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## RESEARCH ARTICLE

# INVESTIGATION OF ANTIBACTERIAL, NUTRITIVE VALUE, ACID AND BILE TOLERANCE PROPERTIES OF *BACILLUS SP.,* ISOLATED FROM INDIAN MAJOR CARP *LABEO ROHITA* (HAMILTON, 1822)

**Rajikkannu, M. and *Nirmala Natarajan**

PG and Research Department of Zoology, Periyar E.V.R College (Autonomous), Tiruchirappalli – 620 023, Tamil Nadu, India

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**ABSTRACT**

*Bacillus sp.,* generally regarded as safe, has emerged as a robust organism that can withstand adverse environmental conditions and grows easily to very high densities. Tolerance and resistance to acidic pH, high osmotic concentration of NaCl, and bile salts were tested in broth medium. A probiotic bacterium isolated from the gut of Indian major carp *Labeo rohita* rendered maximum antagonistic activity against *Aeromonas hydrophila* pathogen and nutritive value were also tested. They were *B. flexus* (M3) and *B. megaterium* (M5) and their characteristics were as given below. *B. flexus* (M3) showed a higher capacity to grow even at 40°C with alkaline- pH 7. It also recorded higher nutrient value in all the biochemical parameters. *B. flexus* (M3) showed maximum inhibitory zone of 22 mm in well diffusion method and 16 mm in disc diffusion method against *B. megaterium* (M5).



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## INTRODUCTION

Probiotics, which are micro-organisms or their products with health benefit to the host, have found use in aquaculture for improving the health of their host and increasing growth rate. The range of probiotics examined for use in aquaculture has encompassed many micro-organisms such as bacteria, bacteriophages, yeasts and unicellular algae (Irianto and Austin, 2002). One interesting strategy that focuses on the use of probiotic microorganisms promotes the welfare of the host by improving its digestion and immune response as well as by inhibiting the growth of pathogenic microorganisms (Verschuere *et al*., 2000; Vine *et al*., 2006; Wang and Xu, 2006; Gatesoupe, 2007; Salem *et al*., 2010; Abdel-Aziz *et al*., 2015; Elghandour *et al*., 2015; Puniya *et al*., 2015). The selection of probiotics is usually based on *in vitro* antagonism or the adhesion, colonization and growth in intestinal mucus (Vine *et al*., 2004). Information on characteristics like growth under different conditions, antibiotic resistance and antipathogenic potential will help to select a safe as well as an effective probiotic strain for species-specific advantages (Nayak and Mukherjee, 2011). Several species of bacteria such as *Bacillus* (Bhatnagar *et al*., 2012), *Lactobacillus* (Balcazar *et al*., 2007), and *Enterococcus* (Wang *et al*., 2008) have been isolated from fish gut and incorporated in formulating diets to study their effect on growth, nutritional quality and immunity. The extracellular protease producer *Bacillus circulans* was isolated from the gut of rohu, *Labeo rohita*, fingerlings. Characterization of the bacterial flora showed its potential for use as a probiotic (Ghosh *et al*., 2002). 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 beneficial to host health, when ingested in sufficient quantities. 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).

***Corresponding author: Nirmala Natarajan,***
PG and Research Department of Zoology, Periyar E.V.R College (Autonomous), Tiruchirappalli – 620 023, Tamil Nadu, India.

*Labeo rohita* has a promising market potential in Asia as well as other areas in the world. Many probiotics have been proposed to improve health quality of rainbow trout (Irianto and Austin, 2002). The stains used were generally antagonistic to pathogens (Joborn *et al*., 1997; Robertson *et al*., 2000), and an important feature was the ability to colonize in the fish gut (Joborn *et al*., 1997; Nikoskelainen *et al*., 2001). Moreover, the immune system of rainbow trout is stimulated by several probiotic (Irianto and Austin, 2002). In the present study, an attempt has been made to isolate a putative probiotic strain from the intestine of *Labeo rohita* and to evaluate the isolates based on various criteria for choosing a potential probiotic and to study its effect on pathogenic inhibition activity, temperature, pH, NaCl and nutrient value of bacteria.

## MATERIALS AND METHODS

### Bacterial strains

The bacterial strain from the gut microflora of *Labeo rohita* were taken as the material for the search of probiotics, as they did not have any obvious.

