Preliminary Study on the Use of *Bacillus* sp., *Vibrio* sp. and Egg White to Enhance Growth, Survival Rate and Resistance of *Penaeus monodon* Fabricius to White Spot Syndrome Virus

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ABSTRACT : Research in low cost feeds with high nutritional value and immunogenicity is important to reduce production cost and increase yields in the shrimp industry. In this study, immunostimulants of bacterial origin (peptidoglycan and lipopolysaccharides) and egg white were incorporated in shrimp diets as feed additives to determine the growth, survival and tolerance of *Penaeus monodon* to white spot syndrome virus (WSSV). Although the results obtained were not statistically significant ($p>0.05$) among the treatments, shrimp fed with bacterial additives and egg white showed higher weight gain, specific growth rate and survival than those fed on commercial shrimp diet. Shrimp fed with artificial diet showed 100% mortality when challenged with WSSV. However, shrimp fed on peptidoglycan supplemented diet had higher survival than their counterpart, whereas shrimp fed on egg white supplemented diet had a higher specific growth rate and better tolerance when challenged with WSSV. Further studies are required to determine the effectiveness and optimization of bacterial strains and egg white as feed additives to increase production and enhance the shrimp immune response to diseases. *(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No.10 : 1477-1482)*

Key Words : *Penaeus monodon*, Immunostimulant, Bacteria, Egg White

INTRODUCTION

Penaeid shrimp farming, due to its high potential profits and generation of foreign exchange, is the most important and extensively cultured crustacean in the world. However, the intensive culture of black tiger shrimp (*Penaeus monodon*) is subjected to various kinds of stress that may lead to immunosuppression leading to development of diseases. Diseases caused by various pathogens have inflicted high mortalities of shrimp larvae resulting in serious losses (Shariff and Subasinghe, 1992). Most of these mortalities and disease outbreaks are as a result of stress due to overcrowding, exposure to pollutants, deterioration of water quality and secondary infections by ubiquitous pathogens (Subasinghe and Shariff, 1994). Thus maintenance of good water quality is crucial, in addition to the enhancement of resistance of the organisms against pathogens.

The use of antibiotics is a common practice for the treatment of diseases. But abuse of antibiotics is leading to widespread pathogen resistance and residues (Lopez, 2000). A recent development to increase growth and resistance in cultured animals is the dietary incorporation of immunostimulants to both fish and crustaceans. Immunostimulants act as effective means of increasing the immunocompetency and disease resistance of fish. Immunostimulants, such as chemicals, bacterial components, polysaccharides, animal or plant extracts, nutritional factors and cytokinins are currently in use in the aquaculture industry (Sakai, 1999).

Yasuda and Taga (1980) anticipated that bacteria would be useful not only as food for cultured aquatic species but also as biological controllers of disease. Recent studies on the use of microorganisms in the control of microbial infections in shrimp larvae rearing have shown promising results (Gil, 1995). Soderhall (1982) proved that non-specific immune responses of shrimp can be evoked by bacterial and yeast cell wall, lipid and detergent. Vargas-Albores et al. (1998) have showed that lipopolysaccharides (LPS) and ß-glucans are involved in the stimulation of cellular functions in shrimp. Both LPS and ß-glucans are capable of simulating shrimp hemocytes to release cellular components. ß-glucan stimulates the pro-phenoloxidase activating system whereas LPS can stimulate both the pro-phenoloxidase system and the coagulation system by activating different hemocyte populations. It has also been shown that immunostimulation of shrimp can be achieved through oral administration of *Vibrio* bacterin and yeast glucan (Devaraja et al., 1998). Egg white is said to contain lactoferrin and lysozyme that have bacteriocidal and bacteriolytic effect (Sohn et al., 2000). Therefore egg white was chosen to serve the dual purpose of binder and as an immunostimulant.

White spot syndrome virus (WSSV) has caused severe

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losses in Asia and Latin America (Flegel, 1997). Since there is no treatment for WSSV, the use of immunostimulants as a possible prophylactic remedial measure would help reduce losses to the shrimp industry. This study was designed to determine the effects of immunostimulants present in indigenous marine bacteria and egg white on the growth, survival and tolerance of *P. monodon* postlarvae challenged with WSSV.

MATERIALS AND METHODS

Bacteria culture

Bacillus sp. (Gram positive) and *Vibrio* sp. (Gram negative) were isolated from a shrimp pond in Kuala Selangor, Malaysia. They were grown at 37°C in particle free (filtered with 0.2 µml membrane filter paper) tryptone soy broth (TSB, Oxoid) supplemented with 2% sodium chloride (NaCl). Bacteria were harvested on the next day by centrifugation at 12,000 rpm for 15 min at 10°C. The resultant pellets were collected and freeze dried for later use.

