BACTERIOLOGICAL ANALYSIS IN THE GUT OF *Cirrhinus cirrhosus* AND SCREENING THE EFFECT OF BACTERIOCIN AGAINST COMMON GRAM-NEGATIVE BACTERIAL PATHOGENS

Karuppasamy Murugan*

PG Department of Microbiology, Sri Paramakalyani College, Alwarkurichi – 627 412, Tirunelveli – District, Tamilnadu, India.

*Correspondence for Author

Karuppasamy Murugan

PG Department of Microbiology, Sri Paramakalyani College, Alwarkurichi – 627 412, Tirunelveli – District, Tamilnadu, India.

ABSTRACT

In the present study focuses, *Cirrhinus cirrhosus* (mrigal fish) was collected from the freshwater fish cultivation pond and the total heterotrophic bacterial population (THBP), the total lactic acid bacterial density and the amylase, protease and cellulase producing bacterial load in the gastro-intestinal tract were enumerated. In this study quantitative wise, amylase and protease producers were found to be in the first two positions and the last position was acquired by cellulase producing bacteria were also determined. Using selective media, common pathogens and beneficial bacteria were isolated for this study. A total of 10 bacterial isolates were selected and taken for identification up to generic level by morphological and biochemical aspects. *Aeromonas* sp., *Vibrio* sp., *Pseudomonas* sp., *Salmonella* sp. and *Lactobacillus* sp. were the identified pathogenic and beneficial bacteria. From this study, the probiotic role of *Lactobacillus* sp. was checked through cross streaking technique and the presence of antagonistic activity in *Lactobacillus* sp. against the *Aeromonas* sp. was confirmed. Production of bacteriocin in the two *Lactobacillus* sp. isolates was noted and the bacteriocin was found to be effective at pH 8 but not at pH 7 was determined. Finally I concluded from this study, revealed the possibility of using bacteriocin as a food preservative and the *Lactobacillus* sp. as a probiotic and antibiotic resistance studies revealed the emergence of resistance invariably in all the studied bacterial pathogens against the tested antibiotics and the need for the inclusion of sustainable friendly techniques such as probiotics, immunostimulatns etc. for managing fish health and diseases.

KEYWORDS: Mrigal fish, Lactic acid bacteria, Nutrient agar medium, Bacteriocin, Probiotics and Antibiotics.

INTRODUCTION

Freshwater 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 the digestive tract of freshwater mrigal fish confers a favorable culture environment for the microorganisms. The importance of intestinal bacteria in the nutrition and well-being of their hosts has been established for homoeothermic species, such as birds and mammals.[1] However, there is limited information for freshwater mrigal fish, the poikilothermic vertebrates. Though the digestive tract of endothermic that is mainly colonized by obligate anaerobes,[2] the predominant bacterial species isolated from most of the fish digestive tracts have been reported to as aerobes or facultative anaerobes.[3,4,5]

Endogenous digestive enzymes in fish have been studied by several workers.[6,7,8] However, information regarding the enzyme producing intestinal bacteria, their source and significance in fish is scarce. In the present study, an attempt has been made to investigate the relative amount of amylase, protease and cellulase producing bacteria in the gastro-intestinal (GI) tracts of *Cirrhinus cirrhosus*. Further intestinal isolates will be evaluated for extracellular enzyme producing capacities.

Yasuda and Taga (1980)[9] suggested that probiotic bacteria would be useful not only as food but also as biological controllers of fish disease and activators of nutrient regeneration. In aquaculture, biological control emerges as an alternate to mrigal fish and since then the research effort has continually increased. *Bacillus* sp. is often antagonistic against other freshwater fish pathogenic bacteria.[10,11] Generally bacteria play two major roles as beneficial bacteria and pathogenic forms. Beneficial bacteria are helpful in nutrient recycling and organic matter degradation and thus clear the environment.[12] Pathogenic bacteria are the causative agents of bad water quality, stress and diseases and could they act as primary and secondary pathogens.[13]

Intensive aqua farming accompanies several disease problems often to opportunistic pathogens as evident from general aquaculture. Austin *et al*. (1995)[14] stated that high stocking densities, more food inputs and other organic loads stimulate the selection and proliferation of opportunistic bacteria.

Bacteriocins are proteinaceous compounds produced by bacteria to inhibit the growth of similar or closely related bacterial strains. They are typically considered to be narrow spectrum antibiotics. They are phenomenological analogous to yeast and *Paramecium* killing factors and are structurally, functionally and ecologically diverse. Bacteriocins were first discovered by Gratia (1925).[15] He involved in the process of searching for ways to kill bacteria, which also resulted in the development of antibiotics and the discovery of bacteriocins all within a span of a few years. He called his first discovery a colicin because it killed *Escherichia coli*.

The genus *Lactobacillus* is well diverse and consists of a number of different species with little commonality. They are Gram-positive rods with a size range of 0.5-1.2 × 1-10 μm and non – spore formers, producing lactic acid as a fermented end product. It includes over 25 species and the first level of differentiation is based on end-product composition. Some are homo-fermentative where as others are hetero-fermentative in nature. Lactic acid bacteria are useful in the food industry. They reduce the pH in food, low enough to inhibit the growth of most of other microorganisms including common human pathogens, thus increasing the shelf life of food.[16]   In the search for a food preservative, investigations on certain antibacterial proteins (bacteriocin) from lactic acid bacteria have been popular.[17] Bacteriocin is proteinaceous compounds of bacterial origin that are lethal to bacteria other than the producing strain. Bacteriocin secreting microbes has selective advantage in a complex microbial niche. Generally, bacteriocins are named according to the genus or species of the strain that produces it. For example, plantaricin produced by *Lactobacillus plantarum*.[18] Bacteriocins produced by lactic acid bacteria have received considerable attention during recent years for their possible as bio-preservative in food, with a resultant reduction in the use of chemical preservatives. *Lactobalillus acidophilus* is one of the most important lactic acid bacteria used for the production of fermented meat, grass and vegetable products reported by Ruiz-Barba *et al*. (1991).[19]

Lactic acid bacteria ferment various carbohydrates mainly to lactate and acetate. Various amino acids, vitamins and minerals are essential for their growth.[20] Ringo and Gatesoupe (1998)[21] have shown that lactic acid bacteria are also part of the normal intestinal flora of fish. Most of the evidence comes from salmonid species like Arctic char (*Salvelinus alpinus*) and Atlantic salmon (*Salmo salar*) reported by Ringo *et al*. (1995)[22] and Gonzalez *et al*. (2000).[23]

Few studies have described lactic acid bacteria in other freshwater fish.[24,25] Kvasnikov *et al.* (1977)[24] described the presence of lactic acid bacteria, including *Lactobacillus* in the intestines of various fish species at larval, fry and fingerling stages inhabiting ponds. However, it was discussed that some human activities like artificial feeding in ponds would have had an effect on the bacterial composition and load in some fish, like carp which showed the highest content of lactic acid bacteria in the intestines. Bacteriocins are bactericidal or bacteriostatic peptides that are mostly active against bacteria closely related to the producer stated by Klaenhammer (1988).[26] The discovery of nisin, the first bacteriocin used on a commercial scale as a food preservative dates back to the first half of last century but research on bacteriocins of lactic acid bacteria has expanded in the last two decades, searching for novel bacteriocin producing strains from dairy, meat and plant products, as well as traditional fermented products.

Lactobacilli are important organisms recognized for their fermentative ability as well as their health and nutritional benefits.[27] They produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocin or bactericidal proteins during lactic fermentations.[28] Bacteriocins are proteinaceous antibacterial compounds and exhibit bactericidal activity against species closely related to the producer strain observed by De Vuyst and Vandamme (1994),[29] many bacteriocins are active against food-borne pathogens especially against *Listeria monocytogenes*.[30,31]

Lactic acid bacteria are widely distributed in various animal intestines stated by Mitsuoka (1980),[32] Sakata *et al.* (1980)[33] and Devriese *et al.* (1987)[34] and some lactic acid bacteria produce probiotics have played an important role in beneficial functions for industrial animals.[35] There have been several reports by Mitsuoka (1990),[36] Perdigon *et al.* (1995)[35] and Salminen and Wright (1998a)[37] of lactic acid bacteria occurring among the major microbial populations in animal intestines. It is well established that some lactic acid bacteria improve the intestinal micro flora and promote the growth and health of animals.[36,35] Most probiotics contain single or multiple strains of lactic acid bacteria and are part of the natural micro flora of many animals; they are generally regarded as safe and may display antagonistic activities against pathogenic bacteria.[38] The intestinal micro flora, especially lactic acid bacteria, may influence the growth and health of fish. However, few studies have reported the composition of intestinal lactic acid bacterial flora in fish. Kandler and Weiss (1986)[39] have classified *Lactobacillus* isolates from temperate regions according to their

morphology, physiology and molecular characters. Lactic acid bacteria from food and their current taxonomical status have been described by many reviewers.[21,40] Ringo and Gatesoupe (1998)[21] have prepared a review of the lactic acid bacteria present in fish intestine. Taxonomic studies on lactic acid bacteria from poikilothermic animal are rare.[21,41,42]

AIM AND OBJECTIVES OF THE STUDY

The main aim of the present investigation is to isolate, the lactic acid bacteria like *Lactobacillus* sp. from the gastro-intestinal tract of common freshwater mrigal fish which is used as a probiotic organisms against fish pathogens like *Aermonas* sp., *Vibrio* sp., *Pseudomonas* sp. and *Salmonella* sp. isolated from the same common freshwater mrigal fish.[43,44,45]

The present investigation falls on the following lines

❖ To enumerate the total heterotrophic bacterial population and *Lactobacillus* sp. in the gastro-intestinal tract of mrigal – a common freshwater fish.

❖ To identify the bacterial pathogens such as *Aeromonas* sp., *Vibrio* sp., *Pseudomonas* sp. and *Salmonella* sp. and friendly bacteria such as *Lactobacillus* sp. through basic microbiological and biochemical analysis.

❖ To screen the load of extracellular enzyme producing bacteria found in the intestinal region of mrigal fish.

❖ To study the quantitative determination of amylase, protease and cellulase production by the bacteria of gastro-intestinal tract of mrigal fish.

❖ Screening the antagonistic effect of *Lactobacillus* sp. over fish bacterial pathogens by cross streak method.

❖ Screening the bacteriocin production in *Lactobacillus* sp. and checking its effect over fish pathogens by agar-well diffusion method.

❖ To screen the influence of pH on the activity of bacteriocin.

❖ To study the determination of antibacterial susceptibility of bacterial pathogens against selected antibiotics.

MATERIALS AND METHODS

Sampling

Omnivore mrigal fish was sampled by gill-net from local fish cultivation pond of Kallidaikurichi Village, Tirunelveli District and Tamilnadu analyzed separately for this

study.[46,47,48,49] During the sampling period, the water temperature was 28^0C and the sample was transported to the Microbiology Laboratory immediately after collection. Then, the sample was subjected to the microbial analysis within six hours to prevent the reduction of bacterial load in the gastro-intestinal tract of the selected freshwater mrigal fish.

Bacteriological Examination

Determination of bacterial population in freshwater mrigal fish

The fish was sacrificed with a blow to the head, opened aseptically and their whole intestines were removed. The intestines were dissected carefully and collected by scraping using a sterile rubber spatula. The intestinal tissues of the fish were weighed to determine the load of bacterial population of the gastro-intestinal tract of the fish.

