Indian J. Fish., **46**(4) : 367-373, Oct.-Dec., 1999

Effect of a probiotic bacterium, *Lactobacillus plantarum* on disease resistance of *Penaeus indicus* larvae

A. UMA, T. JAWAHAR ABRAHAM* AND V. SUNDARARAJ

Fisheries College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Tuticorin - 628 008, India

ABSTRACT

The effect of a probiotic bacteirum, *Lactobacillus plantarum* on the disease resistance of penaeid shrimp, *Panaeus indicus* (H. Milne Edwards) larvae against the antibiotic resistant luminous *Vibrio harveyi* was investigated. *Vibrio harveyi*, isolated from the diseased shrimp larvae, induced mortality in experimentally infected mysis larvae within 12 hours of post-challenge and caused 83% mortality in 72 hours at 1.28 x 10^3 cells/ml level. Addition of the probiont in shrimp larval rearing medium from 10^3 to 10^6 cells/ml improved the growth and survival of *P. indicus* larvae, with 10^6 cells/ml being the most effective level. Bioencapsulation of the probiont (>10^4 cells/g *Artemia* sp.) greatly improved the dietary value of *Artemia* sp. nauplii and increased the disease resistance of shrimp larvae. A significant weight gain (> 70 %) was observed in probiont (10^4 cells/g *Artemia* sp.) fed larvae compared to the control group. Larvae grown in the presence of the probiont resisted *V. harveyi* infection when challenged.

Introduction

Vibrio harveyi, the causative agent of luminous bacterial disease, is considered a serious pathogen of larval shrimp in hatcheries (Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.*, 1994). Antibiotic medication is widely followed to control this pathogen and to improve the production of shrimp larvae (Baticados *et al.*, 1990). The practice of using antibiotics in shrimp hatchery disease management is controversial due to the perceived induction of antibiotic resistance (Baticados *et al.*, 1990), depressive effects on the host immune response (Alberman, 1988), concern over hatchery workers' health and nearshore environmental impacts (Brown, 1989) and chance of the antibiotic resistance being passed onto the human pathogenic bacteria (Aoki *et al.*, 1990). Bioremediation methods involving probiotic microorganisms can be the safe alternative to overcome the said problems. Studies on these aspects in larval rearing of shrimp (*Penaeus monodon*), crab (*Portunus trituberculatus*) (Naeda, 1994) and oyster (*Crassostrea gigas*) (Douillet and Langdon, 1994) showed the efficiency of using probiotics. The purpose of this study was to assess the disease resistance and growth performance of *Penaeus indicus* larvae when fed with

* Address for correspondence: *Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Mohanpur - 741 252, West Bengal, India.*

probiotic bacterium, *Lactobacillus plantarum,* under laboratory conditions.

Material and methods

The lactic acid bacterial (LAB) strain *Lactobacillus plantarum* NDRI-X as a probiotic bacterium was obtained from the National Diary Research Institute, Karnal, India. Penaeid shrimp larval pathogen, luminous *V. harveyi* MA 534, was isolated from hatchery-raised diseased *P. indicus* mysis larvae. De Man Rogosa Sharpe (MRS) medium, either as broth or agar, was used for the growth and maintenance of LAB (Harrigan and McCance, 1976). Probiotic LAB cells were routinely grown at 30°C for 48±3 hours. Sea water (75%), peptone (0.5%) and yeast extract (0.3 %) medium (SPYE), either as broth or agar, was used for the growth of *V. harveyi.* This bacterium was routinely grown at 30°C for 18-24 hours and maintained on SPYE agar slants. Mueller Hinton agar (MHA) supplemented with 1.5% (w/v) sodium chloride was used for antibiotic sensitivity assays. Half strength sea water made from 35‰ aged sea water or 1.0% NaCl solution was used as diluent.

Hatchery-raised *P. indicus* larvae mysis and post-larval (PL) stages were procured, as and when required, from a commercial shrimp hatchery in Tuticorin, Tamil Nadu and acclimatised to laboratory experimental conditions in sea water (salinity: 28-30%; temperature: 29±1°C; pH : 8.0) with continuous aeration. These animals were fed with live feed, *Skeletonema* sp. and/or *Artemia* sp. or a commercial starter feed at a level of 10% of biomass during the experimental period. Nauplii of *Artemia* sp. were produced in the laboratory following Sorgeloos *et al.* (1986) from *Artemia* sp. cysts (Argent Chemicals, USA) at a density of 1.0 g/l of incubation medium. *Skeletonema* sp. cells were collected from a commercial shrimp hatchery.

