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# THE IMPACT OF PROBIOTIC BACTERIUM *Lactobacillus acidophilus* IN GROWTH AND SURVIVAL OF THE JUVENILE FRESH WATER RIVER PRAWN *(Macrobrachium rosenbergii)* INFECTED WITH PATHOGENIC *Vibrio* spp.

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**ABSTRACT**

The present study was performed to investigate the aptitude of *Lactobacillus acidophilus* 04 isolated from home made curd as a probiotic in treating the vibriosis of freshwater river prawn (*Macrobrachium rosenbergii*). Both *in vivo* and *in vitro* analyses were performed to test their efficacy. The antagonistic effect of cell free extract obtained from *L. acidophilus* 04 biomass was tested against three pathogenic *Vibrio* spp. namely *Vibrio harveyi*, *V. vulnificus* and *V. anguillarum* using disk diffusion method. The result showed inhibitory effects of cell-free extract on all three *Vibrio* spp. A feeding experiment was conducted for a period of 30 days by incorporating *L. acidophilus* in shrimp feed in three experimental (T1,T2, T3) and one control groups. The result showed an increased gain in size and weight in experimental groups treated with *L. acidophilus* 04 compared to control group without any *L. acidophilus* 04 addition. The treatment groups were further challenged *in vivo* by inoculating shrimps with $10^4$-$10^6$ cells of pathogenic *Vibrio harveyi*, *V. vulnificus* and *V. anguillarum.* The histopatholgical and microbiological analysis revealed significant variation ($P < 0.05$) in morbidity and mortality rates between the three treatment (T1, T2, T3) and one control (C) groups. The result of the study indicates an obvious improvement in growth and survival rate of juvenile freshwater prawns injected with pathogenic *Vibrio* spp. upon feeding with *L. acidophilus* supplement.

**Keywords:** Probiotic, *Lactobacillus acidophilus*, *Macrobrachium rosenbergii*, Vibriosis, Survival rate

## INTRODUCTION

It is estimated by UN FAO that almost half of the world's seafood demand will be met by aquaculture in the year 2020 as the population of wild fisheries are rapidly declining due to erratic gorging and exploitation of natural aquatic habitats (**Moriarty, 1999**). Aquaculture is considered as one of the fastest growing industry in the arena of food production. It may suffice to understand the magnitude of its expansion that it grew at an annual rate of 16.8% between 1984 and 1995. However, outbreak of diseases beset the aquaculture industry resulting enormous losses. One estimate of World Bank reports that the global losses due to shrimp diseases are around $3 billion (**Vaseeharan and Ramasamy, 2002**). Vibriosis is considered one of the major reasons for the global loss resulted from bacterial diseases. *Vibrio* spp, predominantly occur in various developmental stages of *Penaeus mondon*. Mortality rate resulting from vibriosis is almost around 100% (**Sivakumar *et al.*, 2012**). The people related to aquaculture industry thus opted to antibiotics to save their investments. However, the emergence of antibiotic resistant bacterial species and detrimental effects of various antibiotic residues have surfaced as even a greater concern for the consumers' health. Probiotics have recently attracted more attention as an alternative to antibiotics in aquatic disease management (**Ninawe and Selvin, 2009**). The beneficial effects of some bacterial species are well documented in human, cattle and poultry. Several studies suggest that *Bacillus* spp, *Lactobacillus* spp, *Vibrio alginolyticus*, pseudomonads, algae have potentials to act as probionts to treat bacterial diseases of shrimps and fishes (**Austin *et al.* 1992, 1995; Smith and Davey, 1993**). Most studies conducted so far on probiotic treatment of shrimps involved mostly seawater penaeid shrimps. However, some recent studies suggest that freshwater river prawns (*Macrobrachium rosenbergii*) suffer almost the similar fate with 100% mortality rate within one-day post injection (**Siripornadulsil *et al.*, 2013**). So the present study was designed to evaluate the efficacy of a probiotic bacterial strain *Lactobacillus acidophilus* 04 on survival and growth of freshwater river prawn *M. rosenbergii*. The study aimed to investigate the ability of *L. acidophilus* to thwart the growth of *Vibrio* spp.

