

Full Length Research Paper

Probiotic effect of *Lactobacillus acidophilus* against vibriosis in juvenile shrimp (*Penaeus monodon*)

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The application of effective probiotics in shrimp aquaculture is an excellent alternative for chemicals and antibiotics to prevent disease control. This study was carried out to evaluate the probiotic potential of *Lactobacillus acidophilus* 04 (home made curd isolate) on pathogenic *Vibrio* in shrimp *Penaeus monodon* juveniles. *L. acidophilus* showed antibacterial activity against *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Vibrio harveyi* and *Vibrio alginolyticus*. The probiotic effect of *L. acidophilus* was tested by feeding juvenile shrimp (*P. monodon*) through feed (supplemented with 10^5 CFU g^{-1}) for 30 days before and after an immersion challenge with *V. alginolyticus* at 10^5 CFU mL^{-1} . Shrimp survival was determined after 10 days of challenge. The treatment with *L. acidophilus* 04 resulted in 20% final mortality as compared to 86.7% in the control group. Results of the study validated *L. acidophilus* 04 has potential probiotic principles to control pathogenic *V. alginolyticus* in shrimp aquaculture.

Key words: *Lactobacillus acidophilus*, probiotic, shrimp, vibriosis.

INTRODUCTION

Bacterial diseases may cause a series of problems ranging from growth retardation to mass mortalities. *Vibrio* spp. are aquatic bacteria that are widely distributed in fresh water, estuarine and marine environments (Otta et al., 2001). *Vibrio* spp. are the most important bacterial pathogen in cultured shrimps and they cause mortalities of up to 100% (Karunasaga et al., 1994). Using antibiotics and chemotherapeutic agents to be an important disease controlling measures has developed drug resistance microorganisms (Amabile-Cuevas et al., 1995; Moriarty, 1997; Verschuere et al., 2000). Recently, attention has focused on the use of probiotics in aquaculture. Probiotics are used as dietary supplements in aquaculture, and their role in intestinal microbial balance, growth, nutrition, health status and resistance against infectious agents are already established (Gatesoup, 1999). Probiotics may prevent potential

pathogens from colonizing the gut by production of antimicrobial compounds (Robertson et al., 2000; Balcazar et al., 2006a).

Probiotics that have been examined for use in shrimp aquaculture include bacteria, yeasts and microalgae (Ajitha et al., 2004; Balcazar et al., 2006b). *Lactobacillus*, a genus of lactic acid bacteria (LAB) has been used as probiotic in shrimp aquaculture (Phianphak et al., 1999). Several studies has been demonstrated with different *Lactobacillus* sp. as probiotic in shrimp farming by their nutritional benefits and strong antimicrobial activity against pathogenic microorganisms (Gilliland et al., 1985; Rossland et al., 2003; Qi et al., 2009; Ismail and Soliman, 2010) but no probiotic bacteria are commercially viable for large scale shrimp aquaculture especially against the shrimp pathogen *Vibrio alginolyticus*.

Hence, a study was undertaken to explore the antagonistic property of *Lactobacillus acidophilus* isolated from home made curd on different *Vibrio* sp. *in vitro* and confirmed the probiotic efficacy by *in vivo* challenges against *V. alginolyticus* in juveniles of shrimp *Penaeus monodon*.

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Table 1. Identification of *L. acidophilus* 04.

Character	Results
Morphology	Gram +ve rod, chain
Motility	-
Catalase	-
Growth at 10°C	-
Growth at 42°C	+
Growth in 0.5% NaCl	+
Growth in 7.0% NaCl	+
Acid production in lactose medium (%)	75
Sugar fermentation	
Fructose	A+
Galactose	A+
Glucose	A+
Lactose	A+
Maltose	A+
Mannitol	-
Sucrose	A+

+ = Positive; - = negative, A+ = acid positive.

