CHAPTER 8

EFFECT OF PROBIOTIC BACTERIA ISOLATED FROM FISH INTESTINE TO CONTROL AEROMONAS HYDROPHILA INFECTION AND ENHANCEMENT OF NEUTROPHIL ACTIVITY IN CYPRINUS CARPIO
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8.1. Introduction

Antibiotics used world wide to prevent disease in fish has led to increasing occurrence of resistant bacteria in fish farm (Van der Waaij and Nord, 2000). The strict regulation of using antibiotics in fish and shrimp farming evokes a keen interest in probiotics (Austin et al., 1995; Barker, 1998; Gibson et al., 1998; Rengpipet et al., 1998; Gomez-Gil et al., 2000). Probiotics may provide an alternative method to protect the fish from infectious diseases. Many researchers reported, control of fish pathogens A. salmonicida and Yersina ruckeri by administering probiotics in feed (Nikoskelainen et al., 2001; Irianto and Austin, 2002, 2003; Nikoskelainen et al., 2003; Raida et al., 2003). The selection criteria for probiotic should evaluate colonization methods, competition ability against pathogen, bile resistant, survival in low pH, immunostimulatory, growth effect and adhesion to the intestinal mucus (Gatesoupe, 1999; Gomez-Gill et al., 2000). Although many people have studied the adhesion of probiotic to gut wall, a very few have attempted to isolate and select probiotics based on in vitro growth characterization. A survey of fish and shrimp farms and hatcheries situated on the east coast of Tamil Nadu and Pondicherry clearly shows the farmer's preference for probiotics isolated from the fish or shrimp rather than the commercial product from other hosts (Babu, 2004). The mode of
action of probiotics was found to be inhibiting pathogenic bacteria by bacteriocin production and enhancing the cellular immune response of fish (Irianto and Austin, 2002; Nikoskelainen, 2003; Panigrahi et al., 2005). However no reports were available to control *A. hydrophila* using probiotics.

The present study aimed to isolate and screen the potential probiotic bacteria based on the *in vitro* growth characteristics. The objective of the study was (i) to understand the immune response induced by feeding different probiotic bacteria (ii) to observe the population of probiotics in the gut during the experimental period (iii) to control the *A. hydrophila* infection.

8.2. Materials and Methods

8.2.1. Bacterial strains

A culture of *Carnobacterium divergens* was supplied by NRRL, USA. *A. hydrophila* strain AHPU 13 was isolated from diseased fish. The species level identification of the strain was carried out by biochemical (Joseph and Carnahan, 1994) and polymerase chain reaction (Chilaka, 2001) and pathogenesis routinely tested for by inoculation into common carp (Davis and Hayasaka, 1983). Subculture of *A. hydrophila* and probiotic bacteria were maintained on Tryptone soy agar slopes (Himedia, Mumbai, India) at 5°C. A stock culture in Tryptone soy broth (Himedia, Mumbai, India) was stored at -70°C with 0.85% (w/v) NaCl and 20% (v/v) glycerol to provide stable inocula throughout the study (Yadav et al., 1992; Chabot and Thune, 1991).
8.2.2. Isolation and identification of probiotic bacteria

Freshly caught fish (*Latus calcarifer* and *Mugil cephalus*) and shrimp (*Penaeus monodon* and *P. indicus*) were collected from the local fish market and kept in ice and transported to lab. The skin was then washed with 70% ethanol before opening the ventral surface with sterile scissors. Whole intestine was minced with scissors and 1 g of intestine was taken and suspended in 9 ml of sterile saline diluents (0.85% NaCl). The serial dilution was made up to $10^6$ and 0.1 ml of the solution was suspended onto MRS agar plates. The inoculated plates were incubated at room temperature ($27 \pm 2^\circ$C) for 24 to 48 h. The typical white color colonies obtained were screened by antagonistic activity against *A. hydrophila* by cross streaking and agar spot method (Plate VIII). The probiotic isolates were characterized on the basis of morphological physiological and biochemical by Bergey’s Manual of Systematic Bacteriology (Holt et al., 1993).

