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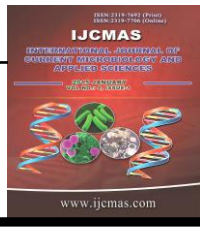


**Original Research Article**

# Isolation, Identification and Characterization of Probiotic Organisms From Intestine of Fresh Water Fishes

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Isolation, Characterization, Probiotic, Intestine, Freshwater fishes

**A B S T R A C T**

The gut microbiota of fresh water fishes *Catla catla*, *Labeo rohita*, *Cirrhinus mirigala* and *Cyprinus carpio* was studied to isolate and identify probiotic bacteria. A total of 27 bacteria were screening out from the fresh water fish intestine. Out of this, five groups were divided in their biochemical characterization. These five isolates were evaluated with probiotic properties. Probiotic bacteria with more ability to inhibit growth of *Aeromonas hydrophila* was selected and identified by conventional and molecular techniques. This strain was able to survive and grow from pH 3 to 8.5 with the highest viability and growth rate at neutral conditions pH 7 for 3 isolates where as another two isolates was pH 6. In addition, all isolates are tolerated 0, 0.15 and 0.3% bile salt concentrations. This isolates also, showed inhibitory activity against tested fish pathogen *A. hydrophila*. Antibiotic sensitivity test indicated that these strains were resistant to Streptomycin and Vancomycin, Intermediate to Kanamycin and sensitive to Methicelin, Pencilin, Chloramphenicol, Gentamycin, Erythromycin and Amphicilin for all isolates.

## Introduction

Probiotic bacteria are essential for beneficial effect on particular organism's health and host nutrition for healthy gastrointestinal function. The action of intestinal flora results in vital benefits, including protection against pathogens and development of immune system. Probiotics are defined as "a live microbial adjunct which has a beneficial effect on the host by modifying the host associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment" Verschuere et al. (2000). Probiotics are also regarded as an environmentally friendly treatment method.

Fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the environment of digestive tract of fish confers a favorable culture environment for the microorganisms.

The gastrointestinal tract of fish is a complex ecosystem possessing a specific micro-biota consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria (Gomez and Balcazar, 2008).

The predominant bacterial species isolated from most of the fish digestive tracts have been reported to be aerobes or facultative anaerobes (Bairagi et al., 2002; Saha et al., 2006). The isolated many lactic acid bacteria are proved to function as probiotics, which are benefit to host health, when ingested in sufficient quantities. The colonization of the gut by probiotic bacteria prevents growth of harmful bacteria by competition exclusion and by the production of organic acid and antimicrobial compounds.

The acid and bile tolerance as well as two fundamental properties that indicate the ability of probiotic microorganism to survive the passage though the upper gastrointestinal tract, particularly acidic condition in the stomach and the presence of bile in the small intestine (Hyronimus *et al*., 2000; Erkkila and Petaja, 2000). Although lactic acid bacteria were not dominant population in fish, it has been well documented in several investigations that lactic acid bacteria are a part of the native microbiota of aquatic animals from temperate regions (Ringo, 2004).

All reports pointed out on the presence of probiotic bacteria from freshwater fish gills, gut, tissue and liver, when artificial feed supplementation of fresh water forms, but dam environment, fresh water fish gut bacteria was analyzed fewer. So, in the present study revealed that

Isolation, enumeration and identification of probiotic bacteria from Parappalaru dam located at Western Ghats near Oddanchathram, Dindigul District.

## Materials and Methods

### Collection and processing of inland fish samples

Fresh-water fishes *Catlacatla, Labeorohita, Cirrhinus mirigala* and *Cyprinus carpio* were collected from parappalaru dam, located at Dindigul District, Tamilnadu in the first week of January 2012 with the help of fisherman net. The fishes were washed with sterile distilled water and then the animals were dissected, to remove the digestive tracts by the sterilization condition. The digestive tracts were homogenized in the same sterile distilled water for centrifugation. After centrifugation the supernatant was taken and serially diluted in sterile distilled water in the test tubes to $10^{-2}$, $10^{-3}$ ,$10^{-4}$ ,$10^{-5}$ ,$10^{-6}$ and$10^{-7}$ dilution and were pour plated on nutrient agar plate and incubated for 24 h at room temperature. Individual colonies were taken with typical characteristics namely pure white, off white, Yellow, small (2–3 mm diameter) with entire margins were picked from each plate, transferred to subculture in three times and quadrant streak for pure culture in single colony. Selective colonies were characterized and identified following Bergey's Manual of Systematic Bacteriology (Whitman et al., 2009) for their colony and cell morphology, gram staining, biochemical and physiological tests (Ghosh et al., 2002). However biochemical and physiological tests are essential tools for identification of bacterial genera and species.

