6. DISCUSSION

Bacterial infections are fairly common in freshwater aquarium fish, especially where the water quality is poor. This may due to inadequate filtration, high stocking density and over feeding or using low quality feed. In addition, high temperature, sudden fluctuation in pH, accumulation of toxic substances like ammonia, nitrite and hydrogen sulphides also deteriorates the water quality. These factors cause stress and leads to disease outbreak in culture organisms. Isolation and identification of pathogenic bacteria associated with diseased animals would broadly benefit to the aquaculture industry. Characterization by both phenotypic and genotypic methods can provide useful epidemiological data of pathogenic bacteria which are responsible for the outbreak of infectious diseases (Nawaz et al., 2003). In the present study, a total of 39 bacteria were isolated from the diseased ornamental fishes. From these, seven Gram negative bacteria (VPG01, VPG05, VPG08, VPG10, APG01, APG02 and FA3) were selected for further study. The biochemical characterization revealed that the selected strains are motile, glucose positive, oxidase positive and catalase positive. Snieszko and Bullock (1976) reported that infections due to Gram negative bacteria are frequent and widespread. According to the previous reports, the most common bacterial fish pathogens are Aeromonas, Acinetobacter, Bacteroides, Citrobacter, Flavobacterium, Pseudomonas, Yersinia, Edwardsiella and Vibrio (Hovda et al., 2007; Kim et al., 2007; Navarrete et al., 2010).

Virulence study plays a major role in the isolation of pathogenic bacteria. The bacterium which shows potential antagonistic activity under in vitro condition may not be efficient under in vivo conditions because of the influence of body metabolism and various responses to the external stimuli. Hence, it is necessary to study the virulence of pathogenic bacterial isolates at a specific concentration under in vivo
condition. In the present study, the VPG01 exhibited 98.33 ± 2.36 % mortality in Koi fingerlings and APG01 showed 100 % mortality in Kenyi cichlid fingerlings at 48th hour of exposure. Thus, VPG01 and APG01 were selected for further investigation. The antibacterial sensitivity of these two isolates was tested with commercially available antibiotic by disc diffusion assay. Antibiotic discs streptomycin, kanamycin, amoxicillin, erythromycin, gentamycin, chloramphenicol and tetracycline inhibited the VPG01 and the zone of clearance ranged from 12 to 27 mm was observed. Similarly, these antibiotic inhibited (ranged from 15 to 35 mm) the growth of APG01. However, VPG01 was resistant to ampicillin and oxacillin, meanwhile APG01 were resistant to oxacillin only. Though, VPG01 and APG01 was highly sensitive to many of antibiotics, the negative impact of using antibiotics to combat the pathogenic bacteria in the aquaculture industry, lead us to think to find out an alternative treatment.

Conventional approaches, such as the use of disinfectants and antimicrobial drugs to control diseases, have had limited success in the prevention or cure of aquatic diseases. The massive use of commercially available antibiotics results in the natural emergence of antibiotic-resistant bacteria, which can transfer their resistance gene to the next generation that have never been exposed to antibiotics (Wand and Xu, 2006). This has lead to apply the non-pathogenic bacteria as a bio-control agent instead of antibiotics (Fuller 1978). In the present study, totally 79 bacteria were isolated from the raw milk, curd, yoghurt and fermented rice. Acid tolerance test, non-pathogenicity and antibacterial activity against pathogens are the few criteria for selecting the potential probiotic strains (Cebeci and Gurakan, 2003; Pan et al., 2008). Hence, before using any organism as a probiotics, it is essential to evaluate its potential,
origin of strain, acid tolerance and production of antimicrobial substances (Salminen et al., 1998).