Enumeration of total heterotrophic bacterial population from the gastro-intestinal tract of freshwater mrigal fish

About one gram of the intestinal content was homogenized in a stomacher and taken, aseptically transferred into a conical flask containing 99 ml of sterile saline solution and it represents 10^{-2} dilution and subsequently, one ml was pipetted out from the homogenate and transferred into a test tube containing 9 ml of sterile saline solution. Care was taken to mix the sample solution of each dilution thoroughly in a vortex mixture for one minute in prior to pipetting out. From the serially diluted sample, one ml of two consecutive dilutions from 10^{-4} and 10^{-5} to be tested was pipette out onto the sterile Petri dishes in replicates and sterile nutrient agar medium was poured. These plates were incubated at 37^0C for 24-48 hours. After incubation, the plates with colonies ranging from 30-300 were selected for counting.

Enumeration of lactic acid bacteria from the gastro-intestinal tract of freshwater mrigal fish

From the above serially diluted homogenate in sterile saline sample, one ml of two consecutive dilutions from 10^{-4} and 10^{-5} to be tested was pipetted out onto the sterile Petri dishes in replicates and sterile *Lactobacillus* MRS (de Man, Rogosa and Sharpe) agar medium was prepared and poured onto the plates and incubated anaerobically at 37^0C for 48-72 hours. After incubation, lactic acid bacteria was understood based on the guidelines of the manufacturer, counted and recorded.[50,51,52] Well isolated colonies with typical characteristics namely pure white, small with entire margins were picked from each plate and transferred to *Lactobacillus* MRS broth and then to *Lactobacillus* MRS agar slants for further analysis of biochemical aspects.

Isolation of bacterial pathogens from gastro-intestinal tract of fresh water mrigal fish by streak plate method

For the selective isolation of *Aeromonas* sp. – Kaper's medium (Hi-Media), *Vibrio* sp. Thiosulphate citrate bile salts sucrose agar medium (TCBS – Hi-Media), *Pseudomonas* sp. – *Pseudomonas* isolation agar medium (PIA – Hi-Media) and *Salmonella* sp. – Xylose-lysine deoxycholate agar medium (XLD – Hi-Media) were prepared and plated into sterile Petri dishes. From the above serially diluted sample, one lapful of inoculums from 10^{-5} dilution was taken and quadrant streaks were made on the above mentioned selective agar media plates and incubated for 24-48 hours at 37^0C. After incubation period, pathogens growth was observed based on the guidelines of the manufacturer and taken for further studies.

Screening of extracellular enzyme producing bacteria found in mrigal fish intestine

To enumerate amylase, protease and cellulase producing bacterial population, from the above serially diluted sample, 0.1 ml of two consecutive dilutions from 10^{-5} was spread plated on starch agar medium (SA), peptone-gelatin agar medium (PGA) and carboxy methyl cellulose agar medium (CMC) plates respectively. The spread plated plates were incubated at 37^0C for overnight and examined for the development of bacterial colonies after the incubation period.[6,7,8,53]

Qualitative studies on enzyme producing bacteria in gastro-intestinal tract of freshwater mrigal fish

a) Determination of amylase production

For extracellular amylase production, isolates were single streaked on starch agar plates and incubated at 37^0C for 48 hours. After incubation, the culture plates were flooded with 1% Lugol's iodine solution and the bacteria, whose amylase producing ability was identified by the formation of transparent zone surrounding the colony.[54,53,55,10]

b) Determination of protease production

Similarly, for extracellular protease,[53,7,56] the isolates were single streaked on peptone-gelatin agar plates and incubated at 37^oC for 15 hours. After incubation, the appearance of clear zone around the colony was observed after flooding the plates with 15% mercuric chloride ($HgCl_2$) solution to understand the presence of proteolytic activity.

c) Determination of cellulase production

Similarly, for extracellular cellulase,[57,58] the isolates were single streaked on carboxy methyl cellulose agar plates and incubated at 37°C for 24 hours. For the determination of cellulase production, the culture plates were then flooded with Congo-red dye prepared with 0.7% agarose and the cellulase production ability of the culture was identified by the appearance of a clear halo zone around the colony due to the hydrolysis of carboxy methyl cellulose found in the media.

Growth characteristics of selective bacterial isolates on selective agar media

Based on the below mentioned characters, bacterial isolates for the present study were selected, identified and taken for further studies.

Lactobacillus MRS agar medium – The colonies of *Lactobacillus* sp. will be luxuriant in growth.

TCBS agar medium – The colonies of *Vibrio* sp. will be yellow coloured and good–luxuriant in growth.

Kaper's medium – The colonies of *Aeromonas* sp. will be luxuriant in growth.

XLD agar medium – The colonies of *Salmonella* sp. will be good–luxuriant in growth and red with black centered colony.

PIA medium – The colonies of *Pseudomonas* sp. will be blue-green coloured and luxuriant in growth.

Enrichment of selective isolates in broth culture

The isolated selective luxuriant colonies from selective agar media were inoculated into the yeast extract broth, incubated at 37°C for 24 hours for enrichment. After 24 hours, the enriched selective isolates were inoculated into the nutrient broth and incubated at 37°C for 24 hours.

Purification of selective isolates on nutrient agar medium

The isolated selective cultures from nutrient broth were streaked onto the nutrient agar medium repeatedly. After repeated streaking on nutrient agar medium, the selective cultures were purified.

Storage of selective isolates on nutrient agar slants

The purified selective fresh culture from nutrient agar medium was streaked onto the nutrient agar slants and stored at 4^oC in the refrigerator for further biochemical analysis.

Identification of selective isolates by morphological and biochemical tests

The purified stored culture from nutrient agar slants were confirmed by the morphological and various biochemical tests and the genus of the selective isolates were identified by using Bergey's Manual of Determinative Bacteriology 9th edition.[59]

Experimental Works

Screening of bacteriocin production using bacterial indicators

Indicator bacterial pathogens used in the study

In the current study, to check bacteriocin production from *Lactobacillus* sp.[60,61,62] isolated from the selected freshwater fish, Gram-negative bacteria pathogens were used as indicators. The used bacteria were *Aeromonas* sp., *Vibrio* sp., *Salmonella* sp. and *Pseudomonas* sp. The above pathogenic bacteria were isolated from the gastro-intestinal tract of the same fish. All the indicator strains were sub-cultured once in every 15 days and stored in nutrient agar slants for further use. Two methods were employed to screen the bacteriocin production in the identified *Lactobacillus* sp. (two isolates) based on Davis and Reeves, (1975);[63] Albano *et al*., (2007).[64] The methods were,

- Cross streak method
- Agar-well diffusion method

Bacteriocin production from *Lactobacillus* species by cross streak method

Overnight culture of the test organisms were streaked linearly in central portion of the dried sterile nutrient agar plate. The overnight broth cultures of the pathogenic organisms were single short streaked perpendicular to the streaked test organisms. These plates were incubated at 37^0C for 24-48 hours. These plates were later carefully observed for the possible presence of zone of inhibition at the points of interception of the perpendicular lines of streak with that of the test isolate and proceed for further analysis.

Bacteriocin production from *Lactobacillus* species by agar-well diffusion method

The 24 hours broth culture of *Lactobacillus* sp. was centrifuged at 10,000 rpm at 4^0C for 30 minutes. From the centrifuged broth, supernatant was collected and loaded in the marked wells cut in nutrient agar media seeded with all the selected indicator organisms separately.

Well loaded with distilled water (50 μl) was used as control. The plates were incubated at 37°C for 24 – 48 hours. After incubation, the plates were observed for zone formation and the *Lactobacillus* sp. isolate whose supernatant produced zone was found to be bacteriocin producer (Mayer-Harting *et al*., 1972).[65]

Screening the effect of bacteriocin at different pH by agar-well diffusion method

Lactobacillus sp. was propagated in MRS broth for 72 hours at 30°C anaerobically. For extraction of bacteriocin, cell-free neutralized supernatant were obtained by centrifugation of the culture at 10,000 rpm for 20 minutes at 4^{0}C. To screen the effect of different pH on the bacteriocin action, the pH of the supernatant was adjusted to 7 and 8 separately using 1N NaOH and the activity of the bacteriocin exposed to different pH tested using agar-well diffusion method proposed by Barefoot *et al*., (1983)[66] against the fish pathogens using them as indicators.

Antibiotic susceptibility test of selective isolates by disc diffusion method

Antibiotic susceptibility test was performed by disc diffusion method formulated by Kirby-Bauer (Bauer *et al*., 1966)[67] using two different antibiotics such as Norfloxacin Nx^{10} (10mcg / disc) and Co-Trimaxazole Co^{25} (25mcg / disc) and compared with the chart given by Hi-Media manual to understand the effect of antibiotics over beneficial bacteria and pathogens.[68,69,70] The zone formation was recorded in the diameter of mm for each antibiotic.

RESULTS AND DISCUSSION

RESULTS

The mrigal fish *Cirrhinus cirrhosus* was collected from freshwater fish cultivation pond and the profile of the test animal was depicted in the Table 1 & Figure 1. Among the studied three cycles, the total heterotrophic bacterial population in the intestine of the selected freshwater fish *Cirrhinus cirrhosus* was found to be in the range of $20{\times}10^4$ to $28{\times}10^4$ CFU/g (Table 2). Using *Lactobacillus* MRS agar media, *Lactoblillus* sp. load was detected in the intestinal region of the test mrigal fish and it was found in the range of $15{\times}10^4$ to $20{\times}10^4$ CFU/g (Table 3).

Table 1: The profile about the test mrigal fish examined

Fish species	Collection spot	Sex	Body weight (gm)	Total length (L_T) (cm)	Weight of the gut (gm)	Gut length (L_G) (cm)	Relative gut length (L_G/L_T) (cm)	Feeding habit
Cirrhinus cirrhosus	Fresh water pond	Female	150	26	2.840	125	99	Microscopic plants, decaying higher plants, vegetable debris, detritus, mut, insects, zooplaktons, insect larvae and smaller fish.

Table 2: Total heterotrophic bacterial population in the gastro-intestinal tract of freshwater *Cirrhinus cirrhosus* (mrigal fish)

S.no.	Sampling cycle	Bacterial load (CFU/g) Nutrient agar medium	Method of sampling
1.	I	28×10^4	
2.	II	20×10^4	Pour plate method
3.	III	24×10^4	

Table 3: Lactic acid bacterial load in the gastro-intestinal tract of freshwater *Cirrhinus cirrhosus* (mrigal fish)

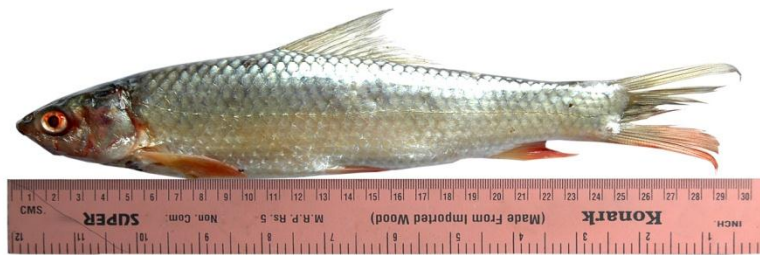| S.no.	Sampling cycle	Bacterial load (CFU/g) *Lactobacillus* MRS agar	Method of sampling
1.	I	16×10^4	
2.	II	20×10^4	Pour plate method
3.	III	15×10^4	

Fig. 1: *Cirrhinus cirrhosus* **(mrigal fish) – A test animal.**

Analysis of extracellular enzyme producing bacteria indicated the presence of amylase, protease and cellulase producers. From the Table 4, it was understood that the load of amylase producer was not uniform during sampling cycle. The range of amylase producer was in between 9 to 12 $\times10^5$ CFU/g. Load of protease producer was determined using peptone–gelatin agar medium. The same trend as in the amylase producers was noted as the protease producer in the intestinal region. The minimum load of protease producer was recorded in the first sampling cycle and the maximum was obtained in the third sample (Table 5). The range of cellulase producer was between 8 to 10×10^5 CFU/gm. The load of cellulase producers was determined using carboxy methyl cellulose agar medium. The maximum load of cellulase producer was recorded in the second sampling cycle (Table 6 & 7).

