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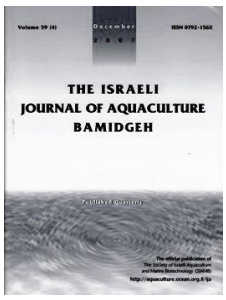
ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA_66.2014.1009, 8 pages



Synergistic Effects of Two Probiotic Bacteria on Growth, Biochemical, and Immunological Responses of *Litopenaeus vannamei* (Boone, 1931)

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(Received 3.8.13, Accepted 21.10.13)

Keywords: probiotics, *Bacillus licheniformis, Bacillus megaterium,* growth, biochemical response, immunological response, *Litopenaeus vannamei*

Abstract

The beneficial effects of two probiotic bacteria, *Bacillus licheniformis* and *Bacillus megaterium,* on growth, biochemical, and immunological responses of *Litopenaeus vannamei* (Boone, 1931) were evaluated both individually and in combination. A 60 day experimental trial was conducted on 1200 post larvae (PL) of *Litopenaeus vannamei* divided into four experimental groups, with three replicates of each treatment, and 100 PL in each replicate. The shrimp were fed a commercial diet (32% protein) supplemented with different probiotics. The first treatment included only *B. licheniformis* @ ~ 1x 10^8 cfu/kg diet (T1), the second consisted of a combination of ~ 0.5 x 10^8 cfu/kg of *B. licheniformis* mixed with a similar concentration of *B. megaterium* (T2), and the third had only *B. megaterium* @ ~1x 10^8 cfu/kg diet (T3). The control diet (C) had neither of the probiotic bacteria. Growth performance, biochemical, and immunological parameters of experimental shrimp were measured at 20 day intervals. Probiotic fed shrimp showed significantly higher growth, biochemical, and immunological response than the control. Among the probiotic treatments the mixture of *B. licheniformis* and *B. megaterium* (T2) performed better than the individual probiotics alone, indicating the synergistic effect of these two functionally different probiotics.

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Introduction

White leg shrimp, *Litopenaeus vannamei* (Boone, 1931) is an important emerging species in shrimp farming. From the beginning of this century there has been a marked shift from the farming of indigenous black tiger shrimp, *Penaeus monodon* to the culture of exotic white leg shrimp, *L. vannamei* in India. The use of probiotics in the culture of aquatic organisms is increasing rapidly with the advent of environmentally-friendly aquaculture practices (Gatesoupe, 1999) especially in shrimp farming. Aquatic probiotics have several modes of action: competitive exclusion of pathogenic bacteria through the production of inhibitory compounds; improvement of water quality; enhancement of immune response of the host, or enhancement of nutrition of host through the production of supplementary digestive enzymes (Thompson *et al*., 1999; Verschuere *et al*., 2000). Among the large number of probiotic products available today, bacteria belonging to the genus *Bacillus* are the most efficient probiotics, because their spores are resistant to external physical and chemical threats (Henriques and Moran, 2000; Nicholson *et al*., 2000). Moreover they secrete many exo-enzymes that contribute to the digestive system of the host (Moriarty, 1996; 1998).

The probiotic bacterium *B. megaterium* produces exo-enzymes having protease, amylase, and lipase activity (Ochoa-Solano and Olmos-Soto, 2006) therefore it is a good feed probiotic for shrimp. *B. licheniformis* can be used as an immunostimulant feed supplement since it enhances shrimp immunity (Li *et al*., 2007). These reports demonstrate that mode of action of each of these bacteria is different. *B. megaterium* influences the digestive system and *B. licheniformis* enhances the immune system. Given this information, the present study aims to compare the performance of these two probiotics both individually and in combination, on growth, biochemical, and immunological responses of *L. vannamei*.

Material and Methods

Probiotic bacteria. *Bacillus megaterium* (MTCC NO. 428) and *B. licheniformis* (MTCC NO. 2447) were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. The probiotics were revived in nutrient broth at 37^0C for 24 hours and the concentrations were determined by serial dilution of the stock followed by spread plate technique on nutrient agar.