### Growth

### Effect of Temperature

Nutrient broth was prepared and dispensed as 5ml aliquots in test tubes. Subsequent to autoclaving, the tubes were inoculated with a loopful of 24 h old broth culture and incubated at various temperatures for a period of 24 h. Growth was expressed in terms of turbidity measurement as optical density in a spectrophotometer (Milton Roy - Spectronic 20) at 660 nm.

### Effect of pH

5 ml aliquots of nutrient broth were adjusted in test tubes. After autoclaving, the pH of nutrient broth was adjusted to various levels: 2, 3, 4, 5, 6, 7 and 8 with either sterile 1N HCL or 1N NaOH. Adjusted pH values were monitored with a digital pH meter (ELICO L1 120). A loopful each of 24 h old bacterial culture was inoculated into each tube and they were incubated at 37°C for 24 h. Growth was measured as mentioned in 2.2.1.

### Effect of NaCl

Nutrient broth was constituted to have sodium chloride concentrations as follows: 1%, 2%, 3% and 4%. 5ml aliquots of these media were distributed in test tubes and autoclaved. A loopful of inoculum (24 h old) was introduced to each tube and the tubes were incubated at 37°C. Growth of bacteria in each tube was assessed, as mentioned in 2.2.1.

### Nutritive value of bacteria

A loopful of bacterial culture (24 h old) was inoculated in 5 ml aliquots of nutrient broth and incubated at 37°C. About 2 ml of log phase cultures was centrifuged at 10,000 rpm for 15 minutes and the bacterial cell pellet was washed with physiological saline and again centrifuged at 10,000 rpm for 15 minutes. 250 µl of a mixture (1:1 v/v) containing 10% Sodium dodecyl sulphate and 1% mercapto ethanol was added to lyse the cells. The bacteria and lysis mixture was vortexed vigorously for 15 minutes. This lysed bacterial cell solution was used for the estimation of biochemical compounds such as protein and carbohydrate. The quantity of lysis mixture and bacteria was scaled up for lipid estimation.

### Protein

The total protein content of the bacteria was estimated following the procedure outlined by Lowry *et al*., (1951). The lysed bacterial mixture was diluted with 1.5 ml of distilled water, followed by the addition of 3ml of freshly prepared Lowry's reagent (50 ml from a 2 g sodium carbonate solution in 100 ml of 0.1 n NaOH + 1 ml solution of 500 mg copper sulphate dissolved in 100 ml of 1% Sodium potassium tartrate). After thorough mixing and reaction for 15 minutes, 0.5 ml of Folin- Ciocalteu reagent (E. Merck) was added. The test tubes were left in the dark for 20 minutes and the intensity of the blue colour developed was read at 720 nm in a spectrophotometer (Milton Roy - Spectronic 20). Standard solution of bovine serum albumin was also subjected to the above reactions to prepare the standard graph.

### Carbohydrate

The carbohydrate content of the bacteria was estimated, following the procedure outline by Roe (1955). 4 ml of freshly prepared anthrone reagent (100 mg anthrone and 1 g thiourea in 100 ml 66% conc. $H_2SO_4$) was added to the lysed bacterial mixture in a test tube. The reaction mixture was placed in a boiling water bath for 10 minutes. Appropriately sized glass marbles were used to cover the mouths of the test tubes to curtail spurting and to facilitate vapour condensation.

### Lipid

Total lipid was estimated, by a modified method of Folch *et al*., (1957). 10 -12 ml log phase culture of bacteria was centrifuged at 10,000 rpm for 15 minutes, washed with saline and lysed with 1250 µl of sodium dodecyl sulphate and mercaptoethanol mixture to the lysed mixture sufficient amount (amount 10 ml) of chloroform. Methanol (2:1) mixture was added and mixed thoroughly. The mixture was transferred to a separating funnel and an aqueous solution of 0.9% NaCl was added to it, the volume of which was approximately $1/5^{th}$ of the reaction mixture.

The separating funnel was shaken vigorously for a few minutes and allowed to stand for several hours to get complete separation of the aqueous and lipid layers. The lipid extracted in the chloroform, methanol mixture was separated and filtered through densely packed glass wool column to remove the cell debris and the filter assembly was washed down with chloroform- methanol mixture repeatedly. The filtrate was evaporated at 60°C in an oven to remove the chloroform-methanol solvent and the amount of lipid was estimated gravimetrically.

