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Assemblage and characterization of Lactobacillus associated with gut of freshwater cultivable fishes of South India

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Abstract: **The gram-positive and non-spore forming gut associated bacterium of *Lactobacillus* in freshwater cultivable fishes were investigated to assess the colonization pattern and characterization using standard microbiological procedures. We analyzed the bacterial diversity in the gastrointestinal tract of *Catla catla, Labeo rohita, Cirrhinus mrigala,* and *Cyprinus carpio*. The results show that the high diversity of bacteria were present and scarcely observed the bacterium *Lactobacillus*. The isolates LrMK2 and CmMK4 of *Lactobacillus* were selected and their morphological, biochemical and molecular characterization were identified. Further, it was selected for the evaluation of probiotic properties concludes that selective isolates LrMK2-*Lactobacillus plantarum* and CmMK4 – *Lactobacillus equicursoris*, which is potentially probiotics. The promising potential probiotic candidates were identified by 16S rRNA sequence and it was edited, aligned and submitted to NCBI**

Keywords: **Intestinal bacteria, Inland fishes, *Lactobacillus sp.*, 16S rRNA profiling, Phylogenetic study.**

1. INTRODUCTION

In dam ecosystem, edible fishes were grown in natural habitats and it was attained maximum size, without artificial feeding [1]. The edible fishes were easily affected by a diseasing causing agent; however, this disease was recovered naturally, because of some beneficial microbes present in the gut of fishes, which activate against disease-causing microbes [2]. The fish intestinal tract contains the digestive sauce, which confers a favorable culture environment of the microorganisms. Fish receives microorganisms in the intestinal tract from the aquatic environment through water and food. Intestinal tract entering microorganisms plays a symbiotic relationship with the host and crucial roles as beneficial forms of fishes. The beneficial microorganisms are usually referred to as probiotics, which can able to colonize and multiply in the gut of fishes.

Previous studies of freshwater fish intestinal bacteria concludes probiotic effects on intestinal isolates, it may also be expected from probiotics such as competition with pathogens for adhesion sites [3], enzymatic activities involved in digestion [4], antiviral effects [5] and trigger the fish immune response [6]. Different type of bacterial isolates are well known once of the beneficial bacteria are capable of inhibiting fish pathogens in the in-vivo study. These were confirmed for *Bacillus sp*, [7], *Lactobacillus sp*. [8],[9], of the bacteria colonizing the intestine, are lactic acid bacteria (LAB) generally considered as favorable microbes due to their abilities to stimulating host intestine development, digestive function, mucosal tolerance, stimulating an immune response, and improved disease resistance [10]. Yasuds and Taga [11] suggested that probiotic bacteria would be useful not only as feed supplements but also as biological controllers of fish disease and activators of nutrient regeneration. Muthukumar and Kandeepan [12] noted that the actions of intestinal flora resulting in vital benefits, including protection against pathogens and development of the immune system.

Presences of LABs and other allied microbes that colonize the intestine of fish are able to evoke immune responses and impart protection against diseases [13], an important role in the digestive process and growth [14]. Bucio et al., [15] have described the highest content of *Lactobacillus sp* from the intestine of *Cyprinus carpio* have fast growth and immune response against pathogens. In the natural presence of lactic acid bacteria in freshwater fish may be of great interest in the growth of dam fishes. Biochemical studies of microbes and potentiality and molecular studies have been studied also several authors [7], [16], [17]. This suggestion is clear that bacterial species present in the intestinal region make influence the health of host animals.

In this sense, we understanding naturally occurring bacteria are successfully isolated from the intestinal tract of fishes, and find out beneficial characteristics against fish intestinal pathogens. Isolation process to be a concern, the content of the different media for isolating fish bacteria; this seems particularly true for the bacteria of the digestive tract. Biochemical and physiological tests are essential tools for identification of bacterial genera and species, immune responses, Auto-aggregation, Antagonistic activity and adhere to the mucus can provide to understanding the potentiality of microbes and also the current 16S rRNA sequencing platforms and bio-informatics tools enable the research on the diversity of intracellular bacteria and phylogenetic studies revealed to understanding the evolutionary relationship of species.