### Isolated bacterial species for probiotic properties

### pH tolerance test

Acidification was measured by selected bacterium was investigated at different pH.

MRS broths with different pH including 3, 4, 5, 6, 7, 8 and 9 were prepared using HCl 1% and NaOH 1 N and divided in universal bottles (Samelis et al., 1994). The broths media along with control bottles were autoclaved at 121°C for 15 min and then inoculated with overnight culture of the selected strain in MRS broth followed by incubation at 30°C. Optical density (OD) as growth rate of bacteria was measured by spectrophotometer at 600 nm after 2 h incubation. The viability of the isolates was also controlled by duplicate inoculation on MRS agar (Balcázar et al., 2008; Kim and Austin, 2008; Allamesh et al., 2012).

**Bile salt tolerance**

Bile salt tolerance was further tested in MRS broth which included 0.0, 0.15 and 0.3% (w/v) Oxgall bile salt. Duplicate bottles of MRS broth containing filtered different concentrations of bile salt were inoculated by 30 µl of cultured strain and incubated at 30°C. Growth rate was assessed by measuring the optical density by spectrophotometer at 600 nm after 0, 2, 4 and 8 h incubation (Balcázar et al., 2008; Kim and Austin, 2008; Allamesh et al., 2012).

**Antibacterial activity of the isolated bacterial strain**

The freshwater fish pathogens, *A. hydrophila* (EU584529.1) (Previously isolated by *Cyprinus carpio* gut and identified the 16s rRNA sequence by Macrogen –Korea) were used to determine the antibacterial effect of the candidate strain by disc diffusion techniques. The pathogenic bacteria were cultured in TSB and incubated at 30°C for 24 h. Thereafter, 30 µl of the cultures with $10^3$ CFU/ml was spread on TSA by swab. At the same time, the selected strains were cultured in MRS broth at 30°C for 24 h. The bacterium cells were harvested by centrifugation at 8000 rpm and 4°C for 5 min and their supernatants were used for antibacterial test using disc diffusion methods (Balcázar et al., 2008; Allamesh et al., 2012).

**Antibiotic sensitivity test**

Antibiotic sensitivity test was carried out for selected strain on the most common antibiotics in aquaculture by disc diffusion technique. They included Methicelin: 5µg/disc, Chloramphenicol: 10µg/disc, Penicillin: 2µg/disc, Vancomycin: 5µg/disc, Amphicelin: 2µg/disc, Erythromycin: 5 µg/disc, Gentamicin: 10µg/disc, Streptomycin: 5µg/disc and Kanamycin: 5µg/disc**.** 50 µl of the 24 h broth culture of the strain was spread on MRS agar and, antibiotic Bio-discs were subsequently placed on plates. Finally, the plates were incubated at 30°C for 24 h to observe and measure the inhibition zone (Kim and Austin, 2008). The interpretations and zone sizes were illustrated based on table of Kirby-Bauer test (Bauer et al., 1966).

**Statistical analysis**

Statistical analysis was conducted to compare the quantitative results of treatments using one-way analyses of variance (ANOVA).

## Results and Discussion

**Total colony count of bacteria in intestine.**

Total number bacterial count/plate in fish intestine was determined on MRS agar medium using serial dilution (up to $10^{-7}$). Total bacterial counts showed that *Catla catla* gut $2.72 \times 10^6$ CFU/ml, *Labeo rohita*gut $1.87 \times 10^6$ CFU/ml, *Cirrhinus mirigala*gut $1.91 \times 10^6$ CFU/mland

*Cyprinus carpio* gut 2.19 × $10^6$ CFU/ml of population in intestine. This result is supported by some reports. Abraham and Banerjee (2007) reported bacterial population in *Labeo rohita* gut 1.84 × $10^6$ CFU/ml, *Cirrhinus mirigala* gut 2.41 × $10^6$ CFU/ml and *Cyprinus carpio*gut 3.50 × $10^5$ to 1.20 ×$10^7$CFU/ml. Uddin and Al-Harbi (2012) reported 1.4 × $10^{10}$ to 1.7 × $10^{11}$ CFU/ml bacterial population levels in *Cyprinus carpio* gut and 2.7 × $10^{10}$ to 1.0 × $10^{11}$ CFU/ml of bacterial population in catfish intestine (*Clarias gariepinus*). Ringø et al. (2006) determined the population levels of adherent bacteria in foregut, midgut and hindgut of Atlantic cod with different diet. The bacterial population level varied between 7 × $10^{3-4}$, 4 × $10^3$ and 4.5 × $10^{4-5}$ in foregut, midgut and hindgut, respectively.