**Pathogenic inhibition**

**Pathogen**

The pathogen strain of *Aeromonas hydrophila* (got from human sources, KAVP medical college, Tiruchirappalli) was grown in the VP agar medium. The selected bacteria were also tested for their growth in '*Aeromonas*' medium. Similarly, the growth of *Aeromonas hydrophila* was tested in nutrient agar medium supplemented with 3% NaCl. *Aeromonas hydrophila* used in this study were identified using standard morphology, physiology and biochemical test (Holt *et al*., 1994).

**Well-diffusion method**

The culture of the *Aeromonas hydrophila* strain was prepared by pouring 2 ml of inoculum ($10^3$ CFU/ml in normal saline at log phase) onto nutrient agar to completely cover the surface of the cover. Excess solution was removed and drained before air-drying for 15 minutes in an incubator set at 30°C. 6 mm diameter wells were punched into the agar using pipette tips, which were cut to obtain a 6 mm diameter bore. Twenty microlitres of inoculums of each bacterial strain ($10^3$ CFU/ml) were carefully pipetted into each well. The diameter of incubation zones around the well was recorded in millimeters after incubating the plates for 24 hours at 30°C (Chythanya *et al*., 2002; Vaseeharan and Ramasamy, 2003).

**Disc-diffusion method**

A cell-free supernatant of each candidate probiotic strain of bacteria ($10^3$ - CFU/ml) was obtained by centrifuging 10-15 log phase culture at 13,400 × g for 10 minutes and filtering through 0.22 µm Millipore membrane (Millipore USA). Blank sterile discs of 6 mm diameter (Himedia, India) were dipped into the cell free supernatant and dried in an incubator for 15 minutes at 37°C. The impregnated discs were placed onto nutrient agar plates, which has been lawn inoculated with 10 ml suspension of *Aeromonas hydrophila* ($10^3$ CFU/ml). Plates were incubated for 24 hours at 30°C. The ability of each strain to inhibit the growth of *Aeromonas hytrophila* was determined by measuring the diameter of the inhibition zones formed around the discs (Chythanya *et al*., 2002).

## RESULTS

**Bacteria**

Among the multiple isolates of *Bacillus* from the gut of *L. rohita*, six species could be identified using biochemical characterization and the *Bacillus* strains isolated were, *B. pumilus* M1, *B. cereus* M2, *B. flexus* M3, *B. licheniformes* M4, *B. megaterium* M5 and *B. subtilis* M6.

**Growth**

**Effect of Temperature**

Growth capacity of the selected bacterial strain in the ambient temperature range of 25°C - 50°C represented as optical density values are depicted in Fig. 1. Although the temperature range selected could effect a fairly appreciable growth of all the bacteria, the maximum growth rate was discernible at the temperature of 40°C for majority of strain. In *B. flexus* M3*, B. cereus* M2 and *B. megaterium* M5, high growth rate was obvious even beyond 40°C then slowly decrease in temperature of 45°C and 50°C.

**Effect of pH**

Effect of pH such as 2, 3, 4, 5, 6, 7 and 8 on the growth of bacterial strain is represented in Fig. 2. Generally the bacterial strain seemed to prefer natural pH. *B. licheniformes* M4 and *B. megaterium* M-5 showed a marked preference for alkaline range of pH. *B. cereus* M2 and *B. flexus* M3 could grow well even at slightly acidic pH 7, then slowly decrease at pH 8.

**Effect of NaCl**

Salt concentration up to 4% did not adversely affect the growth of bacterial strain (Fig. 3). Bacterial strain *B.flexus* M3*, B. megaterium* M5, *B. subtilis* M6, *B. cereus* M2, *B. pumilus* M1 and *B. licheniformes* M4 appeared to prefer medium salt concentration.

**Nutritive value**

The proximate composition of the six species of bacteria is presented in Table 1.

**Dry matter**

Dry weight of bacteria varied from 15.10 to 22.10%. Dry matter was comparatively more in *B. megaerium* M5 (22.10%), *B. cereus* M2 (20.20%) and *B. flexus* M3 (19.20%).

**Protein**

Bacterial protein represented as mg/ml varied between 2.27 and 7.85. *Bacillus* group generally had a lesser range of protein (2.27 – 4.72 mg/ml) while *B. flexus* M3 had 7.85 mg/ml protein and *B. megaterium* M5 having of protein 6.57mg/ml (Table 1).