Experimental design. The experiment was conducted in aerated FRP tanks filled with 200 l chlorine treated seawater (17 ppt). 1200 PL of *L. vannamei* were distributed into four experimental groups in triplicate. Each tank was stocked with 100 PL. T1 group was fed a commercial feed (32% protein, Avanthi feeds India. Pvt. Ltd.) supplemented with *B. licheniformis* alone at ~ 1x 10^8 cfu/kg diet, T2 was fed a mixture of *B. licheniformis* and *B. megaterium* at ~0.5 x 10^8 cfu/kg, T3 was given only *B. megaterium* at ~1x 10^8 cfu/kg diet, and the control group (C) had no supplemented probiotic bacteria.

Preparation of the experimental diet. After 24 hour culture in a nutrient broth the probiotic bacteria were harvested by centrifuge at 10,000 rpm for 10 minutes. The harvested bacteria were washed three times with phosphate buffered saline (PBS pH 7.4) and the cells were re-suspended in PBS and the suspension sprayed uniformly over the pellets. The feed was then dried at 40°C, packed in air tight polythene packs and stored at 4°C. Experimental diets were prepared once every 15 days, since bacterial counts in feeds often increase during storage.

Feeding and sampling. Feed was given at a ratio of 10% of the shrimp body weight. The daily ration was divided into four equal parts and was fed at 08:00 h, 12:00 h, 16:00 h and 20:00 h. Excess feed was removed daily and 50% of the water in each tank was exchanged weekly.

Sampling. Sampling for growth, biochemical and immunological analysis was carried out at 20 days intervals until the 60^{th} day.

Growth related studies. Weight gain, final Average body weight (ABW), Specific Growth Rate (SGR), FCR and survival were calculated according to Ziaei-Nejad *et al*., (2005) and Wang (2007).

Assay of Digestive Enzyme Activity. Intestines of the shrimp were carefully removed, dissected, weighed, and the intestinal contents removed carefully and homogenized with chilled sucrose solution (0.25 M) in glass test tubes using Teflon coated tissue homogenizer. The tubes were kept in ice to maintain low temperatures to avoid any effect on enzyme activity. The homogenate was centrifuged at 5000 rpm for 10 min at 4^0C in a refrigerated centrifuge. The supernatant was stored at -20^0C for further analysis. A 5% homogenate was prepared for enzymatic assay.

Protease activity was determined by the casein digestion method according to Drapeau (1976). The reduced sugars produced due to the action of glucoamylase and α- amylase on carbohydrate was estimated using the dinitro-salicylic-acid (DNS) method (Rick and Stegbauer, 1974). Amylase activity was expressed as moles of maltose released from starch per min at 37^0C. Lipase activity was assayed by the Cherry and Crandall (1932) method using olive oil emulsion.

Immunological assays. Hemolymph from each shrimp was collected from the cephalo-thorax region using a 1-ml syringe with 26-gauge needle, containing pre-cooled (4^0C) anticoagulant solution (450 mM NaCl, 10 mM KCl, 10 mM EDTA-Na, 10 mM HEPES, pH 7.3). Hemolymph from five shrimp was pooled and used for analysis of total hemocyte count (THC), phenoloxidase (PO) activity, and respiratory burst activity. Serum was collected without using anticoagulant and separated from hemolymph by keeping the tubes slanted for ~ 2 h and then centrifuging at 3500 rpm at 4ºC followed by collection with micropipette. The collected serum was stored at -20^0 C until use. Serum was used for the analysis of serum protein and superoxide (SOD) activity. All the tests were conducted in triplicate.

Hemolymph was diluted 2, 4, 8, and 10 times with ice-cold phosphate buffer saline (PBS, pH-7.2). Total hemocytes were counted using a hemocytometer and light microscope at 40X. Hemocytometer counts were made for 5/25 square (volume of each square = 0.2x 0.2x0.1 mm^3) to calculate THC/ml hemolymph according to Sritunyalucksana *et al.* (2005).