8.2.3. Inhibition of fish pathogens by probiotic bacteria

The antagonism against fish pathogens *A. hydrophila*, *A. salmonicida*, *V. parahaemolyticus*, *V. fischeri*, *V. anguillarum* and *V. vulnificus* by probiotic bacteria were assessed using a previously described cross streaking method. For this, a suspension of the pathogen in saline (containing $10^7$ cells / ml) were streaked at right angles across inoculate of the probiotics on TSA agar plates or as appropriate with incubation at $27 \pm 2^\circ$C for 24 h. Antagonism by probiotic bacterium was indicated by interruption in the growth of the pathogens.
Plate VIII. Screening of probiotic bacteria by (A) Cross streaking and (B) Agar spot method.
Antagonistic activity was measured by agar spot method (Gratia, 1946; Kekessy and Piguet, 1970).

8.2.4 Screening for bacteriocin producing strains

Most are based on the diffusion of bacteriocin through solid semisolid culture media to inhibit the growth of sensitive organisms. Deferred antoagonism or indirect method includes the spot on the lawn assays as described by (Gratia, 1946) and the flip streak method of (Kekessy and Piguet, 1970). In the agar spot on the lawn method, the putative bacteriocin producer is spotted on an agar medium and incubated overnight to develop individual colonies; the colonies are then overlaid with sensitive indicator organisms and again incubated to develop zones of inhibition. In the fillip streak method, the putative bacteriocin producing strain is streaked onto the medium, incubated, and a bacteriocin sensitive organism is subsequently streaked perpendicular to the fist on the reverse side of the agar. In a indirect method (Tagg et al., 1976) both the test and indicator organisms are grown simultaneously and demonstration of antagonism depends upon the release into the medium of a diffusible inhibitor early in the growth phase of the test organisms. In the well-diffusion method (Tagg and McGiven, 1971) supernatants from putative bacteriocin producing cultures are placed in wells cut into the agar inoculated with a sensitive microorganisms. The latter method is often used as bioassay for the determination of bacteriocin titers. Finally, the density of the indicator lawn is an important determinant of the sensitive of the assay method used.
8.2.5. PCR amplification of the 16S rDNA of probiotics

The small subunit rDNA gene was amplified using two primers Viz. 16S1 (5'-GAGTTTGATCCTGGCTCA-3') and 16S2 (5'-ACGGCTACCTTGTTACGACTT-3'), complementary to the conserved regions at the 5' and 3' ends of the 16S rDNA of *E. coli* corresponding to positions 9 to 27 and 1498 to 1477, respectively. The PCR amplification reaction mix of 50 μl, contained bacterial DNA (~200 ng), 1 μl (3 units) *Taq* DNA polymerase, 5 μl of *Taq* buffer (10 mM TAPS, pH 8.8, 3 mM MgCl₂, 50 mM KCl and 0.01% gelatin), 5 μl of 2 mM dNTP mix and 5 μl of each primer (10 pM/μl). Amplification was carried out in Eppendorf Thermocycler, programmed for 30 cycles. In each cycle denaturation was done at 94°C for 20 s, annealing was done at 48°C for 20 s and extension was done at 72°C for 40 s. A final extension of 5 min was carried out at 72°C at the end of 30 cycles. In all the reactions, water was used in place of DNA as a negative control. The amplified DNA fragment of approximately 1.5 kb was separated on a 1% agarose gel and purified by using Qiagen spin columns. The purified fragment was used directly for DNA sequencing.