MATERIALS AND METHODS

Bacterial strains

L. acidophilus strain was isolated from home made curd by dilution plating on de Man, Rogosa and Sharpe (MRS) media (Himedia, India). It was identified by using standard morphological and physiological techniques (Bergey's Manual of Determinative Bacteriology, 1989). Table 1 shows the morphological and biochemical examination of the isolate *Lactobacillus* 04, revealed *L. acidophilus*, further, molecular characterization (16S rDNA) required to complete identification. *Vibrio* strains such as *Vibrio parahaemolyticus* SAC 01, *Vibrio cholerae* SAC 04, *Vibrio harveyi* SAC 09 and *V. alginolyticus* SAC 15 (previously isolated from *P. monodon* larvae showing clinical symptoms of vibriosis) were obtained from Microbiology laboratory, Srimad Andavan Arts and Science College, Tiruchirappalli, and were stored in thiosulfate-citrate-bile salts-sucrose (TCBS) agar slants (Himedia, India) at 4°C.

Antibacterial activity assay

A culture of *L. acidophilus* 04 grown in 100 mL MRS broth for 24 h at 30°C was centrifuged at 10,000 g for 10 min. The supernatant was sterilized by passage through a 0.25 µM syringe driven filter (Himedia, India), and neutralized (pH 7.0) with 2 N NaOH. The pathogenic *Vibrio* strains were subculture on trypticase soy agar with 1.0% NaCl (TSA; Himedia) for 12 h at 30°C. Plates of Mueller-Hinton agar (MH; Himedia) were flooded with 100 µL of bacteria (*V. parahaemolyticus* SAC 01, *V. cholerae* SAC 04, *V. harveyi* SAC 09 and *V. alginolyticus* SAC 15) and air-dried Himedia sterile disk (6 mm), impregnated with 20 µL of filtered supernatant, were positioned on them. Disks impregnated with MRS broth (pH 6.5) and neutralized MRS broth was used as controls to determine possible inhibitory activity of the medium. The diameter of the clear zone around each disk was measured after controlled incubation for 24 h at 30°C. All experiments were carried out in triplicate.

Preparation of the feed

L. acidophilus 04 was grown in MRS broth in a shaking incubator at 30°C overnight. After incubation, the cells were harvested by centrifugation (2000 g), washed twice with phosphate-buffered saline (pH 7.2), and re-suspended in the same buffer. The absorbance at 600 nm was adjusted to 0.25 ± 0.05 in order to standardize the number of bacteria (10^6 to 10^7 CFU mL⁻¹). Dilution plating was used to verify the relationship between absorbance at 600 nm and CFU per milliliter. Commercial shrimp feed (starter feed, Godrej, India) was used as the basal diet for the supplementation of *L. acidophilus* 04. In order to reach a final concentration of 10^6 to 10^7 CFU g⁻¹ feed, bacterial suspensions were slowly sprayed onto the feed for mixing. The amount of *Lactobacillus* in the feed was determined by standard plate count method on MRS agar (Ajitha et al., 2004).

Probiotic treatment and *Vibrio* challenging study of shrimp

A total of 250 shrimp (*P. monodon*) larvae were obtained from a commercial shrimp hatchery in Thanjur, India. Shrimp were acclimatized for 7 days. After the acclimation period, the average weight of the shrimp was 0.56 ± 0.02 g and the shrimp were divided into nine 50 L plastic tanks, each containing 25 juvenile shrimps. Three tanks were treated with feed supplemented with 10^6 CFU g⁻¹ of *L. acidophilus* 04 (Experiment 1), another three tanks were treated with feed supplemented with 10^7 CFU g⁻¹ of *L. acidophilus* 04, and called Experiment 2, for 30 days; the other three tanks served as the control group and were fed with a control diet during the entire trial period. Shrimp in all groups were fed twice daily at 5.0% of biomass. The water temperature was held at $28 \pm 1^\circ\text{C}$ during the whole trial. The weight and the survival of the shrimp were recorded and 3 shrimp were removed for microbiological examination at the end of 30 days. After 30 days of probiotic supplementation, the experimental infection was carried out by the immersion method. *V. alginolyticus* was grown for 24 h at 30°C in TCBS broth (Himedia, India). Shrimp in all tanks were exposed to

Table 2. Antibacterial activities of *L. acidophilus* 04 towards pathogenic *Vibrio* species.

Strain	Activity*
<i>V. Parahaemolyticus</i>	++
<i>V. harveyi</i>	++
<i>V. cholerae</i>	+
<i>V. alginolyticus</i>	+++

Culture plates were incubated at 30°C for 12 h. *Clear zone; +, Clear zone of 8-12 mm; ++, 12 to 15 mm; and +++, above 15 mm.