Diet formulation

Artificial diet, in the form of pellets, supplied by Global Star Prawn Feed Company was used as the control diet in the experiment (Diet 1). The proximate composition of control diet was 43% crude protein, 7% crude fat and 12% moisture. The feed size was 1.0 to 1.5 mm. Artificial diet and freeze dried cells of *Bacillus* sp. were mixed in the ratio of 100 g : 0.1 g. After mixing the artificial diet and the bacteria together, 15 ml of egg white was added to bind the bacteria to the pellet (Diet 2). The same procedure was followed for Gram negative bacteria (*Vibrio* sp.) (Diet 3). Diet 4 consisted of artificial feed with egg white only. The diets were dried in an oven below 40°C to avoid denaturation of fatty acid content and stored in bottles at room temperature.

Experiment 1: Growth and survival experiment

Penaeus monodon postlarvae (PL12, 35 day old larvae) were purchased from a nearby shrimp hatchery and stocked in 300 l fiberglass tanks. They were acclimated to laboratory conditions for one week. During this period, the PLs were fed with *Artemia*, microalgae and artificial feed *ad libitum*.

When the post larvae reached PL19, they were randomly selected and 50 individuals were kept in 20 l aquaria tanks provided with constant aeration. The seawater used in the experiments was previously filtered and disinfected with chlorine (10 mg/l). The study consisted of four treatments with three replications per treatment. The shrimp were fed *ad libitum* once a day in the afternoon with the four respective diets for a period of six weeks. Feed intake was observed for 30 min to ensure sufficient feed was given. The weight and survival of the shrimp were recorded at the beginning and at the end of the experiment. To determine the increase in length, 10 shrimp were sampled randomly from each tank every week. Water temperature, ammonia content, dissolved oxygen and salinity were monitored weekly. Data were statistically analyzed by taking the mean of the replicates of the different treatments and using one-way analysis of variance (ANOVA) to determine significant differences between final length, weight gain, survival rate and specific growth rate between the different treatments at $p<0.05$.

Experiment 2: Challenge test with white spot syndrome virus (WSSV)

Resistance of shrimp to WSSV was evaluated after six weeks of feeding with the three experimental diets (Diet 2, 3 and 4) and the control diet (Diet 1). Ten shrimp taken from each tank of experiment 1 were divided into two groups (five in each group). One group was infected with WSSV by feeding on the first day with virus infected shrimp carcass. The other group was kept as control and given feed as mentioned in experiment 1. The shrimp were maintained in 700 ml seawater (salinity 26 ppt). The experiment was run for one week and during the period, both groups were fed as in experiment 1. Data on survival were analyzed using analysis of variance (ANOVA) and chi-square ($p<0.05$).

RESULTS AND DISCUSSION

Growth and survival rate

Growth and survival rates of the shrimp were not statistically different ($p>0.05$) amongst treatments during the 42-day period before the challenge (table 1). However, shrimp fed with *Bacillus* sp. showed the best survival, whilst those fed on egg white had the best growth. In general, there was higher growth and survival rate in the treated group than the control. This is in line with several authors who reported that immunostimulants promoted growth. Boonyaratpalin et al. (1995) showed that 0.01% peptidoglycan (PG) supplemented feed when given to *P. monodon* showed better growth and feed conversion rates than those fed with a normal diet or with 0.1% PG supplemented feed (the highest level administered). Sung et al. (1994) reported that *P. monodon* grew faster with 0.5, 1 and 2 mg/ml glucan immersion than at 0.25 mg/l or in control solutions. Kozasa (1986) also reported that *Bacillus toyi* isolated from soil and used as feed additive increased the growth rate of yellowtail (*Seriola quinqueradiata*). However, Itami et al. (1998) reported that there was no difference in the average body weight in *Penaeus japonicus*

Table 1. Weight, survival and specific growth rate of *Penaeus monodon* fed with different diets after 42 days. Values are means ± SE. n=3 tanks. There is no significant difference ($p>0.05$) amongst the treatments.

	Artificial feed Diet 1	Artificial feed+ *Bacillus* sp.+ egg white Diet 2	Artificial feed+*Vibrio* sp. + egg white Diet 3	Artificial feed+egg white Diet 4
Number of shrimp	150	150	150	150
Final weight (mg)	25.4 ± 2.0	24.7 ± 4.4	29.9 ± 3.3	31.2 ± 5.0
Weight gain (%)	140.5 ± 18.8	133.3 ± 41.9	182.7 ± 31.1	195.0 ± 47.3
Final length (mm)	21.2 ± 0.6	21.9 ± 1.3	21.7 ± 0.6	21.7 ± 1.1
Survival (%)	31.3 ± 2.7	55.3 ± 6.7	48.7 ± 10.0	54.7 ± 4.7
Specific growth rate (%)	4.4 ± 0.8	4.1 ± 1.7	6.1 ± 1.3	6.6 ± 2.0

Weight gain (%) $=(W_f - W_i) / W_i \times 100$, where W_i =initial weight, W_f=final weight.