The desired DNA band from agarose gel was excised, weighed and transferred to a sterile microfuge tube containing thrice the volume of Buffer (300 μl /100 mg) and transferred to a water bath maintained at 65°C for 10 min. The contents were then transferred to a Qiagen column (Catalogue No. 28704, Qiagen Inc. USA) and spin at 8000 rpm for 2 min. Subsequently the column was washed twice with 750 μl of Buffer PE and eluted with 30 μl of sterile water.
8.2.6. Sequencing of 16S rDNA of probiotic bacteria

The purified 1.5 kb DNA product was sequenced using primers listed in Table 3. Sequencing of the purified PCR product (~200 ng/reaction) was carried out using 5 pmole of a given sequencing primer and 3 μl of ready reaction mix from the Big Dye Terminator sequencing kit (Perkin Elmer) in a total volume of 5 μl. Cycle sequencing was carried out in a Gene Amp PCR machine (Perkin Elmer, 9600) for 30 cycles. Each cycle consisted of a denaturation step at 96°C for 10 s, an annealing step at 50°C for 10 s and an extension step at 60°C for 4 min. After the PCR, the products were precipitated using 1 μl of 3 M sodium acetate (pH 4.6) and 50 μl of ethanol and incubated on ice for 15 min. The pellet was recovered by centrifugation at 15000 rpm for 20 min at 4°C, washed with 70% ethanol, dried under vacuum and dissolved in 10 μl of loading buffer [formamide: 25 mM EDTA (4:1)]. About 2 μl of the sample was used to analyze the sequence on an automatic DNA sequencer (ABI prism Model 3700, Applied Biosystems, California, USA).

8.2.7. Sequence similarity analysis

The reference sequences required for comparison were downloaded from the EMBL database using the site http://www.ncbi.nlm.nih.gov/Genebank.

8.2.8. Fatty acid methyl ester (FAME) analyses

Probiotic bacteria were grown on TSA for 24 h at 37°C and loop full cells were suspended in 1 ml of saponification reagent in a clean, dry 13 mm X 100 mm screw cap tube by vortexed for 10 seconds. The tube was then placed in a
100°C water bath and alternatively vortexed for 10 seconds before placing once again in a 100°C water bath for 25 min. The tube was then cooled slowly to room temperature. To this, 2 ml of methylation reagent was added, vortexed and placed in a water bath at 80°C for 10 min. The sample was then cooled rapidly by placing in ice. Extraction buffer (1.25 ml) was added to the sample and mixed well for 10 min. The aqueous lower layer was removed and discarded. To the upper organic phase, 3 ml of base wash reagent was added and mixed well for 5 min. The mixture was then centrifuged at 3000 rpm for 5 min. The upper solvent phase was removed and was analyzed by gas-liquid chromatography (Hewlett-Packard 6890, USA) using capillary column Ultra 2-HP (cross linked 5% phenyl-methyl silicone; 25 m, 0.22 mm; film thickness, 0.33 µm) and hydrogen as the carrier gas. FAME compounds were detected by a flame ionization detector (FID) and identified using the Microbial Identification Software (Sherlock aerobe method and TSBA library version 3.90) developed by MIDI Inc. (Newark, USA).

8.2.9. Isolation of extra cellular products (ECP)

The ECP of probiotics were isolated by cellophane plate assay (Inamura et al., 1984). Three probiotic isolates (MC13, LC149 and PI80) and one reference strain (*C. divergenses*) were inoculated in 5 ml of MRS broth and kept at room temperature for 12 h. 0.2 ml of 12 h culture of these strains was poured on cellophane paper covered MRS agar plates. After 24 h of incubation, the plates were washed with 2 ml of PBS (pH 7.3) and the supernatant was collected. The supernatant was centrifuged at 10,000 rpm for 20 min and the
aqueous layer was collected and filtered through 0.45 μm membrane to remove additional cells present in the solution. The crude ECP was used for the estimation of protein and SDS-PAGE analysis for the determination of bacteriocins.

