7.1. Introduction

Prevention of diseases is a primary goal in aquaculture. The application of hygienic and preventive measures of the environment, such as fish health management, sanitation and disease control procedures are critical factors to prevent fish disease. When faced with disease problems, the common response has been to turn to antimicrobial drugs. Use of antibiotics in aquaculture may lead to the development of drug resistant bacteria and transfer of resistant genes between bacteria (Witte, 2000; Schwarz et al., 2001), the accumulation of residual antibiotics in aquaculture products (Cabello 2006; Hoque, 2014), environmental pollution (Kumari et al., 2007) and detrimental effect on the microbial biodiversity (Zhou et al., 2010). It also has resulted in trade restrictions in export markets. The drawbacks of using antibiotics in
aquaculture evoke a keen interest in probiotics (Wang et al., 2008; Watson et al., 2008; Bloch et al., 2013). They are also an alternative to the use of vaccines to protect the fish from infectious diseases, as vaccines cannot be relied as a universal disease control measure in aquaculture due to their limited availability in few countries and their pathogen specific action of protection against certain bacterial and viral diseases (El-Ezabi et al., 2011).

The term "probiotics" was originally used by Lilley and Stillwell (1965) to describe a substance(s) that stimulate the growth of other microorganisms. The term has now been redefined. The definition of probiotic differs greatly depending on the source, but the most widely quoted definition for probiotics was made by Fuller (1989). He defined probiotic as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. It is a live microbial dietary additive which confers health advantages and had its long history of use in humans and animals (Ige, 2013). It can also be defined as “a viable microorganism which when ingested through the oral cavity in a sufficient quantity confer on the host a beneficial effect due to an improvement in the intestinal microbial balance” (Giorgio et al., 2010). Merrifield et al. (2010) defined probiotic for aquaculture as “a live, dead or component of a microbial cell that when administered via the feed or to the rearing water benefits the host by improving either disease resistance, health status, growth performance, feed utilization, stress response or general vigour, which is achieved at least in part via improving the hosts microbial balance or the microbial balance of the ambient environment”. Another proposed definition of probiotics used in aquaculture is “live microbial cultures added to feed or environment (water) to increase viability (survival) of the host” (Ringo et al., 2010). Probiotics are
biopreparations containing living microbial cells that optimize the colonization and composition of the growth and gut microflora in animals and stimulate digestive processes and immunity (Dhanaraj et al., 2010). Another definition considered appropriate for aquaculture by Ige (2013) is “any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or fish consumer, which is achieved, in part at least, by improving the microbial balance of the fish”. Probiotics may reduce the incidence of disease or lessen the severity of outbreaks in aquaculture.

In aquaculture, the range of probiotics evaluated for use is considerably wider than in terrestrial agriculture. Several probiotics either as monospecies or multispecies supplements are commercially available for aquaculture practices (Decamp and Moriarty 2006; Ghosh et al., 2007). Probiotics protect fish by various mechanisms such as blocking adhesion sites for pathogens, production of organic acids (formic acid, acetic acid, lactic acid) to lower pH and alter protein structure, production of hydrogen peroxide and reactive oxygen species to damage enzyme systems in pathogens and by the activation of innate and adaptive immune responses to amplify killing of pathogenic agents (Bloch et al., 2013). Probiotic microorganisms will of course, have to be non-pathogenic and non-toxic in order to avoid undesirable side-effects when administered to fish. Studies have shown that probiotics are important for weight gain in fish (Aly et al., 2008; Al-Dohail et al., 2009; Sharma et al., 2013).

Enhancement of the immune system seems to be the most promising method for preventing fish diseases. The immune systems of fish have two integral components: the innate, natural or nonspecific defence system and the
adaptive, acquired or specific immune system. The normal micro biota in the gastrointestinal system influences the innate immune system, which is of vital importance for the disease resistance of fish. It is reported that the non-specific immune system can be stimulated by probiotics (Lara-flores, 2011).

The success of probiotics, has laid the foundation for other concepts like “prebiotics” which are the non-digestible food ingredients that selectively stimulate the growth and/or activity of one or limited microbes and “synbiotics”, the nutritional supplements combining probiotics and prebiotics. The obvious potential advantages of such approaches are that they promote specific microbe(s) in the intestine for restoring the intestinal microbial balance and exerting numerous beneficial effects in host (Nayak, 2010).