2. MATERIALS AND METHODS

2.1 Fish sample collection and plating

Fishes of *Catla catla, Labeo rohita, Cirrhinus mirigala,* and *Cyprinus carpio* were caught from Aaliyaru dam and Thirumoorthi dam, located near Pollachi, Coimbatore District, Tamil Nadu, India, with the help of cast net. The alive collected fishes were transported to the laboratory in polythene bags containing oxygenated water. In the laboratory, fishes were aseptically dissected and removed the digestive tract; one gram digestive tract was grind well. One ml of grained digestive sauce was thoroughly mixed then serially diluted and incubated at 37^{o}C for 24 hours and find out the dominant colony. In this study totally 24 (triplicate) intestinal samples were collected and *Lactobacillus sp* were analyzed.

2.2 Isolation and biochemical analysis of Lactobacillus species

Well grown, prevalent bacterial isolates and morphologically different colonies were picked at random from the plates and re-streaked to ensure purity and maintained on MRS agar slants. To identify purified isolates to genus level by basic biochemical were performed following the criteria described in the Practical Microbiology, [18]. Duplicate representatives of each colony type were then streaked on corresponding plates repeatedly until pure cultures were obtained. Specifically, two of them (Isolates LrMK2 and CmMK4) were selected for this study and remaining isolates were purified and inoculated onto Luria Bertani Broth and kept at -4°C for stock; these were re-subculture on slants every 2 weeks.

2.3 Enzymatic activity of Isolates

The enzyme of amylase, protease and lipase activity were detected by the culture was inoculated into appropriate media and observed the zone of clearance. Overnight cultures of isolates were inoculated on the separate starch agar plates and incubate at 37^{o}C for 12-24 hours after flooded with Gram's iodine and allowed to stand for 5 minutes and visualized the clear zone shows positive results. For protease activity isolates were inoculated on skim milk agar plates and lipase activity isolates were inoculated on Tributyrin agar plates, both of them were incubated 37^{o}C for 12-24 hours and visualized the clear zone around the isolates are capable of producing protease and lipase enzymes [18].

2.4 Genomic DNA Extraction, 16S rRNA Amplification, Identification and NCBI submission and construction of Phylogenetic tree:

The genomic DNA was extracted from the promising two potential isolates using C-TAB method, a protocol modified from the method described by Ausubel et al., [19]. The DNA sample was separated according to their molecular weight under electrophoresis system. Finally, the DNA band was visualized under Gel Documentation system (Lark, Germany). The DNA concentration was determined by measuring the absorbance at the ration 260/280 nm and the DNA suspension was stored until it was used for PCR and further analysis. 16S rRNA was amplified by the following condition using eubacterial primers 27F forward primer (5'-AGA GTT TGA TCM TGG CTC AG - 3') and 1492R reverse primer (5'-AAG GAG GTG ATC CAN CCR CA - 3'). The final PCR products were purified by gel elution (Qiagen) and sequenced (Macrogen, Korea), obtained sequences were analyzed by NCBI blast tool and chimera was checked and submitted to

NCBI Gen Bank database and has been assigned the accession numbers. The resulted alignment revealed the identities and the score value of the sequence similarity search. This aligned sequence was a maximum likely hood phylogenetic similarity tree was constructed with the Mega X software.

2.5 Bio-safety assay

Healthy *Labeo rohita* and *Cyprinus carpio* fingerlings of the average weight of 8 – 12 g were acclimatized and used for experimental groups. Well grown isolated strains LrMK2 and CmMK4 were centrifuged (8000 rpm for 15 min) after the pellets were suspended in sterile saline (0.8% NaCl). Experimental fishes were injected intraperitoneal with 100µl of bacterial suspension (Approximately 10^9 CFU/ml), whereas the control groups were injected with sterile saline [20]. Fishes in each group were fed at 5% of the body weight per day for a period of 21 days of study. Treatment fishes swimming behaviour, Pathological symptoms like loss of scales and mucus, lesions, haemorrhage and edema, mortality rate were monitored every day for three-week study.

