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EFFECT OF *BACILLUS SUBTILIS* AND *LACTOBACILLUS RHAMNOSUS* INCORPORATED PROBIOTIC DIET ON GROWTH PATTERN AND ENZYMES IN *PENAEUS VANNAMEI*

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ABSTRACT

The effect of *Bacillus subtilis* and *L.rhamnosus* incorporated diet was investigated on growth pattern and antioxidant enzymes in *Penaeus vannamei* for a period of 3 weeks. 25- day old shrimps were stocked in three circular tanks. One of the tank fed with *Lactobacillus rhamnosus* incorporated diet and other tank was fed with *Bacillus subtilis* incorporated diet. The other tank was fed with commercial diet without probiotic which served as control group. After 3 weeks of treatment SGR, FCR, weight gain%, microbiological analysis and antioxidant enzyme concentration was determined. Statistical analysis was performed using analysis of variance (ANOVA) and Duncan's new multiple range tests at $P \leq 0.05$ using spss 13 version. *B.subtilis* treated groups showed significant increase in WG % of (125 ± 33) followed by *L.rhamnosus* treated groups of (93 ± 0) followed by control group (42.8 ±20).similar trend was observed with SGR. *B.subtilis* treated groups showed significant increase (1.25 ± 0.04) followed by *L.rhamnosus showed* (1.11±0.02) followed by control group (0.98±0.2). *B.subtilis* treated groups showed decreased FCR (1.90± 0.14) followed by *L.rhamnosus* treated groups showed (2.11± 0.52) and control group (2.74± 0.7). Total count of *B.subtilis* showed an increase of 7% compared to *L.rhamnosus* which was 4% after 3 weeks of feeding trial. The SOD activity of the haemolymph in shrimps fed with B.subtilis incorporated diet showed enhanced values of 2.4 auto-oxidation/min/mg of protein compared to *L.rhamnosus* 1.9 auto-oxidation/min/mg of protein that of control 1 auto- oxidation/min/mg of protein . The catalase activity of haemolymph in control was 1.09µ moles H_2O_2 decomposed -/min/mg of protein. The shrimp fed with *B.subtilis* incorporated diet showed the maximum catalase activity of 2.3µ moles H2O2 decomposed /min/mg of protein were as shrimps fed with *L.rhamnosus* incorporated diet showed catlase activity of 1.453µ moles H_2O_2 decomposed /min/mg of protein.

Keywords: *Penaeus vannamei, Bacillus subtilis, Lactobacillus rhamnosus,* catalase, superoxide dismutase.

INTRODUCTION

Globally, shrimp farming has been a significant agro based economic activity since the early 1970s. Because it offered a huge immediate economic return, shrimp farming showed a booming expansion and soon became a multimillion dollar industry (Islam et al.2004). India is one of the major shrimp producing countries along with China, Indonesia and Thailand. But there are also many risks and challenges, especially in the culture techniques and the degradation of environmental quality, biodiversity and natural brood stocks. Pathogenic microorganisms implicated in these outbreaks were viruses, bacteria, algae, fungi and protozoan parasites. Among the pathogenic microorganisms bacterial diseases are considered to be a major cause of mortality in shrimp larviculture

(Wyban et al. 1991) and fish hatcheries (Grisez et al. 1995). The most prevalent bacterial disease is Vibriosis which causes a mass mortality both in larval cultures and shrimp production (Saulnier *et al*. 2000). The major virulent strains of vibrio's in shrimp are *Vibrio alginolyticus, V. anguillarum, V. harveyi* and *V. parahaemolyticus*. Successful shrimp culture requires a combination of factors including pathogen - free larvae, nutritious feed, good aeration, salinity etc., (Stephen et al. 2008).The abuse use of antimicrobial drugs, pesticides, and disinfectants in aquaculture has the evolution of resistant strains of bacteria and concern of the society (Esiobu et al. 2002). Thus, the use of probiotics in the culture of aquatic organisms is increasing with the demand for more environment-friendly aqua-culture practice (Gatesoupe et al. 1999). An effective method is to administer probiotics into the rearing water system or through food (Geir et al. 2001). 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 (Kesarcodi-Watson et al. 2008). *Lactobacillus* is a putative bacterium that enhances phagocytic activity. *Nitrobacter* is said to increases the water quality in shrimp farming Rengpipat. The introduction of *Bacillus* sps in proximity to pond aerators reduced chemical oxygen demand, and increased shrimp harvest (Porubcan et al. 1991). Moriarty (1998) noted an increase of shrimp or prawn survival in ponds where some strains of *Bacillus* sps were introduced. The actual data of Moriarty (1998) showed the inhibitory activity of *Bacillus* sps against luminous *Vibrio* sps 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 we investigated the effect of administering diet formulated probiotic bacteria *Bacillus subtilis* and *Lactobacillus rhamnosus* on growth pattern and antioxidant enzymes in hemolymph of shrimps.