Phenoloxidase (PO) activity was quantified by monitoring the rate of formation of dopachrome from L-3, 4, dihydroxyphenyleamine (L- DOPA) in a colorimetric assay (Tanner *et al*., 2006). PO activity was recorded at one minute intervals as the maximum differences in optical density (OD) during the first 5 min of assay.

Serum protein was estimated by Biuret method (Reinhold, 1953) using a kit (Merck, Germany). Superoxide dismutase was assayed according to Misra and Fridovich, (1972) based on the oxidation of epinephrine-adreno chrome transition by the enzyme. Respiratory burst activity by Nitroblue Tetrazolium Assay was done according to Secombes (1990) modified by Stasiack and Baumana (1996).

Statistical analysis. The data were statistically analyzed using statistical package SPSS version 16. One-way ANOVA and Tukey multiple range tests were employed to determine the significant differences between the means.

Results

Growth parameters. Wet weight of the experimental shrimp were recorded at 20 day intervals (Table 1).

Table 1: Weight gain (g) of *Litopenaeus vannamei* up to 60th day of probiotic treatment

Treatments	***Initial***	***0-20 days***	***0-40 days***	***0-60 days***
C	0.1+0.004^a	1.50±0.005^a	5.42±0.26^a	10.54±0.036^a
T-1	0.1+0.004^a	1.64±0.023^{ab}	5.95±0.0.058^b	10.75±0.2^a
T-2	0.1+0.004^a	1.81±0.007^b	6.04±0.0.034^b	12.00±0.24^{ab}
T-3	0.1+0.004^a	1.81±0.31^b	5.88±0.15^b	11.44±0.97^b

*Mean values ± standard error in the column with different superscripts differ significantly ($p<0.05$)

The average body weight between different treatments differed significantly (P<0.05) at the end of the experimental period. Specific growth rate (SGR), FCR, and survival rates of different experimental groups are shown in Table 2.

Table 2: Biological parameters (mean± SE) of *Litopenaeus vannamei* fed with probiotics for 60 days

Treatments	*Final ABW (g)*	*SGR*	*FCR*	*Survival (%)*
C	10.54±0.036[a]	7.66±0.05[a]	1.81±.06[a]	73.3333[a]
T-1	10.75±0.2[ab]	7.76±0.02[ab]	1.61±.06[b]	81.0000 [a]
T-2	12.00±0.24[c]	7.95±0.01[c]	1.34±.02 [c]	86.6667 [a]
T-3	11.44±0.09[abd]	7.85±0.03[bcd]	1.59±0.14[bd]	78.0000 [a]

*Mean values ± standard error in the column with different superscripts differ significantly (p<0.05)

The specific growth rate was highest in T2 group which was significantly higher (p<0.05) than the other groups. The survival rate (%) ranged from 73.33%-86.65% and there were no significant differences between the treatments.

Digestive enzyme assay. Protease activity (Fig 1.) in the intestine of T2 group shrimp was significantly higher (P<0.05) but other treatment results did not differ from the control throughout the experiment. Amylase activity (Fig 2.) also was significantly higher (P<0.05) in T2 group and lowest in the control throughout the experiment.

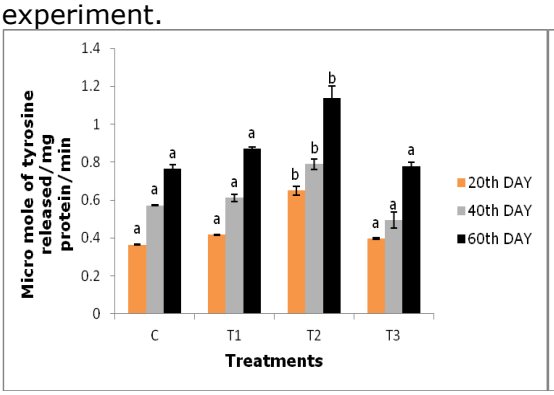

Fig 1: Protease activity of different treatments during 60 days of feeding trial with probiotics. Bars with different superscripts differ significantly (p<0.05) between treatments.