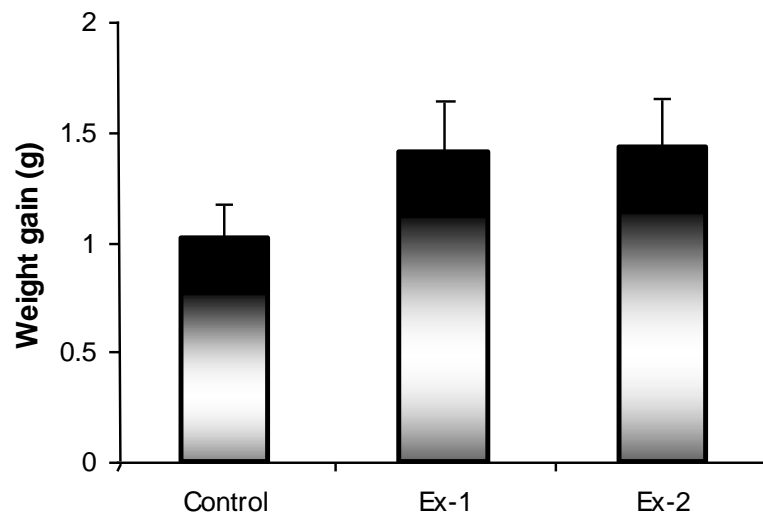


Figure 1. Mean final weight gain of shrimp fed with probiotic *L. acidophilus* 04 supplemented feed. The weight gain was calculated on 30th day (before challenging study). The values are average of three separate experiments with \pm SE (Weight gain = Final weight - initial weight). Ex = experiment.

V. alginolyticus (10^5 CFU ml⁻¹) for a period of 10 days. The accumulated mortality of the shrimp was recorded. Total bacteria, total LAB and total *Vibrio* from the gastro intestinal (GI) tract and tank water were enumerated before and after the vibrio challenging study by growth on marine agar, MRS agar and TCBS agar (Himedia, India), respectively.

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

Antimicrobial activity assay

In this study, antimicrobial activity was evaluated against four different vibrio pathogens. Table 2 gives the results

of inhibition; pathogenic strains inhibited were *V. alginolyticus*, *V. parahaemolyticus* and *V. harveyi*. The diameters of inhibition range between 10 and 16 mm. The highest diameter of 18 mm inhibition was obtained with the extract of *L. acidophilus* 04 on *V. alginolyticus*; the smallest diameter was obtained on the pathogen *V. cholerae*.

Probiotic treatment and infection of shrimp

No shrimp mortality was observed during 30 days before challenging of probiotic experiment. Besides, significant difference ($P > 0.05$) was observed in the mean final weight between the different groups. The mean final weight was 1.43 g in the group fed with probiotic supplemented diet Experiment 2 and 1.02 g in the control groups (Figure 1). In *Vibrio* challenging study, the final mortality of shrimp treated with *L. acidophilus* 04 was low, both Experiment 1 and 2 feed groups shows 20.0%

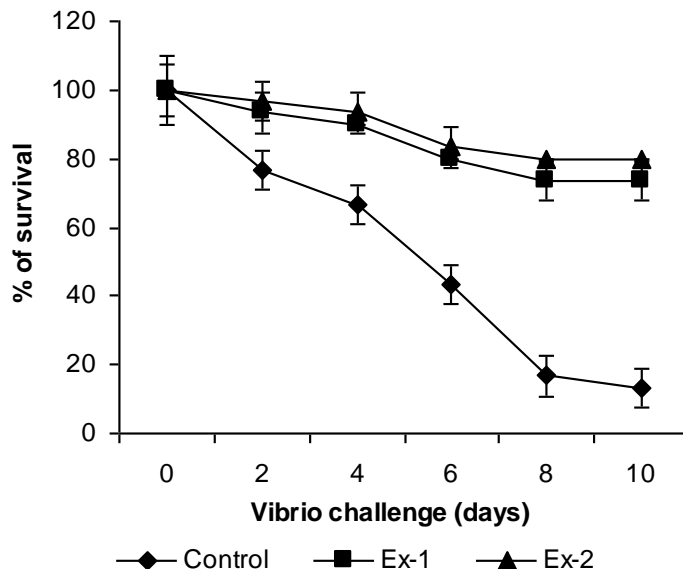


Figure 2. Survival of *P. monodon* juveniles following challenges by immersion with *V. alginolyticus* after feeding with control and probiotic *L. acidophilus* 04 treated feeds (Experiment 1 and 2). The values are average of three separate experiments with \pm SE.

