed supplementation

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Abstract
In the present study was *Vibrio* species from wild marine shrimp, *Penaeus monodon* and to study its effect on the probiotic feed supplemented organism, when artificially in infected. Biochemical changes during infection of *Vibrio parahamolyticus* was evaluated by studying the Superoxide dismutase activity and catalase activity after infection. The superoxide dismutase activity and catalase activity in hepatopancreas was estimated in prawns from all the three feed groups. There was significant variation in the SOD activity and catalase activity in all three groups, after 30 days of feeding, uniformly they recorded gain in the activity. The SOD activity was 2.65 in control animals, 5.8 in *B. coagulans* supplemented animals, and 4.4 in *B. firmus* supplemented animals and catalase activity was 15.8 in control animals, 29.0 in *B. coagulans* supplemented animals, and 26.3 in *B. firmus* supplemented animals. The presence of this *B. coagulans* and *B. firmus* could protect the aquatic animals against the infection by pathogenic bacteria and might be applied as good probiotics in aquaculture.

Keywords: Probiotics, *Penaeus monodon, V. Parahaemolyticus*, SOD and CAT activity

1. Introduction
Aquaculture is a worldwide activity and considered as a major economic and food production sector as it is an increasingly important source of protein available for human consumption. Shrimp farming is an aquaculture business; that is, it exists in either marine or freshwater environment, producing shrimp or prawns. Over the past five years, there have been major developments in shrimp farming [1]. The major virulent strains of *Vibrio's* in shrimp are *V. parahaemolyticus*, *V. alginolyticus*, *V. Anguillarum* and *V. harveyi*. Successful shrimp culture requires a combination of factors including pathogen free larvae, nutritious feed, good aeration, salinity etc. The abuse use of antimicrobial drugs, pesticides, and disinfectants in aquaculture has the evolution of resistant strains of bacteria and concern of the society [2]. Serious viral disease outbreaks of shrimp challenge the shrimp industry to be better prepared in the view of a broadened knowledge about shrimps and their pathogens so that disease prevention methods could be improved [3].
Thus, the use of probiotics in the culture of aquatic organisms is increasing with the demand for more environment-friendly aqua-culture practice [4]. An effective method is to administer probiotics into the rearing water system or through food. The definition of probiotics is for 'life'. Probiotic is defined as a living microbiological dietary supplement that provides a nourishing environment to the friendly flora living in the digestive tract. Many different genera, including photosynthetic bacteria, *Yeast, Bacillus* and *Lactobacillus* have been evaluated as probiotics in fish and shellfish [5].
Moriarty [6] noted an increase of shrimp or prawn survival in ponds where some strains of *Bacillus* sp. were introduced. The actual data of Moriarty [6] showed the inhibitory activity of *Bacillus* sp. against luminous *Vibrio* sp. in pond sediment, but the effect on shrimp/prawn survival might be due either to a probiotic effect, or to an indirect effect on animal health. Probiotics are noticed to prevent pathogens from proliferation, improve health in culture species by improving the balance of intestinal microflora.
In the present study was *Vibrio* species from wild marine shrimp, *Penaeus monodon* and to study its effect on the probiotic feed supplemented organism, when artificially in infected. Biochemical changes during infection of *Vibrio parahamolyticus* was evaluated by studying the Superoxide dismutase activity and catalase activity after infection.

2. Material and Methods
2.1 Experimental design
The juvenile shrimps were weighed accurately in digital electronic balance before the start of the experiment. Post larvae (PI-20) of *Penaeus monodon* was collected from a private farm in Nagapattinam district. Animals were introduced into plastic trough of 45 liter capacity, filled with 40 liter of salinity adjusted sea water. In each plastic troughs, 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 commercial compounded feed two times a day and acclimatized to continuously aerated sea water under laboratory conditions in large plastic tubs.

2.2 Bacterial strains
Vibrio parahaemolyticus were isolated from shrimps collected from the sea water. Shrimps were surface – disinfected by wiping with 75% alcohol. Hepatopancreas was then aseptically removed, and the hepatopancreas tissue was homogenized in 20 ml of 0.85% NaCl solution following the procedure described by Sung and Hong [7]. A series of 10 fold dilutions of each water

and hepatopancreas sample were made using Zobell's agar medium. *Vibrio* sp. were enumerated using thiosulfate-citrate-bilesalt-sucrose (TCBS) agar (Himedia). For the enumeration of total bacteria and *Vibrios*, the inoculated plates were incubated at 25°C in the dark 5 days and 48 h respectively. Identification of *Vibrio* was cried out by biochemical tests described by West and Colwell [8].