The bacterial isolates were screened for their antibacterial activity. Among the 79 isolates, only six bacteria, i.e. PPS02, FA02, BPH1, BPH2, 13FC1 and 21FC2 were exhibited well defined zone of inhibition against the isolated pathogens (VPG01 and APG01) and the reference strain A. hydrophila. Out of six bacteria, two (FA02 and PPS02) of them were isolated from the gut of healthy freshwater fish. Dhanasekaran et al. (2010) isolated 59 Lactobacillus strains from the gut samples of Cat fish, Har fish, Rohu and Tilapia. It has been reported that L. lactis isolated from intestinal microbiota of fish controls the pathogenic strains such as A. hydrophila, A. salmonicida, V. anguillarum and Y. ruckeri (Hong et al., 2005; Balcazar et al., 2008). According to Sugita et al., (1996) an average of 3.2 % of bacterial strains isolated from the fish intestine exhibited antibacterial activity against different pathogenic bacteria including Aeromonas spp. Pseudomonas isolates from the rainbow trout proved their beneficiary effect under in vivo conditions (Korkea-aho et al., 2011; Storm and Wiklund, 2011).

In the present study, BPH1 was isolated from fermented rice and BPH2, 13FC1 and 21FC2 strains were isolated from curd and milk product respectively. The earlier investigations also reported that probiotic bacteria possess inhibitory activity against Aeromonas and Citrobacter were isolated from various freshwater ornamental fishes and aquatic environment (Jawahar and Tirthankar, 2007). Eduardo et al. (2003) reported the antimicrobial activity of bacterial strains isolated from yogurt and milk powder was tested against Staphylococcus, E. coli, Candida albicans and Salmonella strain. Lactobacillus isolates from dairy products act as a potential probiotic feed supplement to Cyprinus carpio (Vignesh et al., 2011).
In the present study, the proposed probiotic strains are Gram negative, motile and positive for glucose fermentation, oxidase and catalase test. Among the potential antagonistic bacterial isolates obtained from ornamental fishes and aquatic environment, 12% were Gram negative with dominance of rods and catalase positive (Jawahar and Tirthankar, 2007). Further, the acid tolerance test is a basic criterion for selecting the potential probiotic bacteria (Cebeci and Gurakan, 2003). The viability and growth of probiotic strains are influenced by the pH. In the present study, among proposed probiotic isolates, 13FC1 and 21FC2 tolerated wide range of pH from 3 to 9. Therefore these two bacteria were selected for further study. The above finding was in conformity with previous studies that the probiotic L. lactis tolerated the pH ranging from 2.5 to 6.5 (Balcazar et al., 2008). Further, the isolated bacteria 21FC2 exhibited minimum tolerance to the pH 2. The above finding was similar to the previous report of Balcazar et al. (2008), where L. plantarum showed tolerance to the pH 2.

A potential probiotic strain needs a thorough evaluation of the required probiotic characteristics and appropriate safety assessment, prior to the application in the field. In the present study, the Koi carp and Kenyi cichlid fingerlings were exposed to all the six isolated bacteria (FA02, PPS02, BPH1, BPH2, 13FC1 and 21FC2) to understand the non-pathogenic nature at different concentrations ($10^5$, $10^6$, $10^7$, $10^8$ and $10^9$ CFU ml$^{-1}$). The fingerlings treated with isolated bacteria exhibited no pathological sign and mortality, hence, the tested bacteria isolated from the food materials and healthy fish guts were non pathogens. The present finding agreed with Aly et al. (2008) who proved the safety of a number of probiotic bacteria via intramuscular and intraperitoneal injection to Atlantic salmon. Similarly, probiotic feed treatment with Bacillus sp. for 60 days showed 100% survival rate in common Carp (Yanbo and Zirong, 2006). Further, Jawahar and Tirthankar (2007) reported that
the Gold fish (*Carrassius auratus*) exposed to *Lactobacillus* and *Bacillus* sp demonstrated 96 % of survival up to 15 days.

In the present study, 13FC1 and 21FC2 strains showed the highest inhibitory activity against tested pathogens (VPG01, APG01 and *A. hydrophila*) in primary screening, tolerant to a wide range of pH and non pathogenic nature, thus the proposed probiotic bacteria considered as a probiotic bacteria and selected for further study to determine its antibacterial activity at different concentrations against isolated pathogenic bacateria. The probiotic cultures and its cell free supernatant (CFS) were tested against the isolated pathogens under *in vitro*.