**Carbohydrate**

Carbohydrate were the least represented nutritive compound and their value was below 0.32 mg/ml. *Bacillus* group generally had a lesser range of carbohydrate (0.18 to 0.32) while *B. subtilis* M6 (0.32) and *B. flexus* M3 (0.27) (Table 1).

**Lipid**

Lipid content of bacteria varied between 0.78 mg/ml (*B. licheniformes* M4) and 1.25 mg/ml (*B. cereus* M2). The lipid content of the *Bacillus* group did not differ considerably and most of them had their lipid level, below 1.25mg/ml (Table 1).

**Pathogenic inhibition test**

The growth inhibition ability of the gut isolates of Indian major carp, *Labeo rohita* against *Aeromonas hydrophila* were

investigated by well diffusion method and disc diffusion method.

### Well-diffusion method

Of the six *Bacillus* species isolated from the gut of *L. rohita* (M1, M2, M3, M4, M5 and M6) inhibited the growth of *A. hydrophila*. Among the strain *B. flexus* M-3 showed maximum inhibitory zone followed by *B. megaterium* M5 and *B. cereus* M-2 with 22mm, 18mm and 11mm respectively (Table 2 and Fig. 4).

### Disc-diffusion method

Among the total six *Bacillus* species isolated from the gut of *L. rohita*, all six species showed inhibitory activity against *Aeromonas hydrophila*. A maximum zone of inhibition (16 mm) was recorded by *B. megaterium* (M5) while, *B. flexus* (M3) and *B. cereus* (M2) showed inhibitory zone of 14 mm and 10 mm, respectively (Table 2 and Fig. 4).

**Table 1. Proximate composition of bacteria (value in mg/ml)**

| Bacteria | Dry matter % | Protein | Lipid | Carbohydrate |
|---|---|---|---|---|
| **M1** | 15.10 | 2.27 | 0.96 | 0.25 |
| **M2** | 20.20 | 3.64 | 1.25 | 0.23 |
| **M3** | 19.20 | 7.85 | 1.14 | 0.27 |
| **M4** | 17.40 | 4.78 | 0.78 | 0.21 |
| **M5** | 22.10 | 6.57 | 0.83 | 0.18 |
| **M6** | 18.10 | 5.37 | 0.97 | 0.32 |

M1- *Bacillus pumilus*, M2- *Bacillus cereus*, M3- *Bacillus flexus*, M4- *Bacillus lichiniformis*, M5- *Bacillus megaterium* and M6- *Bacillus subtilis*.

**Table 2. Growth inhibition zone (mm) for each *Bacilli* species isolated from the gut of *Labeo rohita* against *Aeromonas hydrophila***

| Bacterial strain | Well-diffusion | Disc-diffusion |
|---|---|---|
| **M1** | 2 | 6 |
| **M2** | 11 | 10 |
| **M3** | 22 | 14 |
| **M4** | 6 | 4 |
| **M5** | 18 | 16 |
| **M6** | 8 | 7 |

M1- *Bacillus pumilus*, M2- *Bacillus cereus*, M3- *Bacillus flexus*, M4- *Bacillus lichiniformis*, M5- *Bacillus megaterium* and M6- *Bacillus subtilis*.