Table 4: Enumeration of amylase producing bacterial population in the gastro-intestinal tract of freshwater *Cirrhinus cirrhosus* **(mrigal fish)**

S.no.	Sampling cycle	Bacterial load (CFU/g) Starch agar medium	Method of sampling
1.	I	12×10^5	
2.	II	09×10^5	Spread plate method
3.	III	11×10^5	

Table 5: Enumeration of protease producing bacterial population in the gastro-intestinal tract of freshwater *Cirrhinus cirrhosus* (mrigal fish)

S.no.	Sampling cycle	Bacterial load (CFU/g) Peptone-gelatin agar medium	Method of sampling
1.	I	10×10^5	
2.	II	12×10^5	Spread plate method
3.	III	15×10^5	

Table 6: Enumeration of cellulase producing bacterial population in the gastro-intestinal tract of freshwater *Cirrhinus cirrhosus* (mrigal fish)

S.no.	Sampling cycle	Bacterial load (CFU/g) Carboxy methyl cellulose agar medium	Method of sampling
1.	I	08×10^5	
2.	II	10×10^5	Spread plate method
3.	III	08×10^5	

Table7: Qualitative analysis of extracellular enzyme producing capacities of the bacterial strains isolated from mrigal fish gut (result represents impression of three determinations)

S.no.	Enzyme producing capacity*					
	Strain no.	Amylase	Strain no.	Protease	Strain no.	Cellulase
1.	A_1	+++	P_1	++	C_1	+++
2.	A_2	+++	P_2	+++	C_2	+++

Legend: * - with pure culture of the intestinal isolates and number of '+' sign indicates the intensity of enzyme production.

In the current study, commonly found bacterial pathogens such as *Aeromonas* sp., *Vibrio* sp., *Pseudomonas* sp. and *Salmonella* sp. were isolated using selective media (Hi-Media) such as Kaper's medium, thiosulphate citrate bile salts sucrose agar medium (TCBS), *Pseudomonas* isolation agar medium and xylose-lysine deoxycholate agar medium (XLD). Based on the guidelines of the manufacturer of the media, the targeted organisms were isolated (Figure 2 & 3). The above said pathogens were isolated and stored in nutrient agar slants for further studies (Figure 4). The isolated pathogens (Figure 5-8) from slants were

enriched in yeast extract broth and later transferred to nutrient broth. Further purification of the pathogens was done by repeatedly streaking the cultures from the nutrient broth onto nutrient agar plates. The purified pathogens were stored in nutrient agar slants and taken for various biochemical tests to confirm the pathogens.
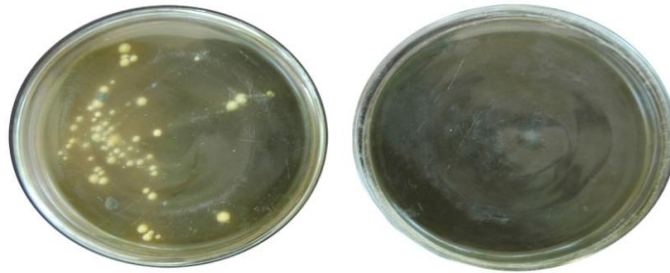

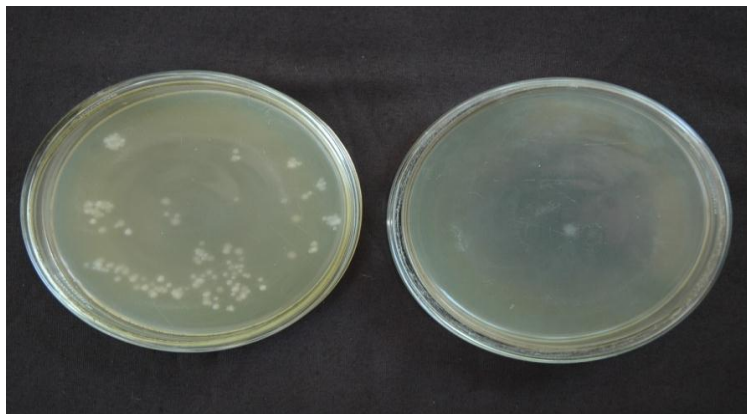**Fig. 2: Selective isolation of *Vibrio* sp. on Hi-Media's TCBS agar medium (left) and control (right).**

Fig. 3: Selective isolation of *Pseudomonas* sp. on Hi-Media's *Pseudomonas* isolation agar medium (left) and control (right).
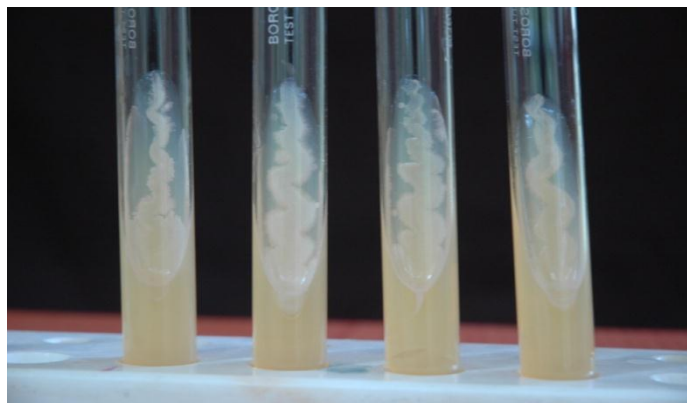
Fig. 4: Growth of fish pathogens for storage on Hi-Media's nutrient agar medium (M001).

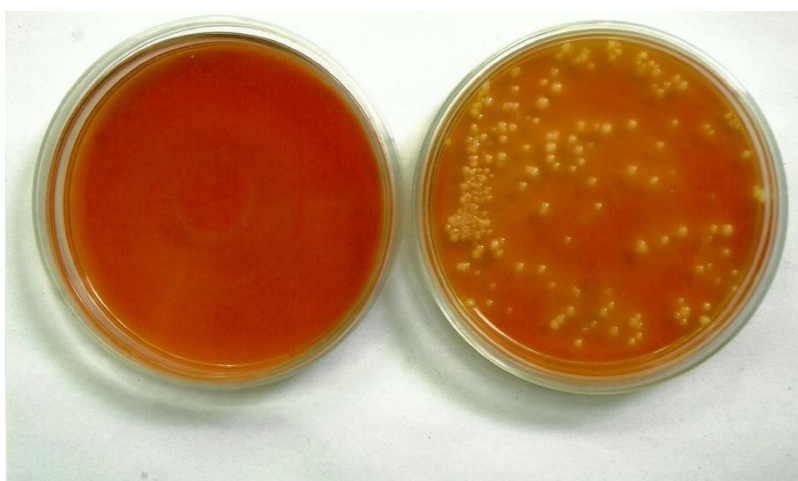**Fig. 5: Growth of *Salmonella* sp. on Hi-Media's hektoen enteric agar medium (M467).**

Fig. 6: Growth of *Vibrio* sp. on Hi-Media's brilliant green agar medium (M016A).

Legend: *Vibrio* sp. I – no growth occur (left) and *Vibrio* sp. II – yellow colour colony (right).

Fig. 7: Growth of *Aeromonas* sp. on Hi-Media's brilliant green agar medium (M016A).

Fig. 8: Growth of *Lactobacillus* sp. on Hi-Media's deoxycholate citrate agar medium (M065).

Legend: *Lactobacillus* sp. I – light pink colour colony and *Lactobacillus* sp. II – yellow with white colour colony.

Identification of bacterial pathogens isolated from the gut of mrigal fish

For identifying the said pathogens, basic morphological and various biochemical tests were conducted to identify the genus of the selective isolates and the results were tabulated (Table 8-12 & Figure 9-25). In lysine iron agar slants, except *Aeromonas* sp. no other pathogens have produced hydrogen sulphide (H_2S) gas. *Salmonella* sp., *Vibrio* sp. and *Pseudomonas* sp. have not produced hydrogen sulphide (H_2S) gas in triple sugar iron agar slants. But *Aeromonas* sp. turned the butt region black due to the release of hydrogen sulphide (H_2S) gas. In connection with hydrogen sulphide (H_2S) gas production, the same trend was noticed from *Aeromonas* sp. and also from other pathogens, when they were inoculated in Kligler iron agar slants. In the same media, *Salmonella* sp. has produced a considerable volume of gas. It was not seen in the other pathogens and it could be understood from the table (Table 8). Sorbitol fermentation ability was tested among the pathogens using sorbitol iron agar slants. The test results revealed that all the pathogens have the caliber of fermenting sorbitol. *Aeromonas* sp. alone stood different by also producing hydrogen sulphide (H_2S) gas compared to the other pathogens (Table 11).

1   2   3   4   5   6
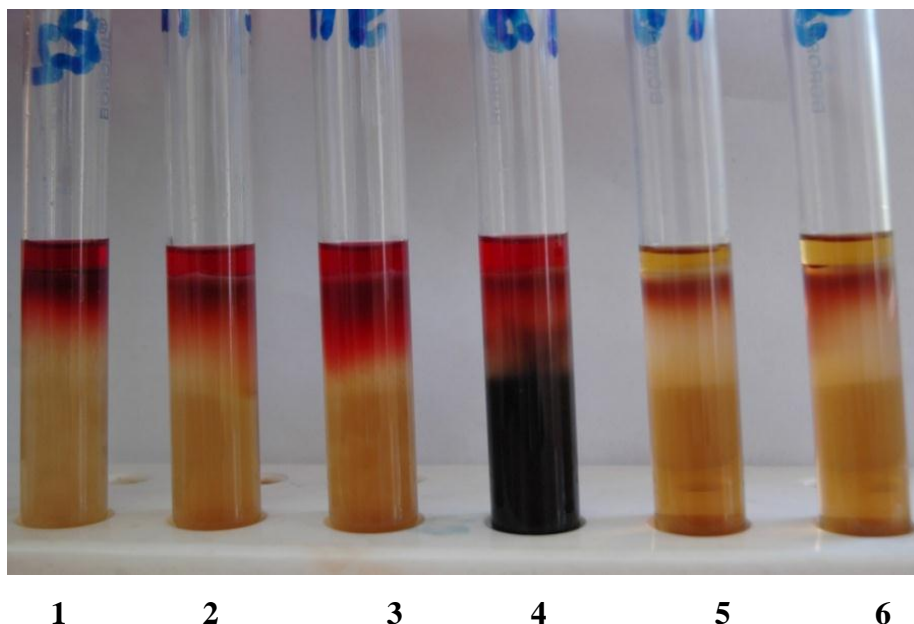
Fig. 9: Sulphide-indole-motility test for selective isolates in Hi-Media's SIM agar medium (M181).