MATERIALS AND METHODS

Penaeus Vannamei 25 (juvneil 30), 1.5 ±0.05 g (mean±SD) body weight, was acclimatized to the Laboratory conditions. The lactic acid bacterium, *Lactobacillus rhamnosus* and Bacillus *subtilis* were collected from IMTECH Chandigarh.The probiotic bacteria *L. rhamnosus* was cultured in Man-Rogosa-Sharpe broth (MRS, Himedia). *Bacillus subtilis* was cultured in Bacillus broth (Himedia) and incubated under continuous agitation of 180rpm at 37°C for 24h. The bacterial cultures were centrifuge at 4000 rpm for 15 min at 4ºC and harvested. The collected bacteria were resuspended in normal saline solution to 5 x 10^{13} CFU/ml of *Bacillus subtilis* and 3x10 ^{5} CFU/ml of *L. rhamnosus*. Feed was oven-dried at 35ºC for 1 - 2 h. The control diet was sprayed with sterile culture medium.

GROWTH PATTERN

25- day old shrimp were stocked in three circular tanks. One of the tank was fed with *Lactobacillus rhamnosus*-supplemented diet and other tank was fed with *Bacillus subtilis* - supplemented with diet. The other tank was fed with commercial diet without supplementation. Water temperature was kept at 28±2ºC and salinity at 5ppt. Bottom sediment removal and 70% water exchange was done every day. The feeding trial was conducted for 3 weeks by sampling 5 shrimps per group and microbiological analysis was also done.

$$\text{Weight gain (\%)} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} \times 100$$

$$\text{Food conversion ratio (FCR)} = \frac{\text{Total feed given (g)}}{\text{Wet weight gain (g)}}$$

$$\text{Specific Growth Rate (SGRday)} = \frac{\text{In final wt} - \text{In initial wt}}{\text{Duration (days)} \times 100}$$

MICROBIOLOGICAL ANALYSIS

5 shrimps were randomly sampled from each tank for microbiological analysis of the digestive tract. Shrimp digestive tracts were removed with tweezers and scalpel, homogenized with sterile saline solution (SSS) in a mortar and serially diluted. Dilutions were spread on the following culture media: MRS agar (lactic bacteria selective), and Bacillus agar (selective for Bacillus bacteria) and incubated at 30°C for 24hrs. Gram staining was performed with the colonies grown in MRS and Bacillus selective agar.

ANTIOXIDANT ENZYMES

HEMOLYMPH COLLECTION

Hemolymph was collected from each treated group animals. 0.5 ml of hemolymph was withdrawn from base of the third walking leg of the shrimp using a syringe containing 1.5 ml of anticoagulant (27mM sodium citrate).

SUPER OXIDE DISMUTASE

SOD assay was performed as described by (Misra and Fridovich, 1972). Briefly 0.1 mL of haemolymph and 0.75 mL of ethanol and 0.15 mL of chloroform (chilled in ice) were added and centrifuged at 10,000 rpm at 4°C for 10 minutes. To 0.5 mL of supernatant 0.5 mL of EDTA (0.6 mM) solution and 1mL of carbonate bicarbonate buffer (0.1 M) P^H 10.2 were added. The reaction was initiated by the addition of 0.5 mL of substrate (Epinephrine 1.8 mM) and the increase in absorbance was recorded at 480 nm at every 30 seconds for 3 minutes. The values are expressed 50 % inhibition of epinephrine auto oxidation /min /mg protein.

CATALASE ASSAY

The Catalase assay was performed as described by Takaharat et al. 1960. 1.2 mL of phosphate buffer (0.05 M, pH 7), 0.2 mL haemolymph was added. Reaction was initiated by addition of 1 mL of substrate H_2O_2 (0.03 M in phosphate buffer). OD at 240 nm was recorded at every 30 seconds for 3 minutes. The enzyme blank was run simultaneously with 1mL of distilled water instead of H_2O_2. A standard contained catalase was carried out simultaneously and expressed as μmoles of H_2O_2 decomposed /min/mg protein.

STATISTICAL ANALYSIS

Statistical analysis was performed using analysis of variance (ANOVA) and Duncan's new multiple range tests at $P \leq 0.05$ using spss 13 version.

RESULTS

GROWTH PARAMETERS

WG, FCR and SGR were used to evaluate the growth performance of cultured shrimp with probiotic incorporated feed applied in different cultured tanks. *B.subtilis* treated groups showed significant increase in WG % of (125 ± 33) followed by *L.rhamnosus* treated groups of (93 ± 0) followed by control group (42.8 ±20).similar trend was observed with SGR. *B.subtilis* treated groups *showed significant increase (*1.25 ± 0.04) followed by *L.rhamnosus* showed (1.11±0.02a) followed by control group (0.98±0.2). *B.subtilis* treated groups showed decreased FCR (1.90± 0.14) followed by *L.rhamnosus* treated groups showed (2.11± 0.52) and control group (2.74±0.7).