## MATERIAL AND METHODS

### Identification of Bacterial strains

Virulent strains of *Vibrio harveyi*, *V. vulnificus*, *V. anguillarum* were obtained from Fish Inspection and Quality Control Laboratory, Department of Fisheries, Chittagong. The strains were obtained in freeze-dried condition. So these strains were subjected to sub-culturing and were confirmed through standard morphological and biochemical techniques. They were further stored at 4°C in TCBS Agar. The probiotic bacterium *L. acidophilus* 04 was obtained from home made curd dilution plating on de Man Rogosa and Sharpe (MRS) Media (Himedia, India). Molecular characterization of all these bacterial strains was performed using 16S rDNA (**Holt, 1994**).

### Antagonism assay

The preliminary antagonistic assay was performed by disk agar diffusion method (**Bauer, 1966**). *V. harveyi*, *V. vulnificus* , *V. anguillarum* were pre-cultured in Luria Bertani (LB) Broth at 28°C for 2 days . The cell free extracts of these bacterial strains were obtained as described previously (**Sivakumar *et al.*, 2012**). The *Vibrio* strains were sub-cultured on TSA containing 1.0% NaCl for 12h at 30°C. The plates of Muller Hinton Agar (Himedia, India) were flooded with 100 µl of *Vibrio* spp. and were air dried. Sterile disks (Himedia, India) were soaked with 20 µl of cell-free extracts of *L. acidophilus* 04 and were placed on the air dried MHA plates containing *Vibrio* spp. Controls were prepared using disks impregnated with MRS broth (pH 6.5) and neutralized MRS broth. Both control and test plates were in triplicate and were subjected to incubation at 30°C for 24h period. Antagonistic activity was defined as clear inhibitory zones around the disks.

### Feed Preparation

*L. acidophilus* 04 was incubated at 30°C in MRS broth with continuous shaking for an overnight period. The cells were subjected to harvesting upon incubation by centrifugation at 2000 x g. The harvest was washed twice using Phosphate

Buffer Solution (pH 7.2) and was re-suspended in the same solution. The absorbance at 600nm was adjusted to 0.25±0.05 in order to standardize the number of bacteria. The number of bacteria was enumerated by plating in 10-fold dilutions. The CFU $ml^{-1}$ in standard plate counts were correlated with absorbance at 600nm. Commercially available shrimp feed was procured to use as basal diet for the supplementation of *L. acidophilus* 04 (ACI Godrej Agrovet, Bangladaesh). The commercially obtained feed was added to each *L. acidophilus* 04 biomass (LAB) and mixed thoroughly over a vortex mixer. A 10-fold dilution of LAB mixed with feed was prepared and the number of bacteria per ml was estimated at regular intervals of 24h by standard plate count method on MRS Agar. The enumeration of bacterial biomass was in suspension was also conducted by measuring the optical density at 600nm. The final concentration *L. acidophilus* was $10^6$ cells $g^{-1}$. A control was also prepared that contained the feed only without any addition of bacterial biomass.

**Feeding experiments**

A total of 600 juveniles of *M. rosenbergii* averaging 0.85±0.1 g in weight and 7±1 cm in length were obtained from a commercial hatchery of Baly Group, Cox's Bazaar. The juveniles were transported in oxygen bags were measured from the tip of the telson to the base of the eye orbit before acclimatization in an aquarium made of fiber glass with capacity of 500 liter for a week. The juvenile shrimps were then allocated into 12 indoor glass aquaria measuring 100x50x50 cm at a rate of 50 animals per aquarium to maintain three replicates of the three treatment and one control groups with continuous aeration at 28 ±2 $^oC$ prior to commencement of the experiment. They were maintained on a pelleted diet supplemented with *L. acidophilus* 04 biomass concentration of $10^6$ cfu $ml^{-1}$ and 15% of body weight in two individual doses per day at 12 hours interval (**Ajitha, 2003**). The feeding experiment was conducted one month prior to challenging the test shrimps with pathogenic *Vibrio* app. Five shrimps from each of the treatment and control groups were randomly sampled every week to measure the size and weight and histopatholgical analysis was performed to check for the signs of morbidity.