7.2. Review of literature
7.2.1. Bacteria used as probiotics in aquaculture

In aquaculture practices, probiotics are used for a quite long time but in last few years probiotics became an integral part of the culture practices for improving growth and disease resistance. This strategy offers innumerable advantages to overcome the limitations and side effects of antibiotics and other drugs and also leads to high production through enhanced growth and disease prevention (Mishra et al., 2001; Das et al., 2008; Sahu et al., 2008; Nayak, 2010).

Most probiotics proposed as biological control agents in aquaculture belong to the Lactic acid bacteria (Lactobacillus, Lactococcus and Carnobacterium), such as Lactococcus lactis, Lactobacillus plantarum, L. rhamnosus, L. sakei, L. delbrueckii, Leuconostoc mesenteroides, Carnobacterium divergens and
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C. inhabens (Balcázar et al., 2007; Balcazar et al., 2008; Hagi and Hoshino, 2009; Merrifield et al., 2010; Pérez-Sánchez et al., 2011; Ige 2013).

Another important group involves Bacillus, such as Bacillus subtilis, B. clausii, B. cereus, B. coagulans, B. circulans and B. licheniformis (Salinas et al., 2005; Ochoa-Solano and Olmos-Soto, 2006; Balcázar and Rojas-Luna, 2007; Newaj-Fyzul et al., 2007; Bandyopadhyay and Das Mohapatra, 2009; Nakayama et al., 2009; Sun et al., 2010; Ai et al., 2011; Olmos et al., 2011; Kamgar et al., 2013).

Vibrio such as Vibrio fluvialis, V. alginolyticus (Fjellheim et al., 2007; Thompson et al., 2010), Pseudomonas (Chythanya, 2002; Das et al., 2006; Preetha et al., 2007; Abd El-Rhman et al., 2009; Ström-Bestor and Wiklund, 2011), and Aeromonas such as Aeromonas hydrophila, A. media (Irianto et al., 2003; Lategan et al., 2004; Lategan et al., 2006) are also used as probiotics.

These probiotics have been used in different aquatic organisms and have been shown to be successful, not only for their ability to prevent disease, but also for improving digestion and growth. Many of these applications have been targeted at the early stages of development of the aquatic organisms, such as the larval stages, because these stages are more susceptible to infections (Bricknell and Dalmo, 2005; Vine et al., 2006; Fjellheim et al., 2007; Dierckens et al., 2009; Fjellheim et al., 2010; Avella et al., 2011).

7.2.2. Probiotic treatments and enhancement of disease resistance and immune response

Bacillus subtilis used as an agent for the control of streptococcosis in Rainbow trout hatchery is reported by Kamgar et al. (2013). Mrigal fed with
probiotics showed protection from epizootic ulcerative syndrome (Sharma et al., 2013). Chabrillón et al. (2006) observed that, the mortality of Gilthead seabream (Sparus aurata) receiving a diet supplemented with potential probiotic was significantly lower, when compared with fishes receiving non-supplemented commercial diet, on challenge with *Listonella anguillarum*. Lategan et al. (2004) studied the control of saprolegniosis in fish by probiotics. Morbidity due to saprolegniosis was low in probiotics treated tanks, in comparison to the non-treated control tanks.

Sharifuzzaman and Austin (2009) observed a significant reduction in mortality in Rainbow trout fed with *Kocuria* supplemented diet when challenged intraperitoneally with *Vibrio anguillarum*. Marzouk et al. (2008) observed a high resistance to challenge with *Pseudomonas fluorescens* in *Oreochromis niloticus* fed with *Bacillus subtilis* and *Saccharomyces cerevisiae*. Effects of probiotics on survival in Japanese flounder (*Paralichthys olivaceus*) were evaluated by Taoka et al. (2006). Pathogen challenge tests with *V. anguillarum* resulted in significantly higher survival in the probiotics treated groups than the control group. Gram et al. (1999) studied the effect of probiotic against infection by *V. anguillarum*, in Rainbow trout (*Oncorhynchus mykiss* Walbaum), and found a significant reduction in mortality in the probiotic fed fishes compared to the control. Gildberg et al. (1997) fed Atlantic cod fry with feed containing *Carnobacterium divergens* and then challenged with a virulent strain of *V. anguillarum*. An improved disease resistance was observed in the probiotic fed fishes.