L. plantarum cells, grown in MRS broth for 48 hours, were harvested by centrifugation at 5000 r/m for 15 minutes, washed twice with sterile 1.0% NaCl solution and finally resuspended in 10 ml sterile diluent. LAB cell suspension was prepared once in two days and stored at 4°C until used. Cells of *V. harveyi* MA 534, grown in SPYE broth, were harvested as done with LAB cells and used immediately for experimental infection studies. Numbers of viable cells of *L. plantarum* and *V. harveyi* in the suspensions were determined on MRS agar and SPYE agar, respectively. Agar disc diffusion method of Bauer *et al.* (1966) was followed for testing the antibiogram of *V. harveyi.* The minimal inhibitory concentration (MIC) of antibiotics was determined by agar dilution method (Abraham *et al.,* 1997). Pathogenicity of *V. harveyi* was tested on *P. indicus* larvae at mysis-3 stage by immersion method (Karunasagar *et al.,* 1994).

Addition of probiont into larval rearing medium : Post-larvae (PL5) of known weight were maintained in a series of experimental troughs (25 larvae/trough) containing 3 l sea water with continuous aeration. Probiont cell suspension was then introduced into the troughs in such a way as to get a concentration of 10^6 (T1), 10^5 (T2), 10^4 (T3) and 10^3 (T4) cells/ml of rearing medium in duplicate. A control group (T5) was also maintained without probiont addition. Probiont cells were added to the larval rearing medium daily for upto 15 days,

after replacing them completely with an equal volume of fresh sea water. Feeding was done daily to all experimental and control groups. At the end, the weight of surviving shrimp larvae (PL-20) in all the experimental categories was recorded individually. The survived PL-20 from all the experimental categories were separated into two lots of 10 numbers each and transferred to fresh troughs containing 2 l sea water to assess their resistance to *V. harveyi* infection. *V. harveyi* cell suspension was introduced into the troughs to get a concentration of 10^4 cells/ml rearing medium. Larval mortality was noted daily for upto seven days.

Bioencapsulation of probiont in Artemia *sp. nauplii:* Hatchery-raised post-larvae of *P. indicus,* 10 numbers each at PL-10 stage, were introduced into a series of glass containers (E1-E4 in triplicate) filled with 2 l sea water and acclimatised for 24 hours. Nauplii of *Artemia* sp. from nauplii separating chamber were harvested by filtering through bolting silk of 60-µm pore size. About 5000 nauplii each were transferred to a series of four containers (A-D) and filled with a litre of sea water. Probiont cell suspension was then added to the containers A, B, and C at a concentration of 10^7, 10^6 and 10^5 LAB cells /ml of the rearing medium, respectively. No probiont was added to the container D. Nauplii were kept in the aerated bacterial suspension for two hours for bioencapsulation and then harvested. Bioencapsulation was done daily. The *Artemia* sp. nauplii exposed to 10^7 LAB cells /ml were fed to the animals in E1, 10^6 LAB cells /ml to E2, and 10^5 LAB cells/ml to E3, and the *Artemia* sp. nauplii without probiont were fed to E4 for 15 days. Growth and survival rates were determined as done before. *Artemia* sp. nauplii exposed to 10^7, 10^6 and 10^5 LAB cells /ml of the rearing medium were asceptically filtered, weighed and macerated using sterile glass rods separately in sterile test tubes. The number of LAB cells in *Artemia* sp. nauplii was determined by pour plating. The survived post-larvae (PL-26) were then challenged with *V. harveyi* as described earlier. However, the challenge dose was kept at 10^5 cell/ml of rearing medium. ANOVA technique (Snedecor and Cochran, 1967) was followed to test the significance of difference among the treatments.