Legend: 1. *Salmonella* sp. I – positive; 2. *Pseudomonas* sp. I – positive; 3. *Lactobacillus* sp. I – positive; 4. *Aeromonas* sp. I – positive; 5. *Vibrio* sp. I – negative; 6. Control – uninoculated.
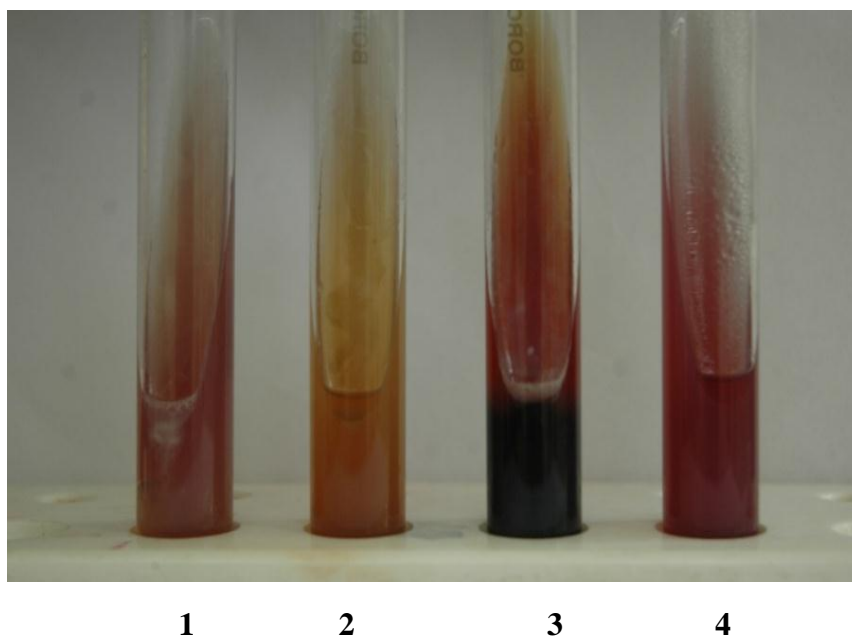

1   2   3   4

Fig. 10: Lysine iron agar test for selective isolates in Hi-Media's lysine iron agar medium (M377).

Legend: 1. *Pseudomonas* sp. I – positive; 2. *Pseudomonas* sp. II – positive; 3. *Aeromonas* sp. II – positive; 4. Control – uninoculated.
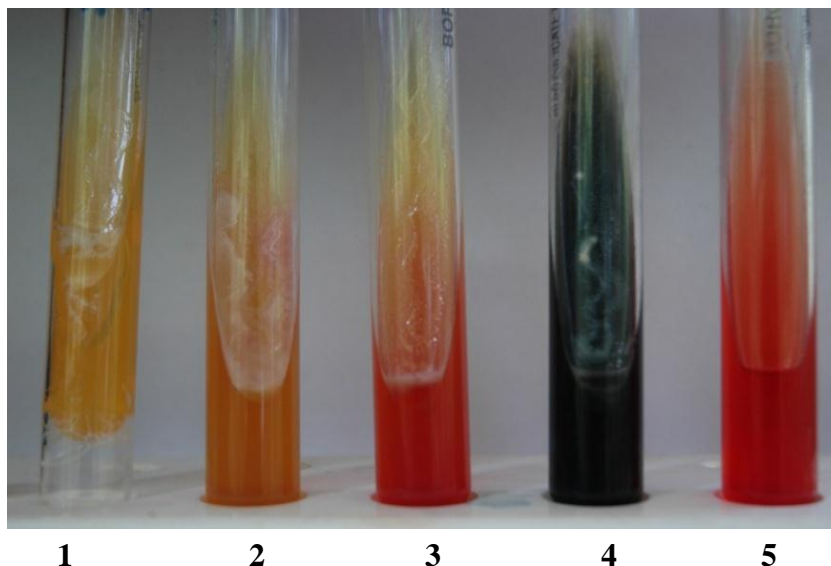
1   2   3   4   5

Fig. 11: Triple sugar iron agar test for selective isolates in Hi-Media's triple sugar iron agar medium (MM021).

Lactobacillus sp. I – positive; 2. *Pseudomonas* sp. II – positive; 3. *Vibrio* sp. I – positive; 4. *Aeromonas* sp. II – positive; Control – uninoculated.
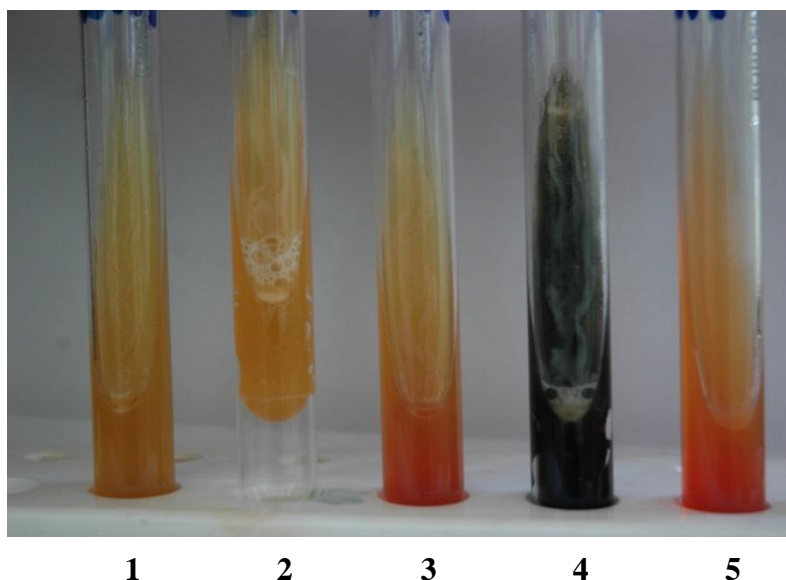

1   2   3   4   5

Fig. 12: Kligler iron agar test for selective isolates in Hi-Media's Kligler iron agar medium.

Legend: 1. *Pseudomonas* sp. II – positive; 2. *Salmonella* sp. II – positive; 3. *Vibrio* sp. I – positive; 4. *Aeromonas* sp. II – positive; 5. Control – uninoculated..
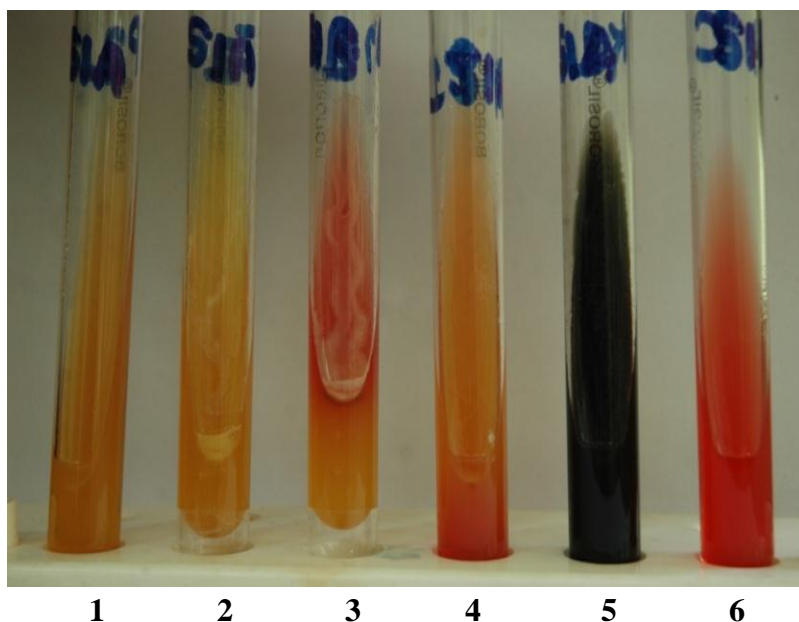
1　　　**2**　　　**3**　　　**4**　　　**5**　　　**6**

Fig. 13: Sorbitol iron agar test for selective isolates in Hi-Media's sorbitol iron agar medium (M299).

Legend: 1. *Pseudomonas* sp. II – positive; 2. *Vibrio* sp. II – positive; 3. *Lactobacillus* sp. II – positive; 4. *Vibrio* sp. I – positive; 5. *Aeromonas* sp. I – positive; 6. Control – uninoculated.
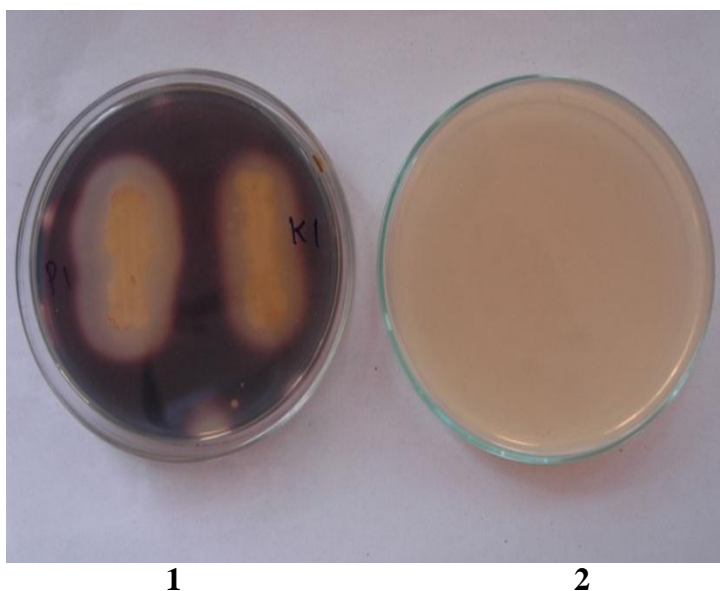

1　　　　　　　　　　　　**2**

Fig. 14: Amylase activity of selective isolates in Hi-Media's starch agar medium.

Legend: 1. *Pseudomonas* sp. I (left) and *Aeromonas* sp. I (right) produces a transparent clear halo zone around their growth; 2. Control – uninoculated.
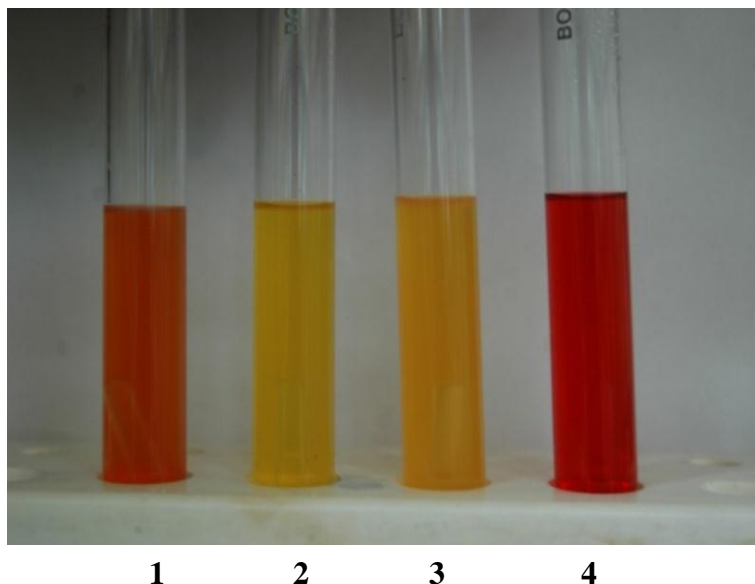
Fig. 15: Carbohydrate fermentation test for selective isolates in Hi-Media's phenol red broth base (M054) with dextrose.