8.2.10. Intestinal mucus growth

Intestinal mucus growth of probiotics was performed as described by Cohen and Laux, (1995) with some modification. The fish, C. carpio weighing 50 g (10 no.) were segregated and placed in separate tank for 15 days starvation. The fish were killed by head blow and cut open the stomach. The mucus was collected from the fish intestine and filtered by 0.22 μm size of Millipore filter and finally stored at -20°C until use. The intestinal mucus was diluted to a protein concentration of 1mg/ml with sterile PBS. 150 μl of diluted mucus sample was added to 96 well microtitre plates. 50 μl of 0.25 OD LAB was added in each well (three well for each strain) and incubated for every 6 h at 37°C for 48 h. The absorbance was measured at 600 nm for every 6 h.

8.2.11. Preparation of experimental diets

All the probiotic bacteria were grown for 24 h at room temperature (27 ± 2°C) in MRS broth (Hi-Media, Mumbai, India). All cells were harvested by centrifugation at 10000 rpm for 20 min. The pellets were re-suspended in PBS at the concentration of 10^{10} cfu/ml and mixed with feed to contain the final count of 10^8 cfu/g of feed. 2% of egg white was used as binder for feed. Control diet received devoid of lactic acid bacteria. The viability of probiotic bacterium was
assessed after storage of feeds in 4°C in different days of storage. Thus 1g quantity of food was homogenized in 9 ml volume of 0.85% (w/v) saline and serial dilution prepared until 10⁻⁸, where upon 0.1 ml was spread on triplicate plates of MRS agar. Colony was determined after incubation at 27 ± 2°C for 24h.

8.2.12. Fish experiments

The experiments were conducted for 60 days using 25 ± 5 g of common carp (C. carpio) obtained from locally available fish farm. Healthy common carp were acclimatized in 15 collapsible tanks (1500 L) and were fed unaltered formulated feed once in a day. The experiment was conducted as a completely randomized design with five treatments (a) fish fed diet mixed with isolate of MC13 (b) fish fed diet mixed with isolate of LC149 (c) fish fed diet mixed with isolate of PI80 (d) fish fed diet mixed with C. divergenes and (e) fish were fed unaltered diet (control). Each treatment had three replicates of 45 fish each. Fish were fed once in a day at 3% of the body weight.

8.2.13. Sample collection

Five fish from each group were randomly sampled from 7th, 15th, 30th and 60th day of feeding for analysis of blood and intestinal probiotic population. The blood was (heparinised 150 IU per ml) collected after anesthetizing the fish with MS-222 from the gill vein of the fish from each experimental tank and tested for the Nitroblue tetrazolium assay. The intestine was collected and the probiotics counts were made as described previously in this paper. Based on
the antagonisms to the same pathogen, similar colony morphology characteristics (Gram stain, shape, color and motility, oxidase and catalase production), natural probiotic present in the gut were eliminated.

8.2.14. Challenge study

Fish fed with the probiotic bacteria supplemented diet (MC13, LC149, PL80 and *C. divergens*) and non-supplemented diet were challenged with *A. hydrophila* on the day of 30 and 60. On day 30, 10 fish in each group were netted out and was given intramuscular injections of *A. hydrophila* and reared in separate tanks. On 60th day the remaining fish in the tanks were netted out and injected with *A. hydrophila*. Challenge study was performed by injecting $10^6$ cells/ml by intra muscular injection into the fish. Observation was made for a period of 15 days and data on mortality was calculated according to Amend (1981).

8.3. Results

8.3.1. Isolation and screening of probiotic bacteria

A total of 160 numbers of lactic acid bacteria have been isolated from fish (*L. calcarifer*, 43 and *M. cephalus*, 67) and shrimp (*P. indicus*, 32; and *P. monodon*, 18) intestine. Among these, 16 isolates were showing antagonistic activity against *A. hydrophila*. Finally, three isolates of MC13 (*M. cephalus*), LC149 (*L. calcarifer*), PL80 (*P. indicus*) and one standard strain of *C. divergens* have been selected for the in vivo study by maximum zone of inhibition against *A. hydrophila* and wide spectrum activity against other fish pathogen such as
A. salmonicida, V. anguillarum, V. fischeri, V. vulnificus and V. parahaemolyticus. The zone of inhibition against A. hydrophila was 31 mm, 27 mm, 23 mm and 12 mm in respect of MC13, LC149, PI80 and C. divergens (Table 12).