Balcazar et al. (2007) analysed the effect of probiotic strains on the immune responses of Rainbow trout (*Oncorhynchus mykiss*). Rainbow trout
fed with probiotic supplemented diet exhibited survival rates ranging from 97.8 to 100%, whereas survival was 65.6% in fish not treated with the probiotics, when challenged with *A. salmonicida*. Newaj-Fyzul *et al.* (2007) observed that in Rainbow trout, the survival rate of fish challenged with *Aeromonas* spp. ranged from 65 to 100% for the probiotic fed as compared to 5 to 15% in the control fish. Austin *et al.* (1995) evaluated the effect of administering *Vibrio alginolyticus* strain in a bath treatment to Atlantic salmon and the results revealed that application of *Vibrio* led to a reduction in mortality after exposure to *A. salmonicida*.

Kamgar *et al.* (2013) observed significant difference in the serum total protein, serum albumin, IgM and lysozyme of probiotic fed Rainbow trout, compared to control. Kim *et al.* (2012) investigated the effect of a probiotic, *Enterococcus faecium*, on the immune responses against infection with *Lactococcus garvieae* in Olive flounder (*Paralichthys olivaceus*). The lysozyme activity, complement activity and antiprotease activity was found elevated on probiotic treatment. El-Ezabi *et al.* (2011) investigated the effect of *Bacillus subtilis* and *Lactobacillus plantarum*, a mixture of both bacterial isolates and the yeast, *Saccharomyces cerevisiae* on the immune response of the Nile tilapia (*Oreochromis niloticus*). The results showed significantly higher phagocytic activity, acid phosphatase activity, lysozyme activity and total immunoglobulin activity in probiotic fed fish as compared with the control. Al-Dohail *et al.* (2011) observed the protective effect of probiotics in African catfish against infection by *Staphylococcus xylosus, Aeromonas hydrophila* and *Streptococcus agalactiae*. They found that serum total immunoglobulins concentration was significantly higher in fishes fed with probiotic supplemented diet, compared to the control.
Marzouk et al. (2008) observed that *Bacillus subtilis* and *Saccharomyces cerevisiae* improved the non specific immune response of *O. niloticus*, through the stimulation of macrophage cells and increased phagocytic activity. Probiotic fed fishes exhibited an increase in the number of lymphocytes, monocytes and total white blood cell count and also a high resistance to the challenge with *Pseudomonas fluorescens*.

Balcazar et al. (2007) analysed the effect of probiotic strains on the cellular and humoral immune responses of Rainbow trout (*Oncorhynchus mykiss*). The fish supplemented with probiotic diets were more resistant to challenge with *A. salmonicida*. In comparison to untreated control fish, the phagocytic activity of leukocytes and the alternative complement activity in serum were significantly greater in the probiotic fed group. Panigrahi et al. (2007) studied the immune modulation including the expression of cytokine genes following dietary administration of probiotic bacteria, *Lactobacillus rhamnosus, Enterococcus faecium* and *Bacillus subtilis* to Rainbow trout (*Oncorhynchus mykiss*). Production of superoxide anions and leukocytes were found to be enhanced after feeding with probiotics. There was also an improvement in the alternate complement activity of serum, in the probiotic fed fishes. Besides this, there was an up regulation of cytokine genes, in this group. Newaj-Fyzul et al. (2007) observed that in Rainbow trout, probiotic feeding provided protection against *Aeromonas* challenge and it also stimulated the immune parameters like serum and gut lysozyme activity, peroxidase activity and phagocytic activity besides resulting in an increase in lymphocyte populations.

Effects of probiotics on growth, stress tolerance and non-specific immune response in Japanese flounder (*Paralichthys olivaceus*) were evaluated by Taoka
et al. (2006). Growth and survival against *Vibrio anguillarum* challenge, of Flounder treated by supplying commercial probiotics either in the diet, or into the rearing water, were higher compared to the control group. They have also observed an increase in plasma lysozyme activity in the probiotic treated group. Sakata (2006) investigated the effect probiotics on the non-specific immune system of Tilapia (*Oreochromis niloticus*). Probiotics were introduced by feeding either in the form of live or dead cells, or supplying live cells to the rearing water in a closed recirculating system. The probiotics treatment enhanced non-specific immune parameters such as lysozyme activity, migration of neutrophils and plasma bactericidal activity, resulting in improvement of resistance to *Edwardsiella tarda* infection. Sakai *et al.* (1995) observed that, oral administration of *Clostridium butyricum* bacteria to Rainbow trout enhanced the resistance of fish to vibriosis by increasing the phagocytic activity of leucocytes.