Results and discussion

Results of antibiotic sensitivity and pathogenicity of *V. harveyi* MA 534 are summarised in Tables 1 and 2, respectively. *V. harveyi* was resistant to six out of ten antibiotics tried, suggesting that numerous shrimp pathogens with multiple antibiotic resistance (MAR) may be present in shrimp hatchery. Incidence of MAR among the shrimp pathogens is well known phenomenon and is amply documented (Baticados *et al.,* 1990; Karunasagar *et al.,* 1994; Abraham *et al.,* 1997). The recorded MICs for chloramphenicol, sulphadiazine and trimethoprim against *V. harveyi* were high (>100µg/ml) possibly due to the abundant use of these drugs to control bacterial infection in shrimp hatcheries. Development of resistance to the said antibiotics was associated with increased virulence of *V. harveyi* and mass mortality of penaied larvae in hatcheries (Karunasagar *et al.,* 1994). Likewise, the MAR isolate of the present study was also highly virulent and capable of causing 83% larval mortality at 1.28×10^3 cells/ml level within 72 hours (Table 2). This clearly shows the potential danger of regular and

TABLE 1. *Results of antibiogram and minimal inhibitory concentration (MIC) of antibiotics against* Vibrio harveyi *MA 534*

Antibiotic	Concentration/disc (µg)	Antibiogram group	MIC (µg/ml)
Chloramphenicol	30	R	100.00
Ciprofloxacin	5	IM	0.39
Furazolidone	50	R	50.00
Gentamycin	10	IM	6.25
Neomycin	30	R	50.00
Nalidixic acid	30	S	6.25
Oxytetracycline	30	S	6.25
Streptomycin	10	R	>100.00
Sulphadiazine	300	R	>500.00
Trimethoprim	5	R	100.00

R=Resistant; IM=Intermediate; S=Sensitive.

continuous use of antibiotics in shrimp hatcheries and their impact on larval pathogen, and ultimately on the reared animal.

Effect of addition of *L. plantarum* cells into larval rearing medium on the survival, growth and disease resistance of *P. indicus* larvae are presented in Table 3. Survival rates (72 - 94%) were significantly high ($P<0.01$) in larvae reared in probiont added medium (T1 - T4) than in control (T5 : 68%). Highest mean survival (94 ± 2%) was recorded in treatment T1 wherein LAB was introduced at 10^6 cells/ml. Growth rate in terms of weight increment was the highest in T1 group and differed significantly among the treatments ($P < 0.001$). Weight of larvae in T1 group increased by 70% in 15 days of experimental period over the control group. Mortality rate observed in T1 group (50%) was comparatively lower than in other treatments (60%) and control (65%) which, however, differed significantly only at 10% level. These results corroborate with the observations made on shrimp larvae (Maeda and Liao, 1992; Maeda, 1994) and oyster larvae (Douillet and Langdon, 1994) when grown in the presence of beneficial bacteria. According to Douillet and Langdon (1994), this effect was due to the essential nutrients supplied by probionts besides enzymes that could enhance the digestive process. Colonisation of probiont in the gut of shrimp, possibly from ingestion of probiont from the surrounding water, and modification of gut flora, particularly *Vibrio* spp. could be the probable reason for the observed improvement in growth and survival of *P. indicus* larvae.

Bioencapsulation method using *Artemia* sp. nauplii was proposed as an innovative way of delivery to the shrimp larvae (Mohney *et al.*, 1989). This concept was modified and extended to

TABLE 2. *Pathogenicity of* V. harveyi *MA 534 on* P. indicus *larvae*

Concentration of *V. harveyi* (cells/ml)	Number dead out of 100 challenged larvae in		
	24 h	48 h	72 h
1.28×10^6	29	48	100
1.28×10^5	16	43	90
1.28×10^4	11	36	88
1.28×10^3	12	29	83
Unchallenged control	5	11	17

TABLE 3. *Effect of addition of* Lactobacillus plantatum *in rearing medium on growth survival and disease resistance of* P. indicus *larvae*

Treatment	Concentration *L. Plantarum* (cells/ml)	Survival rate (%) Mean ± SD*	Weight increment (g)+ Mean ± SD**	Mortality (%) in challenged larvae Mean ± SD***
T1	10^6	$94.0 \pm 2.0^{a,b}$	0.0239 ± 0.0031	50.0 ± 0.0
T2	10^5	$90.0 \pm 2.0^{a,c}$	0.0174 ± 0.0024^{A}	60.0 ± 0.0
T3	10^4	$90.0 \pm 2.0^{b,c}$	0.0170 ± 0.0011^{A}	60.0 ± 0.0
T4	10^3	72.0 ± 4.0^{d}	0.0152 ± 0.0032^{B}	60.0 ± 0.0
T5	Nil	68.0 ± 4.0^{d}	0.0140 ± 0.0017^{B}	65.0 ± 5.0