In respect of biochemical identification, three isolates belong to the group of cocci, gram positive, oxidase and catalase negative. The probiotic isolates utilizes the sugar of glucose, lactose and sucrose. MC13 utilized the sugar of raffinose but others were not. MC13 and LC149 belong to the group of Enterococcus and Lactococcus identified by 16S rRNA sequencing (Figure 9 &10) and fatty acid methyl ester (FAME) methods. Partial sequence of MC13 (AY751462) and LC149 (AY751463) has been submitted to National Center for Biotechnological Information, USA (NCBI). Isolate PI80 was identified as Streptococcus by 16S rRNA sequencing (Figure 11). In respect of antibiotic sensitivity test, MC13 and C. divergens were sensitive to chloramphenicol and LC149 and PI80 were resistant. Similarly MC13 was resistant to rifambicin but LC149 and PI80 were sensitive. All the probiotic strains were sensitive to tetracycline, except LC149 which showed the intermediate activity.

8.3.2. Molecular weight of proteins from ECP of probiotics

The crude ECP of the four probiotic bacteria was concentrated and analyzed using SDS-PAGE (Plate IX). The gel shows four distinct bands with the molecular weigh range of 38.33, 31.30, 22.96 and 21.81 kDa in the MC13 isolate ECP. Four distinct bands also were observed whose molecular weight were 45, 31.3, 30.69 and 20 kDa in LC149 and PI80 isolates. Whereas in the
Table 12. Antagonistic activity (zone of inhibition) of probiotics bacteria against fish pathogens.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>MC13 (mm)</th>
<th>PI80 (mm)</th>
<th>LC149 (mm)</th>
<th>CD (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hydrophila</td>
<td>31</td>
<td>23</td>
<td>27</td>
<td>12</td>
</tr>
<tr>
<td>A. salmonicida</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>V. anguillarum</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>V. vulnificus</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>V. fischeri</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>30</td>
</tr>
<tr>
<td>V. parahaemolyticus</td>
<td>NI</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

CD – Carnobacterium divergenes
Figure 9. 16S rDNA sequence of probiotic isolate (LC149) of *Enterococcus* sp.

```
GCCACATTGGGACTGAAACACGGCCCAAACTCCTACGC 70
AGGGTGAGTAGACAGGTGGGTAAAGGCTTCAGCTGAC 140
TATAACAATCGAGACCCGATATTTTGATTTGAAAGCCCTTTCGCTGAGTATGGATGGACCGCG 210
GTGATTAGCTAGTTGTTGAGTTAACGGCTCAACAGCGAGTTAACCGCAATCTGATGGATGGAC 280
GCCACATTGGGACTGAAACACGGCCCAAACTCCTACGC 350
CAACGTCTGACGAGCAACGCCGCGTGAGTGAAGAAGGCTTTCGTCGTCTCTGTTGTTAG 420
AACAAGGATGAAGTA 435
```
Figure 10. 16S rDNA sequence of probiotics isolate (MC13) of *Lactococcus* sp.
Figure 11. 16S rDNA sequence of probiotic isolate (P180) of *Streptococcus* sp.
Plate IX. SDS-PAGE analysis of bacteriocin from probiotic bacteria. The crude bacteriocin was extracted and subjected to gel electrophoresis.
ECP of *C. divergens* only two distinct bands of molecular weight range 45 and 20 kDa were present. All these proteins were suspected to be bacteriocins as bacteriocin activity was found in all the ECP of the probiotics.