**Table 1** The Physicochemical Parameters of the Glass aquaria

| Parameter | Condition |
|---|---|
| Salinity | 31.5±2 % |
| Oxygen | 4.5±0.5 mg/l |
| Temperature | 28±1$^o$ C |
| pH | 7.8±0.5 |

***In vivo* Challenging with pathogenic *Vibrio* spp.**

After one month of regular feeding with *L. acidophilus* probionts, 20 shrimps from each of the treatment groups were injected with $10^4$-$10^6$ cells of *V. harveyi*, *V. vulnificus* and *V. anguillarum*, respectively. They were maintained segregated for next two weeks of post-infection in same conditions and were fed every second day and was observed for signs of morbidity and vibriosis. Ten shrimps surviving from each treatment and control groups were sampled and were microbiologically and histopathologically tested every week of post-infection period (**Bell and Lightner, 1988**). The feeding and observation for morbidity continued in the second week. The remaining inoculated shrimps were sacrificed and were subjected to microbiological and histopathological analysis. The uninoculated shrimps were also subjected to microbiological and histopathological analysis. Total viable count, total *L. acidophilus* and total *Vibrio* from the gastro intestinal (GI) tract and tank water were counted before and after the vibrio challenging study by growth on marine agar, MRS agar and TCBS agar (Himedia, India),respectively.

**Detection of Lactic acid production**

*Lactobacillus acidophilus* 04 strain was inoculated into 10 ml of MRS broth at an $O.D._{600}$ of 0.05 and incubated statically at 37°C in a microaerobic incubator for 48 h. Supernatants were collected from one ml of culture by centrifugation followed by passage through a 0.22 µm filter to remove bacteria. The concentrations of D- and L-lactate in the cell-free supernatants were measured using stereo-specific D- and L-lactate assay kits (Eton Bioscience, San Diego, CA, USA). The measurements were performed in triplicate for reproducibility. Assays were performed with pure solutions of D- and L-lactate to ensure that the kits were stereo-specific. (**Neal-McKinney et al., 2012**)

**Statistical analysis**

The results were analyzed by the Student's t-test to determine differences ($P < 0.05$) between tested groups. All statistics were performed with SPSS for Windows, version 11.5 (SPSS Inc, Chicago, IL, USA).

**RESULTS**

**Antagonism Assay**

The cell-free extracts of the *L. acidophilus* showed inhibitory activity against all three *Vibrio* spp. The inhibitory effect was highest against *V. harveyi* followed by *V. anguillarum* and *V. vulnificus* respectively. The inhibitory effect was measured by the diameter of the clear inhibitory zones around the impregnated disks soaked with *L. acidophilus* extracts. The largest diameter of clear zone obtained was 21 mm against *V. harveyi.* The smallest diameter obtained was 13 mm for *V. vulnificus.* The control plates on the other hand formed no clear zone.

**Feeding experiment**

No morbidity was observed during the period of probiotic treatment of the shrimps. The juveniles that were procured were in sound health prior to acclimatization. All of the shrimps in the feeding experiment gained in size and weight during the trial of probiotic treatment. The mean final weight of the treatment groups were 1.52 g and that of the control group was 1.44 g. The average gain in size of the treatment groups was 5.43 cm. The same for the control group was 4.88 cm. The histopathological analysis revealed no sign of bacterial diseases.

***In vivo* challenging**

All three groups started to show the signs of morbidity due to vibriosis in the first week of *in vivo* challenging with *Vibrio* spp. However, the highest rate of morbidity was observed in the control group that received no probiotic treatment. All of the three test groups showed reduced mortality. The mortality rate was also highest in the control group. The histopathological analysis of the first week revealed both localized and systemic lesions in the form of hemocytic nodules in gills, hemocel spaces and loose connective tissues. Histological analysis of hepatopancreas revealed no visible anomaly in the first week of infection. The morbidity and mortality rates increased in the final week of infection. The rate of mortality and morbidity was still highest in the control group. The uninoculated shrimps also developed the signs of morbidity in the final week. The histopathological analysis revealed multifocal lesions throughout the shells of the infected shrimps. Multifocal menalized hemocytic nodules were most common in the heart, lymphoid organ and gills. Table 2 describes the developing morbidity and mortality rates in the post infection period in all of the treatment and control groups.