The isolated probiotic bacteria (21FC2 and 13FC1) exhibited the highest antibacterial activity (39 ± 0.0 mm and 35.5 ± 0.7 mm) against VPG01 and APG01 respectively in all the antibacterial assays. The zone of inhibition significantly increased (*P* < 0.05) when the pathogenic VPG01 and APG01 treated with probiotic 13FC1 and 21FC2 than other probiotic bacteria. Further, the antibacterial activity was increased corresponding to the concentration of probiotic bacteria. The above findings proved that the isolated bacteria showed better antibacterial activity than the previously reported probiotic strains. Earlier reports suggested that the probiotic cultures of *L. lactis*, *B. coagulans* and *M. luteus* and *Pseudomonas* sp. inhibited the growth of *A. hydrophila* and *V. harveyi* and the zone of inhibition was ranged between 4 and 14.77 (Azza *et al.*, 2009; Zhou *et al.*, 2010; Gupta *et al.*, 2014).

The CFS of probiotic isolates demonstrated less antibacterial activity (9.5 ± 0.7 mm to 19 ± 1.41) against the isolated pathogens than the probiotic cultures. The highest zone of inhibition was registered by CFS of 13FC1 and 21FC2 against pathogenic APG01 and VPG01 respectively. These findings are in agreement with the previous reports that the CFS of *S. phocae*, *E. faecium*, *S. flexneri* and *Lactobacilli*
effectively controlled the growth of *E. coli, V. parahaemolyticus, V. Angullarum, L. Monocytogenes, S. sonnei* and *S. intritidis* in the agar well diffusion assay and the zone of inhibition was ranged from 11 to 22 mm (Davoodabadi et al., 2015). In another study, the CFS of *L. plantarum* strongly inhibited *V. harveyi* on 18th hour in the microdilution assay (Kongnum and Hongpattarakere, 2012). The inhibitory activities of CFS are mainly caused by the extracellular products of the probiotic bacteria. The antibacterial effect of probiotic bacteria might be due to the secretion of bacteriocin or bacteriocin-like compounds (Khunajakr et al., 2008; Kanmani et al., 2010). The bacteriocins are ribosomally synthesized peptides shows antagonistic properties against Gram negative bacteria (Cotter et al., 2005). These findings are in agreement with the present study that the CFS of 21FC2 and 13FC1 exhibited highest antibacterial activity against VPG01 and APG01 respectively, and the zone of inhibition was 19 ± 1.41 and 16 ± 0.0 mm respectively. It shows that the CFS of isolated probiotic bacteria contains strong antibacterial substances.

The potential probiotic bacteria (13FC1 and 21FC2) and virulent pathogens (VPG01 and APG01) were selected for 16S rDNA gene sequencing. The VPG01 isolated from Koi carp showed 100 % similarity with *Citrobacter freundii* (KF769539). The *Citrobacter* species have been isolated from various freshwater fishes, intestine of farm raised catfish, intestine of grass carp (Nawaz et al., 2008; Aijun et al., 2011). Frederiksen (2005) reported twelve distinct *Citrobacter* species based on the biochemical profiles and molecular studies. The *Citrobacter* strains (*C. freundii, C. amalonicus*, and *C. braakii*) isolated from the intestine of farmed catfish showed tetracycline-resistance (Nawaz et al., 2008).

According to the earlier literature, *C. freundii* is generally regarded as an opportunistic pathogen and most of the *Citrobacter* species are isolated from human
clinical sources (Borenshtein and Schauer, 2006). In fish, *C. freundii* has been isolated predominantly from mixed microbial population and it is recognized as an opportunistic secondary pathogen (Akoachere et al., 2009). Molecular characterization and phylogenetic analysis identified the virulence associated fimC gene in *Citrobacter* (Lu et al., 2011).

Jeremic et al., (2003) reported that the carp infected with *C. freundii* (intraperitoneal injection) showed disease symptoms and 50 % mortality on the 8th day. This pathogen caused gastroenteritis in Rainbow trout, hemorrhagic septicemia in Cyprinids and in wild Zebra fish petechial haemorrhages on the gills, bleeding on the skin are reported (Jeremic et al., 2003; Lu et al., 2011) and red leg syndrome in *L. catesbeianus* (Sergio et al., 2011). The *Citrobacter* isolated from the infected humans and animals have been characterized extensively, however, only limited reports are available in the aquaculture environment (Akoachere et al., 2009). In the present study, *Citrobacter* was isolated from the infected freshwater ornamental fish.