**Isolation, selection, biochemical characterization and identification of probiotic bacteria**

In the present study 4 different freshwater fish, such as *Catla catla, Labeo rohita, Cirrhinus mirigala* and *Cyprinus carpio* from gut sample were screened for bacterial species. Among 4 different varieties of fish, the presence of number of different bacteria. From these bacteria 27 bacterial species were selected test against the probiotic properties. It shows the resemblance with the findings of other researcher who reported maximum population of *Lactobacillus* was selected in fresh water fishes, Dhanasekaran et al. (2008, 2010). The 27 isolates were culturally, morphologically and biochemically characterized in five groups; Isolated strain -1 (11 isolates), Isolated strain-2 (8 isolates), Isolated strain-3 (4 isolates), Isolated strain-4 (3 isolates) and Isolated strain-5 (1 isolate). All the isolates were Gram positive rods, entire margin and able to grow 20°C to 45°C. Isolates showed that same in their sugar fermentation pattern (Table.2). Among the biochemical test such as V.P, Citrate utilization and Casein Hydrolysis are positive and Gelatin hydrolysis are negative. The growth was optimum between 1 and 9% (w/v) NaCl for isolated species. The results obtained from the pattern of carbohydrate fermentation all isolates are positive. However, in the present study different organisms were selected. This may be the result of different hosts, different habitats, or totally different identification and further probiotic characteristic procedures.

**pH tolerance**

Each of the organisms gave promising results to *in vitro* selection probiotic criteria such as pH and bile salt tolerance tests. pH -3 proved to be more harmful than bile salt test for three isolates i.e. isolate -1, isolate-3 and isolate-5. (Fig.1). In these three isolates did not have any activity at pH 3 after 2 h incubation but, present activity and growth at pH 4 up to pH 7 than slowly decrease at pH-8 and pH 9. This results shows that the pH could significantly affect activity and growth of selected 3 Isolates. But in the Isolate -2 and 4 were grown in pH 3 to pH 6 and slowly decrease in pH 7 and pH 8, but there was no activity in pH 9. There is no activity and growth was obtained at pH 2 and the highest at pH 7; since isolates from intestine that has neutral condition and the highest activity was observed at pH 7. The same results were obtained from *L. mesenteroides*, and it's isolated from *Channa striatus* intestine that has neutral condition and the highest activity was observed at pH 7 (Allamesh et al., 2012). According to this reports, one of the most important criteria for probiotic organisms is potential viability at low pH. Kim and Austin (2008) reported growth of probiotic Carnobacterial strains that had been isolated

from rainbow trout intestine which occurred at pH 5 to 10. In this study, survival and growth at low pH confirm that these isolate can transit through stomach.

**Bile salt tolerance**

Tolerance to detrimental actions of bile salts (0.0, 0.15 and 0.3% at 2 h incubation period) recorded for 5 isolates. It results shows not only activity but also growth in all three concentrations for 2 h incubation period. From the isolates 1, 3, 5 bile salt concentration increased, the growth rate of isolates were decreased significantly ($p<0.5$), But in the isolates 2 and 3 bile salt concentration increased the growth rate of isolates also increased significantly, (Figure 2). In this study, the bile salt affected the growth rate of isolates1, 3, 5 in limited its ability, but in the bile salt induce the growth rate of isolates 2, 3 maximum its ability. Furthermore, these strains show different ability to survive and grow in bile salt. Bile salt tolerance is required for probiotic bacterial to grow and survive in fish intestine (Salminen et al., 2004). Cebeci and Gurakan (2003) determined that *L. plantarum* as a probiotic could survive in 0.3% of bile salt. The probiotics that can tolerate low pH and bile salt means they not only can transit through stomach and be active in intestine but also are able to be alive and survive in stress conditions (Cebeci and Gurakan, 2003). In the present study, all 5 isolates had bile and acid-tolerant and may appear to have high potential to adhere to 4 fresh water fish mucus as a desirable probiotic.