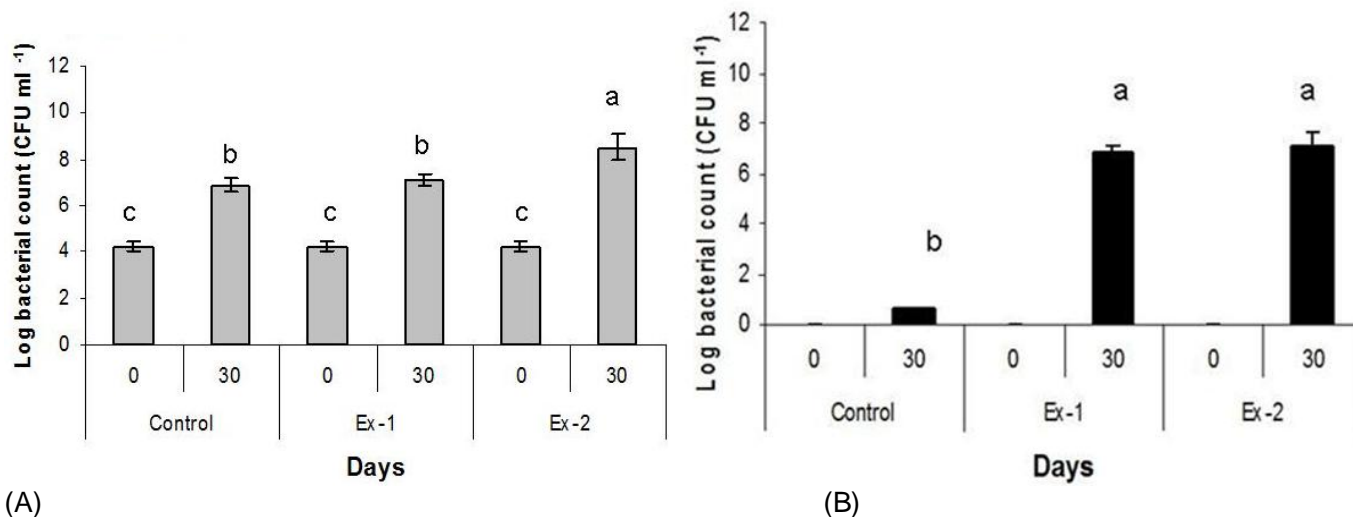


Figure 3. Average bacterial count in the shrimp intestinal tract before challenging with *Vibrio* sp. The values are average of three separate experiments with \pm SE. The mean values with different superscripts are significantly different ($P \leq 0.05$). (A) Total bacterial count, (B) *Lactobacillus* count. Ex = experiment.

mortality, whereas mortality was high (86.67%) in control feed group (Figure 2). Statistical analysis demonstrated significant differences ($P < 0.05$) in mortality between probiotic and control groups.

Figure 3 shows the total bacteria and *Lactobacillus* count in the shrimp GI tract before challenging. Higher total bacterial count was recorded in shrimp fed with feed Experiment 2 ($5.2 \pm 0.53 \times 10^8$ CFU g⁻¹), total

Lactobacillus count of the GI tract was also higher in Experiment 2. The total bacterial count in GI tract was significantly varied from the second week of experiment and it was continued until the end of this study. At the beginning, there were no observed total *Lactobacillus* in GI tract of shrimps, but the total *Lactobacillus* count were increased significantly ($P < 0.05$) in probiotic feed groups, Experiment 1, $8.2 \pm 0.33 \times 10^6$ CFU g⁻¹ and Experiment 2,