2.6 Congo red binding assay

The Congo red agar plate was prepared by adding 50 g of sucrose and 0.8 g of Congo red, both of which had been previously autoclaved separately into Brain heart infusion agar medium. The Congo red agar plate was inoculated with the isolates from an overnight culture plate and incubated at 37^{o}C for 24 – 48 hours. Positive results were indicated by block colonies with a dry crystalline consistency and isolate develop red or pink colonies indicated as negative results [21].

2.7 Probiotic potential test of isolates-In-vitro

2.7.1 pH tolerance test: pH tolerance of selected bacterium was investigated at different pH: 2- 9 were prepared using HCl 1% and NaOH 1 N and divided into 25 ml bottles [22]. The broth media along with control bottles were autoclaved at 121°C for 15 min soon after cooling and then inoculated with an overnight culture (30µl) of the selected strain in MRS broth followed by incubation at 37°C. Optical density (OD) as a growth rate of bacteria was measured by spectrophotometer at 600 nm after 2, 4 and 8-hour incubation. The viability of the isolates was also controlled by duplicate inoculation on MRS agar [23], [24].

2.7.2 Bile tolerance test: Bile salt tolerance was further tested on LrMK2 and CmMK4 in MRS broth which included 0.0, 0.15 and 0.3 % (w/v) Oxgall bile salt. Triplicate bottles of MRS broth containing filtered different concentrations of bile salt were inoculated by 30µl of cultured strains and incubated at 37^{o}C. The growth rate was assessed by measuring the optical density by spectrophotometer at 600 nm after 2, 4 and 8 hours incubation (Balcazar et al., 2008; Kim and Austin, 2008)

2.7.3 Growth at different NaCl concentration: The Growth rate of the bacterial strain at different NaCl concentration was determined in MRS broth by adding 0, 1, 2, 3 and 4% NaCl. The duplicate conical flask (25 ml Medium) containing different levels of NaCl were inoculated with 30µl of an overnight MRS broth culture and incubated at 37^o C. OD was measured at 600nm 24 h of incubation as described by Balcazer et al., [23], and Kim & Austin [24].

2.7.4 Temperature tolerance test: Growth of LrMK2 and CmMK4 bacterial isolates was evaluated at different temperature 10^o C - 50^o C. 30µl of an overnight MRS broth culture was transferred to duplicate MRS broth conical flasks and incubated at a different temperature. Optical density was measured at 600 nm after 0, 2, 4, 8, 16 and 24 h of incubation according to Balcazer et al., [23], and Kim & Austin [24].

2.8 Antibacterial activity of bacterial strain

Disc-diffusion method was employed for the determination of the antibacterial activity of selected two bacterial strains (LrMK2 and CmMK4) against three pathogenic bacteria. The freshwater fish pathogens, *Staphylococcus aureus* (KY123795), *Aeromonas veronii* (KY123797) and *Aeromonas hydrophila* (KY123799) (Previously isolated by *Cirrhinus mrigala* gut and identified the 16s rRNA sequence by Macrogen –Korea) were used to determine the antibacterial effect of the selective bacterial strain by disc diffusion techniques. The pathogenic bacteria were cultured in TSB and incubated at 37°C for 24 h. Thereafter, 50 µl of the cultures with 10^3 CFU/ml was spread on TSA by the swab. At the same time, the selected strains were cultured in MRS broth at 37°C for 24 h. The bacterium cells were harvested by centrifugation at 8000 rpm and 4°C for 10 min and their supernatants were used for an antibacterial test using disc diffusion methods [25].

2.9 Antibiotic sensitivity test by disc diffusion method

An antibiotic sensitivity test was carried out for the selected two bacterial strains on the common antibiotics by disc diffusion technique [26]. They included Methicillin: 5µg/disc, Chloramphenicol: 10µg/disc, Penicillin: 2µg/disc, Vancomycin: 5µg/disc, Ampicillin: 2µg/disc, Erythromycin: 5µg/disc, Gentamicin: 10µg/disc, Streptomycin: 5µg/disc and Kanamycin: 5µg/disc. 50 µl of the 24 h broth culture of the strain was spread on MRS agar and antibiotic Bio-discs were subsequently placed on plates. Finally, the plates were incubated at 37°C for 24 h to observe and measure the inhibition zone. The interpretations of the inhibition zone were determined according to the zone size of the chart of Kirby-Bauer test results [27].