+ : Weight (g) = (final weight of shrimp at the end of experiment + wet weight of dead shrimp) – initial weight of shrimp.
* : $F_{4,4} = 25.367$; $P < 0.005$; CD = 11.984.
** : $F_{4,4} = 293.816$; $P < 0.001$; CD = 0.00124.
*** : In 7 days of experimental period after challenging with *V. harveyi* at 10^4 cells/ml of rearing medium. $F_{4,4} = 6.0$; $P < 0.10$. *No mortality was observed in unchallenged control group.*
a-d: A,B : Values sharing common superscripts differ insignificantly at 5% level.

feed fish larvae with bioencapsulated probiont in live food (Gatesoupe, 1991). Results presented in Table 4 show that the bioencapsulation process greatly improved the dietary value of *Artemia* sp. nauplii. Incubation of *Artemia* sp. nauplii in aerated probiont suspensions at 10^7, 10^6 and 10^5 cells/ml rearing medium for two hours during bioencapsulation process yielded the LAB count of $> 10^4$, $>10^3$ and $>10^2$/g *Artemia* sp. respectively. The highest mean survival rate was observed in E1 (76.66 %) followed by E2 (73.33 %). Among the treatment and control groups insignificant ($P > 0.005$) differences in survival rates were noticed. But, significant differences ($P > 0.005$) in growth rates were observed. Highest mean weight increment of 0.0162g was observed in E1 group larvae fed with bioencapsulated probiont, i.e., 10^4 LAB cells/g *Artemia* sp. followed by E2 and E3. Larval weight in E1 group was increased by 70% over the control group in 15 days of feeding. Mortality rates

TABLE 4. *Effect of feeding bioencapsulated probiont in* Artemia *sp. nauplii on growth, survival and disease resistance of* P. indicus *larvae*

Treatment	Concentration of *L. plantarum (cells/ml)*	Survival rate (%) Mean ± SD*	Weight increment (g) Mean ± SD**	Mortality (%) in challenged larvae Mean ± SD***
E1	10^4	76.66 ± 4.71	0.0162 ± 0.0026	45.0 ± 5.0^{A}
E2	10^3	73.33 ± 4.71	0.0153 ± 0.0005	$55.0 \pm 5.0^{A,B}$
E3	10^2	63.33 ± 4.71	0.0109 ± 0.0009^{a}	60.0 ± 0.0^{B}
E4	Nil	63.33 ± 4.71	0.0095 ± 0.0002^{a}	75.0 ± 5.0

* : $F_{3,6} = 4.499$; $P>0.05$; Not significant.
** : $F_{3,6} = 13.29$; $P<0.05$; CD = 0.0048.
*** : $F_{3,6} = 25.00$; $P<0.001$. In 7 days of experimental period after challenging with *V. harveyi* at 10^5 cells/ml level.
A, B, a : Values sharing common superscripts differ insignificantly at 5% level.

in experimentally infected larvae of E1 (45%), E2 (55%) and E3 (60%) groups were significantly ($P < 0.01$) lower than in control (E4 : 75%) group. It can be inferred from these reults that the concentration of probiont plays an important role in improving the dietary value of *Artemia* sp. and on performance of larvae. A concentration of $>10^4$ probiont cells/g *Artemia* sp. nauplii appeared essential for feeding shrimp larvae. Likewise, a significant weight gain was observed in *Scophthalmus maximus* (turbot) larvae when fed with bioencapsulated LAB and *Bacillus toyoi* due to the displacement of bacterial pathogens such as *Vibrio* spp. in live larval feed, rotifer (*Brachionus plicatilis*) and/or improvement in the dietary value of rotifer (Gatesoupe, 1991)

Shrimp larvae resisted *V. harveyi* infection when they were fed with bioencapsulated probiont or reared in the presence of probionts (Tables 3 and 4). The observed degree of protection against bacterial challenge could be attributed to the activation of the haemolymph constituents or phagocytes of penaeid larvae. LAB were reported to exhibit immunomodulatory role by increasing phagocytosis in man, livestock and mice (Fuller, 1989) in a similar fashion as suggested for immunostimulants used in aquaculture (Yoshida *et al.*, 1993). It is concluded from the results of this study that the probiotic bacteria, which offer a certain degree of protection against bacterial pathogens, may play an important role in shrimp aquaculture in the nearfuture, especially in the light of increasing number of antibiotic-resistant strains of bacteria, cost effectiveness and strict environmental regulations of treatments.