**Table 2** Morbidity and Mortality rates in treatment and control groups during in vivo challenging

| Group | Morbidity rate (%) | | Mortality rate (%) | |
|---|---|---|---|---|
| | First week post infection | Second week post infection | First week post infection | Second week post infection |
| T1 | 41.50 | 56.54 | 7.50 | 14.20 |
| T2 | 36.90 | 48.30 | 6.20 | 11.30 |
| T3 | 43.00 | 57.25 | 7.80 | 15.30 |
| C | 79.50 | 98.60 | 57.00 | 86.90 |

**Legends:** T1= Treatment group 1, T2= Treatment group 2, T3= Treatment group 3, C= Control group

The histopathological findings of the all four groups are also summarized in Table 3. The total viable count of the tail and hepato pancreas showed an increase in the bacterial population in the first week of post infection period. The $OD_{600}$ analysis of the bacterial suspension grown in Nutrient Broth upon growing on agar media revealed the exponential growth of bacterial population during the first week. The following week showed a decline in the total plate count. Figure 1 shows the total bacterial growth at different stages.

**Table 3** Histopathology of the treatment and control groups upon inoculation of pathogenic *Vibrio* spp.

| Histopathological findings | Number of developing pathological cases in each treatment and control group upon inoculation with pathogenic *Vibrio* spp. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Treatment group 1 (T1) | | Treatment group 2 (T2) | | Treatment group (T3) | | Control Group (C) | |
| | Week 1 | Week 2 | Week 1 | Week 2 | Week 1 | Week 2 | Week 1 | Week 2 |
| Melanized hemocytes forming capsules | 13 | 28 | 16 | 34 | 10 | 26 | 24 | 46 |
| Multifocal melanized hemocytic nodules | 6 | 14 | 8 | 17 | 6 | 16 | 21 | 43 |
| Multifocal non-melanized hemocytic nodules | 9 | 19 | 11 | 28 | 11 | 30 | 23 | 44 |
| Multifocal necrosis and hemocytic inflammation of Hepatopancreatic tubules | Nil | Nil | Nil | Nil | Nil | 1 | 7 | 15 |

**Table 4** Total *L. acidophilus* count in GI tracts of shrimps

| Groups | Total *L. acidophilus* count (cfu/ml) | | | |
|---|---|---|---|---|
| | Before feeding | After feeding for 30 days | First week after inoculation | 2nd week after inoculation |
| T1 | Nil | $9.6 \times 10^6$ | $11.4 \times 10^6$ | $10.7 \times 10^6$ |
| T2 | Nil | $7.8 \times 10^6$ | $9.4 \times 10^6$ | $8.8 \times 10^6$ |
| T3 | Nil | $9.2 \times 10^6$ | $10.5 \times 10^6$ | $9.4 \times 10^6$ |
| C | Nil | Nil | Nil | Nil |

**Legends:** T1= Treatment group 1, T2= Treatment group 2, T3= Treatment group 3, C= Control group

All of the *Vibrio* spp and *L. acidophilus* 04 were identified in the juveniles tested at the end of final week of post-infection. The comparison of uninoculated shrimps tested both microbiologically and histopathologically of the treatment groups with that of uninoculated control group showed a massive difference. The rate of morbidity and mortality of the uninoculated treatment groups were very low (23%) while the same was almost 96% for control group. Despite developing morbidity, the shrimps of the treatment groups gained in both size and weight. The mean of the weight gained by the animals of the treatment groups was 2.69 g and the mean of the size gained by the treatment groups was 2.14 cm. At the beginning, there was no observable *L. acidophilus* in the GI tract of the shrimps, however, the count increased significantly in the probiotic treatment groups. There was a slight decline in the *L. acidophilus* count at the final week of infection with *Vibrio* spp. The count for *L. acidophilus* in the water of the treatment group increased with the weeks of feeding. After challenging with *Vibrio* spp., the highest count of *Vibrio* was observed in the control groups. All three different *Vibrio* spp, were identified (*V. harveyi*, *V. vulnificus and V. anguillarum*). The treatment groups, however, had a subtle increase in the count for vibrios. The count for *Vibrio* spp. reached its peak at the end of final week after challenging. Figure 2 depicts the growth of pathogenic *Vibrio* spp. at different stages ranging from the very beginning of the *in vivo* challenging experiment till the end of the experiment.