The 16S rDNA gene sequencing of APG01 isolated from Kenyi cichlid fish showed 99 % similarity with *Plesiomonas shigelloides* (KF769536). It is a fish pathogen and mostly isolated from the intestine of freshwater fish (Arai et al., 1980; Gonzalez et al., 1999). *P. shigelloides* are usually present in natural water, fish and shellfish, but epizootics occur only in poor environmental conditions (Krovacek et al., 2000). According to the ninth edition of Bergey’s manual of Determinative Bacteriology, the genus *Plesiomonas* was included in the family *Vibrionaceae* together with the genera of *Vibrio, Aeromonas, Photobacterium* and *Enhydrobacter* (Holt et al., 1994). It can cause gastroenteritis and the symptoms are resembled to other enteric pathogen (Gonzalez et al., 2000).
In the present study, Kenyi cichlid fish infected with *P. shigelloides* registered 100 % mortality at $10^5$ CFU ml$^{-1}$. The previous studies reported that the *P. shigelloides* caused mortality in Asian Arowana and rainbow trout (Jin et al., 2011; Cruz et al., 1986). Nadirah et al. (2012) isolated the *P. shigelloides* from the digestive tract and muscle of red hybrid tilapia, *Oreochromis niloticus*. The *P. shigelloides* infected fish showed various symptoms of catarrhal and hemorrhagic enteritis, hepatopancreatic degeneration, ventricular hemorrhage, renal edema, gall bladder dilation and skin pathology (Bardon, 1999). Diagnosis and treatment of bacterial infections in fishes require isolation and identification of the causative agent from the infected animal. In the present study also pathogens were isolated from the diseased fish to find out the causative agents and to initiate the preventive measures.

The BLAST analysis of 16S rDNA gene sequencing of probiotic isolates (13FC1 and 21FC2) showed 99 % to 100 % similarity with *Enterobacter cloacae* and *Enterobacter cloacae* Subsp. *Dissolvens* respectively. The *E. cloacae* complex has been divided into 12 genetic clusters and one sequence crowd. Nine of the clusters correspond to species: *E. asburiae*, *E. kobei*, *E. ludwigii*, *E. hormaechei* Subsp. *oharae*, *E. hormaechei* Subsp. *hormaechei*, *E. hormaechei* Subsp. *steigerwaltii*, *E. nimipressuralis*, *E. cloacae* Subsp. *cloacae*, and *E. cloacae* Subsp. *Dissolvens* (Hoffmann and Andreas, 2003). The strains of both *E. cloacae* and *E. cloacae* Subsp. *dissolvens* come under the same DNA relatedness (Grimont and Grimont, 1992; Lindh and Ursing, 1991). In the present study, RAPD analyses using different primers showed molecular variation between the *E. cloacae* Subsp. *Dissolvens* and *E. cloacae*. The unique banding patterns of two probiotic species showed little dissimilarity in their genetic information.
*E. cloacae* commonly occurred in water, soil, food, skin and gastrointestinal tract (Dalben *et al*., 2008; Lee *et al*., 2002). This strain occurs as commensal microflora in the intestinal tracts of humans and animals (Dalben *et al*., 2008). However, only a few reports are available on the occurrence of *E. cloacae* in yoghurts and fermented milk (Al-Zoreky *et al*., 1991). *E. cloacae* are resistant to low pH and produce large amounts of viscous exopolysaccharides (Wang *et al*., 2013). Limited reports were available on the probiotic efficacy of *E. cloacae* Subsp. *Dissolvens* and *E. cloacae*. It is an ideal bio-pesticide for controlling insect in the agricultural fields. Watanabe *et al.* (2000) reported that the *E. cloacae* control the mulberry pyralid larvae in agricultural fields (Tang, 2001).

It is important to know the molecular diversity and characterization of *Enterobacter* species for understanding the probiotic efficiency. Several microorganisms have emerged as organisms of considerable public health significance that are present in many foods and able to survive in milk and yoghurts (Pazakova *et al*., 1997). In the present study, under *in vitro* condition, the bacterial isolates from natural food sources such as curd and milk product showed maximum antibacterial activity against the pathogenic strains such as *C. freundii* and *P. shigelloides*. However, it is necessary to understand the role of proposed probiotic under *in vivo* conditions before taking it to field application. Thus, the probiotic ability of the isolates on the growth, water quality, immunology and histopathology of the ornamental fishes were evaluated under *in vivo* conditions.