Administration of probiotics can also cause alteration in the number of immunohaematological parameters in fishes. Selvaraj *et al.* (2005) studied the effects of yeast glucan administration on immune modulations in *Cyprinus carpio* against the bacterial pathogen *A. hydrophila*, and they observed significant increase in total blood leucocyte counts and an increase in the proportion of neutrophils and monocytes.

Al- Dohail *et al.* (2009) noticed an elevation in red blood cell count, white blood cell count and packed cell volume in African cat fish fingerling fed on a diet containing *Lactobacillus acidophilus* compared with the normal control. Mehrim (2009) recorded a similar observation in Nile tilapia fed on probiotics compared with the normal control. Tantawy *et al.* (2009) found that
application of *Lactobacillus acidophilus* in common carp feed showed a significant increase in total and differential leucocytic count.

Infections cause haematological changes in fishes. Study by Haniffa and Mydeen, (2011) evaluated the haematological changes in *Channa striatus* intramuscularly administered with *A. hydrophila*. Fish injected with *A. hydrophila* showed a higher mean corpuscular volume and packed cell volume than control fish. White blood cells and lymphocytes numbers increased significantly in fish injected with *A. hydrophila* when compared to non-injected control. Zorriezhahra et al. (2010) observed an increase in total leucocyte count and a decrease in lymphocytes and neutrophils in diseased Rainbow trout (*Oncorhynchus mykiss*) compared with control. Thoria (2010) noticed a significant decrease in red blood cell count, haemoglobin concentration and packed cell volume with leukocytosis in Nile Cat fish experimentally infected with *A. hydrophila* compared with normal non infected group. The study of Harikrishnan et al. (2003) in Carp infected by *A. hydrophila* showed an increase in the leukocyte number. But the opposite was found in Pacu (*Piaractus mesopotamicus*) experimentally infected with *A. hydrophila* (Garcia et al., 2007). Rafiq et al. (2001) did not observe any alteration in the differential counts of white blood cells in Tilapia challenged with *A. hydrophila*. Stoskopf (1993) found leukocytosis, neutrophilia, monocytes and lymphopenia in naturally infected common carp with *A. hydrophila* compared with the normal non infected group.

Martins *et al.* (2008; 2009) have reported that Tilapia injected with *Enterococcus* showed an increase in white blood cells, lymphocytes and thrombocytes when compared to non-injected fishes. In *Oreochromis aureus*
infected with *Corynebacterium* sp., Silveira-Coffigny *et al.* (2004) observed an increased number of lymphocytes. Pathiratne and Rajapakshe (1998) observed high number of neutrophils in Chichlids with epizootic ulcerative syndrome. Lamas *et al.* (1994) and Balfry *et al.* (1997) found a reduced number of lymphocytes in the blood of Rainbow trout and Tilapia, infected with *V. anguillarum* and *V. parahaemolyticus*, respectively.

### 7.2.3. Control of *Aeromonas* infections in fishes through probiotics

The protective effects of oral administration of *Bacillus coagulans* and chitosan oligosaccharides, single or combined, on the resistance of *Cyprinus carpio* koi against *A. veronii* were observed by Lin *et al.* (2012). The control of *A. hydrophila* infection in *Cyprinus carpio* by bacteria *Enterococcus faecium* isolated from fish *Mugil cephalus* intestine was observed by Gopalakannan and Arul (2011). Feeding Nile Tilapia with the probiotic *Micrococcus luteus* was found to exert an inhibitory effect against *A. hydrophila* (Abd El-rhman *et al.*, 2009). Zhou *et al.* (2010) observed inhibitory ability of probiotic, *Lactococcus lactis*, against *A. hydrophila* in *vitro*. Kumar *et al.* (2008) assessed the use of probiotic *Bacillus subtilis* in *Labeo rohita* against *A. hydrophila* infection and found it to be effective in controlling infection. The *in vitro* antimicrobial assay by Aly *et al.* (2008) showed that *Bacillus subtilis* and *Lactobacillus acidophilus* inhibited the growth of *A. hydrophila*. They have observed that Tilapia nilotica (*Oreochromis niloticus*) fed with this bacterial mixture exhibited better protection against *A. hydrophila* challenge infection. Abdel-Tawwab *et al.* (2008) showed that the addition of probiotic *Saccharomyces cerevisiae* in diet decreased the mortality of Nile Tilapia challenged by *A. hydrophila*. Newaj-Fyzul *et al.*
(2007) observed that *Bacillus subtilis* controls *Aeromonas* infection in Rainbow trout.