Legend: 1. *Vibrio* sp. I – slightly positive; 2. *Pseudomonas* sp. II – positive; 3. *Lactobacillus* sp. I – positive; 4. Control – uninoculated (no colour change).
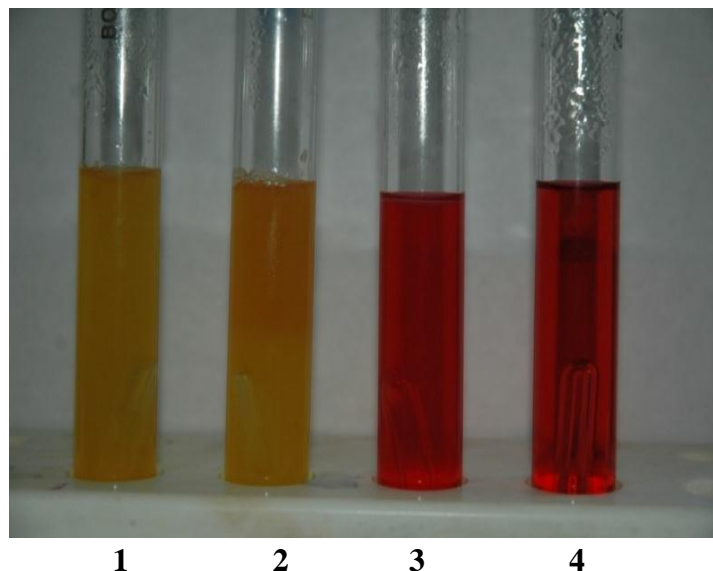

Fig. 16: Dulcitol fermentation test for selective isolates in Hi-Media's phenol red dulcitol broth (M617).
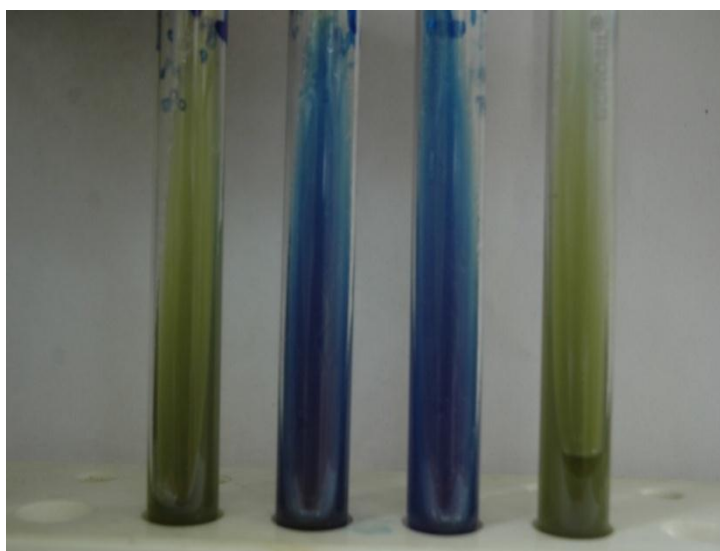
Legend: 1. *Aeromonas* sp. I – positive; 2. *Lactobacillus* sp. II – positive; 3. *Vibrio* sp. I – negative; 4. Control – uninoculated (no colour change).

1   2   3

Fig. 17: Indole production test for selective isolates in Hi-Media's tryptone broth (RM014).

Legend: 1. *Lactobacillus* sp. II – positive; 2. *Vibrio* sp. I – negative; 3. Control – uninoculated.

1   2   3   4

Fig. 18: Citrate utilization test for selective isolates in Hi-Media's Simmon's citrate agar medium (M099).

Legend: 1. *Vibrio* sp. I – negative; 2. *Pseudomonas* sp. II – positive; 3. *Lactobacillus* sp. I – positive; 4. Control – uninoculated (no colour change).

Fig. 19: Citrate utilization test for selective isolates in Hi-Media's Koser citrate broth (M069).

Legend: 1. *Salmonella* sp. I – positive; 2. *Vibrio* sp. I – negative; 3. *Pseudomonas* sp. I – positive; 4. *Aeromonas* sp. I – positive; 5. Control – uninoculated.

Fig. 20: Urealytic activity of selective isolates in Hi-Media's urea agar base (M112) with urea 40%.

Legend: 1. *Lactobacillus* sp. I – negative; 2. *Vibrio* sp. II – positive; 3. *Aeromonas* sp. II – positive; 4. Control – uninoculated (no colour change).
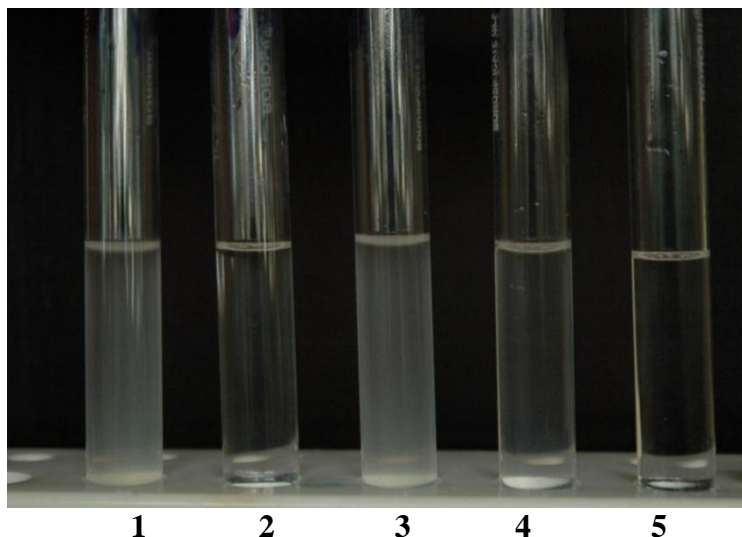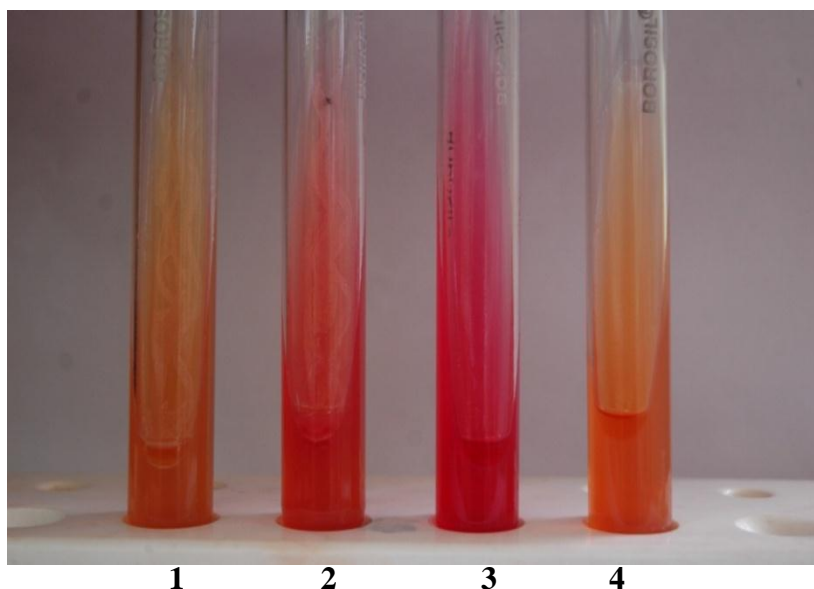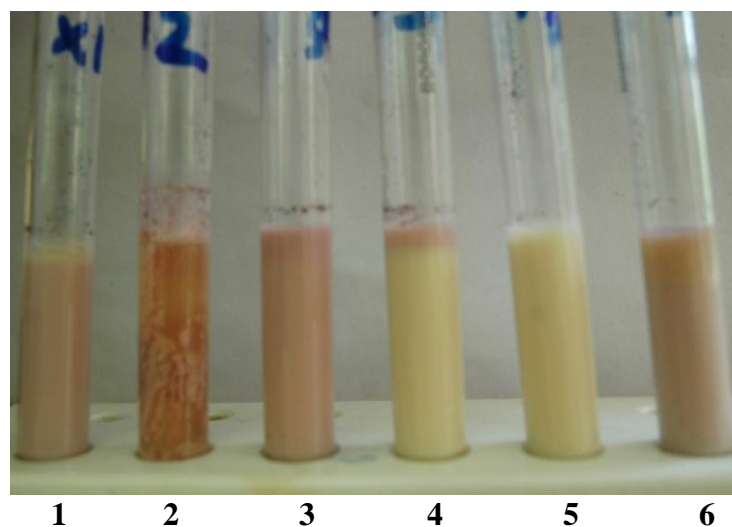
Fig. 21: Litmus milk reaction test for selective isolates in Hi-Media's litmus milk broth (M609).

Legend: 1. *Salmonella* sp. I – positive; 2. *Vibrio* sp. II – positive; 3. *Pseudomonas* sp. II – positive; 4. *Aeromonas* sp. II – positive; 5. *Lactobacillus* sp. II – positive; 6. Control – uninoculated.

Fig. 22: Nitrate reduction test for selective isolates in Hi-Media's nitrate broth (M439)

Legend: 1. *Salmonella* sp. I – positive; 2. *Vibrio* sp. I – positive; 3. *Aeromonas* sp. I – positive; 4. *Pseudomonas* sp. II – negative; 5. Control – uninoculated.
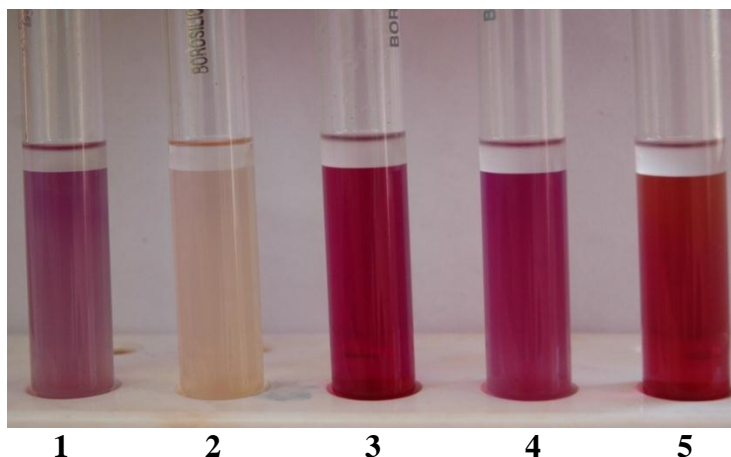
Fig. 23: Lysine decarboxylation test for selective isolates in Hi-Media's lysine decarboxylase broth (M376).

Legend: 1. *Salmonella* sp. I – positive; 2. *Pseudomonas* sp. I – positive; 3. *Vibrio* sp. I – negative; 4. *Lactobacillus* sp. II – positive; 5. Control – uninoculated (purple colour).

Fig. 24: Oxidative-fermentation test for selective isolates in Hi-Media's OF basal medium (M395) with sucrose.

Legend: 1. *Salmonella* sp. II – positive; 2. *Lactobacillus* sp. II – positive; 3. *Pseudomonas* sp. II – positive; 4. *Vibrio* sp. I – slightly positive; 5. Control – uninoculated (green colour).