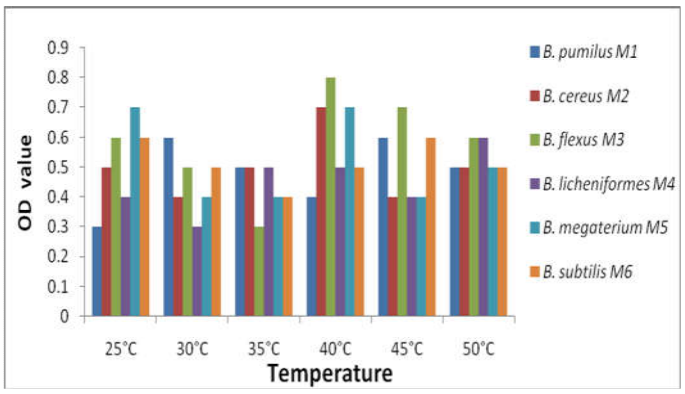


**Fig. 1. Effect of temperature on the growth of bacteria**

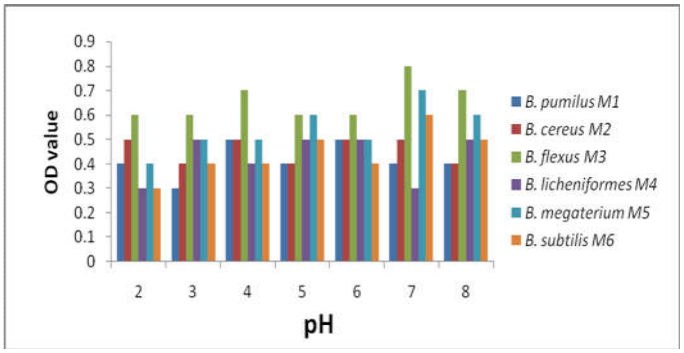


**Fig. 2. Effect of pH on the growth of bacteria**

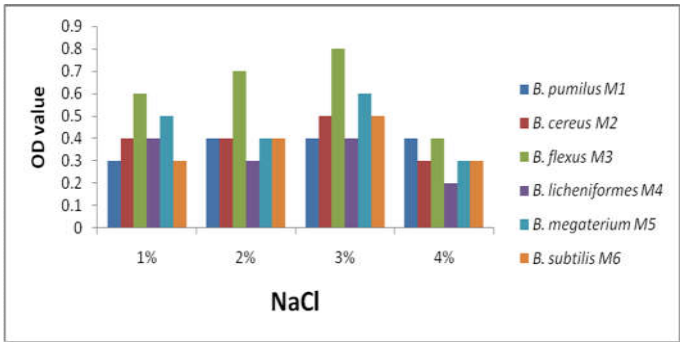


**Fig. 3. Effect of NaCl on the growth of bacteria**

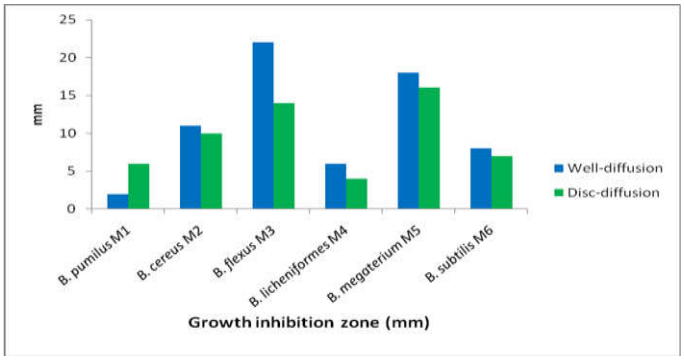


**Fig. 4. Growth inhibition zone (mm) for each Bacillus species isolated from the gut of *Labeo rohita* against *Aeromonas hydrophila***

## DISCUSSION

Among the multiple isolates of *Bacillus* from the gut of *L. rohita*, six species were identified using biochemical characterization techniques (Bergey's Mannual, 1989). The *Bacillus* strains isolated were, *B. pumilus* (M1), *B. cereus* (M2), *B. flexus* (M3), *B. licheniformes* (M4), *B. megaterium* (M5) and *B. subtilis* (M6). The strains of *Bacillus* spp. used as probiotics for terrestrial livestock has telluric origins. They are not autochthonous in the gastrointestinal tract but may be active during intestinal transit (Gournier-Chateau *et al*., 1994). Similar findings were reported for *Labeo rohita* (Hossain *et al*., 1999); rainbow trout (Spanggaard *et al*., 2000); grass carp (Hatha *et al*., 2000) and *Catla catla* (Bhatnagar *et al*., 2012).
In the present study, the physical and biochemical characters of potential probiotic bacterial isolates were identified as *Bacillus* sp. according to Bergey *et al*., (1994). By further physiological