Table 2. Lipid and fatty acid composition of *Bacillus* sp. and *Vibrio* sp

	Bacillus sp.	*Vibrio* sp.
Lipid (% dry weight of sample)	8.0	4.5
Fatty acids (% of total fatty acid)		
Palmitic acid	55.6	32.6
Palmitoleic acid	-	14.0
Stearic acid	9.8	4.5
Vaccenic acid	-	4.5
Oleic acid	-	15.6

between PG fed and the control group until day 60. But at the end of day 95, the body weight of PG fed and control were 2.5 and 2.0 g respectively. In the present study, no significant difference in growth and survival rates were observed among treatments probably due to the relatively short feeding period of 42 days.

When the survival rates of all the treatments with egg white were pooled (52.9%) and compared with the control (31.3%), before the challenge, they were found to be significantly different ($p<0.05$). Mortality is a common problem in larval and postlarval stages fed with processed diets (Jones et al., 1979). Mortality can be minimized if diets have the proper energetic balance and better retention mainly of the protein components (Bages and Sloane, 1981). In our experiment, the factor that could have contributed to the increased survival may be the presence of protein in the egg white. Egg white contains 9.4 g of protein per 100 g of edible raw liquid (Cotterill et al., 1977). Use of egg white as binder could have increased the protein content in the feed leading to an increase in the survival rate of shrimp compared to the control group.

In the present experiment, lipid content in the *Bacillus* sp. and *Vibrio* sp. was 8% and 4.5% respectively (table 2). In addition, the bacteria, *Bacillus* sp. and *Vibrio* sp. contained saturated and monounsaturated fatty acids (MUFA) (table 2). Use of dietary lipids in shrimp diets has been recognized for many years not only as a source of energy but also as a source of essential fatty acids (EFA), sterols, phospholipids and fat soluble vitamins (Lim et al., 1997) for normal growth and survival. Chen (1993) has documented the beneficial effects of increased growth and/or survival due to inclusion of dietary phospholipids for larval and juvenile stages of *P. monodon*. Recommended lipid levels for commercial shrimp feeds range from 6% to 7.5% and a maximum level of 10% has been suggested by Akiyama et al. (1991). In our experiment, *Bacillus* sp. and *Vibrio* sp. had lipid contents of 8% and 4.5% respectively. Although there was no significant difference ($p>0.05$) in growth among the different treatments, there was an overall improvement in growth and survival rate in shrimp fed diets supplemented with bacteria and egg white. The result also showed that peptidoglycan supplemented feed (Diet 2) did not perform well to promote a better growth among the feed additives but had the highest survival rate with regards to the rest of the treatments. Studies conducted by Kanazawa et al. (1985) have also demonstrated significant effects of phospholipid supplementation on survival in larval *Penaeus japonicus*.

Challenge trials

The results of ANOVA and chi-square analyses did not show any significant difference in the survival rate amongst the treatments ($p>0.05$). However survival rates were comparatively higher among shrimp which received the experimental diets (Diets 2, 3 and 4) prior to challenge compared to the control group of shrimp receiving artificial diet without any feed additives. In fact, when all the treatments having egg white were pooled and compared with the control, the survival rate was significantly higher ($p<0.05$) in the diet containing additives. The highest survival rate was found in shrimp fed artificial feed coated with egg white (35%, Diet 4) followed by *Bacillus* sp. (15%, Diet 2) and *Vibrio* sp. (8%, Diet 3) supplemented feed respectively. There was 100% mortality in WSSV challenged shrimp fed with the artificial diet (Diet 1) only (fig. 1). This is comparable to the result of Itami et al. (1994) who reported that oral administration of ß-1,3-glucan tends to enhance disease resistance in *Penaeus*