Acknowledgment

The authors thank the Dean, FCRI, Tuticorin, for providing necessary laboratory facilities. The award of a Junior Research Fellowship by ICAR, New Delhi, during the tenure of this work is gratefully acknowledged by Uma.

References

Abraham, T.J., R. Manley, R. Palaniappan and K. Dhevendaran 1997. Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. *J. Aqua. Trop.*, **12**(1) : 1-8.

Alderman, D.J. 1988. Fisheries chemotherapy: a review. In: Recent Advances in *Aquaculture*, Vol. 3, p. 1-61. J.F. Muir and R.J. Roberts (Eds.), Croom Helm, London.

Aoki, T., K. Takami and T. Kotao 1009. Drug resistance in a non-haemolytic *Streptococcus* sp. isolated from cultured yellow tail *Seriola quinqueradiata*. *Dis. Aquat. Orgs.*, **8** : 171-177.

Baticados, M.C.L., C.R. Lavilla-Pitogo, E.R. Cruz-Lacierda, L.D. de la Pena and M.A. Sunaz 1990. Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. *Dis. Aquat. Orgs.*, **9** : 133-139.

Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Turck 1966. Antibiotic susceptibility testing by standarized single disk method. *Am. J. Clin. Patho.*, **45** : 493-496.

Brown, J.H. 1989. Use and abuse of antibiotics in aquaculture. *World Aquacult.*, **20** : 34-43.

Douillet, P.A. and C.J. Langdon 1994. Use of a probiotic for the culture of larvae of the pacific oyster (*Crassostrea gigas* Thunberg). *Aquaculture*, **199** : 25-40.

Fuller, R. 1989. Probiotics in man and animals. *J. Appl. Bacteriol.*, **66** : 365-378.

Gatesoupe, F.J. 1991. The effect of 3 strains of lactic bacteria on the production rate

of rotifers *Brachionus plicatillis* and their dietary value for larval turbot *Scophthalmus maximus*. *Aquaculture*, **96** : 335-342.

Harrigan, W.F. and M.E. McCance 1976. *Laboratory Methods in Food and Dairy Microbilogy*, 452 pp, Academic Press, London.

Karunasagar, I., P. Pai, G.R. Malathi and I. Karunasagar 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. *Aquaculture*, **128** : 203-209.

Lavilla-Pitogo, C.R., M.C.L. Baticados, E.R. Cruz-Lacierda and L.D. de la Pena 1990. Occurence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. *Aquaculture*, **91** : 1-13.

Maeda, M. 1994. Biocontrol of the larvae rearing biotope in aquaculture. *Bull. Natl. Res. Inst. Aquaculture*, Suppl., **1** : 71-74.

Maeda, M. and I.C. Liao 1992. Effect of bacterial population on the growth of a prawn larva, *Penaeus monodon Bull. Natl. Res. Inst. Aquaculture*, **21** : 25-29.

Mohney, L.L., D.V. Lightner and M. Bauerlein 1989. Bioencapsulation of therapeutic quantities of the antibacterial Romet-30 in nauplii of the brine shrimp *Artemia* and in the nematode *Panagrellus redivivus*. *J. World Aquacult. Soc.*, **21** : 186-190.

Snedecor, G.W., W.C. Cochran 1967. *Statistical Methods*, 593pp, Oxford and IBH Publishing Co., Calcutta.

Sorgeloos, P., P. Lavens, P. Leger, W. Tackaert and D. Versichele 1986. *Manual for the Culture and Use of Brine Shrimp, Artemia in Aquaculture*, 319 pp., Artemia Reference Center, State University of Ghent, Ghent.

Yoshida, T., M. Sakai, T. Kitao, M.S. Khlil, S. Araki, R. Saitoh, I.T. Ineno and V. Inglis 1993. Immunomodulatory effects of the fermented products of chicken egg, EF 203, on rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, **109** : 207-214.