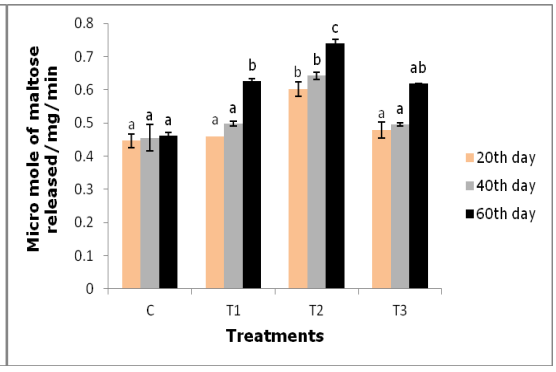

Fig 2: Amylase activity of different treatments during 60 days of feeding trial with probiotics. Bars with different superscripts differ significantly (p<0.05) between treatments.

Lipase activity (Fig. 3) was significantly higher (P<0.05) in all the probiotic treated shrimp compared to the control at the end of the experiment.

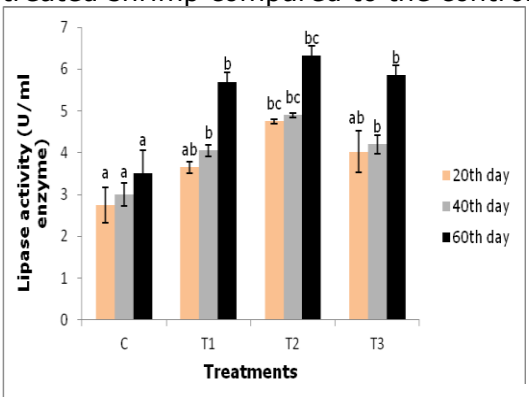

Fig 3: Lipase activity of different treatment groups during 60 days of feeding trial with probiotics. Bars with different superscripts differ significantly (p<0.05) between treatments.

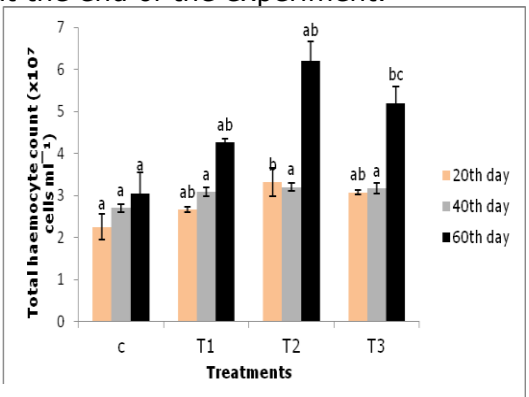

Fig 4: Total hemocyte count of different treatments during 60 days of feeding trial with probiotics. Bars with different superscripts differ significantly (p<0.05) between treatments.

Immunological parameters. Total hemocyte count (Fig 4.), which is the indicator of cellular immunity in crustaceans was higher in all the probiotic treated shrimp than the control throughout the experiment. T2 was significantly higher than the control on day 60. Total serum protein expressed in g/dl (Fig 5) did not differ significantly among probiotic treated shrimp but was always higher than the

control group. Phenoloxidase activity (Fig 6) of T2 group was significantly (<0.05) higher than the other groups by the end of the experiment.

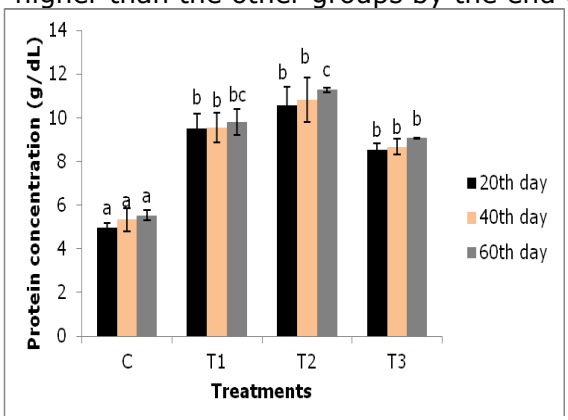

Fig 5: Total serum protein of different treatments during 60 days of feeding trial with probiotics. Bars with different superscripts differ significantly (p<0.05) between treatments.