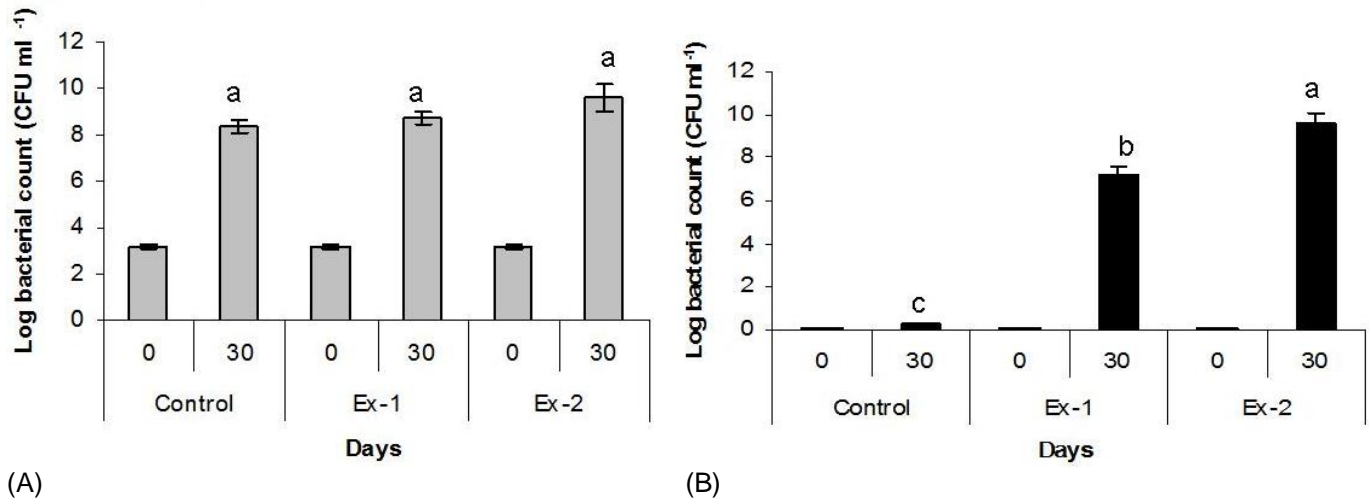


Figure 4. Average bacterial count in the tank water during probiotic study. The values are average of three separate experiments with \pm SE. The mean values with different superscripts are significantly different ($P \leq 0.05$). (A) Total bacterial count, (B) *Lactobacillus* count. Ex = experiment.

$1.4 \pm 0.53 \times 10^7$ CFU g⁻¹, before challenging. Figure 4 shows the total bacterial counts in tank water before challenging (30th day). Total bacterial counts in tank waters were ranged from $1.5 \pm 0.2 \times 10^3$ to $3.6 \times 0.2 \times 10^8$, $2 \pm 0.02 \times 10^3$ to $7.2 \pm 0.2 \times 10^8$ and 1.6×10^3 to $6.1 \pm 0.63 \times 10^8$ CFU ml⁻¹ for control, Experiment 1 and 2, respectively (Figure 4). There were significance differences for total *Lactobacillus* in tank water of Experiment 1 and 2 from day 15 to 30 (Figure 4).

After challenging with *V. alginolyticus*, higher total bacterial load was observed in control tank water $49 \pm 0.4 \times 10^{11}$, it was lower in experimental tank water, Experiment 1 ($40 \pm 0.3 \times 10^{11}$) and Experiment 2 ($12 \pm 0.02 \times 10^{11}$ CFU ml⁻¹). Total *Lactobacillus* count was higher in the probiotic supplemented feed groups (Experiment 2, $11 \pm 0.23 \times 10^2$ CFU ml⁻¹) but it was low in control feed group. Total *Vibrio* count was higher in control groups ($45 \pm 0.55 \times 10^{11}$ CFU ml⁻¹), but it was lower in probiotic groups, Experiment 2 about $9 \pm 0.23 \times 10^4$ CFU ml⁻¹ (Figure 5). Average total *Lactobacillus* count in the GI tract after *Vibrio* challenging study were decreased in both probiotic and control groups. But significant variation of total *Vibrio* count was recorded in GI tract of control group ($50 \pm 0.21 \times 10^{10}$ CFU g⁻¹). Total *Vibrio* count was decreased in GI tract of shrimp fed with probiotic feed, but it was significantly high in control group (Figure 6).