2.10 Microbial adherence to hydrocarbon (MATH)

MATH was performed as described by Klayraung et al.,[28] with modifications. Solvents used in this study were Xylene (apolar), Chloroform (monopolar, Lewis –acid) and Ethyl acetate (monopolar, Lewis - base). Isolated strains were grown in broth at 37^{o}C for 24 hours, after centrifuging at 5000 rpm for 15 minutes, then the pellets were washed twice with PBS (pH 7.0) and OD600 nm of the bacterial sample was measured (A1). Respective solvents (1 ml) were added separately to every 1 ml of cell suspension, incubated at 37^{o}C for 30 min and OD600 nm measured against a solvent-extracted PBS blank (A2). Percentage of adhesion (% Adhs) was expressed as

$$\% \text{ of Adhs} = [(\text{A1 value at OD600} - \text{A2 value at OD600})/ \text{A1 value at OD600}] \times 100$$

2.11 Autoaggregation Assay

Auto-aggregation assay was measured according to Del Re et al., [29] and Kos et al., [30] with certain modifications. The cells of overnight grown broth cultures of isolates LrMK2 and CmMK4 were centrifuge at 6000 rpm for 15 minutes, and the pellets were washed and re-suspended in PBS (pH 7.0) to adjust an OD600nm, 0.25±0.05 [31], to give viable counts of approximately 10^{8} CFU/ml. 4 ml of the cell suspension were mixed by vortexing for 10 Sec, and incubated at room temperature for different times (0h, 2h, 4h, 6h, 8h, 10h). End of each hour interval, 100µl of the upper suspension was transferred to another tube with 3.9 ml of PBS and the absorbance (A) was measured at OD 600 nm. The autoaggregation percentage was calculated according to the equation:

$$A\% = (A0 - At) / A0 \times 100.$$

Where at represents the optical density at time **t**=2, 4, 6, 8 and 10 hours and **A0** represents the absorbance at t=0.

2.12 Coaggregation Assay in vitro

Co-aggregation assay was performed according to Jena et al. [32] with certain alterations. The bacterial pathogens used in this study were *Staphylococcus aureus*, *Aeromonas veronii*, and *Aeromonas hydrophila*. Equal volumes (1: 1) of the isolates suspension (Probiotics) and pathogenic isolate suspension were thoroughly mixed together using vortex for 10 s. The absorbance (A) at OD600 nm of the suspension was measured after 0h, 2h, 4h, 6h, 8h, and 10h of incubation at room temperature [30]. The Coaggregation percentage is measured using the equation:

$$CA\% = (A0 - Amix/A0) \times 100,$$

Where Amix represents the absorbance at time t = 2, 4, 6, 8, and 10 hours; A0 represents the absorbance at t=0.

2.13 Hemolytic Activity of isolates

The hemolytic activity of the isolates with promising anti-pathogenic activity was tested using blood agar plates, containing erythrocyte suspension of C.catla [33]. Overnight grown MRS broth culture of the isolates were a spot done on the blood agar plates (pH 7.2) and incubated for 48 hours at 37^{o}C; thereafter, the plates were observed for the formation of any clean (β-hemolysis) or green-hued (α-hemolysis) hemolytic zones, or no zone (γ-hemolysis) around the isolated colonies.

2.14 Statistical analyses

In this study, all the experiments were performed in triplicates and the results were analyzed to one-way analysis of variance (ANOVA). Data were analyzed by SPSS for windows version 15.0 software. The P values less than 0.05 were considered to be statistically significant.

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3. RESULTS

3.1. Identification of Lactobacillus species

Present study, bacterial compositions of the intestinal tract of fishes in Aaliyar dam and Thirumoorthi dam, mostly similar type of bacteria were obtained. A total number of 55 bacterial strains were selected from MRS agar plates, among them, two distinct colonies (Isolates LrMK2 and CmMK4) were selected based on morphological and biochemical characterization (TABLE. 1). Selective isolates were Gram-positive, Catalase negative, Oxidase positive and CO_2 fermentation positive rod-shaped bacteria occurring predominantly.

