http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (9): 1481-1485, 2007
ISSN 1028-8880

Effect of Feeding *Bacillus* sp. As Probiotic Bacteria on Growth of Giant Freshwater Prawn (*Macrobrachium rosenbergii* de Man)

[1]Sirirat Deeseenthum, [2]Vichai Leelavatcharamas and [3]John D. Brooks
[1]Graduate School, Khon Kaen University, Thailand 40002
[2]Department of Biotechnology, Faculty of Technology, Khon Kaen University, Thailand 40002
[3]Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand

Abstract: The effects of feeding two *Bacillus* spp. isolated from the intestine of the giant freshwater prawn on the growth of Giant Freshwater Prawns (*Macrobrachium rosenbergii* de Man) was examined. The isolated *Bacillus* KKU02 and *Bacillus* KKU03 (~10^7 CFU mL^{-1}) were mixed into commercial prawn feed (200 mL kg^{-1}). After rearing shrimp with the bacteria in four feed treatments, (*Bacillus* KKU03, *Bacillus* KKU02, mixed culture and control groups) for 120 days, body length and weight of the prawns in mixed culture tanks were significantly higher ($p = 0.05$) than in the control tanks (7.48 cm and 3.32 g, vs 6.6 cm and 2.1 g, respectively). Both isolates were found to produce amylase and protease. The stabilities of the single *Bacillus* sp., mixed culture and commercial probiotic in the feeds were examined during storage at 4°C and room temperature. The percentage viability of *Bacillus* KKU02, *Bacillus* KKU03 and mixed culture stored at room temperature declined dramatically to 2.54, 21.88 and 10.92% within 2 weeks, respectively. At 4°C however, the percentage viability of the tested probiotics reduced slowly. The survival of the commercial probiotics was the same at both temperatures about 50% after 70 days' storage.

Key words: Giant freshwater prawn, probiotic, *Bacillus* sp.

INTRODUCTION

During the past 20 years, the aquaculture industry, especially the production of shrimps, has grown tremendously. Recently, shrimp culture all over the world has been frequently affected by viral and bacterial diseases. Disease is a major problem for the shrimp farming industry, which currently is the fastest growing food-protein producing sector, with an annual increase of approximately 9%. Pathogenic microorganisms implicated in these outbreaks were viruses, bacteria, rickettsia, mycoplasma, algae, fungi and protozoan parasites. Infectious bacterial diseases in aquaculture are of major concern to the industry. Although vaccines are being developed and marketed, they cannot be used alone as a universal disease control in aquaculture. Treatment with antibiotics and chemotherapeutics continues to be an important disease control measure, though the illegal and excessive use of chemicals has caused governments to increase testing and to ban imports of some fish and crustacean products. Development of bacterial resistance to antibiotics has been well documented and the fear of the spread of this resistance to human pathogens (Amabile-Cuevas *et al*., 1995) has recently led to the banning of several antibiotics as so-called growth promoters in animal husbandry within the European Union. This concern has also been raised in the aquaculture industry and has led to suggestions for other disease control measures, including the use of live non-pathogenic bacteria as probiotics (Westerdahl *et al*., 1991; Smith and Davey, 1993; Austin *et al*., 1995; Bly *et al*., 1997; Gatesoupe, 1994; Graam *et al*., 1999).

Recently, attention has focused on the use of probiotics, using methods developed for agriculture for which the mechanisms of action have been well defined. Most probiotics are supplied as live supplements in feed and must have the ability to survive passage through the intestinal tract. The benefit to the host may arise as a nutritional effect, whereby the bacteria are able to break down toxic or otherwise non-nutritious components of the diet, which the host can then digest. Alternatively, the probiotic may prevent potential pathogens from colonizing the gut by production of antimicrobial compounds, or by out-competing them for nutrients or mucosal space (Roberson *et al*., 2000).

The objective of this research was to study the response of giant freshwater prawns to different modes of probiotic treatment in the feed.