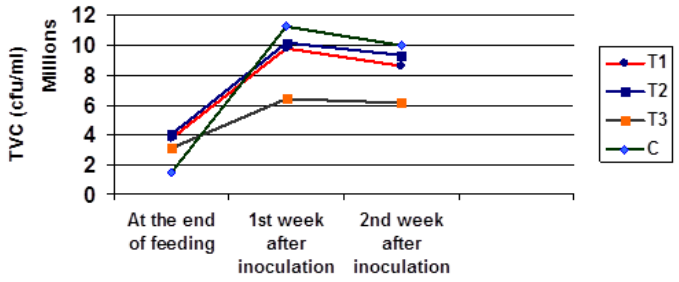


**Figure 1** Total bacterial growth before and after inoculation of shrimps with pathogenic *Vibrio* spp.

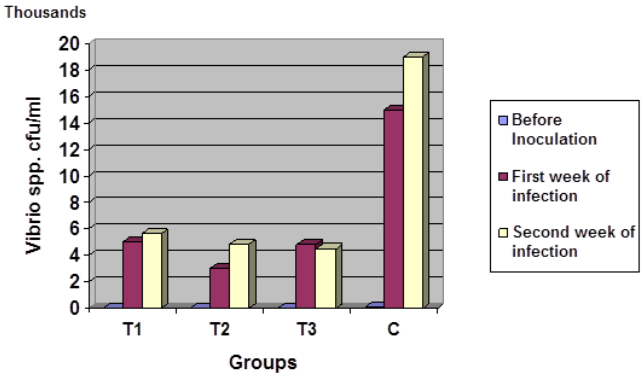


**Figure 2** Growth of pathogenic *Vibrio* spp. after in vivo challenging

**Lactic acid produced by *L. acidophilus* inhibits *Vibrio* spp.**

To determine latic acid production, the amount of D- and L-lactic acid produced by *L. acidophilus* was measured using colorimetric enzymatic assay. The amount of lactic acid produced by *L. acidophilus* was 128±3.4 mM D-lactate and 143±2.1 mM L-lactate, respectively. The inhibition *Vibrio* spp. by lactic acid was determined by performing a survival experiment. *Vibrio* spp. were grown in LB broth supplemented with NaCl. LB broth was treated with lactic acid and HCl to reach pH level 5.12, 4.32, and 3.46, respectively. Control ledia were also maintained that were not treated. Five ml of each broth were inoculated with *Vibrio* spp. in triplicate, and samples were taken at 1, 2, 4, and 8 h for bacterial enumeration. There was a complete inhibition of *Vibrio* growth in media containing 100 mM lactic acid in 1 h. Media containing 25 mM lactic acid resulted in the death of all *Vibrio spp.* by 2 h, while treatment media at pH 4.32 (HCl) did not kill all *Vibrio* until 8 h. Media containing 10 mM lactic acid or pH 5.12 (HCl) inhibited *Vibrio* spp. growth slightly compared to the untreated medium.

## DISCUSSION

Vibriosis has been recognized as one of the major causes responsible for mortality of cultured shrimp world wide. The potential negative consequences and reduced efficacy of antimicrobial agents necessitated the search for an alternative (**Farzanfar, 2006**). The use of probiotics has increasingly being recognized as a safer and more effective method of controlling diseases. *Lactobacillus* spp. has always been at the center of interest due to its beneficial features on animal and human (**Verschuere *et al.*, 2000**). Several studies suggest that probiotic bacteria are capable of exerting antagonistic effect against pathogenic vibrios in penaeid shrimps (**Vaseeharan and Ramasamy 2003, Rengipat 2000, Sivakumar *et al.*, 2013**). The present study showed similar inhibitory action as the cell-free extract obtained from *L. acidophilus* 04 formed clear zones around the disks applied on the surface flooded with pathogenic *Vibrio* spp. There are reports suggesting the ability of *Lactobacillus* to alter the pH and production peptide antibiotic, bacteriocins capable of inhibiting the growth of both gram-positive and gram negative bacteria (**Chaurasia *et al.* 2005; Yilmaz *et al.*, 2006; Enserink, 1999; Zhou *et al.*, 2005**). The bacteriocin producing strain *L. acidophilus* was isolated from the gut of *Penaeus monodon* (**Karthikeyan and Santhosh, 2009**).