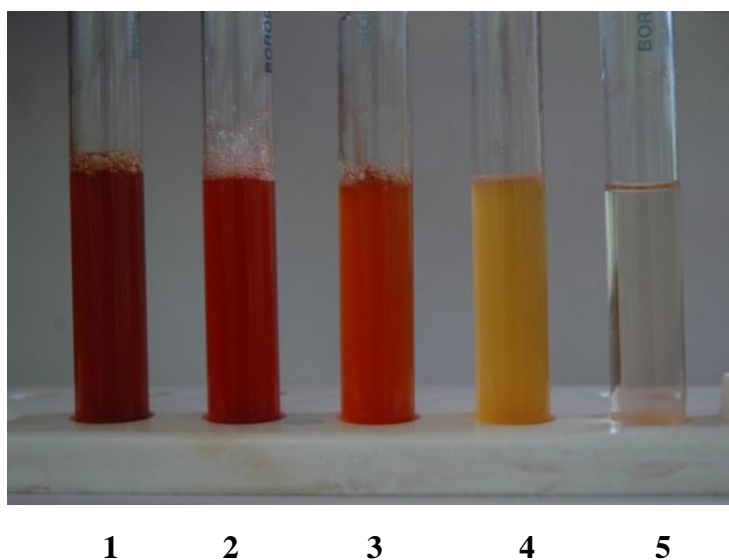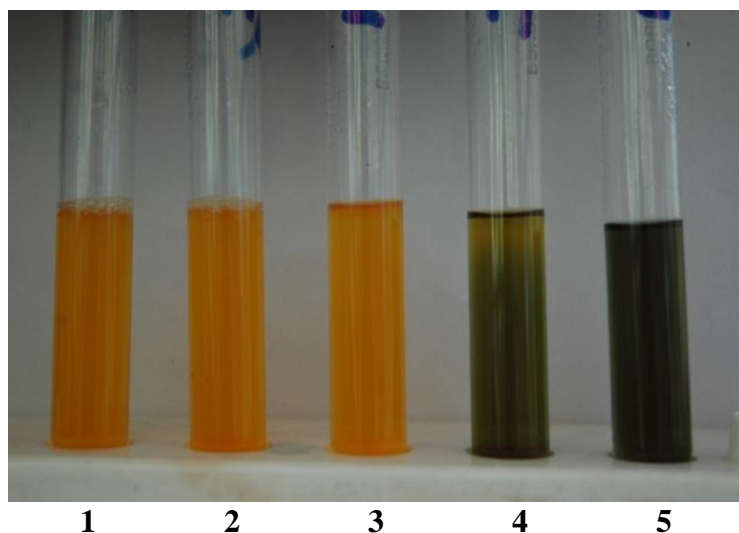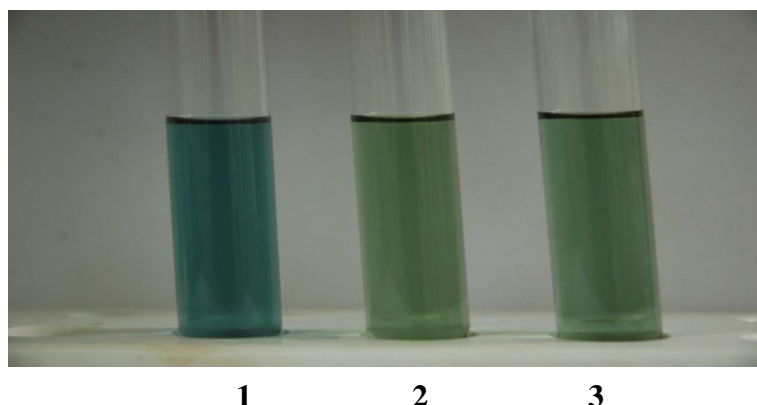**Fig. 25: Malonate utilization test for selective isolates in Hi-Media's malonate broth (M382).**

Legend: 1. *Salmonella* sp. II − positive; 2. *Vibrio* sp. I − negative; 3. Control − uninoculated (green colour).

Table 8: Interpretation of selective isolates for lysine iron agar slants

S.no.	Name of the selective isolates	Slant	Butt	Gas	H$_2$S	Inference
1.	*Salmonella* species I	Red orange	Red orange	-	-	Dextrose and lysine fermentation not occur
2.	*Salmonella* species II	Red orange	Red orange	-	-	Dextrose and lysine fermentation not occur
3.	*Vibrio* species I	Slight yellow	Slight yellow	-	-	Slightly dextrose and lysine fermentation can occur
4.	*Vibrio* species II	Red orange	Red orange	-	-	Dextrose and lysine fermentation not occur
5.	*Pseudomonas* species I	Yellow	Yellow	-	-	Dextrose and lysine fermentation can occur
6.	*Pseudomonas* species II	Yellow	Yellow	-	-	Dextrose and lysine fermentation can occur
7.	*Aeromonas* species I	Pink red	Yellow	+	-	Dextrose and lysine fermentation, H$_2$S gas production in the butt region can occur
8.	*Aeromonas* species II	Pink red	Black	-	-	Dextrose and lysine fermentation, H$_2$S gas production in the butt region can occur
9.	*Lactobacillus* species I	Red orange	Black	+	-	Dextrose and lysine fermentation not occur
10.	*Lactobacillus* species II	Red orange	Red orange	+	-	Dextrose and lysine fermentation not occur
11.	Control	Red orange	Red orange	-	-	Uninoculated

Legend: Gas produced (+); no gas and no H$_2$S gas produced (-).

Table 9: Interpretation of selective isolates for triple sugar iron agar slants

S.no.	Name of the selective isolates	Slant	Butt	Gas	H$_2$S	Inference
1.	*Salmonella* species I	Yellow	Yellow	+	-	Dextrose and lactose / sucrose fermentation can occur
2.	*Salmonella* species II	Yellow	Yellow	+	-	Dextrose and lactose / sucrose fermentation can occur
3.	*Vibrio* species I	Yellow	Orange red	-	-	Dextrose fermentation can occur
4.	*Vibrio* species II	Yellow	Yellow	(+)*	-	Dextrose and lactose / sucrose fermentation can occur
5.	*Pseudomonas* species I	Yellow	Yellow	++	-	Dextrose and lactose / sucrose fermentation can occur
6.	*Pseudomonas* species II	Yellow	Yellow	-	-	Dextrose and lactose / sucrose fermentation can occur
7.	*Aeromonas* species I	Black	Black	+	+	Dextrose and lactose / sucrose fermentation, abundant H$_2$S gas production can occur
8.	*Aeromonas* species II	Black	Black	-	+	Dextrose and lactose / sucrose fermentation, abundant H$_2$S gas production can occur
9.	*Lactobacillus* species I	Yellow	Yellow	++	-	Dextrose and lactose / sucrose fermentation can occur
10.	*Lactobacillus* species II	Yellow	Yellow	(+)*	-	Dextrose and lactose / sucrose fermentation can occur
11.	Control	Orange red	Orange red	-	-	Uninoculated

Legend: More gas produced (++); slightly gas produced (+); no gas and no H$_2$S gas produced (-).*

Table 10: Interpretation of selective isolates for Kligler iron agar slants

S.no.	Name of the selective isolates	Slant	Butt	Gas	H$_2$S	Inference
1.	*Salmonella* species I	Yellow	Yellow	++	-	Dextrose and lactose fermentation can occur
2.	*Salmonella* species II	Yellow	Yellow	++	-	Dextrose and lactose fermentation can occur
3.	*Vibrio* species I	Phenol red	Phenol red	-	-	Dextrose and lactose fermentation not occur
4.	*Vibrio* species II	Pink	Pink	-	-	Slightly dextrose and lactose fermentation can occur
5.	*Pseudomonas* species I	Yellow	Yellow	(+)*	-	Dextrose and lactose fermentation can occur
6.	*Pseudomonas* species II	Yellow	Yellow	-	-	Dextrose and lactose fermentation can occur
7.	*Aeromonas* species I	Black	Black	-	+	Dextrose and lactose

S.no.	Name of the selective isolates	Slant	Butt	Gas	H$_2$S	Inference
						fermentation, abundant H$_2$S gas production can occur
8.	*Aeromonas* species II	Black	Black	-	+	Dextrose and lactose fermentation, abundant H$_2$S gas production can occur
9.	*Lactobacillus* species I	Yellow	Yellow	++	-	Dextrose and lactose fermentation can occur
10.	*Lactobacillus* species II	Yellow	Yellow	++	-	Dextrose and lactose fermentation can occur
11.	Control	Phenol red	Phenol red	-	-	Uninoculated

Legend: More gas produced (++); slightly gas produced (+); no gas and no H$_2$S gas produced (-).*

Table 11: Interpretation of selective isolates for sorbitol iron agar slants

S.no.	Name of the selective isolates	Slant	Butt	Gas	H$_2$S	Inference
1.	*Salmonella* species I	Yellow	Yellow	++	-	Sorbitol fermentation can occur
2.	*Salmonella* species II	Yellow	Yellow	++	-	Sorbitol fermentation can occur
3.	*Vibrio* species I	Yellow	Red orange	-	-	Slightly sorbitol fermentation can occur
4.	*Vibrio* species II	Yellow	Yellow	++	-	Sorbitol fermentation can occur
5.	*Pseudomonas* species I	Yellow	Yellow	(+)*	-	Sorbitol fermentation can occur
6.	*Pseudomonas* species II	Yellow	Yellow	-	-	Sorbitol fermentation can occur
7.	*Aeromonas* species I	Black	Black	-	+	Sorbitol fermentation and abundant H$_2$S gas production can occur
8.	*Aeromonas* species II	Black	Black	-	+	Sorbitol fermentation and abundant H$_2$S gas production can occur
9.	*Lactobacillus* species I	Yellow	Yellow	++	-	Sorbitol fermentation can occur
10.	*Lactobacillus* species II	Red orange	Yellow	++	+	Slightly sorbitol fermentation and less H$_2$S gas production can occur
11.	Control	Red orange	Red orange	-	-	Uninoculated

Legend: More gas produced (++); slightly gas produced (+); no gas and no H$_2$S gas produced (-).*

Table 12: Morphological and biochemical characterization of selective isolates in gastro-intestinal tract of freshwater mrigal fish

S.no.	Name of the selective isolates	Gram staining	Shape of bacteria	Motility test	SIM test	LIA test	TSI test	KIA test	SIA test
1.	*Salmonella* species I	Gram negative	Rod shaped	+	+	-	+ GP	+ GP	+ GP
2.	*Salmonella* species II	Gram negative	Rod shaped	+	+	-	+ GP	+ GP	+ GP
3.	*Vibrio* species I	Gram negative	Comma shaped	+	-	(+)	+ NGP	- GP	+ NGP
4.	*Vibrio* species II	Gram negative	Comma shaped	+	+	-	+ SGP	+ GP	+ GP
5.	*Pseudomonas* species I	Gram negative	Rod shaped	+	+	+ GP	+ GP	+ SGP	+ GP
6.	*Pseudomonas* species II	Gram negative	Rod shaped	(+)	+	+ NGP	+ NGP	+ NGP	+ NGP
7.	*Aeromonas* species I	Gram negative	Rod shaped	+	+	+ H₂S GP	+ GP	+ H₂S GP	+ NGP
8.	*Aeromonas* species II	Gram negative	Rod shaped	+	+	+ H₂S GP	+ NGP	+ H₂S GP	+ NGP
9.	*Lactobacillus* species I	Gram positive	Rod shaped	+	+	-	+ GP	+ GP	+ GP
10.	*Lactobacillus* species II	Gram positive	Rod shaped	(+)	+	-	+ SGP	+ GP	+ GP

Legend: + - positive; - - negative; (+) – slightly positive; GP – gas produced; NGP – no gas produced; SGP – slightly gas produced; H₂S GP – Hydrogen sulphide gas produced.

Table 12: Continuation.

S.no.	Name of the selective isolates	Starch hydrolysis	Gelatin hydrolysis	Dextrose test	Lactose test	Sucrose test	Maltose test	Mannitol test	Dulcitol test
1.	*Salmonella* species I	+	+	+ GP	+ GP	+ GP	+ GP	+ GP	+ GP
2.	*Salmonella* species II	-	+	+ GP	+ GP	+ GP	+ GP	+ GP	+ GP
3.	*Vibrio* species I	+	(+)	(+) NGP	(+) NGP	(+) NGP	-	(+) NGP	-
4.	*Vibrio* species II	(+)	-	+ GP	+ NGP	+ GP	+ GP	+ GP	(+) NGP
5.	*Pseudomonas* species I	+	+	+ GP	(+) NGP	+ GP	+ GP	+ GP	(+) NGP
6.	*Pseudomonas* species II	+	(+)	+ NGP	+ NGP	+ NGP	+ NGP	+ NGP	-
7.	*Aeromonas* species I	+	+	+ GP	+ GP	+ GP	+ GP	+ GP	+ GP

8.	*Aeromonas* species II	+	+	+ GP	+ GP	+ GP	+ GP	+ GP	+ GP
9.	*Lactobacillus* species I	-	-	+ GP	+ GP	+ GP	+ GP	+ GP	+ GP
10.	*Lactobacillus* species II	-	-	+ GP	+ GP	+ GP	+ GP	+ GP	+ GP

Legend: + - positive; - - negative; (+) – slightly positive; GP – gas produced; NGP – no gas produced.