In this study, bacteria isolated from natural sources were subjected to *in vitro* and *in vivo* tests to prove its probiotic efficacy. Most probiotics used in aquaculture are lactic acid bacteria or bacterial strains belong to genus *Vibrio, Bacillus* and *Pseudomonas*. These probiotics have been tested for their role in the animal health by applying
Discussion

through food or culture water (Gatesoupe, 1999). In the present study, *E. cloacea* Subsp. *Dissolvens* and *E. cloacea* was supplemented through feed and water to evaluate their antibacterial efficacy against *C. freundii* and *P. shigelloides* in Koi carp and Kenyi cichlid fishes respectively. In addition, the effect of these probiotic on the water quality parameters and immune response were also investigated.

The infected fishes treated with probiotic *E. cloacea* Subsp. *Dissolvens* enhanced the survival rate by reducing the virulence of pathogenic *C. freundii*. The infected fish treated with probiotic and prebiotic group showed weight gain of 0.81 ± 0.20 gm after 60 days of trial. The previous reports also supporting the present investigation, that the Common carp fed with *Bacillus* sp. showed 0.76 ± 0.02 gm weight gain after 60 days (Yanbo and Zirong, 2006). Administration of probiotic *Lactobacillus, Bacillus coagulans* and *Paenibacillus polymyxa* through feed enhanced the growth and survival rate of *Cyprinus carpio* (Vignesh et al., 2011; Gupta et al., 2014). In the present study, the Koi carp fishes treated with probiotic and prebiotic showed highest specific growth rate (SGR) of 1.34 ± 0.34 %. The above findings are coinciding with the earlier report that the freshwater carp administrated with dietary probiotic (*B. licheniformis*) enhance the specific growth rate of 1.45 ± 0.22 % (Gupta et al., 2014).

Aly *et al.* (2008) reported that the Tilapia supplemented with feed probiotic (*L. acidophilus*) showed maximum weight gain of 35 gm. Similarly, 90 days feeding of basal diet with *Pseudomonas* sp. showed 7.8 ± 0.05 gm weight gain and 100 % survival rate in Nile tilapia (El-Rhman *et al.*, 2009). Gram *et al.* (1999) reported that *P. fluorescence* isolated from freshwater fish improved the growth rate and reduction (46 %) of mortality in rainbow trout when the fish treated with probiotic through a water medium. In the present study, the Kenyi cichlid fish treated with combinations
of probiotic (E. cloacae) and prebiotic showed the maximum weight gain of 0.87 ± 0.17 gm.

The physico-chemical parameters of the rearing water are an important concern in aquaculture. The bacteria with probiotic properties can support to maintain the water quality parameters in aquaculture. However, little information’s are available on the probiotic effects of water quality parameters in the freshwater ornamental fish culture. Previous studies have demonstrated that several B. subtilis strains produced a variety of extracellular enzymes and antimicrobial peptides (Sutyak, 2008). The secretion of these substances not only control pathogenic bacteria, but also supports to improve the water quality parameters (Zokaeifar et al., 2014).

Increased water pH can affect the fish either directly or indirectly by increasing the toxicity of ammonia and other components (Svobodova et al., 1993). pH values above 8.5 may increase the toxic metabolic substances in the carp rearing environment (Raskovic et al., 2010). In the present study, probiotic application in water significantly maintained (P < 0.05) the water pH within the limit (below 8) in Koi carp experiment, whereas the water pH level was more than 8.0 in control and infected Kenyi cichlid group. .