and biochemical test the strain was identified as *Bacillus* genus six species (M1, M2, M3, M4, M5 and M6). The use of *Bacillus* species in aquaculture practices has also been reported previously (Moriarty, 1998; Rengpipat *et al*., 1998; Ghosh *et al*., 2007; Salem *et al*., 2010; Nayak and Mukherjee, 2011; Elghandour *et al*., 2015; Puniya *et al*., 2015). Growth capacity of the selected bacterial strain in the ambient temperature range of 25°C – 50°C represented as optical density values are depicted in Fig. 1. Although the temperature range selected could affect a fairly appreciable growth of all the bacteria, the maximum growth rate was discernible at the temperature of 40°C for majority of strain. In *B. flexus* (M3)*, B. cereus* (M2) and *B. megaterium* (M5), high growth rate was obvious even beyond 40°C then slowly decrease at 45°C and 50°C. The different results obtained in the study of Falguni and Sharma (2012) who stated that the optimum temperature for growth of *B. flexus* (M3) were 40°C. The difference in the optimum temperature for *B.megaterium* (M5) might occur due to the physiological nature of the strains. Effect of pH on the growth of bacterial strain is represented in Fig. 2. Generally the bacterial strain seemed to prefer natural pH. *B. licheniformes* (M4) and *B. megaterium* (M5) showed a marked preference for alkaline range of pH. While *B. cereus* M2 and *B. flexus* (M3) could grow well even at slightly acidic pH 7, then slowly decrease at pH 8. Allamesh *et al*., (2012) have determinate that *L. mesenteroides* isolated from *Channa striatus* intestine that has neutral condition and showed the highest activity at pH 7. According to this report, 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 the rainbow trout intestine, which occurred at pH 5 to 10. Alkalophilic bacteria were microorganisms that lived well at alkaline pH (above pH 7). Results of research conducted by Magdi *et al*., (2010) in *Bacillus subtilis* KO obtained an optimum pH value of 6.5 - 7. The optimization results for *Bacillus firmus* was carried out by Roosdiana *et al*., (2013) that obtained an optimum pH value of 7-8. pH, it was in line with the results of this research. Salt concentration up to 4% did not adversely affect the growth of bacterial strain (Fig. 3). Bacterial strain *B. flexus* (M3)*, B. megaterium* (M5), *B. subtilis* (M6), *B. cereus* (M2), *B. pumilus* (M1) and *B. licheniformes* (M4) appeared to prefer medium salt concentration. Bile salt tolerance is required for probiotic bacteria to grow and survive in the fish intestine (Salminen *et al*., 2004). Cebeci and Gurakan (2003) determined that *L. plantarum* as a probiotic could survive on 0.3% of bile salt. The probiotics that can tolerate low pH and bile salt means they not only can transit through the stomach and be active in the intestine, but also are able to be alive and survive in stress conditions (Cebeci and Gurakan, 2003). In feeding studies, useful information on larval requirements to essential components can be achieved by salinity challenging when assessing their physiological condition (Dhert *et al*., 1992a and 1992b). Challenge tests are proposed as meaningful tools for assessing fish quality in the aquaculture industry, environmental resources management and in research (Wedemyer and McLeay, 1981). The concept is based on the presumptions that stress loading above the acclimation capacity of an organism will weaken it and reduce performance in growth, survival and reproduction, and that the reduction in performance can be quantified by assessing tolerance to reference stresses (Wedemyer and McLeay, 1981). The influence of probiotic *Bacilli* spp. on the resistance of Persian Sturgeon Larvae against challenge tests, including salinity experiment, and showed that the mortality of larvae fed with probiotic was significantly lower than the control group (Faramarzi *et al*., 2012). The proximate composition of six species of bacteria is presented in Table 1. Carcass chemical composition measurements have been used as a reliable index to estimate nutritional conditions and growth of fish larvae (Rengpipat *et al*., 1998; Hevroy *et al*., 2005). Aubin *et al*., (2005) demonstrated that dietary supply of the yeast increased muscle lipids and red pigmentation. Dry weight of bacteria varied from 15.10 to 22.10%. Dry matter was comparatively more in *B. megaerium* M5 (22.10%), *B. cereus* M2 (20.20%) and *B. flexus* M3 (19.20%).Bacterial protein represented as mg/ml varied between 2.27 and 7.85. *Bacillus* group generally had a lesser range of protein (2.27 – 4.72 mg/ml) while *B. flexus* (M3) had 7.85 mg/ml of protein and *B. megaterium* (M5) having of protein 6.57 mg/ml (Table 8). It has been reported that, *M. rosenbergii* fed with *B. subtilis* incorporated diets, there was significant improvement in the proximate composition of body tissues (Seenivasan *et al*., 2012). Seenivasan *et al*., (2011) have reported that, the commercial probiotic, Binifit$^{TM}$ supplemented diets; there was significant improvement in the proximate composition of body tissues in *M. rosenbergii* PL. The beneficial effects of probiotics in *M. rosenbergii* PL culture has been supported by Venkat *et al*., (2004), Suralikar and Sahu (2001) and Shinde *et al*., (2008). Spirulina is a cyanobacterium that has been commercially cultivated for more than 10 years due to its high nutritional content consisting of protein, amino acids, vitamin, minerals, essential fatty acid and β- carotene (Abdul Kadhar *et al*., 2012). It is reported that the digestive organs are very sensitive to food composition and cause immediate changes in the activities of the digestive enzymes (Bolasina *et al*., 2006; Shan *et al*., 2008), which is finally reflected in fish health and growth. Moreover, bacteria also secrete proteases to digest the peptide bonds in proteins and therefore break down the proteins into their constituent monomers and free amino acids, which can benefit the nutritional status of the animal (MacFarlane and Cummings, 1991). Majority of probiotics are capable of secreting lipase, which triggers production and assimilation of essential fatty acids resulting higher growth and immunity in fish. Feed supplementation of essential fatty acid not only boosts the immunity, but also triggers the growth (Sharma *et al*., 2009).