In another study, Vendrell *et al.* (2008) showed that feeding Rainbow trout with *Lactobacillus rhamnosus* supplemented feed reduced fish mortality caused by *A. salmonicida*. The beneficial effect of probiotic strains, *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Lactobacillus sakei* in Rainbow trout against *A. salmonicida* infection was noticed by Balcazar *et al.* (2007). Protective effect of probiotics against *A. salmonicida* infection in Rainbow trout has been reported (Irianto and Austin, 2002; Brunt *et al.*, 2007). It has been found that the addition of a probiotic bacteria *L. rhamnosus* to Rainbow trout diet could reduce mortality of fish challenged with a virulent strain of *A. salmonicida* (Nikoskelainen *et al.*, 2001). A strain of *Carnobacterium* sp. isolated from the intestine of Atlantic salmon was effective in controlling infections caused by *A. salmonicida*, in fry and fingerlings of Salmonids (Robertson *et al.*, 2000). Application of the probiotic *V. alginolyticus* led to a reduction in mortality after exposures to *A. salmonicida* in Atlantic salmon (Austin *et al.*, 1995). The use of combination of putative strains of *A. sobria* and *Brochothrix thermosphacta* was able to prevent fin rot caused by *A. bestiarum* in Rainbow trout (Pieters *et al.*, 2008).

7.3. **Objectives of the study**

Review of literature amply reflects the role of *A. hydrophila* and *A. sobria* as fish pathogens and the role of probiotics in combating/controlling the disease caused by them. Unlike in developed countries regulatory control over the use/misuse of antibiotics in aquaculture system is weak in India. The same is reflected in our studies on prevalence of antibiotic resistance among
motile aeromonads from ornamental fishes in the study area. Hence the study has been taken up with the following objectives:

1) To study the effect of probiotic treatment (Bacillus NL 110) on the survival of Cyprinus carpio, post challenge with A. hydrophila.

2) To study the effect of probiotic treatment (Bacillus NL 110) on the immune parameters of C. carpio, post challenge with A. hydrophila.

3) To study the effect of probiotic treatment (Bacillus NL 110) on the histopathology of C. carpio, post challenge with A. hydrophila.

7.4. Material and Methods

7.4.1. Experimental fishes

Koi carp (Cyprinus carpio), the fresh water ornamental fish, obtained from an aquarium shop in Kerala, India was used for the study. Fishes weighing ~1.5g ± 0.2g were brought to the laboratory, acclimatized in tanks containing dechlorinated water over a period of two weeks until feed consumption and general behaviour became normal. The tanks in which the fishes were maintained had water temperature ranging from 27 to 29°C, dissolved oxygen concentrations from 6.8 to 7.8 mg/L, pH from 7.0 to 7.5, and unionized ammonia concentration from 0.04 to 0.14mg/L. After the period of acclimatization, the fishes were transferred to the experimental tanks and were allowed to acclimatize for another week.

7.4.2. Experimental diet

Pure culture of Bacillus NL110 (probiotic strain, lab stock, Rahiman et al., 2010) was used for experimental diet preparation. Bacillus NL110 was inoculated into sterile nutrient broth and incubated on a shaking incubator for
24 hours at 37°C. The cells were then harvested by centrifugation at 3,000 rpm for 15 minutes, washed thrice and resuspended in physiological saline. The cells were thoroughly mixed with commercial fish feed to obtain $10^9$ cells/g feed. The feed was aseptically spread out and dried overnight at 37°C. Feed thus prepared was stored at 4°C and fed to the fishes maintained in respective experimental tanks (Shariffuzaman and Austin 2009; Rahiman et al., 2010).

7.4.3. Addition of probiotic through water

Pure culture of *Bacillus* NL110 was inoculated into sterile nutrient broth and incubated on a shaking incubator for 24 hours at 37°C. The cells were then harvested by centrifugation at 3,000 rpm for 15 minutes, washed thrice, resuspended in distilled water, added to the experimental tank to obtain a final concentration of ~$10^6$ cells/ml.

7.4.4. Experimental design

After acclimatization, fishes were randomly divided into two groups. One group was kept as control and was fed with commercial feed. The other group was fed with experimental feed (probiotic supplemented diet). Each group consisted of 12 animals in each tank. Experiment was conducted in triplicate. Fishes in each group were fed at 2% of the body weight per day for a period of 14 days (Shariffuzaman and Austin 2009).