DISCUSSION

At present, *Lactobacillus* sp. widely used as probiotics in aquaculture is still increasing (Guerra et al., 2007). Many studies have reported that different species of the genus *Lactobacillus* produce bactericidal proteins (Verschuere

et al., 2000; Farzanfar, 2006) which exhibit strong antimicrobial activity against many pathogenic microorganisms (Rossland et al., 2003; Sanni et al., 1999). The results of the present study also revealed similar effect, in which the culture filtrate of *L. acidophilus* 04 effectively inhibits the growth of *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus*. Balcazar and Rojas-Luna (2007) reported the same inhibitory effect of *Vibrio* species against *Bacillus*. Moreover, previous studies have suggested that the inhibitory effects of probiotics might be due to either alteration of pH in the growth medium, utilization of essential nutrients, or production of volatile compounds (Chaurasia et al., 2005; Yilmaz et al., 2006). In addition, several studies have reported that *Lactobacillus* produces peptide antibiotics like bacteriocins, which are active against a wide range of Gram-positive and Gram-negative bacteria (Enserink, 1999; Zhou et al., 2005). Karthikeyan and Santhosh (2009) reported that bacteriocin producing *L. acidophilus* strain was isolated from the gut of marine prawn (*P. monodon*). This bacteriocin has broad range of antibacterial activity against major food borne pathogens.

The administration of probiotics might constitute a valuable mechanism to increase shrimp growth (biomass) and survival rates. In the present study, average shrimp weight gain of the treatment group were significantly ($P < 0.05$) greater than those of the control after 30 days (Figure 1). However, there were no observed significant differences ($P > 0.05$) for survival in between the experimental groups. This finding supports the earlier finding with other probiotics for the same purposes (Rengpipat et al., 1998; Ajitha et al., 2004; Li et al., 2006; Far et al., 2009).

The shrimp *P. monodon* challenged with the pathogenic bacterium *Vibrio alginolyticus* showed