**Antibacterial test**

The study examined the antibacterial activities of the isolated strains against the test bacteria *Aeromonas hydrophila*. Isolate -1 shows the inhibition zone 7 .20 mm in scale likewise Isolate 2 (5.00 mm), Isolate 3 (5.00 mm), Isolate 4 (4.80 mm) and Isolate 5 (1.00mm) Fig: 3. this result shows that probitic strains whose safety was taken i.e. antimicrobial properties against the pathogenic organisms. The same trend was observed Kaynar and Beyatli (2012) the antagonistic activities of the *B. subtilis* (5.80 mm), *B. pasteurii* (4.95 mm) and *B. licheniformis* (5.00 mm) form a zone of inhibition against *P. fluorescens.* Aly et al. (2008) reported that the growth of *A. hydrophila* was inhibited by three species of *Bacillus* bacteria that used as probiotic and also, Rengpipat et al. (2008) confirmed growth inhibition on *A. hydrophila* using a cell-free cultured broth of five LAB. Kim and Austin (2008) determined the antibacterial ability of two probiotic strains that were isolated from rainbow trout intestine against *A. hydrophila* and *A. salmonicida*. These strains inhibited the growth of both *A. hydrophila and A. salmonicida*. Moreover, similar results for *Leuconostoc mesenteroides were* reported by Allamesh et al. (2012). In general, probitic bacterial species have different ability to inhibit growth of pathogenic bacteria. Therefore, the findings in this study suggest that isolated bacteria may have high potential probiotic and anti adhesion effect against pathogens.

**Antibiotic sensitivity test**

The susceptibility and resistance pattern obtained with the 5 isolated species against 9 antibiotics is shown in Table: 3 and Fig : 4. Observations indicated that 5 isolated species were susceptible to most of the antibiotics and low resistance was found which can be considered a positive trait for bacteria employed in probiotics. The interpretations of inhibition zone were determined according to zone size of chart of Kirby-Bauer test results (Bauer et al., 1966).

**Table.1** Incidence of bacterial isolates were selected from each serial dilution

| S.No | Fish Variety | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
|---|---|---|---|---|---|---|
| 1 | *Catla catla* | 2 | - | 2 | 2 | - |
| 2 | *Labeo rohita* | 1 | 2 | 1 | 2 | 1 |
| 3 | *Cirrhinus mirigala* | - | 1 | 3 | 2 | 1 |
| 4 | *Cyprinus carpio* | 1 | 1 | 2 | 3 | - |