2.3 Compounded feed

For the sustenance of prawn under, laboratory conditions essential nutrients in adequate composition should be given. A pelleted feed based on the recommendations of Tacon [9] was compounded in the laboratory. Prawns 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 prawns, 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 prawns.

2.4 Selection of probiotics

The probiotic strains were isolated from the gut of wild marine prawns *Penaeus monodon* and identified by biochemical test. Putative probiotic strains of *B. coagulans* was identified and pure culture was isolated and mass cultured at 37°C for 24 hours in temperature controlled shaker. Bacterial pellets were harvested every 24 hours and stored in a sterile container. Selected strains of probiotics *B. coagulans* and *B. firmus* 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 bended with the moltenagar containing plant extract. The mixture was stridden well with sterile glass rods to have a uniform coating of the bacteria over the feed pellets. Similarly, the probiotic cells of *B. coagulans* and *B. firmus* were coated on feed pellets, with molten agar.

2.5 *Vibrio parahaemolyticus* challenge test

To study the bacterial clearance, the prawns, were infected with *Vibrio parahaemolyticus*. The prawn were challenged with known sublethal concentration of *V. parahaemolyticus*. The challenge trials were conducted in duplicates. To study the bacterial clearance of prawns, animals were stocked in 45litre troghs with a stocking density of 8 prawns per trough. All the prawns were injected intramuscularly with an LD^{50} dosed of 100ul *V. parahaemolyticus* in saline adjusted to 1.0 OD animal between 5^{th} and 6^{th} abdominal segments. The infected animals showed signs of red discolouration, erratic swimming, and lethargy and swam near the water surface of trough before death.

2.6 Protein estimation

Total protein content in the tissue extract was estimated using Lowry *et al*. [10]. 100 mg of tissue extract was taken and mixed with five ml of 10% TCA and homogenized and then centrifuged at 3000 rpm for 15minutes. The precipitate was dissolved in 4ml of distilled water. Then 5.5 ml of reagent C was added and mixed thoroughly, and allowed to stand for 10 - 15 minutes. Finally 0.5 ml of Folin-Ciocalteu reagent was added and mixed rapidly. The test tubes were left as such for 20 minutes and the appearance of blue colour was measured at 720 nm in UV-visible spectrophotometer (Systronics, 118). A proper blank solution containing 4 ml of distilled water, 5.5 ml of reagent C and 0.5 ml of Folin-Ciocalteu reagent was also prepared. Bovine serum albumin (BSA) was used as the standard. The protein concentration was expressed in milligram/100mg of tissue.

2.7 Tissue extracts

Hepatopancreas, gill, muscle, heart and eye stalk with eyes attached were dissected from the healthy and *Vibrio*-infected shrimp. Haemolymph (1 ml 26G 1/2) containing (400 ul) an anticoagulant 0.94 mM/L EDTA in isotonic NaCl solution [11]. The haemolymph and 100 mg of each tissue from each of six replicate animals were homogenized with TN buffer (20 mM Tris-HCl, 400 mM NaCl pH 7.4). Homogenates samples were centrifuged at 8000xg for 10 min at 4°C. The supernatant fractions (tissue extracts) and haemolymph were collected and protein was estimated by using Lowry *et al*. [10]. The supernatant fractions and haemoymph were stored at - 40°C prior to analyses.

2.8 Superoxide dismutase

Superoxide dismutase activity was assayed according to the method of Misra and Fridovich [12], which is based on the oxidation of pyrogallol to adrenochrome by the enzyme. 0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml of ethanol and 0.15 ml of chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant were assed 0.5 ml of EDTA solution and 1 ml of buffer. The reaction was initiated by the addition of 0.5 ml of pyrogallol and the increase in absorbance at 480 nm was monitored at 30-s intervals for 3 min. the enzyme activity was expressed as 50% inhibiton of pyrogallol auto-oxidation/min/mg protein.

2.9 Catalase assay

Catalase activity was assayed according to the method of Takahara *et al*., [13]. To 1.2 ml of phosphate buffer, 0.5 ml of tissue homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of hydrogen peroxide solution. The decrease in absorbance was monitored at 240 nm every 30-s up to 3 min. The enzyme activity was expressed as umoles of hydrogen peroxide decomposed/min/mg protein.