Corresponding Author: Vichai Leelavatcharamas, Department of Biotechnology, Faculty of Technology, Khon Kaen University, Thailand 40002 Tel: 66-815443172 Fax: 66-843362121

MATERIALS AND METHODS

Experimental conditions: The experiment was run for 120 days at the Department of Biotechnology, Khon Kaen University, Khon Kaen, Thailand. The experiment was divided in four experimental groups, each with triplicates, in 12 concrete tanks (1.50×2.00×1.00 m). The total water in each tank was maintained at 1,500 L and aeration was continuously provided to all the containers. All the tanks were cleaned and about 30% of water was replaced with fresh water every week.

The details of the experimental groups were as follows: T_1 Commercial diet + *Bacillus* KKU03; T_2 Commercial diet +*Bacillus* KKU02; T_3 Commercial diet +*Bacillus* KKU02 +*Bacillus* KKU03 and T_4 Commercial diet (control). The commercial feed used was from Charoenpokapan Company, Samut Sakhon, Thailand (CP No. 9041) The feed was autoclaved at 110°C 15 min before mixing with the cultures.

Bacterial strains and feeding regime: *Bacillus* KKU 03 and *Bacillus* KKU 02 used as probiotics in this research were obtained from the culture collection, Department of Biotechnology, Khon Kaen University. The isolated *Bacillus* sp. were grown in nutrient broth (NB) for 24 h at 37°C on a shaker at 150 rpm to a final concentration of about 10^7 CFU mL^{-1} and mixed at the rate of 200 mL to 1 kg feed. Prawns were fed twice daily, at 8.00 and 16.00 h. The daily feeding rate was ~10% of total body weight.

Preparation of experimental animals: Post larvae of giant freshwater prawn (PL-15) were obtained from a hatchery located in Supanburi, Thailand. The prawns were acclimatized in the tanks for 2 weeks feeding with only commercial feed. Two hundred PL-15 of uniform size (0.11-0.12 g) were kept in each tank.

Analysis of samples: Ten randomly collected live prawns from each tank were measured for lengths and weights once every 30 days.

Water quality was checked weekly for pH, dissolved oxygen and temperature. The amount of ammonium, nitrate, nitrite and total alkalinity were also determined by test kits obtained from Prima Tech Company, Bangkok, Thailand.

Statistical analysis: One-way analysis of variance (ANOVA) to determine any significant differences among the treatment groups and comparison between four treatments were done using Duncan's Multiple Range Test (DMRT) using SPSS for window XP.

Longevity of the probiotics in prawn feed: Aerobic plate counts were performed on nutrient agar plates and incubated at 37°C for 24 h for comparison of the test feeds and the commercial probiotic mixed feed. The commercial probiotic (Biopro®) was obtained from M.D. Synergy Co. Ltd., Bangkok, Thailand. The probiotic (10^7CFU mL^{-1}) was mixed in the feed at the rate of 200 mL to 1 kg feed.

Enzyme production by the probiotic cultures: Amylase, lipase and protease production by the selected cultures, *Bacillus* KKU02 and *Bacillus* KKU03, were tested using starch agar, tributyrin agar and skim milk agar, respectively.

The formulations of agars were as follows:

Starch agar: Soluble starch (Diffco) 10.0 g L^{-1}, beef extract 3.0 g L^{-1} (BDH), agar 12.0 g L^{-1} adjust volume by deionised water to 1,000 mL, pH 7.5.

Tributyrin agar: peptone from meat (Oxoid) 2.5 g L^{-1}, proteose peptone (Oxoid) 2.5 g L^{-1}, yeast extract (Diffco) 3.0 g L^{-1}, agar 12.0 g L^{-1}, tributyrin (Oxoid) 100 mL. Adjust volume to 1 L by deionised water, pH 7.5.

Skim milk agar: skim milk 100 g L^{-1}, agar 20 g L^{-1} adjust volume to 1,000 mL by deionised water, pH 6.3.

The strains were grown in nutrient broth at 37°C on a shaker at 150 rpm for 12 h before testing by the well diffusion method and by streaking on the specific agar plate. One hundered milliliter of cultured medium were loaded into each well (diameter 7 mm). The plates were held at room temperature for 24-48 h (72 h for tributyrin agar). After growth, the starch agar plates were flooded with iodine solution.