Table 1

Effect of Bacillus subtilis and Lactobacillus rhamnosus incorporated diet on growth performance of L. vannamei (Mean ± S.D.)

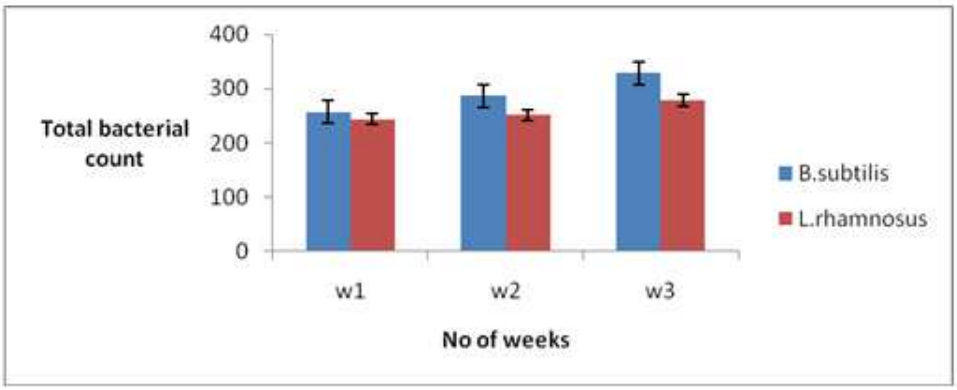
Variables	Control	*Bacillus subtilis*	*Lactobacillus rhamnosus*
a) Initial mean weight (g)	2.8± 0.05 [a]	3.1± 0.03[a]	3.0± 0.02 [a]
b) Final mean weight(g)	4± 0.06[a]	7.0± 0.04[b]	5.8±0.02[ab]
c) Mean weight	1.2±0.01[a]	3.9±0.03[a]	2.8± 0[a]
d) weight gain %	42.8 ± 20[ab]	125 ± 33[ab]	93 ± 0[ab]
e) FCR	2.74± 0.7[a]	1.90± 0.14 [a]	2.11± 0.52[a]
f) SGR	0.98±0.2[a]	1.25±0.04 [a]	1.11±0.02 [a]

MICROBIOLOGICAL ANALYSIS

Total bacterial count in digestive tract of *Penaeus Vannamei* after 3 weeks of probiotic treatment was significantly higher in groups treated with *B.subtilis* followed by *L.rhamnosus* followed by control group. Total count of *B.subtilis* showed an increase of 7% compared to *L.rhamnosus* which was 4% after 3 weeks of feeding trial.

Graph 1

Propagation levels of probiotics in the gut of Peneaus Vannamei over different time periods (weeks)



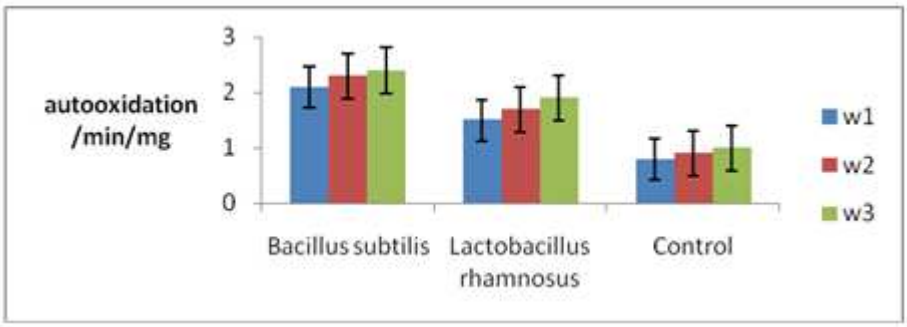
ANTIOXIDANT ENZYMES

SUPER OXIDE DISMUTASE

The SOD activity of the haemolymph in shrimps fed with B.subtilis incorporated diet showed enhanced values of 2.4 auto-oxidation/min/mg of protein compared to L.rhamnosus 1.9 auto-oxidation/min/mg of protein that of control 1 auto- oxidation/min/mg of protein .