2.10 Statistical Analysis:

One way ANOVA of the results were carried out using a statistical package (SPSS version. 10).

3. Results

3.1 Super oxide dismutase activity in tissues

Superoxide dismutase activity in hepatopancreas was estimated in prawns from all the three feed groups. There was significant variation in the SOD activity was 2.65 (SOD activity Units/min/mg of protein) in control animals, 5.8 in *B. coagulans* supplemented animals, and 4.4 in *B. firmus* supplemented animals (Table 1). Similar to that of differential count in this also there was gradual decrease in SOD activity after infection. Maximum reduction was observed in control animals with 1.2, 3.2 in *B. coagulans* supplemented animals. Thus probiotic supplementation has disease combating ability.

3.1.2 Superoxide dismutase activity in gills (SOD)

The Superoxide dismutase activity in gills was estimated in prawns from all the three feed groups. There was significance variation in the SOD activity in all three groups, uniformly they recorded gain in the activity. The SOD activity was 3.4 (SOD

activity Units/min/mg of protein) in control animals, 6.4 in *B. coagulans* supplemented animals, and 5.1 in *B. firmus* supplemented animals (Table 2). Similar to that of differential count in this also there was gradual decrease in SOD activity after infection. Maximum reduction was observed in control animals with 2.0, 3.4 in *B. coagulans* supplemented animals, and 2.7 in *B. firmus* supplemented animals. Thus probiotic supplemented animals. Thus probiotic supplementation has disease combating ability.

3.1.3 Superoxide dismutase activity in Muscle (SOD)

The superoxide dismutase activity in muscle was estimated in prawns from all the three feed groups. There was significant variation in the SOD activity in all three groups, uniformly they recorded gain in the activity. The SOD activity was 5.6 (SOD activity Units/min/mg of protein) in control animals, 7.8 in *B. coagulans* supplemented animals, and 6.5 in *B. firmus* supplemented animals (Table 3). Similar to that of differential count in this also there was gradual decrease in SOD activity after infection. Maximum reduction was observed in control animals with 2.4, 5.5 in *B. coagulans* supplemented animals, and 4.6 in *B. firmus* supplemented animals. Thus probiotic supplementation has disease combating ability.

3.1.4 Superoxide dismutase activity in Eye (SOD)

The superoxide dismutase activity in muscle was estimated in prawns from all the three feed groups. There was significant variation in the SOD activity in all three groups, uniformly they recorded gain in the activity. The SOD activity was 3.4 (SOD activity Units/min/mg of protein) in control animals, 7.3 in *B. coagulans* supplemented animals, and 5.9 in *B. firmus* supplemented animals (Table 4). Similar to that of differential count in this also there gradual decrease in SOD activity after infection. Maximum reduction was observed in control animals with 2.0, 4.5 in *B. coagulans* supplemented animals, and 3.3 in *B. firmus* supplemented animals. Thus probiotic supplementation has disease combating ability.

3.2 Catalase activity in tissues

3.2.1 Catalase activity in hepatopancreas

The catalase activity in hepatopancreas was estimated in prawns from all the three feed groups. There was significant variation in the catalase activity in all three groups, uniformly they recorded gain in the activity. The catalase activity was 15.8 (catalase activity Units/min/mg of protein) in control animals, 29.0 in *B. coagulans* supplemented animals, and 26.3 in *B. firmus* supplemented animals (Table 5). Similar to that of differential count in this also there was gradual decrease in catalase activity after infection. Maximum reduction was observed in control animals with 8.7, 19.4 in *B. coagulans* supplemented animals, and 17.9 in *B. firmus* supplemented animals. Thus probiotic supplementation has disease combating ability.

3.2.2 Catalase activity in gills

The catalase activity in gills was estimated in prawns from all the three feed groups. There was significant variation in the catalase activity in all three groups, uniformly they recorded gain in the activity. The catalase activity was 20.0 (catalase activity Units/min/mg of protein) in control animals, 38.0 in *B. firmus* supplemented animals (Table 6). Similar to that of differential count in this also there was gradual decrease in catalase activity after infection. Maximum reduction was observed in control animals with 11.9, 26.2 in *B. coagulans* supplemented animals, and 22.0 in *B. firmus* supplemented animals. Thus probiotic supplementation has disease combating ability.