The higher amount of organic matter in culture tanks can also promote the occurrence of disease outbreaks. The food pellets are extremely friable and decomposed rapidly when it contacts with water. Accumulation of excess feed and waste materials in the culture environment may increase the toxic metabolites. It may cause stress to the rearing animals and leads to bacterial disease outbreak (Cruz et al., 1986). It has also been reported that use of Bacillus sp. improved water quality, survival, growth rates and health status of juvenile Penaeus monodon and reduced the pathogenic Vibrios (Dalmin et al., 2001). Probiotic B. subtilis added to the rearing water of shrimp
culture for 8 weeks showed significant decrease in the nitrite concentration (Zokaeifar et al., 2014). In contrast, Yanbo and Zirong (2006) reported that no obvious effect was found in the use of Bacillus sp. and photosynthetic bacteria on water quality parameters such as total ammonium, nitrite and pH in C. carpio tank. However, Bacillus sp. is the most commonly reported probiotic bacteria which improves the water quality parameters such as ammonia and nitrite (Abdul et al., 2010). In the present study, the fishes infected with C. freundii and P. shigelloides (without probiotic application) showed significant ($P < 0.05$) increase in ammonia and nitrite concentration which indicated the accumulation of waste material in the culture tanks that may not be broken down by the beneficial bacteria. However, application of probiotic E. cloacea Subsp. Dissolvens and E. cloacea through water and feed either alone or in combination significantly maintain the level of ammonia and nitrite ($P > 0.05$) in Koi carp and Kenyi cichlid tanks.

The enhancement of the fish defence mechanism by probiotic supplement has been reported by several authors (Nikoskelainen et al., 2003; Brunt and Austin, 2005; Panigrahi et al., 2007). Study of haematological parameters in fish blood is one of the important tools for disease diagnosis. Leucocytes play an important role in innate immunity of fish during inflammation and their count is considered as an indicator of its health status (Secombes, 1996). In general, infections reduce the number of circulating WBCs (Pickering and Pottinger, 1987) and/or suppress their activity (Ellsaesser and Clem, 1986). The above reports are in agreement with the present study, less number of WBCs in Koi carp (86.36 ± 0.32 $10^3$ mm$^3$) and Kenyi cichlid (36.44 ± 2.63 $10^3$ mm$^3$) was observed in infected with pathogen alone (group 3) whereas the group treated with the probiotic and prebiotic (group 7) exhibited highest WBC count, followed by other probiotic treated groups.
The Kenyi cichlid infected with *P. shigelloides* and treated with probiotic and prebiotic (2 % of mannanoligosaccharide) increased the WBC count. The earlier studies supported the present findings, that supplementation of 1 % fructooligosaccharide with basal feed for 75 days increased the level of WBC count (13.31 ± 0.27 × 10^3 mm⁻³) in juvenile Stellate sturgeon (Akrami *et al*., 2013). 1 % prebiotic insulin supplements significantly increased the WBC count in Beluga juveniles (Ahmadifar *et al*., 2010). However, Razeghi *et al*., (2012) reported that 1 % mannanoligosaccharide showed no impact on the WBC level in Beluga juveniles. In the present study, fishes supplemented with 2 % of prebiotic along with probiotic. Hence, this might be the reason for the enhancement of WBC count in treated groups.

The non-specific immune system can be stimulated by probiotics. The respiratory burst activity is the main indicator of non-specific innate immune system where O₂⁻ is the first product to be released (Miyazaki, 1998). It is mainly due to increase in oxidation level in the phagocytes, stimulated by foreign agents. In fish the O₂ production is generally accepted after the activation of phagocytes since it triggers the production of superoxide anion (O₂⁻) and its active derivatives (*i.e.* hydrogen peroxide and hydroxyl radicals) associated with intense oxygen consumption called respiratory burst (Secombes and Fletcher, 1992). The reactive species are capable of destroying the invading pathogens (Hassett and Cohen, 1989). Phagocytes are crucial in the host defence mechanism against invading microorganisms through reactive oxygen species (ROS) production. Measurement of ROS production by phagocytosis is of critical importance to investigate the physiological consequences resulting from cellular mechanisms that lead to the oxidative burst.