Table 12: Continuation.

S.no.	Name of the selective isolates	Indole test	Methyl red test	VP test	Citrate test	Koser broth	Urease test	Litmus milk test	Nitrate broth test
1.	*Salmonella* species I	+	+	+	+	+	(+) NGP	+	+
2.	*Salmonella* species II	+	+	+	+	+	-	+	+
3.	*Vibrio* species I	-	-	-	-	-	-	+	+
4.	*Vibrio* species II	+	+	(±)	+	+	+ NGP	+	+
5.	*Pseudomonas* species I	+	(+)	+	+	+	-	+	-
6.	*Pseudomonas* species II	+	+	+	+	(+)	-	+	-
7.	*Aeromonas* species I	+	+	+	+	+	+ NGP	+	+
8.	*Aeromonas* species II	+	(+)	+	+	+	+ NGP	+	+
9.	*Lactobacillus* species I	+	+	+	+	+	-	+	+
10.	*Lactobacillus* species II	+	+	-	+	+	-	+	+

Legend: + - positive; - - negative; (+) – slightly positive; (±) slightly positive or negative; NGP – no gas produced.

Table 12: Continuation.

S.no.	Name of the selective isolates	Lysine test	Ornithine test	Arginine test	Aspargine test	Malonate test	Bushnell Haas broth	Pigment production test	C.L.E.D. agar slant test
1.	*Salmonella* species I	+	+	+	+	+	+	White with yellow	+ NGP
2.	*Salmonella* species II	+	+	+	+	+	+	Light yellow	+ GP
3.	*Vibrio* species I	-	(+)	-	-	-	(+)	Yellow with white	+ NGP
4.	*Vibrio* species II	+	+	+	+	+	+	White	+ NGP
5.	*Pseudomonas* species I	+	+	+	+	-	+	No growth occur	+ NGP
6.	*Pseudomonas* species II	+	+	+	+	-	+	Sandal colour	+ GP
7.	*Aeromonas* species I	+	+	+	+	+	(+)	Light yellow	+ NGP
8.	*Aeromonas* species II	+	+	+	+	+	+	Light yellow	+ GP
9.	*Lactobacillus* species I	+	+	+	+	+	+	Yellow with white	+ NGP
10.	*Lactobacillus* species II	+	+	+	+	+	+	Yellow with white	+ NGP

Legend: + - positive; - - negative; (+) – slightly positive; GP – gas produced; NGP – no gas produced.

Isolation and identification of *Lactobacillus* species

The most commonly used probiont – *Lactobacillus* sp. was isolated from the intestine of the test mrigal fish with the help of *Lactobacillus* MRS agar media following the guidelines of the manufacturer. As mentioned above, the same protocol was followed for the confirmation of *Lactobacillus* sp. The results of *Lactobacillus* sp. for various tests were shown in Table 8-11. A total of two *Lactobacillus* sp. isolates were identified and they were labeled as LAC I and LAC II.

Screening of antagonistic activity of *Lactobacillus* species against common mrigal fish bacterial pathogens

Through cross streaking method, the antagonitstic activity of the isolated *Lactobacillus* sp. was tested. From the plate, it was very clear that *Lactobacillus* sp. has the potential to suppress the growth of *Aeromonas* sp. But no significant effect was noted against the other

tested pathogens. A similar action was found between the two isolates of *Lactobacillus* sp. over the tested pathogens (Figure 26).
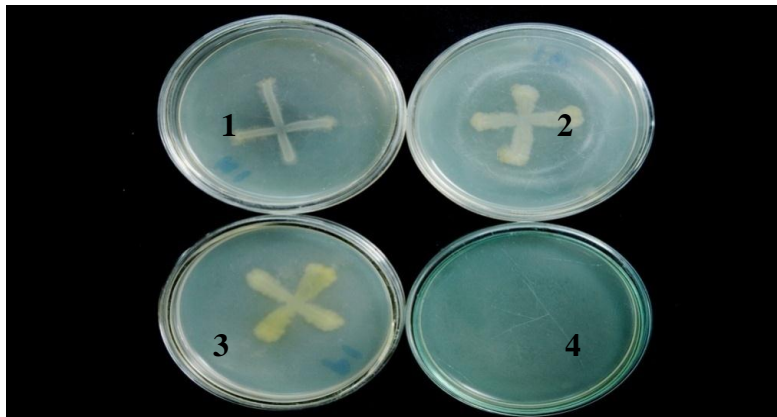

Fig. 26: Cross streak method for fish pathogens against *Lactobacillus* species I in Hi-Media's nutrient agar medium (M001).

Legend: 1. *Aeromonas* sp. I – retarted growth occur; 2. *Salmonella* sp. I and 3. *Pseudomonas* sp. I – inhibited growth occur; 4. Control – uninoculated.

Legend: 1. *Salmonella* sp. I and 2. *Vibrio* sp. II – inhibited growth occur; *Aeromonas* sp. II – retarted growth occur; 4. Control – uninoculated.

Determination of the influence of pH on bacteriocin activity

Bacteriocin production was tested in the two isolates of *Lactobacillus* sp. (LAC I and LAC II) using agar-well diffusion method and its activity was tested at different pH such as 7 and 8. All the bacterial fish pathogens were able to grow around the well loaded with supernatant obtained from *Lactobacillus* sp. It indicated the loss of activity of bacteriocin at pH 7 prepared from both of the test isolates. Zone formation against the test pathogens around the well loaded with supernatant set with pH 8 indicated that bacteriocin (in both isolates)

activity was possible at in both isolates at pH 8. Even though the pH 8 was found to be supportive for retaining the activity of bacteriocin, the activity of the bacteriocin was not found to be uniform against all the tested pathogens. Bacteriocin collected from two *Lactobacillus* sp. showed variation in its action against different pathgoens.   In LAC I, bacteriocin produced was found to be active at pH 8 against *Vibrio* sp. but not against *Aeromonas* sp., *Pseudomonas* sp. and *Salmonella* sp. *Salmonella* sp. was inhibited by the bacteriocin of *Lactobacillus* sp. LAC II. The same soup did not exhibit any effect over other pathogens. *Aeromonas* sp. and *Pseudomonas* sp. was not inhibited by the soup obtained from both the *Lactobacillus* sp. isolates even at pH 8.

Screening antibiotic resistance in mrigal fish bacterial flora

Strategy for prevention of disease outbreak in aquaculture system may be planned depending on several factors. Although management of health of fish under culture system is a matter of top most priority for an aqua culturist, the use of various chemicals and drugs are age old practices.   Most of the pathogens have been uncontrollable because of resistance development and its transfer. All the ten bacterial isolates including *Lactobacillus* sp. from the test mrigal fish through three cycles were screened against two antibiotics (Norfloxacin Nx^{10} and Co-Trimoxeazole Co^{25} – Hi-Media) to cheek antibiotic resistance and their status and the results were presented in Table 13 where is table for antibiotic resistance. Both the isolates of *Lactobacillus* sp. were found to be resistant to Co-Trimoxazole Co^{25} at the selected concentration (Figure 27 & 28).

Table 13: Antibacterial susceptibility tests of selective isolates in gastro-intestinal tract of freshwater mrigal fish

S.no.	Name of the selective isolates	Diameter of zone of inhibition in mm	
		Norfloxacin Nx^{10} (10mcg)	Co-Trimoxazole Co^{25} (5mcg)
1.	*Salmonella* species I	10 (R)	04 (R)
2.	*Salmonella* species II	16 (I)	05 (R)
3.	*Vibrio* species I	15 (I)	10 (R)
4.	*Vibrio* species II	10 (R)	08 (R)
5.	*Pseudomonas* species I	13 (I)	09 (R)
6.	*Pseudomonas* species II	12 (R)	09 (R)
7.	*Aeromonas* species I	14 (I)	07 (R)
8.	*Aeromonas* species II	15 (I)	08 (R)
9.	*Lactobacillus* species I	08 (S)	08 (R)
10.	*Lactobacillus* species II	20 (S)	07 (R)

Zone size interpretative chart indicates: 12 mm or less – resistant; 13-16 mm – intermediate;
17 mm or more – sensitive for Norfloxacin and 10 mm or less – resistant; 11-15 mm –
intermediate; 16 mm or more – sensitive for Co-Trimoxazole.

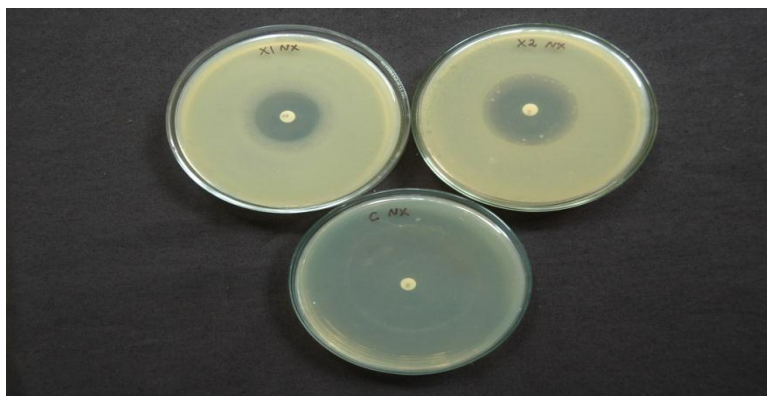*Legend: R – resistant; I – intermediate; S – sensitive; mm – millimeter.*

Fig. 27: Antibacterial activity of *Salmonella* sp. against Norfloxacin Nx[10] (10mcg).
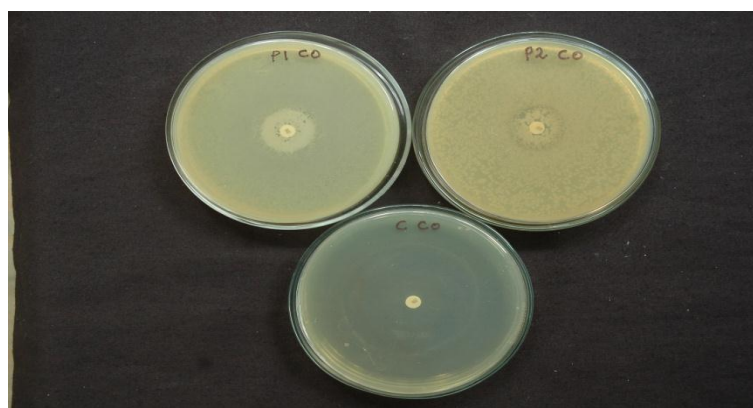Legend: *Salmonella* sp. I (top left); *Salmonella* sp. II (top right); Control (bottom).

Fig. 28: Antibacterial activity of *Pseudomonas* sp. against Co-Trimoxazole Co[25] (5mcg).
Legend: *Pseudomonas* sp. I (top left); *Pseudomonas* sp. II (top right); Control (bottom).