3.2.3 Catalase activity in Muscle

The catalase activity in muscle was estimated in prawns from all the three groups, uniformly they recorded gain in the activity. The catalase activity was 18.6 (catalase activity Units/min/mg of protein) in control animals, 32.7 in *B. coagulans* supplemented animals, and 27.2 in *B. firmus* supplemented animals (Table 7). Similar to that of differential count in this also there was gradual decrease in catalase activity after infection. Maximum reduction was observed in control animals with 9.4, 21.2 in *B. coagulans* supplemented animals, and 17.8 in *B. firmus* supplemented animals. Thus probiotic supplementation has disease combating ability.

3.2.4 Catalase activity in Eye

The catalase activity in eye was estimated in prawns from all the three feed groups. There was significant variation in the catalase activity in all three groups, uniformly they recorded gain in the activity. The catalase activity was 13.8 (catalase activity Units/min/mg of protein) in control animals, 26.5 in *B. coagulans* supplemented animals, and 22.8 in *B. firmus* supplemented animals (Table 8). Similar to that of differential count in this also there was gradual decrease in catalase activity after infection. Maximum reduction was observed in control animals with 7.7, 18.4 in *B. coagulans* supplemented animals, and 14.5 in *B. firmus* supplemented animals. Thus probiotic supplementation has disease combating ability.

Table 1: Superoxide dismutase activity in hepatopancreas of normal and *V. parahaemolyticus* challenged *Penaeus 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	2.65 ± 0.2	5.8 ± 0.2	4.4 ± 0.2
2	Infected	1.2 ± 0.2	3.2 ± 0.1	2.5 ± 0.3

Table 2: Superoxide dismutase activity in gills of normal and *V. Paradhaemolyticus* challenged *Penaeus monodon*, supplemented with live probiotic *B. coagulans* and *B. firmus* in (mean values ± Standard deviation)

Organs	Treatment	Control	*B. coagulans*	*B. firmus*
Gill	Normal	3.4 ± 0.3	6.4 ± 0.2	5.1 ± 0.1
	Infected	2.0 ± 0.2	3.4 ± 0.3	2.7 ± 0.1
Muscle	Normal	5.6 ± 0.3	7.8 ± 0.2	6.5 ± 0.3
	Infected	2.4 ± 0.1	5.5 ± 0.3	4.6 ± 0.3
Eye	Normal	3.4 ± 0.2	7.3 ± 0.2	5.9 ± 0.3
	Infected	2.0 ± 0.2	4.5 ± 0.2	3.3 ± 0.2

Table 3: Catalase activity in hepatopancreas of normal and *V. parahaemolyticus* challenged *Penaeus 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	15.8 ± 0.5	29.0 ± 0.2	26.3 ± 0.3
2	Infected	8.7 ± 0.4	19.4 ± 0.4	17.9 ± 0.3

Table 4: Catalase activity of normal gill, muscle & eye and *V. parahaemolyticus* challenged *Penaeus monodon*, supplemented with live probiotic *B. coagulans* and *B. firmus* in (mean values ± Standard deviation)

Organs	Treatment	Control	*B. coagulans*	*B. firmus*
Gill	Normal	15.8 ± 0.5	29.0 ± 0.2	26.3 ± 0.3
	Infected	8.7 ± 0.4	19.4 ± 0.4	17.9 ± 0.3
Muscle	Normal	18.6 ± 0.4	32.7 ± 0.2	27.2 ± 0.4
	Infected	9.4 ± 0.2	21.2 ± 0.3	17.8 ± 0.5
Eye	Normal	13.8 ± 0.3	26.5 ± 0.8	22.8 ± 0.4
	Infected	7.7 ± 0.3	18.4 ± 0.2	14.5 ± 0.3

Fig 1: Experimental set up

Fig 2: Penaeus monodon-infected and uninfected animals

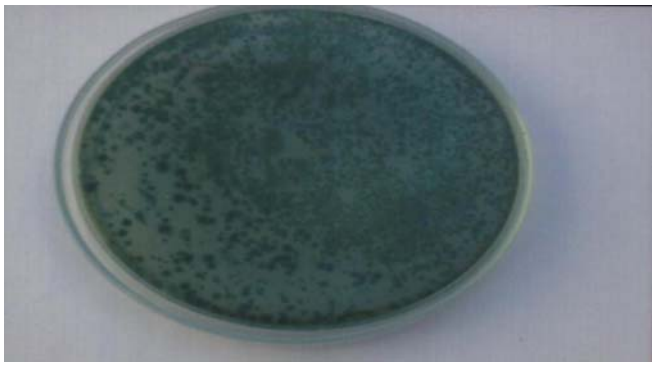

Fig 3: Vibrio parahaemolyticus

4. Discussion

Changes in the biochemical alteration of the *Vibrio* challenged prawns lead to structural manifestations of disruptions in the absorptive, storage and secretary functions at heptopancreas and in the osmoregulartory, respiratory, and physiological mechanisms. Even low levels of bacterial infection can result in such deleterious changes and hence, it is imperative that bacterial infection should be prevented.