Since O₂⁻ is the first product released during the respiratory burst, which has been accepted as an accurate parameter to quantify the intensity of a respiratory burst
(Harikrishnan et al., 2011). Nikoskelainen et al. (2003) reported that the administration of a lactic acid bacterium *L. rhamnosus* stimulated the respiratory burst activity in Rainbow trout (*Oncorhynchus mykiss*). The *Cyprinus carpio* administrated with *Enterococcus faecium* through oral and intraperitoneal showed anti-infective activity against *A. hydrophila* (Ayyaru and Venkatesan, 2011). In another study, the fish supplemented with *B. coagulans, B. licheniformes* and *Paenibacillus polymexa* for 80 days significantly increased the respiratory burst activity (Gupta et al., 2014). In addition, Indian major carp treated with andrographolide supplements through feed showed highest NBT level. Dietary administration of *L. lactis* and *L. plantarum* significantly increased the NBT level in Olive flounder (Beck et al., 2015). The above reports are supporting the present findings that the respiratory burst activity was significantly increased (0.349 ± 0.033 to 0.383 ± 0.0007) in Koi carp and Kenyi cichlid fishes treated with probiotic (group 5 and 6) alone and combination of probiotic and prebiotic (group 7). The groups treated with the combination of probiotic and prebiotic were exhibited highest respiratory burst activity than probiotic alone.

Fish intestine is the first organ to be infected when they are exposed to pathogenic bacteria. The gastroenteritis of Rainbow trout and hemorrhagic septicaemia of Cyprinids were caused by *C. freundii* (Jeremic et al., 2003). The microbiota of the gastrointestinal tract of aquatic animals can be modified by several factors, therefore manipulating the microbial population constitutes a viable tool to reduce or eliminate the incidence of opportunistic pathogens (Balcazar, 2002; Vine et al., 2004a). Although competition for adhesion sites has been widely suggested as a mode of action, there is a little evidence in the literature to demonstrate this. Hansen and
Olafsen (1999) reported the adhesion properties of certain bacteria to the intestinal mucus and no supporting evidence was noticed under *in vivo* condition.

In this study, the microbiota of the intestine of Koi carp and Kenyi cichlid fishes were investigated. The TPC level was high in fish infected with pathogen alone (group 3) and fish treated with probiotic through water and feed (groups 6) and combined treatment of probiotic (water and feed) and prebiotic (group 7). These findings are in conformity with the earlier report of Kim *et al.* (2010), registered higher probiotic bacteria in the intestine of Olive flounder fed with probiotic *Zooshikella sp.* for 16 weeks. Similarly, Standen *et al.* (2013) demonstrated that the dietary *Pediococcus acidilactici* led to high population of the same bacterium (1.59 x 10\(^5\) CFU gm\(^{-1}\)) in the intestine of Tilapia. The same trend was also reported in Tilapia (Ferguson *et al.*, 2010) and Rainbow trout (Merrifield *et al.*, 2011; Merrifield *et al.*, 2010). In the present study, the count of pathogens in the fish intestine was estimated with TCBS and Aeromonas isolation agar media, since *C. freundii* and *P. shigelloides* were grown in the same respectively. Fish treated with probiotic and prebiotic showed higher TPC and low bacterial colonies recorded on TCBS and Aeromonas selective medium. It is clear that, the bacterial colonies obtained in the intestine of fish might be beneficial group.

Stimulation of specific indigenous microflora by supplementing through fish feed with indigestible carbohydrates that act as prebiotic that could be an interesting approach to increase the proportion of health promoting bacteria in the gut. In the present study, application of prebiotic supported the growth of probiotic bacteria. Akrami *et al.* (2013) also suggested that the supplementation of 1 % prebiotic fructooligosaccharide to Stellate sturgeon improved the growth of total bacteria and lactic acid bacteria in the intestine.
The probiotic administration may alter the composition of gut microflora that could help the culture animal to combat against pathogens, improve the immune system, provide nutritional benefits and assist the intestinal mucosal barrier (Vaughan et al., 2002). The adhesion property of probiotic bacteria in the fish intestine is an important feature (Nikoskelainen et al., 2001). Intestinal microbiota may serve as a supplementary source of food and vitamins or essential amino acids (Dall and Moriarty, 1983). In addition, probiotic bacteria may inhibit the growth of pathogen by producing antimicrobial compounds, namely bacteriocins, lysozymes, hydrogen peroxide, formation of ammonia, diacetyl, and altered the pH by the production of organic acids (Gullian et al., 2004).