**Table.2** Biochemical characterization selected 5 isolates

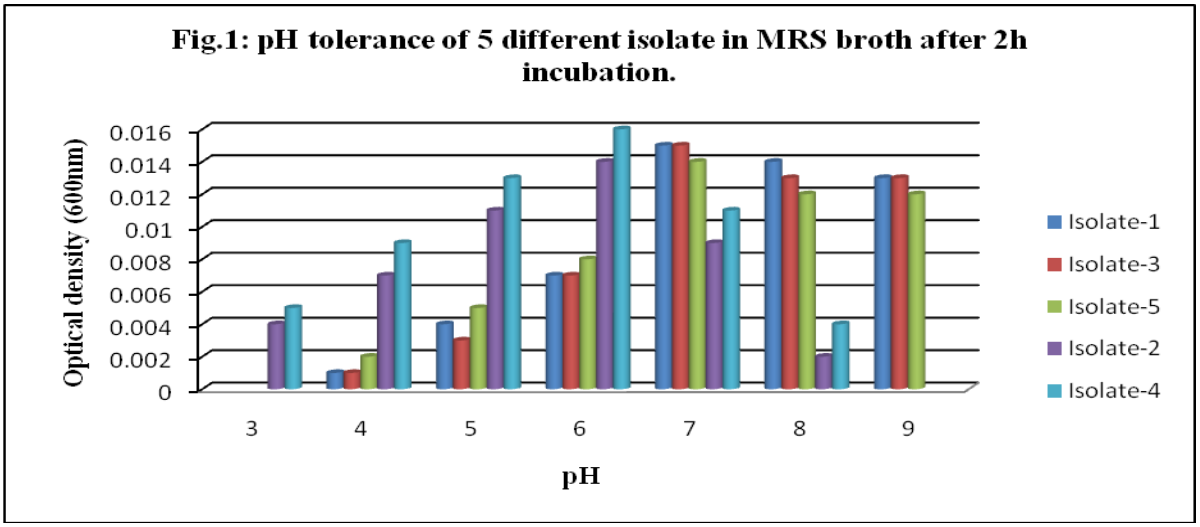
| **Characteristics** | **Isolate-1** | **Isolate- 2** | **Isolate- 3** | **Isolate- 4** | **Isolate- 5** |
|---|---|---|---|---|---|
| **Colony appearance** | White | White | Cream White | Yellow | Golden yellow |
| **Morphology** | Coccid Rods | Unicellular rods | Long rods | Long bent Rods | Coccid |
| **Gram's test** | + | + | + | + | + |
| **Motility** | Non-motile | Motile | Motile | Non-motile | Non-motile |
| **Catalase test** | - | + | + | - | + |
| **Oxidase test** | - | + | - | - | - |
| **Indole Production** | + | + | + | - | - |
| **Methyl Red Test** | + | + | + | + | - |
| **V P Reaction** | + | + | + | + | + |
| **Citrate utilization** | - | + | + | + | + |
| $CO_2$ Fermentation | | | | | |
| **Glucose** | +,G | +,A | +,G | +, A | +,G |
| **Fructose** | +,G | +,A,G | +,G | +, A | +,A |
| **Sucrose** | +,G | +,A,G | +,G | +, A | +,A |
| **Mannitol** | +,A,G | +,G | +,G | -,A | -,A |
| **$H_2S$ Production** | - | + | + | - | - |
| **Nitrate Reduction** | + | + | - | - | - |
| **Starch Hydrolysis** | + | + | + | + | + |
| **Casein Hydrolysis** | - | + | + | + | + |
| **Gelatin Hydrolysis** | - | + | - | - | - |
| **TSI** | ACS/ACB | ALS/ACB | ALS/ACB | ACS/ACB | ALS/ACB |
| **Possible Micro-Organisms** | **Lactobacillus spp** | **Bacillus spp.** | **Bacillus spp.** | **Lactobacillus spp** | **Micrococcus spp** |

ALS-Alkaline slant, ACB-Acid butt, A-Acid production, G-Gas production

**Table.3** Antibacterial susceptibility of isolated 5 different strains

| S.No | Antibiotic | µg/disc | Isolated strains 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|
| 1 | Methicelin | 5 | +++ | +++ | +++ | +++ | +++ |
| 2 | Pencilin | 2 | +++ | +++ | +++ | +++ | +++ |
| 3 | Vancomycin | 5 | - | - | - | - | - |
| 4 | Chloramphenicol | 10 | ++ | +++ | ++ | +++ | ++ |
| 5 | Amphicilin | 2 | +++ | +++ | +++ | +++ | +++ |
| 6 | Streptomycin | 10 | - | - | ++ | - | - |
| 7 | Gentamicin | 10 | +++ | +++ | +++ | +++ | +++ |
| 8 | Erythromycin | 5 | +++ | +++ | +++ | +++ | +++ |
| 9 | Kanamycin | 5 | ++ | ++ | ++ | ++ | ++ |