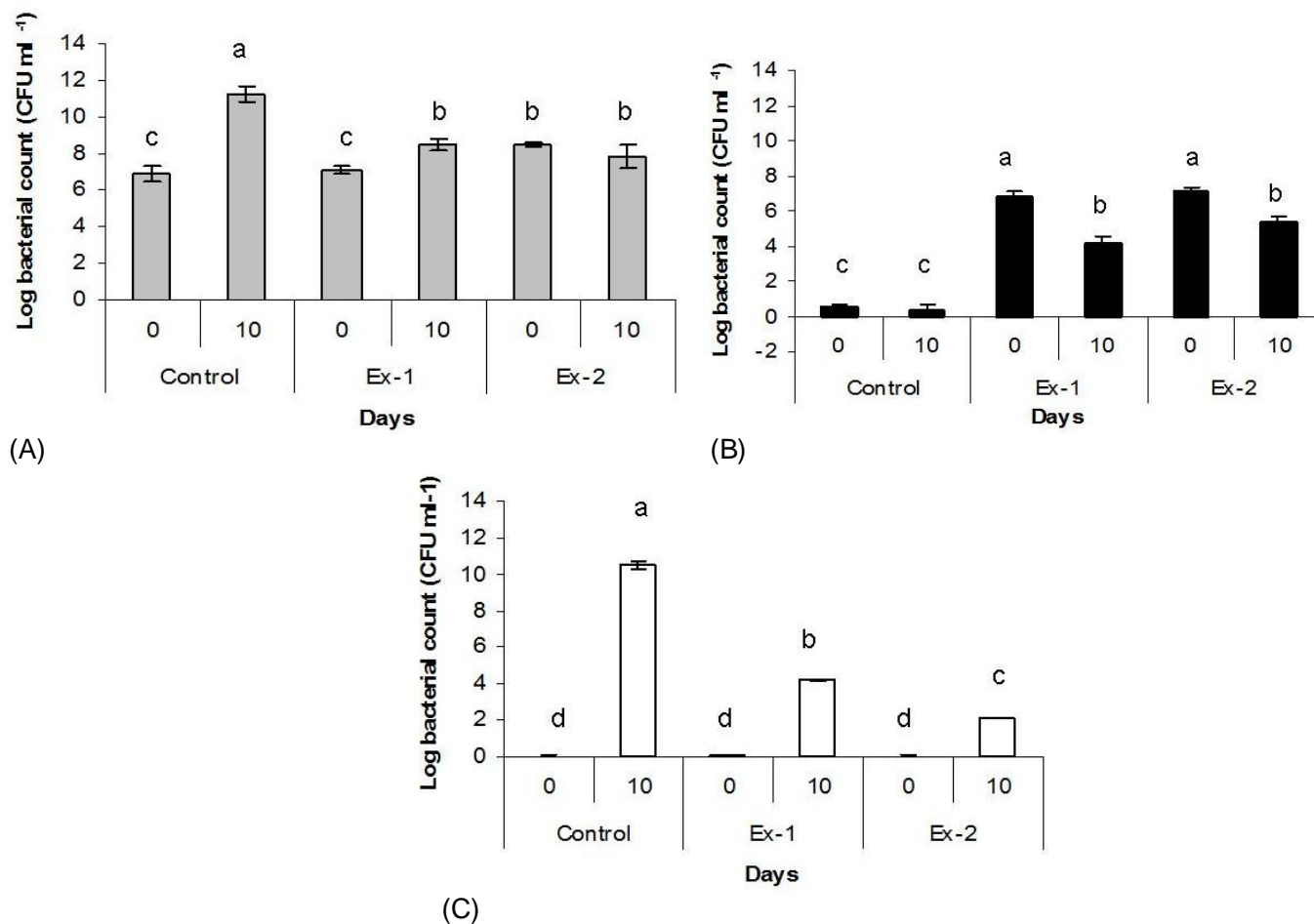


Figure 5. Total bacterial flora in shrimp intestinal tract during pathogen study. The values are average of three separate experiments with \pm SE. The mean values with different superscripts are significantly different ($P \leq 0.05$). (A) Total bacterial count, (B) *Lactobacillus* count, (C) *Vibrio* count. Ex = experiment.

significant ($P < 0.05$) reduction of mortalities at probiotic *Lactobacillus* treated groups and their survival was six fold higher when compared to the untreated group (Figure 2). Similar kind of result was reported by Phianpak et al. (1999). Mortality of the juvenile shrimp was effectively reduced in probiotic treatment during *V. alginolyticus* infection. It is therefore reasonable to speculate that action mechanism of *L. acidophilus* is based on secretion of antibacterial peptide and competitive exclusion against the pathogen. Uma et al. (1999) reported that addition of LAB in *P. indicus* larvae rearing water improve survival rates significantly. Ajitha et al. (2004) reported better survival of shrimp *P. indicus* (56 to 72%) when probiotic *Lactobacillus* supplemented feed groups challenged with *V. alginolyticus*.

In aquatic animals, gastrointestinal tract bacterial flora is mainly composed of Gram-negative bacteria which include *Vibrio*, *Aeromonas* and *Pseudomonas* (Vine et al., 2006). Several researchers reported that administration of Gram-positive bacteria can modify the

GI tract microbiota (Ziaei-Nejad et al., 2006; Vieira et al., 2007) and the culture water (Rengpipat et al., 2000). In the present study, the total count of bacteria and *Lactobacillus* in the GI tract of shrimp before and after challenge with *V. alginolyticus* clearly demonstrate the reduction of shrimp *P. monodon* mortality in treated groups. Shrimp fed with *L. acidophilus* 04 supplemented (treated) diets showed less total bacteria, and high *Lactobacillus*. After challenging with *V. alginolyticus*, total bacteria and *Vibrio* count was comparatively low in treated groups this could be due to inhibition by the secretion of antibacterial peptides and competitive inhibition.

Several mechanisms of bacterial inhibition in GI track of shrimp have been investigated, which include adhesion to digestive track wall to prevent colonization of pathogens (competitive inhibition) (Gomez-Gil et al., 2000) and increased immune competence (Fernandez et al., 2011). Previous studies revealed that LAB produces acid end product and antimicrobial peptides, which may