RESULTS AND DISCUSSION

Prawn production: There were significant differences ($p = 0.05$) of body weight and length gain between T_3 and the control group during probiotic feeding (Fig. 1A and B). The prawns fed mixed culture exhibited the highest Figures at 3.325 g and 7.487 cm, respectively. However, the probiotic treatment groups T_1 and T_2 were not significantly different from each other ($p \geq 0.05$) or the control group. There have been previous reports that using a mixed culture feed resulted in better shrimp growth. Thimmalapura *et al.* (2002) investigated shrimp (*Penaeus monodon*) production in ponds treated with two commercial mixed microbial products. There were significant differences in feed conversion ratio in the pond treated with a product containing *Bacillus* sp. and *Saccharomyces* sp. compared with ponds treated with

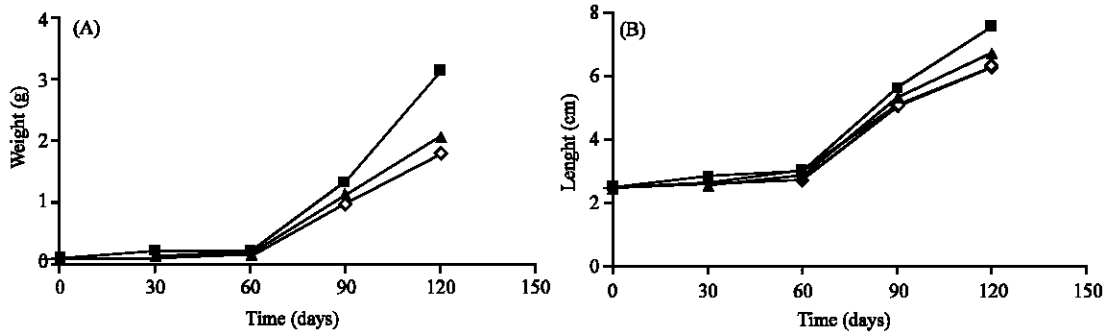

Fig. 1: The body weight (A) and length (B) of Giant freshwater prawn after rearing for 120 days with isolated bacteria in four feed treatments : ♦ = T_1, (*Bacillus* KKU03); ◇ = T_2, (*Bacillus* KKU02); ■ =T_3 (*Bacillus* KKU02 + *Bacillus* KKU03) and ▲ = T_4, Control

Table 1: Ranges of water quality parameters during experiments

Treatments	pH	Temperature (°C)	DO (mg L^{-1})	Ammonia (mg L^{-1})	Nitrite (mg L^{-1})	Total Alkalinity (mg L^{-1})
T_1	6.83-8.25	24-29	4.41-9.80	0-0.33	0.1-0.3	79.33-170
T_2	6.51-8.05	24-29	4.75-9.50	0-0.40	0.1-0.3	73.67-125
T_3	7.05-8.24	24-29	4.68-9.5	0-0.30	0.1-0.3	73.64-164
T_4	7.0-8.82	24-30	4.6-10.3	0-0.33	0.1-0.3	73.67-136

Table 2: Enzyme production of *Bacillus* KKU02 and *Bacillus* KKU03

	Diameter of clear zone (mm)		
Strain	Amylase activity	Protease activity	Lipase activity
Bacillus KKU 02	10	5	0
Bacillus KKU 03	12	11	0

Nitrosomonas sp. and *Nitrobacter* sp. and untreated controls. Likewise, Gatesoupe (1991) reported a significant difference in turbot larvae weight when feeding with bioencapsulated Lactic acid bacteria (LAB) and *Bacillus toyoi*.

Water quality: The water quality in each shrimp culture ponds is shown in Table 1. Saxena (2003) reported that the optimum parameters for giant freshwater prawn culture were pH = 7.5-8.5, Temperature = 25-32°C, Total alkalinity concentration = 50-150 mg L^{-1}, DO (Dissolved oxygen) = 4 mg L^{-1}, Ammonia concentration = 0.1 mg L^{-1}, Nitrite concentration = 0.1 mg L^{-1}. Our results revealed that all of the measured parameters were in the acceptable ranges suggested by Amstrong *et al.* (1976) Hsieh *et al.* (1990) and New (1990). However, the season changed to winter 60 days into the experiment and the water temperature was slightly low at 24°C for two weeks and then increased.