Further, probiotic ability to modulate fish intestinal microbiota has been reported for both Gram positive and Gram negative bacteria (Garrido et al., 2005). These reports are corroborated with the present study, the concentration of pathogens (C. freundii and P. Shigelloides) were highly controlled by the isolated probiotic strains (E. cloacea Subsp. Dissolvens and E. cloacea). The in vivo results confirmed that the fish treated with probiotic (feed and water) and combined application of probiotic and prebiotic enhanced the growth and survival rate of the organisms by controlling the pathogenic bacteria.

Histological changes in the intestine may vary depending on the composition of feed used. Replacement of fish meal with protein sources has increased the length of the villi and height of enterocytes (Bozidar et al., 2011). In the present study, C. freundii infection caused widening of intestinal villi and central stroma. The Kenyi cichlid fish infected with P. shigelloides without probiotic treatment group showed partial degradation of microvilli and absorptive vacuoles were cystic in nature. Numerous studies have reported that the exposure of epithelium to fish pathogens can
result in severe tissue damage, disorganised and damaged microvilli and lamina propria (Ringo et al., 2007). It is accepted that the changes in the intestinal morphology are mediated by anti-nutritional factors which disrupt the intestinal barrier by altering membrane permeability. Subsequently, these histological changes in the intestine can increase susceptibility to bacterial infection.

In the present study, dietary administration of *E. cloacea* Subsp. *Dissolvens* and *E. cloacea* caused beneficial changes in the intestine morphology. The infected Koi carp treated with probiotic alone and combination of probiotic and prebiotic (group 7) showed improved intestinal morphology without shortening of the villi and disruption of microvilli. These findings are in agreement with earlier reports on the intestine of Red drum (Zhou et al., 2010), and Rainbow trout (Heidarieh et al., 2013) showed alteration in the intestinal morphology by the administration of prebiotics. In contrast, dietary administration of mannanoligosaccharide caused no remarkable changes in the intestine of Gulf sturgeon (Pryor et al., 2003) and European sea bass (Torrecillas et al., 2007). Similarly prebiotic xylooligosaccharide showed no alteration in Caspian white fish (Hoseinifar et al., 2014). In the present study, the prebiotic was used in combination with probiotics. It has improved the intestine morphology and bear resemblance to the control groups. The application of 2% of mannanoligosaccharide with probiotic might be reason for remarkable recovery on the intestine morphology.

The natural immunostimulants seems to be the most promising method of preventing fish diseases. Natural immunostimulants are biocompatible, biodegradable and safe for the environment and animal health (Ortuno et al., 2002). Probiotics are live organisms which can improve the intestinal microflora and support the animal health. Further, probiotics protect against infections, alleviate lactose intolerance,
reduce blood cholesterol levels, improve weight gain and feed conversion ratio, and also stimulate the immune system (Salminen et al., 2004; Agrawal, 2005).

The proposed probiotic strain should undergo the following rigorous evaluation process such as acid tolerance, *in vitro* and *in vivo* antibacterial activity, immune response, adhesion properties and production of antimicrobial substances for field application. The efficiency of probiotic bacteria may be differ in relation to fish species, stage of development, dosage, duration of feeding, feed composition, mode of supplementation and/or environmental/rearing conditions. In the present investigation, the probiotic bacteria (*E. cloacae* Subsp. *Dissolvens* and *E. cloacae*) tolerated wide range of pH, exhibited highest antibacterial activity, improved fish growth, controls infection, maintained the water quality parameters, increased the WBC and NBT values, increased the adhesion properties and improved the intestine architecture. Comparatively, *E. cloacae* Subsp. *Dissolvens* showed highest probiotic efficiency than *E. cloacae*. In addition, the synergistic effect of the probiotics and prebiotic significantly improved the health of Koi carp and Kenyi cichlid fishes. Therefore, the selected bacterial strains *E. cloacae* Subsp. *Dissolvens* and *E. cloacae* can be used as a potential probiotics in freshwater ornamental fish culture. Further, addition of prebiotic to these probiotics provides more beneficial effects on growth and disease prevention ability of freshwater ornamental fish. However, the selected isolates should be further evaluated in larger scale field trials and an in-depth assessment of the mode of action, prior to progress towards commercial products.