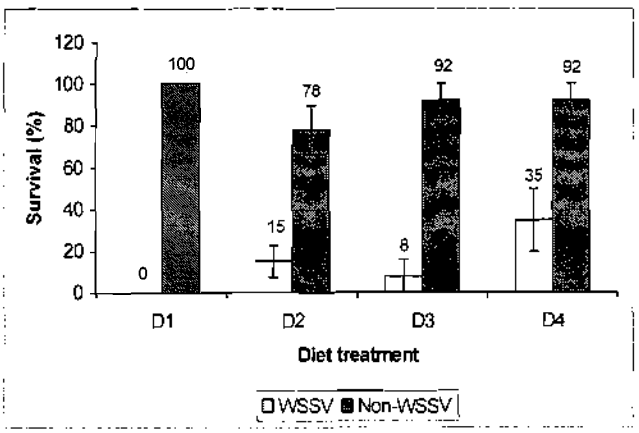

Figure 1. Survival of *Penaeus monodon* fed different diets and challenged on day 42 with white spot syndrome virus. Number of shrimp per treatment =15
D1- Artificial feed
D2- Artificial feed + *Bacillus* sp. + egg white
D3- Artificial feed + *Vibrio* sp. + egg white
D4- Artificial feed + egg white

japonicus and the results were not statistically different. Performance of Diet 4 was better, probably because of the presence of lysozyme in egg white. Lysozyme from chicken egg white has been found to possess activity against human immunodeficiency virus type 1 (HIV-1) (Lee-Huang et al., 1999). In addition, the lysozyme can destroy bacterial cells due to its ability to cleave peptidoglycan bonds (Garrett and Grisham, 1995). Furthermore, studies conducted by Yoshida et al. (1993) showed that rainbow trout (*Oncorhynchus mykiss*) fed with fermented products of chicken egg had higher phagocytic activities and higher resistance to natural bacterial infection.

Performance of shrimp fed with Diet 2 and Diet 3 containing PG and LPS respectively was comparatively better than the control group (Diet 1) in the present experiment (fig. 1). This result is analogous to those reported for rainbow trout fed with PG at different dosages which provided significant protection against challenge with *Vibrio anguillarum* (Matsuo and Miyazono, 1993). Itami et al. (1998) also reported that PG derived from *Bifidobacterium thermophilum* and fed to Kuruma shrimp (*Penaeus japonicus*) for 95 days were better able to withstand challenge than non-treated ones.

The continuing effort to improve animal performance (growth and survival) and feed efficiencies has stimulated a search for new additives for shrimp feeds. These additives are used in small amounts and could improve performance and feed efficiency by 10% to 25% (Pillay, 1990). Immunostimulants can protect fish from several infectious diseases and decrease mortality rates (Sakai, 1999). However, Sakai (1999) added that the efficacy of the immunostimulant will depend on several factors such as right feeding regimes, dosages, methods of administration, and physiological condition of fish.

Our present result showed that the indigenously isolated bacteria and the egg white increased the resistance of shrimp to WSSV infection. It is likely that the bacterial feed additives and egg white stimulated the non-specific immune system of shrimp (Soderhall and Hall, 1984; Anderson, 1992; Sung et al., 1996). However, there are several factors such as feeding frequency and duration, dosage, and additional additives which may influence the bacteria and egg white to function as immunostimulants. Kitao et al. (1987) reported that high doses (10 μg/kg) of the short chain peptide immunostimulator FK 565, did not increase the number of plaque forming cells (PFC) against *Yersinia ruckeri* in rainbow trout (*Salmo gairdneri*) and Atlantic salmon (*Salmo solar*) although the optimum dose (5 μg/kg) increased PFC. The long term effects of oral administration of immunostimulants are still not clear. Yoshida et al. (1995) reported that the number of potential killing activity (NBT)-positive cells in African catfish (*Clarias gariepinus*) increased following oral administration of glucan or oligosaccharide over 30 days but not over 45 days. In another trial Matsuo and Miyazono (1993) reported that rainbow trout (*Oncorhynchus mykiss*) treated with peptidoglucan orally for 56 days did not show protection when challenged with *Vibrio anguillarum*, although fish treated for 28 days showed increased protection. Newman (1997) had proposed several different feeding regimes. Among these are feeding every five days on and five days off for first two months or every second day for two months. Thus the effective administration period for each immunostimulant should be thoroughly investigated which Sakai (1999) has also emphasized. Purity of product (PG and LPS) may also influence the desired/maximum effect of immunostimulants. The use of whole bacterial cell and raw egg white in our experiment may not be as efficient as the use of pure extracted product. Whole bacterial cell and raw egg white may contain other compounds that may inhibit immunogenic reactions.

The use of immunostimulants is a new concept and their mode of action is poorly understood (Sohn, 2000). Much work remains to be done to determine their cost effectiveness and to optimize their use (Newman, 1997). In our experiments it appeared there was a benefit in terms of increased disease resistance, higher survival and growth rate in *P. monodon* PL fed with indigenous isolated bacteria as a source of LPS and PG and egg white compared to the control group fed with commercial shrimp feed without additive. The indigenous bacteria and egg white holds potential to enhance the immune response of shrimp. Future trials are needed to evaluate factors for enhancing the effectiveness of bacteria and egg white as immunostimulants.

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