Enzyme production by the isolates: Both the probiotic strains gave positive results in starch agar (amylase) and skim milk agar (protease) but lipase activity was not detected in tributyrin agar (Table 2). These extracellular enzymes are also the main enzymes in the digestive tract involved in breaking down organic material in the feed matter. Increased growth yield of shrimps might be partly the result of better digestion and assimilation of nutrients in the feed resulting from the exoenzymes produced by the modified gut microflora. Bacteria, by virtue of their exoenzymes, have been reported to play an important role in the process of digestion in turbot larvae (Munilla-Moran *et al.*, 1990). However, an alternative explanation for the increased yields observed in the present trials might be the suppression of pathogenic micro-organisms in the gut by the probiotic strains.

Shelf life of the probiotic in prawn feed: The stability of the pure *Bacillus* and mixed *Bacillus* spp. and commercial probiotic were examined in the feeds, which were stored at 4°C and room temperature (Fig. 2). The viability of all the probiotics was detected consistently for 70 days. It was found that the % viability of *Bacillus* KKU03, *Bacillus* KKU02 and mixed culture stored at room temperature declined dramatically to 21.88, 2.54 and 10.92% within 2 weeks respectively. Under 4 °C storage, however, the % viabilities of the tested probiotics were reduced more slowly and the *Bacillus* KKU03 behaved similarly to the commercial probiotics. The percentage survival of the commercial probiotics was the same at both temperatures. The average percentage viability of the commercial probiotic was about 50% after 70 days' storage. It appears that the commercial preparation is more stable at room temperature than *Bacillus* KKU02. This was possibly because it has been formulated for having

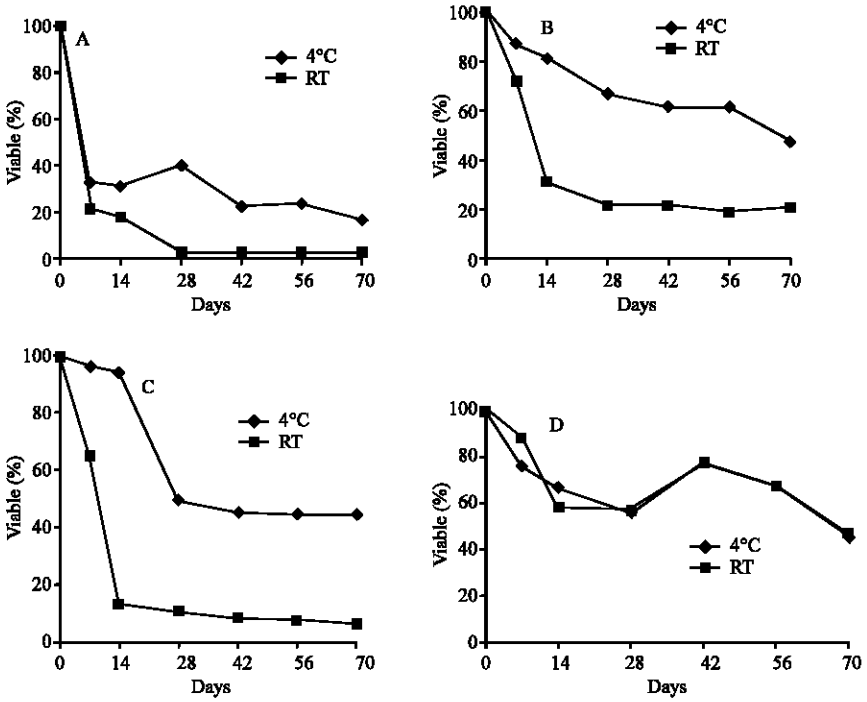

Fig. 2: Viability of probiotics in prawn feed stored at 4°C and room temperature for 70 days. (A) *Bacillus* KKU02; (B) *Bacillus* KKU03; (C) *Bacillus* KKU02+*Bacillus* KKU03; (D) commercial probiotic

a longer shelf life. Before commercialization can be undertaken, it is important to study and optimize the extended survival of the *Bacillus* KKU02 probiotics in shrimp feed.