Carbohydrate were the least represented nutritive compound and their value was below 0.32 mg/ml. *Bacillus* group generally had a lesser range of carbohydrate (0.18 to 0.32) while *B. subtilis* M6 (0.32) and *B. flexus* M3 (0.27) (Table 1). The changes in carbohydrate, protein and lipid in fish body could be related to the changes in their synthesis and deposition rate in muscles (Abdel-Tawwab *et al*., 2006). The biochemical analyses often provide vital information for health-assessment and management of cultured fish (Cnaani *et al.*, 2004; Ghosh *et al*., 2003). Bacterial enzymatic hydrolysis have been shown to enhance the bioavailability of protein and fat (Ling and Hanninen, 1992), which may result in higher growth and nutrient utilization as observed in the present study. It is reported by the early workers that the

increase in nutrient digestibility may be because of better availability of exoenzymes produced by probiotics (Vine *et al*., 2006) or better health condition (Yanbo and Zirong, 2006) when probiotic- supplemented diets are fed to the fish. Bairagi *et al*., (2002) reported that the *Bacillus* species isolated from the gut of *Cyprinus carpio* were found to a have high amount of extracellular amylolytic, proteolytic and lipolytic activity. Lipid content of bacteria varied between 0.78 mg/ml (*B. licheniformes* M4) and 1.25 mg/ml (*B. cereus* M2). The lipid content of the *Bacillus* group did not differ considerably and most of them had their lipid level, below 1.25 mg/ml (Table 1). Fish can use lipid, protein and carbohydrate as energy sources (Cho, 1992; Hidalgo *et al*., 1993; Wilson, 1994). The improvement is seen in agreement to increase in lipid content; this fact was also observed by Bromley (1980) and Watanabe (1982). The major biochemical activity of the heterotrophic bacteria is the dissemination of organic matter.

In the present investigation biochemical characterization indicated that both the isolated organisms were capable of hydrolyzing proteins such as casein and gelatin. In addition, the strains were able to utilize a wide variety of carbohydrates including cellulose. Enzymes produced by intestinal fish microbiota might have a significant role in digestion, especially for substrates such as cellulose, which few animals can digest, and also for other substrates (Smith, 1989). Luczkovich and Stellwag (1993) opined that the gastrointestinal microbiota of finfish (*Lagodon rhomboides*) might contribute to the breakdown of plant materials. Recent observations have documented that the fish having harbored proteolytic, amylolytic and cellulolytic bacteria in their digestive tracts (Bairagi *et al*., 2002; Ghosh *et al*., 2002; Saha *et al*., 2006), which is in agreement with the present study. In addition to utilizing various proteins and carbohydrates, both the strains were ornithine decaroboxylation and urease positive. The enzyme ornithine decarboxylase has been reported to catalyse the synthesis of polyamines such as spermine and spermidine, which are used in DNA packaging and required in large amounts in rapidly dividing cells (Lehninger *et al*., 1993). The ability to decarboxylate ornithine for the intestinal isolates may be indicative of their growth and colonization potential in the GI tract. In general, urease catalyzes hydrolysis of urea into carbon dioxide and ammonia, thereby raising the pH of the media. The urease positive nature of the isolated strains could be helpful in maintaining a neutral or alkaline environment in the GI tract to facilitate microbial growth.