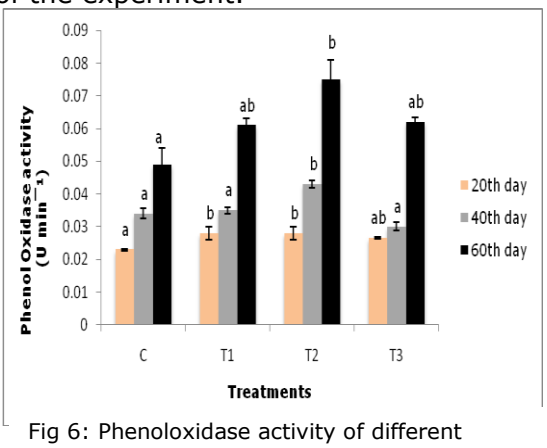

Fig 6: Phenoloxidase activity of different treatments during 60 days of feeding trial with probiotics. Bars with different superscripts differ significantly (p<0.05) between treatments.

Superoxide dismutase activity (Fig. 7) of T2 was elevated significantly (p<0.05) compared to all the other treatments at day 60. The respiratory burst activity (Fig. 8) of phagocytes of T2 was always higher than the other treatments at all the sampling points.

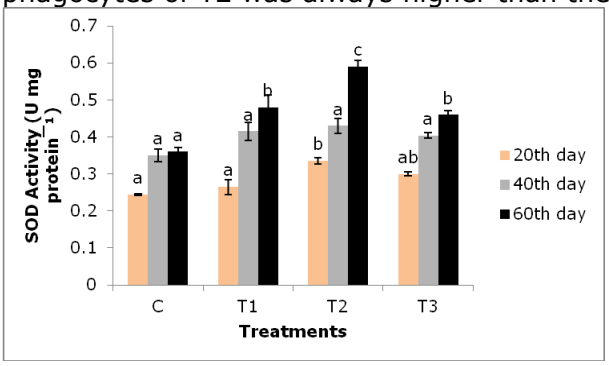

Fig 7: Superoxide dismutase activity of different treatments during 60 days of feeding trial with probiotics. Bars with different superscripts differ significantly (p<0.05) between treatments.

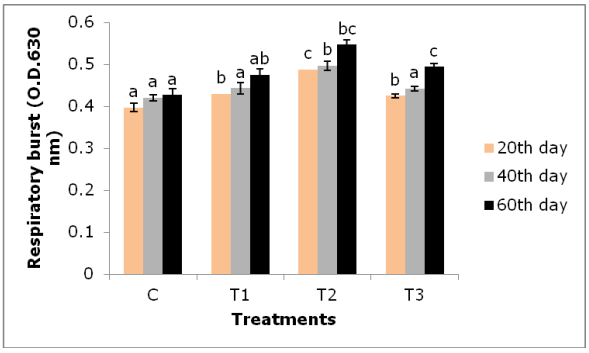

Fig 8: Respiratory burst activity of different treatments during 60 days of feeding trial with probiotics. Bars with different superscripts differ significantly (p<0.05) between treatments.

Discussion

Many previous studies report that crustaceans fed with probiotic treated feed grew better than those fed with untreated feed (Ziaei-Nejad *et al*., 2006; Venkat *et al*., 2004). The same has been reported for *L. vannamei* (Wang, 2007). Similarly in the present study all the probiotic treatments resulted in higher average body weight, specific daily growth rate, and lower FCR compared to the control. The combined addition of two probiotics produced the best results in terms of weight gain, SGR, and lower FCR. The combined probiotics (photosynthetic bacteria and *Bacillus* sp. isolated from carp ponds) also induced better growth performances in carp than a single probiotic bacterium (Wang and Xu, 2006). This may be due to the colonization of probiotic bacteria that are known to secrete exo-enzymes that improve digestion and utilization of feeds.