CONCLUSIONS

The results from these experiments indicate that a significant improvement of growth of *M. rosenbergii* occurred when the feed included a mixed culture of *Bacillus* KKU02 and *Bacillus* KKU03 previously isolated from the intestinal contents of shrimps. There was no difference between the groups fed single strain probiotics and the controls. However, the optimum concentration ratio of these probiotics in the prawn feed is still needed to be determined. These results should also be confirmed in outdoor, earthen pond trials before they are applied commercially. Further investigation of the effect of probiotics on the prawn immune system and a risk assessment to human consumers is continuing and will be reported in a future publication.

ACKNOWLEDGMENT

The authors would like to thank the Thailand Research Fun (TRF), Thailand Toray Science Foundation (TTSF) and the Agro-industry Consortium of Thailand for the financial support of this research. Thank you to the Department of Biotechnology, Khon Kaen University; Department of Biotechnology, Maha Sarakham University; Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand and the Fermentation Research Center for value added agricultural products, Khon Kaen University, Thailand for providing facilities for this research.

REFERENCES

Amabile-Cuevas, C.F., M. Gardenas-Garcia and M. Ludgar, 1995. Antibiotic resistance. Am. Sci., 83: 320-329.

Armstrong, D.A., M.J. Stephenson and A.W. Knight, 1976. Acute toxicity of nitrite to larvae of the giant Malaysian prawn, *Macrobrachium rosenbergii.* Aquaculture, 9: 39-46.

Austin, B., L.F. Stuckey, P.A.W. Robertson, I. Effendi and D.R.W. Griffith, 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. J. Fish Dis., 18: 93-96.

Bly, J.E., S.M.-A. Quiniou, L.A. Lawson and L.W. Clem, 1997. Inhibition of Saprolegnia pathogenic for fish by *Pseudomonas fluorescens*. J. Fish Dis., 20: 35-40.

Gatesoupe, F. J., 1991. The effect of three strains of lactic acid bacteria on the production of rotifers, *Brachinus plicatilis* and their dietary value for laval turbot, *Scopthalmus maximus*. Aquaculture, 91: 342-353.

Gatesoupe, F.J., 1994. Lactic acid bacteria increase the resistance of turbot larvae, *S. maximus*, against pathogenic vibrio. Aquat. Living Res., 7: 277-282.

Graam, L., J. Melchiorsen, B. Spanggaard, I. Huber and T. F. Nielsen, 1999. Inhibition of *Vibrio anguillarrum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. Applied Environ. Microbiol., 65: 969-973.

Hsieh, C.H, N.H. Chao, D. Oliviala, L.A. Gomes and I.C. Liao, 1990. Culture practices and status of the giant freshwater prawn *Macrobrachium rosenbergii* (de Man), in Taiwan. In: Proceedings of the 3rd Brazilian shrimp framing congress, 15-20 October 1990. Joao Pessoa-PB. Brazil.

Munilla-Moran, R., J.R. Stark and A. Barbour, 1990. The role of exogenous enzymes in digestion in cultured turbot larvae (*Scopthalmus maximus* L.) Aquaculture, 88: 337-350.

New, M.B., 1990. Freshwater prawn culture: A review. Aquaculture, 88: 99-144.

Robertson, P.A.W., C. O'Dowd, C. Burrells, P. Williams and B. Austin, 2000. Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). Aquaculture, 185: 235-243.

Saxena, V., 2003. Scientific Guidelines for farmers engaged in freshwater Prawn farming in India. Aquacult. Asia Mag., 8: 17-18.

Smith, P. and S. Davey, 1993. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonas. J. Fish Dis., 16: 521-524.

Thimmalapura, N.D., M.Y. Fatimah and S. Mohamed, 2002. Changes in bacterial populations and shrimp production in ponds treated with commercial microbial products. Aquaculture, 206: 245-256.

Westerdahl, A., J.C. Olsson, S. Kjelleberg and P.L. Conway, 1991. Isolation and characterization of turbot *Scophtalmus maximus*-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied Environ. Microbiol., 57: 2223-2228.