The biochemical induced by stress may lead to disturbance in metabolism. Changes such as reduction in protein and globulin content of haemolymph and inhibiton of activity of certain important enzymes at cellular level lead to retardation of growth, reduction in the fecundity and longevity of organism [14, 15]. The reduction in the SOD and Catalase activity in tissues like muscle, gills, eye and hepatopancreas may be attributed to the bacterial infection in the present study.

In tiger shrimp *P. monodon* temperature induced stress causes, decreased respiratory burst and SOD activity I 24 h. this fact indicated that the activity of NADPH-oxidase, responsible for the release of superoxide anion decreased together with a decrease in the activity of SOD responsible for scavenging superoxide anion. Influence of temperature induced stress on the activities of catalase and peroxidasde for *P. monodon* has to probed [16, 17]. In the present study we evaluated the impact of *Vibrio*-induced stress on the SOD and Catalase activity and found that our result was in accordance with the earlier studies.

The release of superoxide anion and hydrogen peroxide was considered to play a more important role in shrimp microbial activity than hypochlorite's and myeloperoxidase during phagocytosis [18]. The semi-granular haemocytes and hyaline cells are considered as phagocytes [19]. Dopamine decreased the release of hypochlorite's from 2 to 8 h, and decreased the activity of SOD at 8h. It is suggested that the reduction of hypochlorite's may result from the decrease of semi-granular cell count and NADPH oxidase activity of haemocytes, and the respiratory burst decrease also results in the decrease of SOD activity in haemocytes of shrimp that received DA in a short time [20]. Similar trend of decreased SOD and Catalase activity was observed in the present study after *Vibrio*-infection.

Superoxide dismutase (SOD) converts superoxide anions into hydrogen peroxide and oxygen. Superoxide dismutase has been reported to contain arginine and histidine residues at its active site [21]. Free radicals attack these highly reactive amino acids resulting in chemical modification of the protein structure and loss of enzyme activity. This may have been the cause of low SOD activity we observed. Hydrogen peroxide is decomposed by catalase and glutathione peroxidase. Glutathione peroxidase is considered to play a major role in the removal of hydrogen peroxide that is generated in vertebrate tissues undergoing oxidative stress. Catalase is thought to be more important in invertebrates [22].

The phagocytic activities of shrimp insignificantly differed among different treatments. Respiratory bursts of haemocytes increased in shrimp following of *Lac. Plantarum*-containing diet from 48 to 168 h, the superoxide dismutase (SOD) activity and peroxinectin increased with the dose of *Lac. Plantarum*. These facts suggesft that the difference in tespiratory bursts of shrimp administered different levels of *Lac. Plantarum*-contained diets was a consequence of increased in the activity of SOD, which catalyses the superoxide anion to hydrogen peroxide inducing an increase in PE gene transcription. In the present study also feed supplementation with two probiotic bacteria *B. coagulans* and *B. firmus* showed enhanced level of SOD and Catalase activity. Thus the supplementation of probiotics has immunomodulatory effect on *P. monodon* prior and after infection of *Vibrio.*

5. Conclusion

The superoxide dismutase activity and catalase activity in hepatopancreas was estimated in prawns from all the three feed groups. There was significant variation in the SOD activity and catalase activity in all three groups, after 30 days of feeding, uniformly they recorded gain in the activity. The SOD activity was 2.65 in control animals, 5.8 in *B. coagulans* supplemented animals, and 4.4 in *B. firmus* supplemented animals and catalase activity was 15.8 in control animals, 29.0 in *B. coagulans* supplemented animals, and 26.3 in *B. firmus* supplemented animals. The presence of this *B. coagulans* and *B. firmus* could protect the aquatic animals against the infection by pathogenic bacteria and might be applied as good probiotics in aquaculture.

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