Each aquarium was supplied with compressed air using aquarium air pumps. Fish wastes settled at the bottom of the tanks were siphoned out daily along with three quarters of the aquarium water, which was replaced by aerated water from the storage tank. In the experimental tank, probiotic was incorporated in water to get a final concentration of ~$10^6$ cells/ml. The basic physico-
chemical parameters of water viz. temperature, dissolved oxygen, NH$_3$-N, NO$_2$-N and NO$_3$-N were maintained at optimal levels.

7.4.5. Challenging with *Aeromonas hydrophila*

7.4.5.1. Bacterial culture

Pure culture of *Aeromonas hydrophila* (Genbank accession number: JX987236) originally isolated from aquarium fish samples (John and Hatha, 2013) was used for challenge experiment. The isolate was grown in nutrient broth for 24 h at 28°C. The broth cultures were harvested by centrifugation at 5000 × g for 15 min at 4°C. The bacterial pellet was washed by resuspension in sterile phosphate buffered saline (PBS-pH 7.4) and centrifugation as above and the final pellet was resuspended in PBS to get a cell density of 7.5 x 10$^6$ cells/ ml. The viable count of the suspension was confirmed by spread plate technique.

7.4.5.2. Challenge experiment

At the end of 14 days feeding, fishes from each experimental group were injected intra-peritoneally (IP) with 0.1 ml dose (7.5 x 10$^6$ cfu/ml) of *Aeromonas hydrophila*. All groups were kept under observation for 7 days to record clinical signs of the disease and mortality. The cause of death was confirmed by reisolating the organism from body parts of dead fishes (10% of dead fishes were used for reisolation) using starch ampicillin agar.

7.4.6. Relative Percentage Survival

Fish mortality was recorded for 7 days following bacterial challenge and percentage survival was calculated employing the following formula:
% survival = \frac{\text{No. of surviving fish after challenge}}{\text{No. of fish injected with the pathogen}} \times 100

Relative Percentage Survival (RPS) (Amend, 1981) was determined using the following equation

\[ \text{RPS} = 1 - \left( \frac{\text{\% mortality in treated group}}{\text{\% mortality in control group}} \right) \times 100 \]

7.4.7. Serum collection

The procedure for serum collection was same as described in section 6.4.7. Serum bactericidal efficiency was determined as described in section 6.4.7.1.

7.4.8. Statistical analysis

Statistical analysis was performed using student’s \( t \) test to determine differences between treatments, at significance level of 0.05. Standard errors of treatment means were also estimated. All statistics were carried out using SPSS 13.

7.4.9. Histopathological analysis

Gill, liver and intestine from the body of infected fishes were removed and fixed in 10% buffered formalin for 24 hours. Tissues were then washed in running tap water overnight. Tissues were dehydrated in ascending grade of alcohol; for 30 minutes in 50% alcohol and for 45 minutes each in 80 and 90% alcohol and then transferred to absolute alcohol (two changes) for one hour each.

Tissues were cleared in xylene until they became translucent. They were then transferred to a mixture of xylene and paraffin wax and left overnight. The tissues were infiltrated in 2-3 changes of molten paraffin wax of melting
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point 60-62°C for one hour each. They were then embedded in paraffin wax of melting point 60-62°C. Paraffin blocks were cut in a rotary microtome to prepare sections of thickness 4 to 6 microns.

Staining technique using Haematoxylin-Eosin stain was carried out as described in 6.3.9.1.

7.5. Results

7.5.1. Survival of *Cyprinus carpio* treated with probiotic NL110

After the challenge with *A. hydrophila* significant increase in survival (*p*<0.05) was observed in *Cyprinus carpio* fed with the probiotic diet compared to the control (Figure 7.1) (Appendix 8.1).

![Bar chart showing survival of *Cyprinus carpio*](image)

**Figure 7.1.** Survival (%) of *Cyprinus carpio* in control and experimental group after challenge with *A. hydrophila*

* Represents significant difference (*p*<0.05) between control and experimental group

Relative percentage survival observed was 59%. RPS values over 50 indicate positive effect of the probiotics (Amend, 1981).
7.5.2. Serum bactericidal efficiency

Bactericidal efficiency of serum was significantly ($p<0.05$) higher in the probiotic treated group compared to the control as is evident from the survival rate of bacteria after incubation with serum (Figure 7.2) (Appendix 8.2).