(+++) Sensitive, (+) Intermediate Sensitive, (-) Resistance



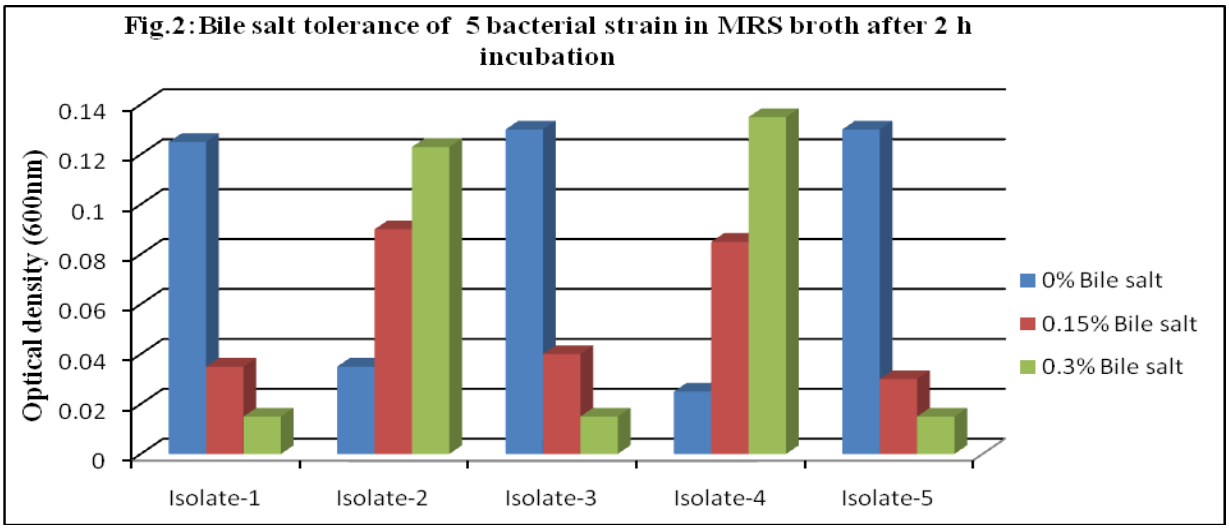



**Fig.3** Zone of inhibition of *Aeromonas hydrophila* against 24h fresh culture supernatant of different 5 isolated strain in disc diffusion method

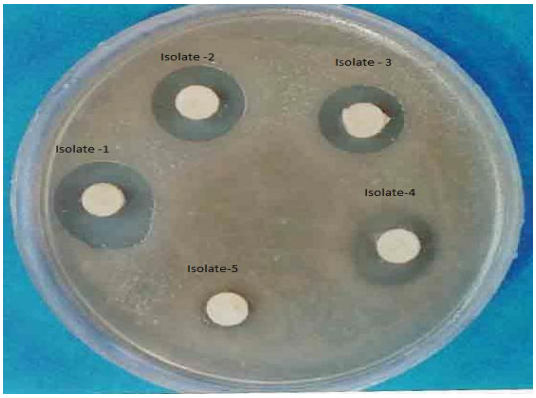
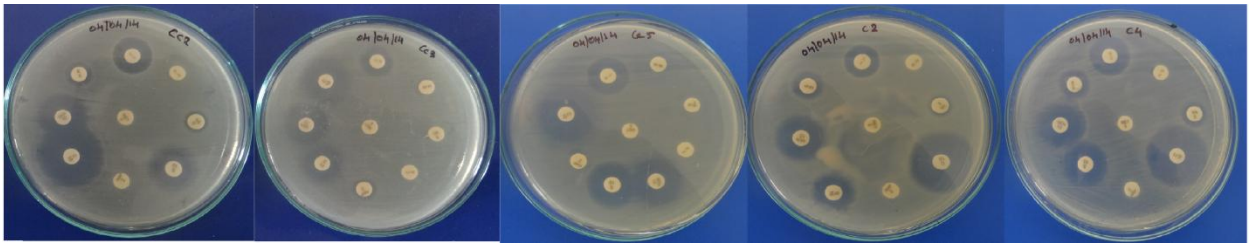



**Fig.4** Antibiotic sensitivity test against 5 isolates



Resistance to specific antibiotic means that, the probiotic can be given at the same time when antibiotic treatment is required. Secondly, microflora of intestine can recover more quickly (Cebeci and Gurakan, 2003; Kim and Austin, 2008). Kim and Austin (2008) determined the antibiotic susceptibility of *Carnobacterium* strains.

They reported resistance to Ampicillin, Gentamycin, Kanamycin, Streptomycin and Penicillin G but sensitivity to Chloramphenicol, Tetracycline and Cotrimaxazole. They also believe that antibiotic-resistant probiotic may be advantageous in the case of administration of antibiotics to fish and the establishment of the beneficial microorganisms in the intestine for prolonged periods.

The present study concluded that the 5 isolates were normal in microflora in fresh water fishes *Catla catla, Labeo rohita, Cirrhinus mirigala* and *Cyprinus carpio*. From the above said results were compare to the Bergey's Manual of Systematic Bacteriology, Sec. Ed., Vol.3 (Whitman et al., 2009) the Isolate – 1 is *Bacillus spp*, Isolate -2 has *Lactobacillus spp*, Isolate -3 has *Bacillus spp*, Isolate-4 has *Lactobacillus spp* and Isolate -5 has *Macrococcus spp*. These isolates may have high potential to adhere to fish mucus. In addition, these isolated organisms show high ability to inhibit growth of freshwater fish pathogens particularly *A. hydrophila*. Therefore, it seems that isolated organisms have high potential probiotic, so these organisms are further studied by molecular characterization 002E.

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