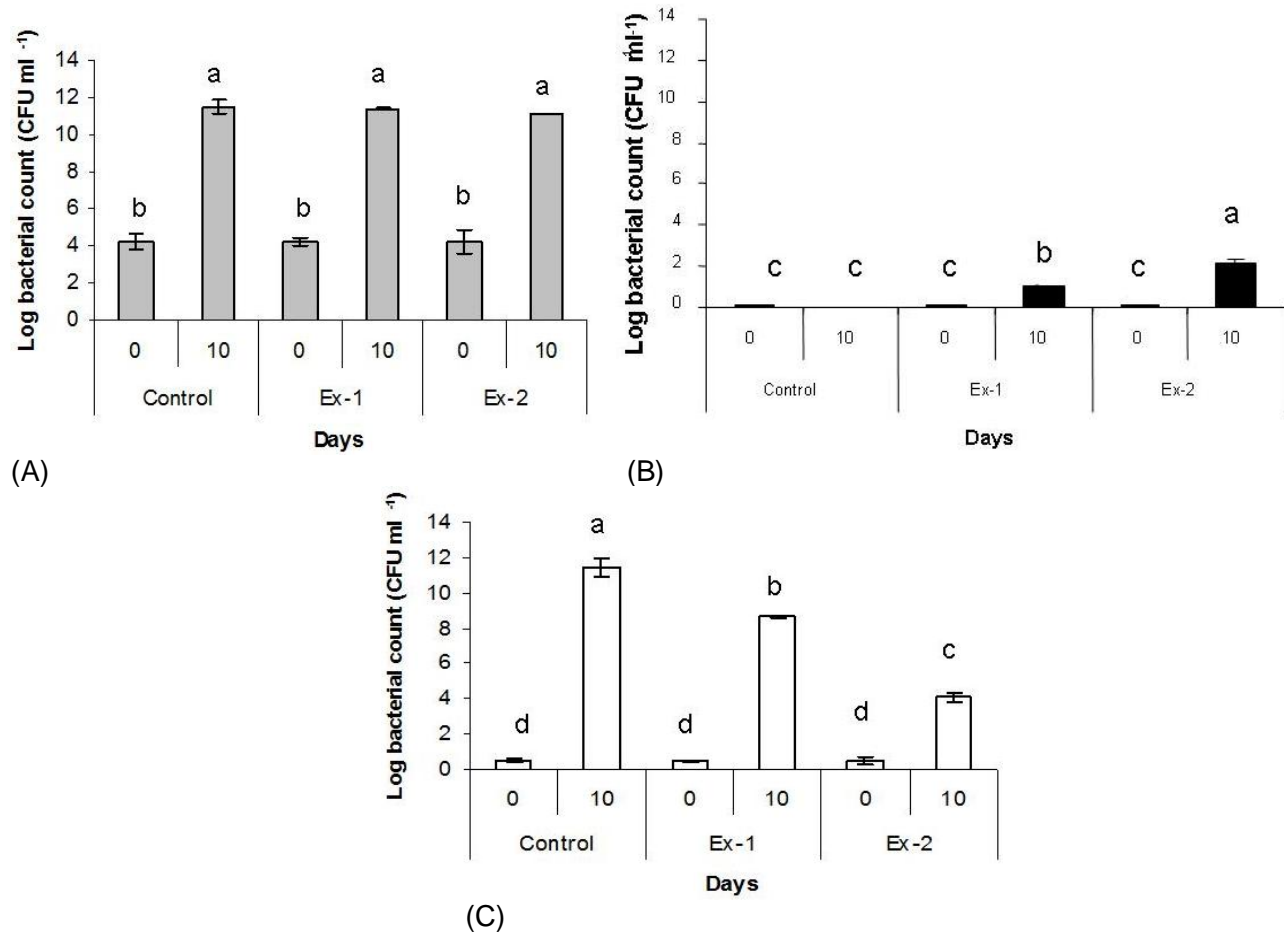


Figure 6. Average bacterial count in the tank water during challenging experiment. The values are average of three separate experiments with \pm SE. The mean values with different superscripts are significantly different ($P \leq 0.05$). (A) Total bacterial count, (B) *Lactobacillus* count, (C) *Vibrio* count. Ex = experiment.

inhibit and reduce the other bacteria in GI tract (Marteau et al., 2001; Gill et al., 2003; Vine et al., 2006; Vinothkumar et al., 2011). In addition, lactic acid bacteria showed a great capacity to inhibit *in vitro* the growth of *V. harveyi* (Vaseeharan and Ramasamy, 2003; Vieira et al., 2007).

The study concluded that the *L. acidophilus* 04 isolate will be helpful in the management of *V. alginolyticus* related bacterial disease in tiger shrimp *P. monodon*. Introducing such specifically screened strains bound to favor the application of probiotics, particularly in shrimp production. The molecular identification, characterization of antibacterial peptide, colonization ability and *in vivo* studies will be a further course of study.

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