DISCUSSION

Atlas (1984)[71] reported the total heterotrophic bacterial population (THBP) is a group of organisms which require performed organic matter as a source of carbon. The total heterotrophic bacterial population always dominates the other groups in the natural environments. The total heterotrophic bacterial population includes both the beneficial and pathogenic bacteria. The total heterotrophic bacterial population in the intestinal region of the selected mrigal fish was in the range of 20×10^4 to 28×10^4 CFU/g. The total heterotrophic bacterial population was analyzed in the gut of *Labeo rohita* (Hamilton) and *Channa*

punctatus (Bloch) by Kar and Ghosh (2008)[53] and they also reported that bacterial load in the intestine is diet dependent, fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria and being rich in nutrient, the environment of the digestive tract of fish confers a favorable culture environment for the microorganisms.

Constructive role of *Lactobacillus* sp. in the intestinal tract is confirmed by many findings (Lenzner, 1973; Mitsuoka, 1992; McCartney *et al*., 1996 and Tannock, 1998).[72,73,74,75] The load of *Lactobacillus* sp. in the intestinal region was found in the range between 15×10^4 to 20×10^4. Major occurrence of lactic acid bacteria in the animal intestine has been reported by Perdigon *et al.* (1995)[35] and Salminen and Wright (1998a).[37] It is well established that some lactic acid bacteria improve the intestinal micro flora and promote the growth and health of animals observed by Mitsuoka (1990)[36] and Perdigon *et al*. (1995).[35] The intestinal micro flora, especially lactic acid bacteria, may influence the growth and health of fish. However, few studies explaining the concentration of lactic acid bacteria in the intestine of fish are meager (Kar and Ghosh, 2008).[53]

Endogenous digestive enzymes in fish have been studied by several workers (Dhage, 1968; Kawai and Ikeda, 1972; Das and Tripathi, 1991).[6,7,8] However, information regarding the enzyme producing intestinal bacteria, their source and significance in fish is scarce (Kar and Ghosh, 2008).[53] In the present investigation, quantitative analysis of bacteria producing amylase, protease and cellulase was carried out. From the data, it was understood that the proteolytic bacterial load was found to be dominant compared to the other two types of bacteria such as amylase producers and cellulase producers. Since it is an omnivorous fish, the more occurrences of all three types of enzyme producers were possible. Presence of proteolytic, cellulolytic and amylolytic bacteria in the gut of rohu suggests an omnivorous feeding aptitude of the fish as has been studied by Creach (1963)[55] and Ghosh *et al.* (2002).[10] The relationship between the feeding habit and nature of bacterial presents has been explained by Kar and Ghosh (2008).[53] The maximum density of proteolytic bacteria was detected by *Channa punctatus* by Kar and Ghosh (2008).[53] Thus, the occurrence of proteolytic bacteria in the gut of *Cirrhinus cirrhosus* in high density also seems to support the presence of diet dependent microbial population indicating their feeding towards animal matter. The presence of considerable quantity of amylolytic 9×10^5 to 12×10^5 CFU/g and cellulolytic bacteria stood as the evidence for its colonization potential and their high

intensity may suggests that supplementation of amylase and cellulase sense as the basis for the symbiotic (mutual) relationship between the bacterial flora and the fish species. The reports on microbial amylase activity in fish gut are scanty. The current work explained the quantity of amylase producers and its relationship with other enzymes. The presence of considerable cellulolytic bacterial population has been observed in fish digestive tracts in the present investigation. Such abundance of cellulolytic bacteria has gained further support from the reports made by Das and Tripathi (1991)[8] in carp, Saha and Ray (1998)[76] and Ghosh *et al*. (2002)[10] in rohu fingerlings.

Distribution of cellulotytic bacteria in omnivore's fish such as mrigal is supported by Stickney (1975)[77] who looked at cellulase activity in a number of freshwater species and concluded that herbivores are unlikely to have the enzyme, but omnivores and carnivores may pick it up from invertebrates that harbor the bacteria producing the enzyme. The current study explained that the bacteria present within the gut of mrigal fish were capable of producing various extracellular enzymes. The information generated from the present investigation might contribute to the incorporation of these bacteria in commercial aquaculture as a probiotic and in the formulation of feed. However, further research has to be conducted to evaluate the ability of the bacteria to produce different enzymes while feeding with several kinds of feed.

Generic analysis studies among the isolates derived from the intestinal tract of the mrigal fish indicated the presence of both commensally and pathogenic (*Aeromonas* sp., *Vibrio* sp., *Pseudomonas* sp. and *Salmonella* sp.) and beneficial bacteria (*Lactobacillus* sp.). Results on bacterial analysis coincided with the findings of McCarthy and Roberts, (1980)[78] and several workers have observed bacterial load in different organs of fishes from various sources (Okuzumi and Hories, 1969; Rahim *et al*., 1984; Balasubramanian *et al*., 1992).[79,80,81] Bacterial nature in kidney, liver and spleen of several fishes have been reported (Nieto *et al.,* 1984; Lindsay, 1986, Cahill, 1990; d Sousa, 1996).[82,83,84,85]

Generic composition of bacteria in fresh water fishes has been studied by Rajeswari Shome and Shome (1999).[70] Detailed information about the microbial load and types of bacteria in the internal organs of apparently healthy fish is essential in order to recognize and correct the abnormal conditions (such as those attributed to adverse water and food quality factors or unfavorable management aspects) which can be a prelude to the appearance of disease.

In addition to that the indigenous micro flora of fish in aquaculture has previously been studied for many other reasons (Joseph *et al*., 1988; Horsley, 1973; Allen *et al*., 1983; Moriarty, 1990; Rajeswari Shome and Shome, 1999; Cahill, 1990; Toranzo *et al*., 1993; Santos *et al*., 1991; Toranzo *et al*., 1992).[86,87,88,89,70,84,90,91,92] In the current study, Gram-negative bacteria pathogens were isolated and identified as *Aeromonas* sp., *Vibrio* sp., *Pseudomonas* sp. and *Salmonella* sp. *Aeromonas hydrophila* is recognized as a scourge of freshwater fish farming worldwide and considered to be a major economic problem. It is the causative agent of hemorrhagic septicemia and Epizootic Ulcerative Syndrome (EUS) of freshwater fishes of all Asian countries (Haley *et al*., 1967).[93] *Aeromonas* sp. also shoots troubles at humans and for lower vertebrates, including amphibians and reptiles (Janda and Abbott, 1998).[94] Ishiguro and Trust (1980)[95] reported that in humans, it leads to diarrhea.

In the current work presence of *Aeromonas* sp., *Vibrio* sp., *Salmonella* sp. and *Pseudomonas* sp. have been traced. Along with commensally and pathogenic bacteria, *Lactobacillus* sp. was also identified and its probiotic candidature was tested against the isolated pathogens using cross streak method. The probiotic effect of *Lactobalillus* sp. was proved against the most potent pathogen – *Aeromonas* sp. through cross streaking method and also found ineffective against other pathogens. Existence of *Lactobacillus* species in fish intestine (Ringo *et al*., 1995; Ringo and Gatesoupe, 1998; Gonzalez *et al*., 2000)[22,21,23] and its probiotic role are well known from various literatures. The inhibitory activity of *Lactobacillus* sp. over *Aeromonas* sp. may be due to the release of organic acids (Sorrells and Speck, 1970),[96] hydrogen peroxide (Wheater *et al*., 1952; Price and Lee, 1970 and Gilliland and Speck, 1977)[97,98,51] or through the destruction of basic molecular structures of nucleic acid and cell proteins (Dahl *et al*., 1989).[99]

Through the present investigation, the efficacy of bacteriocin was screened at two different pH (pH 7 and pH 8) and the bacteriocin was found active against pathogens at pH 8. The response of the bacteriocin of two *Lactobacillus* sp. was not uniform; it could be understood from the different inhibitory role of the two isolates.

Health promoting role of *Lactobacillus* sp. through bacteriocin production by compacting *Helicobacter pylori*, *Escherichia coli* and *Salmonella* sp. have been documented by Luc De Vuyst and Frederic Leroy (2007).[100] Arrest of bacterial pathogens by bacteriocin produced from *Lactobcillus* sp. explaining the promising role of *Lactobacillus* sp. in probiotic technology which has been an indispensable tool in sustainable aquaculture. Disease

outbreaks are being increasingly recognized as a significant constraint on aquaculture production and trade, affecting the economic development in many countries (Verschuere *et al*., 2000).[101]

The massive use of antimicrobials for disease control and growth promotion in animals increase the selective pressure exerted on the microbial world and encourages the natural emergence of bacterial resistance. Most important aspect of the present study was the detection of very high rate of natural resistance to different antibiotics in *Aeromonas hydrophila.* Antibiotic resistance has been shown to occur more frequently in bacterial species. For example, *Pseudomonas* sp., *Escherichia coli* and *Aeromonas hydrophila* (Jones *et al*., 1986 and Rajeshwari Shome and Shome, 1999).[68,70]

In the present investigation deals with *Lactobacillus* sp. was found to be sensitive to the selected antibiotics. It reveals the possibility of elimination of friendly strain while taking the checked antibiotics for the related problems. The disappearance of *Lactobacillus* sp. from the gut will not only hamper the health of the individual but, also increase the risk of infection from pathogens of water and food-borne. *Salmonella* sp., *Vibrio* sp. and *Pseudomonas* sp. were found to be fully resistant against Co-Trimaxazole (Co^{25}). 50% in the total strains was found to be resistant against Norfloxation (Nx^{10}) and rest of the strains was found to be intermediate to the selected antibiotics. From the previous study, antibiotic resistance has been shown to occur more frequently in bacterial species such as *Escherichia coli* and *Aeromonas* sp. (Jones *et al*., 1986; Rajeshwari Shome and Shome, 1999).[68,70] The same response against the selected antibiotics was found in the most potent agent of EUS in freshwater fishes - *Aeromonas* sp. Resistant development in *Aeromonas hydrophila* against the commonly described antibiotics such as ampicillin, erythromycin, gentamycin, streptomycin, tetracycline and trimethoprim have been reported previously by Rahim *et al*., (1984); Pathak *et al*., (1993) ; Rajeshwari Shome and Shome, (1999).[80,102,70] The current results distinctly indicating the emergence of multidrug resistance in common fish pathogens such as *Aeromonas* sp., *Vibrio* sp., *Pseudomonas* sp. and *Salmonella* sp.

From the previous report, multidrug resistance development may be because of the fact that bacteria in common environment are likely to exchange genetic material through plasmid exchange. The mechanism of resistance is a complete event, more or less evenly distributed in bacterial population in an environment (Rajeshwari Shome and Shome, 1999).[70] The highlight of the present study was the very high rate of natural resistance to different

antibiotics in *Aeromonas hydrophila* population associated with mrigal fish. The impending danger lies with human health as these resistance genes may find their way to humans through aquatic food chain and other ways. Further antibiotics, may themselves protect DNA from degradation and their presence may even enhance DNA uptake by bacteria (Webb and Davies, 1994).[103] This amply supports the need of alternative eco-friendly technique such as probiotics in aquaculture.

CONCLUSION

From the present investigation, finally concluded that two lactic acid bacteria (LAB) isolates from gastro-intestinal tract of mrigal fish, capable of producing good amount of bacteriocins and have been anticipated to have enormous potential for food applications as bio-preservatives. Bacteriocins produced by lactic acid bacteria have the potential to cover a very broad field of application, including both the food industry and the medical sector. With respect to medical applications, antimicrobials produced by probiotic lactic acid bacteria might play a role during *in vivo* interactions occurring in the fish gastro-intestinal tract, hence contributing for gut health.

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