Graph 2

Super oxide Dismutase activity in hemolymph of P.vannamei fed with Bacillus subtilis* and *Lactobacillus rhamnosus incorporated diet

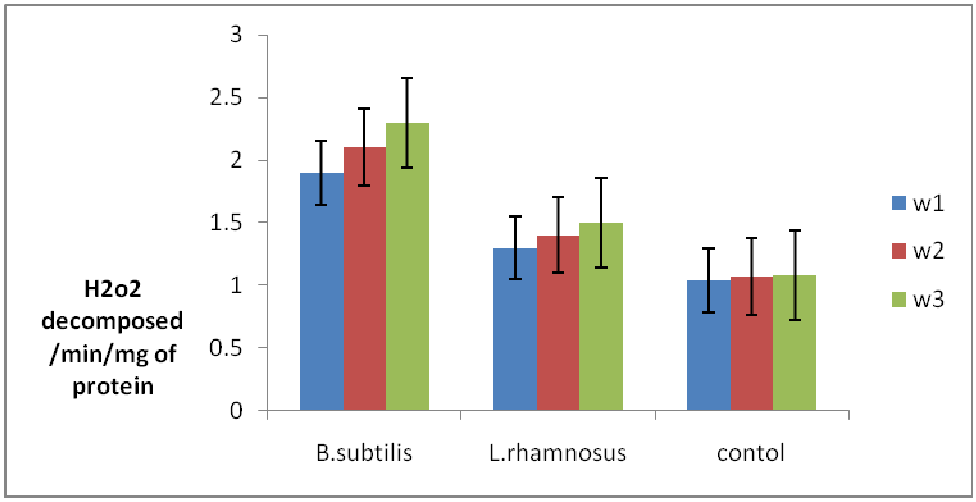


CATALASE ACTIVTY

The catalase activity of haemolymph in control was 1.09µ moles H2O2 decomposed - /min/mg of protein. The shrimp fed with B.subtilis incorporated diet showed the maximum catalase activity of 2.3 µmoles H2O2 decomposed /min/mg of protein were as shrimps fed with L.rhamnosus incorporated diet showed catlase activity of 1.453 µmoles H2O2 decomposed /min/mg of protein .

Graph 3

Catalase activity in hemolymph of P.vannamei fed with Bacillus subtilis* and *Lactobacillus rhamnosus incorporated diet



DISCUSSION

It is important to provide healthy shrimp with higher production and probiotics has a great deal of potential (Gomez-Gil et al. 2000). Effects of commercial probiotic on aquaculture has been investigated by researchers, and some of these research have not shown any positive effects on growth parameters or survival rate or any promising result on the cultural condition. For instance, (Shariff et al. 2001) found that treatment of *P. monodon* with a commercial *Bacillus.* Probiotic did not significantly increase survival. (Dennis et al. 2000) used a commercial bacteria supplement to culture on *L. vannamei* and did not showed increase mean final weight and FCR of the shrimps. But in our present studies we observed increase growth pattern of *Penaeus vannamei* when fed with *B.subtilis* incorporated diet and *L.rhamnosus* compared to control groups. *Bacillus* bacteria are able to out-compete other bacteria for nutrients and space and can exclude other bacteria through the production of antibiotics (Verschuere et al. 2000). Many different antibiotic compounds are produced naturally by a range of *Bacillus* bacteria. Result of amplification of gut incorporated bacteria showed significant difference ($P<0.05$) between *B. subtilis* and *L.rhamnosus.* Total count of *B. subtilis* showed significant increase of 7% compared to *L.rhamnosus of* 4%. Super oxide dismutase (SOD) is one of the main antioxidant defence enzymes generated in response to oxidative stress. (Sarathi et al. 2007) and Mohankumar and Ramasamy (2007) observed the activity of SOD was significantly lowered in WSSV-infected *F. indicus.* Where as M. Madhumathi (2011) showed *D. salina* incorporated diet -treated shrimp significantly increased in (SOD) when compared with control animals. Chang *et al.,* 2003 observed that the shrimp fed with b-glucan (BG) diets showed significantly higher levels of O_2 concentration than the BG free group as observed in shrimp treated with *C. dactylon* plant extract. Holmblad and Soderhall (1999) observed that SOD is related to immunity in crustacean. The high level of O_2 in *P. vannamei* fed with *B. subtilis and L.rhamnosus incorporated* diets indicated that probiotics are potential immunostimulant. Hydrogen peroxide is toxic to cells and catalase is a major primary antioxidant defense component that catalyses the decomposition of H_2O_2 which is produced by the action of superoxide dismutase to H2O. The present study revealed that the catalase assay of haemolymph of *P.vannamei* fed with different probiotic incorporated diets showed increased levels of catalase when compared to control.

CONCLUSION

In conclusion, significant increase in SGR, Weight gain% and antioxidant enzyme activity in shrimps fed up on probiotic incorporated diet forms a basis to understand the efficiency of *B. subtilis* and *L.rhamnosus* in enhancing the growth pattern and antioxidant concentration in

P.vannamei. There is a current worldwide interest in finding new and safe antioxidants from natural sources to prevent oxidative deterioration of food and to minimize oxidative damage to living cells (Pralt et al. 1992).Since the crustaceans are lacking a well-defined acquired immunity the dietary supplementation of natural antioxidants possessing health promoting and antimicrobial properties may be an effective alternative.

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