### 8.3.3. Growth in intestinal mucus

Among the four probiotic isolates (MC13, LC149, PI80 and *C. divergens*) and two fish pathogen (*A. hydrophila* and *V. fischeri*) selected for the fish intestine mucus growth study, the higher growth was observed in *A. hydrophila* at 42 h (1.22 ± 0.021 OD) followed by *V. fischeri* (Figure 12). All the four probiotics strains grew slower than fish pathogens and maximum growth was observed between 18-24 hours after incubation in the intestinal mucus. Thereafter their growth was started to decline. However, the growth of both fish pathogens remained in the stationary phase even until 48 hours.

### 8.3.4. LAB count in intestine

Before starting the experiment, fish had minimum number of natural *Lactobacillus* sp. in the fish intestine. The number of viable *Lactobacilli* increased significantly (P<0.05) in the entire probiotic fed group except control. The probiotic count was increased two logs at the end of first week in MC13 (2.46 ± 0.34X10^5), LC149 (2.3X10^5), PI80 (2.31± 0.18X10^5) *C. divergens* (2.3 ± 0.16 X10^5) fed fish and there was no increment in control fish (3.3 ± 0.46X10^3) (Figure 13). Significant growth stability was observed on 60th day of probiotic fed fish in all the experiment but highest count was observed in PI80 (3.97 ± 0.32X10^6) followed by MC13 (3.74 ± 0.51X10^6), CD (3.72 ±
Figure 12. Growth of probiotics and fish pathogenic bacteria in *C. carpio* intestinal mucus at different duration (h) conducted in flat bottomed ELISA plates. Each value (mean ± SD) is the average performance of five wells / treatment for a period of 54 h.
Two way analyses of variance were performed to find out the significance to compare treatments and duration (days). The LAB count significantly varied between the five treatments ($F_2$ 6.46; $P<0.01$; df 4) and the duration ($F_2$ 5.8; $P<0.01$; df 4) of the treatment. Subsequently SNK test was performed to compare the means. There is a significant difference between control and the treatment means. The treatment means were then ranked. The treatment of PI80, MC13, CD and LC149 yielded the maximum LAB count in the gut ($P<0.01$) proving the maximum efficacy of probiotics (Figure 13).

### 8.3.5. Neutrophil activity

The neutrophil activity of probiotic fed *C. carpio* was significantly ($P<0.01$) increased in all the experimental groups on day 7 (Figure 14). On 60th day of probiotic feeding, significant higher neutrophil activity was observed in MC13 fed fish ($1.5245 \pm 0.379$) followed by CD ($1.10 \pm 0.0492$), LC 149 ($0.933 \pm 0.09$) and PI80 ($0.827 \pm 0.0431$) than control fish ($0.2915 \pm 0.0734$). Two way Analyses of variance were performed to find out the significance to compare treatments and duration (days). The NBT activity significantly varied between the four treatments ($F_2$ 9.19; $p<0.05$; df 4) and days ($F_2$ 6.28; $p<0.01$; df 4) (days) of the treatment.