![Figure 7.2. Serum bactericidal efficiency in control and experimental groups after challenge with *A. hydrophila*](image)

* Represents significant difference ($p<0.05$) between control and experimental groups

7.5.3. Histopathological analysis

Histopathological analysis of gill tissues of fishes injected with *A. hydrophila* exhibited lamellar hyperplasia, clubbing and epithelial desquamation in the probiotic treated group. However, the pathological changes were more severe in fishes fed with control diet, these fishes exhibited severe damage of secondary lamella. Fishes injected with saline showed normal gill architecture (Plate 7.1 A-C).

Histopathological analysis of liver tissues is given in Plate 7.2 A-C. Fishes injected with saline showed normal architecture of liver. Fishes injected with
A. hydrophila exhibited areas of necrosis in the probiotic treated group, vacuolization and pyknosis of nuclei was more prominent in fishes fed with control diet.

Histopathological analysis of intestinal tissues is given in Plate 7.3 A-C. Fishes injected with saline showed normal architecture of intestine. Fishes injected with A. hydrophila exhibited shrinkage of enterocytes in the probiotic treated groups; severe necrosis of enterocytes was seen in fishes fed with control diet.

Plate 7.1.
A. Photomicrograph of gill of C. carpio injected with saline (control) showing normal gill architecture (H&E X 400)
B. Photomicrograph of gill of C. carpio fed on probiotic diet and injected with A. hydrophila showing hyperplasia (a), lamellar clubbing (b) and epithelial desquamation (c) (H&E X 400)
C. Photomicrograph of gill of C. carpio fed on normal diet and injected with A. hydrophila showing damaged secondary lamella (a), lamellar shortening (b), hyperplasia (c), lamellar curling (d) and epithelial desquamation (e) (H&E X 400)
Plate 7.2.

A. Photomicrograph of liver of *C. carpio* injected with saline (control) showing normal architecture (H&E X 400)

B. Photomicrograph of liver of *C. carpio* fed on probiotic diet and injected with *A. hydrophila* showing focal areas of necrosis between the hepatocytes (H&E X 400)

C. Photomicrograph of liver of *C. carpio* fed on normal diet and injected with *A. hydrophila* showing pyknotic nuclei (a) and prominent vacuolization in hepatocytes (b) (H&E X 400)
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Plate 7.3

A. Photomicrograph of intestine of *C. carpio* injected with saline (control) showing normal architecture (H&E X 400)

B. Photomicrograph of intestine of *C. carpio* fed on probiotic diet and injected with *A. hydrophila* showing shrinkage (a) and necrosis (b) of enterocytes (H&E X 400)

C. Photomicrograph of intestine of *C. carpio* fed on normal diet and injected with *A. hydrophila* showing architectural loss (a) and complete necrosis (b) of enterocytes (H&E X 400)
7.6. Discussion

With the increasing intensification and commercialization of aquaculture production, disease is now a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries (Oke et al., 2013). Prevention or control of diseases is essential to the success of the large-scale, intensive production of fish in culture. The enhancement of the immune system of fish is considered to be the most promising method of preventing fish diseases in aquaculture. Modulation of host immune system is one of the most commonly purported benefits of the probiotics. Probiotics either individually or in combination can enhance both systemic as well as local immunity in fish (Nayak, 2010).

Influence of probiotic feeding duration on disease resistance and immune parameters in Rainbow trout was evaluated by Sharifuzzaman and Austin (2009). The results revealed that a two-week feeding regime led to the maximum reduction in mortalities and higher disease protection, with protection linked to stimulation of immune parameters compared to one, three and four week feeding regimes. Based on this literature and several other reports (Balcazar et al., 2007; Newaj-Fyzul et al., 2007; Kumar et al., 2008; Kim et al., 2012) a feeding regime of 14 days was selected in this study and the results showed that feeding Cyprinus carpio with Bacillus NL110 had significantly improved the survival rate of fishes against Aeromonas hydrophila infection.