In order to compare the ability of probiotics to influence the digestive system of shrimp, enzymatic activity in the gut of treated shrimp was evaluated. Even though *Bacillus sp.* are known to enhance protease activity in the intestine, in the present study significantly higher activity (P<0.05) was observed in the mixed probiotic treatment only. Amylase activity, one of the major carbohydrases which hydrolyzes glycosidic links between sugar residues in large carbohydrate

molecules, varied significantly among all the treatments on day 60. It was highest in the mixed probiotics treatment and lowest in the control throughout the experimental period. Lipase activity was also higher in probiotic treated shrimp and was highest in the mixed probiotics treatment.

The digestive enzyme assay of the present investigation confirms that *Bacillus* spp enhanced the digestive physiology in *L. vannamei*. As indicated in previous reports, Gram-positive bacteria, particularly the members of the genus *Bacillus* secrete a wide range of exo-enzymes (Moriarty, 1996; 1998; Zhou *et al.* 2007). It appears that the combination of more than one supplemented probiotic bacterium, rather than one individual probiotic, improves digestive enzyme activity in shrimps.

The effect of probiotic treatments on immunity in shrimp was evaluated with indicators such as THC, serum protein level, PO assay, SOD and respiratory burst activity. THC is one of the most widely used cellular parameters to describe the health status of crustaceans (Bachère, 1995; Li *et al.,* 2007). As indicated in the previous report by Gullian *et al*. (2004), THC did not differ significantly during the initial phase of the experiment (until day 40). But on day 60 there was a sharp increase ($P<0.05$) in THC in the treated groups compared to the control and was highest in group which received the combined probiotic treatment. Increase in serum protein content is considered to have a protective effect of the immune system of shrimp (Downs *et al.,* 2001; Campa-Cordova *et al.,* 2002; Zhao et al. 2013). In this study the serum protein content remained stable from day 20 to day 60 in all the treatments but was always higher in the probiotic treatments. Similar observations were observed in *L. vannamei* treated with *B. licheniformis* (Li *et al.* 2007). In the present study, twice the protein concentration in the serum was found in the mixed probiotic treated shrimp than in the control group. Phenoloxidase (PO) is considered as one of the most important indicators of immune enhancement in shrimp (Gullian *et al.,* 2004, Tseng *et al.,* 2009; Rengpipat *et al*., 2000). In the present study PO activity differed significantly ($P<0.05$) in the treatment groups from the control. It was highest in mixed probiotic fed shrimp and lowest in the control group.

Superoxide dismutase (SOD) is one of the main enzymes in antioxidant defense pathways to oxidative stress (Fridovich, 1995). There was a significant difference in SOD values ($p<0.05$) among all the treatments at day 60. Highest rate of SOD was found in shrimp fed combined probiotic bacteria. Increased respiratory burst activity correlates with increased bacterial pathogen killing activity of phagocytes (Sharp *et al*., 1993). In the present study, respiratory burst activity differed significantly between the different treatments. After 40 days the mixed probiotics treatment produced the highest increment in activity. *L. vannamei* fed with one probiotic bacteria alone exhibited no significant difference in respiratory burst activity even after 98 days (Tseng *et al.,* 2009). This demonstrated that a mixture of probiotics is superior to individual bacteria.

These immunological parameters indicate that combined rather than individual probiotic treatments have a greater impact on immunity. Moreover many parameters improved during the initial phase itself (~ 20 days) with the exception of THC and SOD where significant effects were seen later on.

The present study clearly demonstrates that probiotic treatments especially when two applied probiotic bacteria are supplemented simultaneously could effectively enhance both digestive enzyme activity and non-specific immunity in *L. vannamei*. This suggests that the combination of bacteria had a synergistic effect, which boosted both the digestive and immune system simultaneously. Even though there have been some reports regarding the effect of probiotics on digestive enzymes and immunity of shrimp, little research into the comparison of the two functionally different probiotic bacteria supplemented individually and in combination has been made. The findings in this study will help in formulating new combinations of probiotics in shrimp farming to enhance both the digestive system and the immune system in shrimp.

Acknowledgements

The authors are grateful to Dr. W. S. Lakra, The Director, Central Institute of Fisheries Education, Mumbai for the continuous encouragement to carry out the research work.

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