### 8.3.6. Relative Percentage of Survival

During the challenge test, high survival was observed in MC13 and LC149 fed groups on 30th and 60th day of challenge (Table 13). The highest
Figure 13. Mean probiotic bacterial counts in *C. carpio* intestine in different treatment and control. Each value (mean ± SD) is the average performance of five fish / treatment for a period of 60 days.
Figure 14. NBT activity of probiotics and control fed diet at 7, 15, 30 and 60th day of feeding. Each value (mean ± SD) is the average performance of five fish / treatment for a period of 60 days.
Table 13. Survival (%) of *C. carpio* after feeding with probiotics and challenging by *A. hydrophila*.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of challenged fish</th>
<th>Mortality to <em>A. hydrophila</em> (%)</th>
<th>Survival (%)</th>
<th>Relative Percentage Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>30th Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC 13</td>
<td>10</td>
<td>2 (20)</td>
<td>80</td>
<td>75*</td>
</tr>
<tr>
<td>LC149</td>
<td>10</td>
<td>3 (30)</td>
<td>70</td>
<td>62.5*</td>
</tr>
<tr>
<td>PI80</td>
<td>10</td>
<td>6 (60)</td>
<td>40</td>
<td>25*</td>
</tr>
<tr>
<td>CD</td>
<td>10</td>
<td>4 (40)</td>
<td>60</td>
<td>50*</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>8 (80)</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>60th Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC 13</td>
<td>15</td>
<td>3 (20)</td>
<td>80</td>
<td>78**</td>
</tr>
<tr>
<td>LC149</td>
<td>13</td>
<td>3 (23)</td>
<td>77</td>
<td>74.7**</td>
</tr>
<tr>
<td>PI80</td>
<td>15</td>
<td>8 (53.33)</td>
<td>46.67</td>
<td>41**</td>
</tr>
<tr>
<td>CD</td>
<td>14</td>
<td>5 (35.71)</td>
<td>64.29</td>
<td>61**</td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>10 (90.9)</td>
<td>8.33</td>
<td>-</td>
</tr>
</tbody>
</table>

* *p < 0.01; ** *p < 0.001; ns: not significant

*Fish were challenged by intramuscular injection with the *A. hydrophila* strain.

*Relative percent of survival = 1 - [% mortality in the probiotic fed group /% mortality in the control group] X 100. RPS values over 50 indicate positive effect of the probiotics (Amend, 1981).
relative percentage survival was observed in MC13 fed fish on both 30\textsuperscript{th} (75\%) and 60\textsuperscript{th} (77.8\%) day of challenge followed by LC149 fed fish on the day of 30 (62.5\%) and (74.7\%). However high mortality rate was found in groups fed with other two probiotics CD and PL80. Some of the fish in the experimental as well as controls developed the symptoms of \textit{A. hydrophila} infection on the external body surface. However the infected area of the body surface was smaller in probiotic fed fish compared to control and slowly disappeared after continuously fed with probiotics.

8.4. Discussion

In this study the role of the isolated beneficial bacteria from fish intestinal micro flora are potential competitors of the pathogenic bacteria was investigated. Hemorrhagic septicemia is one of the major diseases in fresh water fish farms in Asia and other countries. Currently, chemotherapy agents were used to protect fish in hatchery as well as in farm condition against hemorrhagic septicemia and other bacterial diseases. Vaccine was available for few diseases only. The use of probiotics in aquaculture is a new potential and natural prevention mechanism against fish disease caused by pathogenic bacteria. This possibility has already been reported by many workers (Olsen et al., 1992; Austin et al., 1995; Joborn, 1998; Robertson et al., 2000).

The good adhesion ability to mucosal surfaces is a prerequisite for bacterial colonization, and has been well documented for \textit{L. rhamnosus} (ATCC 53103) to fish mucus as well as to human mucus (Kirjavainens et al., 1998, Ouwehand et al., 1999). In this study all the four probiotic bacteria, MC13, PL80,
CD and LC149 grew well but slower than the pathogenic bacteria in the intestinal mucus. We suggest that, these probiotic may have the ability to resist bile and adhere to intestinal wall and utilize the mucus as a growth medium. The probiotic strains of *L. rhamnosus* ATCC53103, *L. bulcarius*, *B. lactis*, Bb12 and *L. johnsonii* La1 tended to adhere in high numbers to the different fish mucus types (Nikoskeleinan et al., 2001). Similarly, Vine et al. (2004) reported that pathogenic bacteria *A. hydrophila* and *V. alginolyticus* had high growth than isolated probiotics in clown fish intestinal mucus. To avoid constant flushing from the intestine during gut evacuation and to compete with pathogens for attachment, in the mucus of the intestinal wall, a fast growing probiotic bacteria should be selected or probiotics has to be fed to fish daily at a higher concentration to maintain the population in the intestine to compete for the space with pathogens in the mucus (Cahill, 1990). However discontinuation of probiotics feeding in *C. carpio* for ten days drastically reduces the probiotic population in the intestine (Khan, 2002). The population was restored after continuation of probiotic feeding. Similar observation was made by Nikoskeleinan et al. (2003) and Panigrahi et al. (2005) in rainbow trout. Joborn, (1998) observed a full wash out of probiotic bacteria in three days. Hence in this study probiotics were administered daily and two of the probiotics CD and LC149 though had a slow growth in vitro study was found to be in large numbers in the gut.