Earlier investigations have suggested that microorganisms have a beneficial effect in the digestive processes of fish (Ringo *et al*., 1995). Characterization of microbial populations in the intestinal micro environment of fish and understanding the physiological interactions between the indigenous microbiota and the host may have important implications (Silva *et al*., 2005). The use of such beneficial bacteria as probiotics has a long tradition in animal husbandry (Stavric and Kornegay, 1995). These beneficial bacteria could be introduced in commercial aquaculture by incorporating them into formulating fish diets, or in the form of bacteria biofilm to achieve colonization in the fish GI tract at a higher degree. The growth inhibition ability of the gut isolates of Indian major carp *Labeo rohita* against *Aeromonas hydrophila* were investigated by well diffusion method and disc diffusion method. Of the six *Bacillus* species isolated from the gut of *L. rohita* (M1, M2, M3, M4, M5 and M6) inhibited the growth of *A. hydrophila*. Among the strain *B. flexus* M3 showed maximum inhibitory zone followed by *B. megaterium* M5 and *B. cereus* M2 with 22mm, 18mm and 11mm respectively (Table 2 and Fig. 2). The growth inhibition ability of the gut isolates of Indian major carp *Labeo rohita* against *Aeromonas hydrophila* were investigated by well diffusion method and disc diffusion method. Of the six *Bacillus* species isolated from the gut of *L. rohita* (M1, M2, M3, M4, M5 and M6) inhibited the growth of *A. hydrophila*. Among the strain *B. flexus* M3 showed maximum inhibitory zone followed by *B. megaterium* M5 and *B. cereus* M2 with 22mm, 18mm and 11mm respectively (Table 2 and Fig. 4). According to Moriarty (1998), *Bacilli* are not associated with aquatic organism pathology and are widely accepted and used as probiotics advocating the use of selected M3 and M5 as feed probiotic. 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 the 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). Among the total six *Bacillus* species isolated from the gut of *L. rohita*, six species showed inhibitory activity against *Aeromonas hydrophila*. A maximum zone of inhibition (16 mm) was recorded by *B. megaterium* (M5) while, *B. flexus* (M3) and *B. cereus* (M2) showed inhibitory zone of 14 mm and 10 mm (Table 2 and Fig. 4). Compared to the well diffusion method the inhibitory zone in disc diffusion method was slightly smaller (Table 9). Similar results were observed by Swain *et al*., (1996) and Ghosh *et al*., (2003) in Indian major carps. *B. cereus* has also been studied for its probiotic action in *Cyprinus carpio* (Lalloo *et al*., 2007), red tilapia (Bernard *et al*., 2013) and silver catfish (Moreira de Souza *et al*., 2012).

The interpretations of inhibition zone were determined according to zone size of the chart by Kirby-Bauer test results (Bauer *et al*., 1966). Resistance to specific antibiotic means that, the probiotic can be given at the same time when antibiotic treatment is required. Secondly, the micro flora of the intestine can recover more quickly (Cebeci and Gurakan, 2003; Kim and Austin, 2008). Kim and Austin (2008) determined the antibiotic susceptibility of *Carnobacterium* strains. 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. In general, probiotic 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.

## Summary and Conclusion

The putative probionts (M3 and M5) by virtue of their prominent traits such as pH, temperature, salinity and nutrient

composition two putative probionts were recruited in the present study. They were *B. flexus* (M3) and *B. megaterium* (M5) and their characteristics were as given below: *B. flexus* (M3) showed a higher capacity to grow even at 40°C. M3 prepared alkaline- pH 7. It also recorded higher nutrient value in all the biochemical parameters. *B. flexus* (M3) showed maximum inhibitory zone of 22 mm in well diffusion method and 16 mm in disc diffusion method for *B. megaterium* (M5) (Table 2 and Fig. 4). Thus, after screening all the positive selection criteria for putative probionts, these two strains M3 and M5 were selected as putative probionts for further Invivo experiments in the Indian major carp fish, *Labeo rohita*. The two putative probionts, *B. flexus* (M3) and *B. megaterium* (M5) chosen on the basis of selection of procedure results were alone selected for further study. Wholesome evaluation of these bacterial species as a probiotic candidate under laboratory condition is essential before their application in the field.

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