In the present study, Cyprinus carpio fed on feed supplemented with Bacillus NL110 for two weeks exhibited survival rate of 63%, whereas survival was only 11% in fish not treated with the probiotics, when challenged with A. hydrophila. Similar to the present study, Kumar et al. (2008)
administered *Bacillus subtilis* to *Labeo rohita* for two weeks and challenged intraperitoneally with *A. hydrophila*. The results suggested that *B. subtilis* can enhance immune responses in the fishes and improve the survival rate. Improvements in immunity and disease resistance in *Cyprinus carpio* koi against *A. veronii* on oral administration of *Bacillus coagulans* and chitosan oligosaccharides, single or combined was reported by Lin et al. (2012). In another study, Vendrell et al. (2008) showed that feeding rainbow trout with *Lactobacillus rhamnosus* supplemented feed reduced fish mortality caused by *A. salmonicida*. Balcazar et al. (2007) and Newaj-Fyzul et al. (2007) observed high survival rates in Rainbow trouts (*Oncorhynchus mykiss*) administered with probiotics compared to control fishes when challenged with *Aeromonas*. The control of *A. hydrophila* infection in fishes on feeding with probiotics and immunostimulants is reported by several authors (Selvaraj et al., 2005; Abdel-Tawwab et al., 2008; Aly et al., 2008; Abd El-rhman et al., 2009; Maqsood et al., 2010; Gopalakannan and Arul 2011). Protection against *A. salmonicida* infection is also reported (Austin et al., 1995; Robertson et al., 2000; Nikoskelainen et al., 2001; Irianto and Austin, 2002; Brunt et al., 2007).

Significant increase in the serum bactericidal activity in the probiotic treated group in comparison to the control is observed in the present study. The survival rate of bacteria after incubation with serum was found to be 39% in the case of control fish, whereas it was 18.8% in the case of probiotic fed fishes. This is similar to the observation of Maqsood et al. (2010), who reported increase in the serum bactericidal activity in common carp challenged with *A. hydrophila* on feeding with the immunostimulant chitosan. Reneshwary et al. (2011) reported that the increase in resistance against *A. hydrophila* in fish fed with *Bacillus thuringiensis* can be explained on the basis
of increased bactericidal activity of serum. Kumar et al. (2008) indicated significant increase in the serum bactericidal activity of *Labeo rohita* fed with *Bacillus subtilis*. Similar observations are also made by Nayak et al. (2007) and Aly et al. (2008). Taoka et al. (2006) reported that the probiotic treatment enhanced the non-specific immune parameters such as plasma bactericidal activity, resulting in the improvement of fish resistance against *Edwardsiella tarda* infection. The increased serum bactericidal activity in *Achyranthes* treated *Labeo rohita* infected with *A. hydrophila* indicated that various humoral factors involved in innate and/or adaptive immunities are elevated in the serum to protect the host effectively from infection (Rao et al., 2006). Misra et al. (2006 b) mentioned that, serum bactericidal activity in the fish injected with different dosages of β-glucan was significantly higher than in controls.

Probiotics also modulate various immunohaematological parameters in fishes; interact with the immune cells such as mononuclear phagocytic cells (monocytes, macrophages) and polymorphonuclear leucocytes (neutrophils) and natural killer cells to enhance innate immune responses. Probiotics, in both *in vitro* and *in vivo* conditions, actively stimulate the proliferation of B lymphocytes in fish (Nayak, 2010).

Histopathological analysis of gill tissues of *Cyprinus carpio* in the present study revealed severe architectural loss of gill filaments in the control fishes compared to the probiotic treated fishes. In the liver, vacuolization and pyknosis of nuclei was more prominent in the fishes fed with control diet. In the intestine, necrosis of enterocytes was more prominent in the fishes fed with control diet. The histopathological studies by Nouh et al. (2009) revealed no remarkable pathological alterations in the gill arch and lamellae of Nile tilapia.
infected with *A. hydrophila*. The liver revealed congestion and vacuolation of some hepatic cells with nuclear pyknosis. In the intestine mucinous degeneration in the epithelial lining was observed. Focal epithelial desquamation was also seen in the intestine.

When looking at probiotics for an aquatic usage, it is important to consider certain influencing factors that are fundamentally different from terrestrial based probiotics. Aquatic animals have much closer relationship with their external environment. Therefore there are many differences between terrestrial and aquatic animals in the level of interaction between the intestinal microbiota and the surrounding environment. The larval forms of most fish and shellfish are released in the external environment at an early ontogenetic stage. These larvae are highly exposed to gastrointestinal microbiota-associated disorders, because they start feeding even when the digestive tract is not yet fully developed, and the immune system is still incomplete (Lara-flores, 2011). Thus, probiotic treatments are particularly desirable during the larval stages (Gatesoupe, 1999).

Improved water quality has especially been associated with probiotics. Research also shows that the use of commercial probiotics in aquaculture ponds can reduce concentrations of nitrogen and phosphorus and increase the production yield (Wang *et al*., 2008). The multipronged attack by probiotics is more efficient than just relying on antibiotics to disrupt cell wall structures and/or poison metabolic pathways in pathogenic agents.

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