Electrophoretic analysis of concentrated ECP of probiotics shows presence of bacteriocins having various molecular weights ranging from 20.5 kDa to 45 kDa. Though most of the bacteriocins characterized from probiotics
were below 10 kDa, bacteriocin with large molecular weight (122 kDa) have been detected and characterized from *Lactobacillus plantarum* (Lash et al., 2005). This bacteriocin inhibits the growth of Gram-positive and Gram-negative bacteria including *Staphylococcus aureus, Escherichia coli, Listeria innocua* and *Pseudomonas aeruginosa*. In the present study we have not identified which bacteriocins inhibit the pathogenic bacteria *A. hydrophila*.

It has been shown with humans that certain probiotic bacteria are able to stimulate phagocyte activity (Scchiffrin et al., 1997; Arunachallam et al., 2000), as well as complement receptor expression (Pelto et al., 1998). The present study demonstrates that administration of probiotic bacteria through fish feed stimulated the NBT activity in all groups after two weeks of feeding. In our study the significant NBT activity was observed in all the groups fed with probiotics. Maximum reduction of NBT was observed in the group fed with MC13. The reason might be extra cellular products of probiotic bacteria like bacteriocin and nisin like compounds may induce the immune system of the fish. Nisin is a potent immunostimulants which induce the headkidney macrophages, chemiluminecent and serum lysozyme activity in turbot (*Scophthalmus maximus* L) at the concentration between 2.5 and 0.025 μg ml⁻¹ (Villamil et al., 2002). Enhancement of phagocytosis, lysozyme activity, macrophages and immunoglobulin were also observed in rainbow trout *Oncorhynchus mykiss* by administration of probiotics like *Carnobacterium* sp. *Vibrio fluvialis* *Lactobacillus rhamnosus*, and *L. rhamnosus* JCM1136 (Nikoskelainen et al., 2001; Irianto and Austin, 2002, 2003; Panigrahi et al., 2005).
In vitro bacterial activity results were well correlated with in vivo observation. Our experiments showed that higher relative percentage survival was observed on 60th day of challenge compared with 30th day of challenge when fish were fed with probiotics MC13, LC149 and CD. Similarly the mortality of rainbow trout was reduced when challenged with *A. salmonicida* and *Yersinia ruckeri* by administration of probiotics (Nikoskelainen et al., 2001, Irianto and Austin, 2002, 2003, Nikoskelainen et al., 2003; Raida et al., 2003). There are several ways by which probiotic bacteria can induce bacterial antagonism, by producing antimicrobial agents, such as antibiotics, antimicrobial peptides or siderophore substances (Sugita et al., 1998). Bacillus has been found to produce polymyxin, bacitracin and gramicidin antibiotics (Rhodehamel and Harman, 1998). However Bacillus bacterial antagonisms also can be provoked by competition to obtain nutrients with other fast growing bacteria (Moriarty, 1998). The results demonstrated that MC13, LC149, and *C. divergens* could themselves function as probiotics in the prevention of *A. hydrophila* infection in *C. carpio* by competitive exclusion or by stimulation of defense reaction in the host. So, these probiotic bacteria may introduce into the field level application for the further study.