The supplementation of feed with probiotic bacteria might positively influence the growth and survival of freshwater prawns. The treatment groups in the present study gained size and weight significantly during the period of 30 days of feeding experiment. The retarded growth of the juvenile freshwater prawns of the control group is a strong indication that the probiotic supplement had a positive impact on the growth and survival rate of the shrimps in test. The finding of **Seenivasan *et al.* (2012)** is in agreement with the result of the present study.

They observed that the growth parameters such as survival, weight gain, geed conversion efficiency and protein efficiency rate of *M. rosenbergii* were significantly higher ($p<0.05$) in 3% *Lactobacillus sporogenes*, *Bacillus subtilis* and *Saccharomyces cerevisiae* incorporated diet fed.

The *in vivo* challenging experiment showed significant reduction in morbidity and mortality rates in the groups fed with *L. acidophilus* 04 supplement. The morbidity and mortality rates were much higher in the control group. The mortality rate was 8-10 folds higher in all three treatment groups compared to untreated control groups. **Sivakumar *et al.* (2012)** reported six-fold higher survival rate in treatment groups of penaeid shrimps (*Penaeus monodon*) fed with probiotic bacterium *L. acidophilus* compared to untreated control groups upon exposure to pathogenic *Vibrio* spp. The increase in the *Vibrio* spp. and *L. acidophilus* count in the first week following the inoculation of shrimps with pathogenic bacteria suggests exponential growth phase. The slump of bacterial count at the end of experiment can be result of nutrition attrition (**Llorens *et al.*, 2010**).

Results of the histopathological examination revealed melanized hemocyte forming plugs in all groups at the first week of post-exposure period of the study. Such lesions are suggestive of localized vibriosis (**Baticados *et al.*, 1990**). However, there were no multifocal melanized hemocytic nodules at the end of first week of infection in the treatment groups. The control groups did develop some multifocal melanized hemocytic nodules in the first week but it saw a massive upsurge in the second week of infection. All of the groups developed multifocal melanized hemocytic nodules and non- melanized hemocytic nodules at the end of second week upon inoculation with pathogenic *Vibrio* spp. These nodules are considered as principal diagnostic feature of systemic vibriosis (**Lightner, 1996**). The control groups developed such chronic inflammatory lesions 10-fold higher than in the treatment groups. There were no multifocal melanized hemocytic nodules in the hepato pancreas of the shrimps in the treatment groups even after exposure for two weeks. The control groups however, did develop such inflammatory lesions in the hepato pancreatic tubules suggesting septic hepatopancreatitis (**Ambipillai, 2003**). Pathogenic *Vibrio* spp. were isolated from the hepato pancreas of shrimps in control group. The Vibrio count of the treatment groups was low compared to that of control group, which could be the result of secretion of inhibitory peptides. Reports suggest that probiotic bacteria colonize the GI tract of the shrimp to prevent the adhesion of pathogenic bacteria (**Gomez-Gill *et al.*, 2000**). Acids produced by the LAB can also be another factor that inhibits the growth of pathogenic *Vibrio* spp. (**Vinothkumar *et al.*, 2011**). Our present study indicates that lactic acid produced by *L. acidophilus* inhibits the growth of pathogenic *Vibrio* spp. through both pH-dependent and pH-independent mechanisms. Previous study also suggests that supplementation of Luria-Bertani broth containing 0.3% agar with norepinephrine and dopamine (100 µM) significantly induced the motility of tested strains of *Vibrio* spp., which is in concurrence with our present investigation (**Suong, 2013**). The impact of *Lactobacillus acidophilus* to induce host response to the infection by pathogenic *Vibrio* spp. is yet to be revealed.

## CONCLUSION

The study concluded that the bacterial biomass of *L. acidophilus* can be used as a dietary supplement of freshwater prawn *M. rosenbergii* to deter infection by pathogenic *Vibrio* spp. The influence of probiotics to induce host immune response could be a further course of study. The specific roles of antibacterial products produced by the *L. acidophilus* can also invite researchers to investigate deeper into the matter.

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