TABLE 1: Morphological and Biochemical Characterization of freshwater fish intestinal Isolates

Characteristics	LrMK2	CmMK4	Characteristics	LrMK2	CmMK4
Size	Long rod	Curved rod	**CO_2 Fermentation Glucose**	+, A	+,A
Shape	Round end	Irregular	**Fructose**	+, A	+,A
Colour	off white	White	**Sucrose**	+, A, G	+,A, G
Texture	Smooth	smooth	**Mannitol**	+,A, G	+,A, G
Elevation	raised	Convex	**H_2S Production**	-	-
Margin	entire	entire	**Nitrate Reduction**	+	+
Opacity	Opaque	translucent	**Starch Hydrolysis**	+	+
Growth at	15°C to 45°C	15°C to 45°C	**Casein Hydrolysis**	+	+
Gram's test	+ve	+ve	**Gelatin Hydrolysis**	-	+
Motility	Non-motile	Non-motile	**Urease**	-	-
Spore forming	Non –spore	Non –spore	**TSI**	ACS/ACB	ACS/ACB
Catalase test	-	-	**Arabinose**	+, A	+, A
Oxidase test	-	+	**Cellobiose**	+, A, G	+,A, G
Indole Production	-	-	**Lactose**	+,A	+, A
Methyl Red Test	-	+	**Maltose**	+, A, G	+, A, G
V P Reaction	-	-	**Rhamnose**	-, A	+(w)
Citrate utilization	-	-	**Hemolysis**	γ- hemolysis	γ- hemolysis

3.2. Enzyme activity of isolates

Enzyme production rates of amylase, protease and lipase activity was determined for isolates LrMK2 and CmMK4 are shown in TABLE. 2. The two isolates showed the positive results were obtained from the enzymes amylase production and it was observed by the zone of clearance around the colony when treated with iodine solution. Isolate LrMK2 maximum amylase production with an average diameter of 1.8 mm, whereas isolate CmMK4 was amylase production diameter 1.2 mm. For the protease activation both isolates was observed by the zone of clearance around the colony with an average diameter of 2mm and 1.4mm respectively. As in the lipase activation, isolate LrMK2 was observed by the zone of clearance around the colony on Tributyrin agar plate with an average diameter of 1.3 mm whereas isolate CmMK4 lipase activation was not detected.

TABLE 2: Enzyme activity of isolates showing the zone appearance ± standard error of 3 determinants.

Bacterial isolates	Enzyme activity on agar plates (zone appearance in mm)		
	Amylase	Protease	Lipase
LrMK2	1.8±0.23	2.0±0.11	1.3±0.12
CmMK4	1.2±0.16	1.4±0.09	ND*

*ND-Not Detected

3.3 Molecular characterization of Isolates

Molecular identification of potential isolates LrMK2 and CmMK4 was based on using 16S rRNA gene sequencing analysis. The Genomic DNA of two isolates was extracted and used as a template for further studies. PCR products were (isolated DNA) was separated in a 1% agarose gel electrophoresis and visualized in-gel documentation (Lark, Germany), purified and photographed (Fig.1). Approximately 1500bp of 16S rRNA gene fragment was amplified from each isolates

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as shown in Fig.2. The NCBI BLAST, the amplified partial 16S rRNA sequences the isolates LrMK2 and CmMK4 further indicated that strains belonged to the *Lactobacillus* Genus and strain LrMK2 revealed 99% identity to *Lactobacillus plantarum* strain CIP103151 (MH571418.1) and CmMK4 revealed 99% identity to *Lactobacillus equicursoris* DSM19284=JCM 14600=CIP 110162 strain DI70 (NR_112652.1).

The obtained sequences were deposited in the Genbank database and the following accession numbers were obtained: LrMK2 (*Lactobacillus plantarum* strain PMCK11) - MK396609 and CmMK4 (*Lactobacillus equicursoris* strain PMCK10) - MK396608. The almost chimera removed complete gene sequence of the two strains were determined aligned and found to be two different species. The obtained sequences were used to construct a phylogenetic tree using the Neighbor-Joining method in Mega X software and evolutionary history was inferred by neighbor-joining using the bootstrap method. A tree depicting the phylogenetic position of strains LrMK2 and CmMK4 within the *Lactobacillus* genus is highlighting with arrow mark shown in Fig.3 **(a)**.

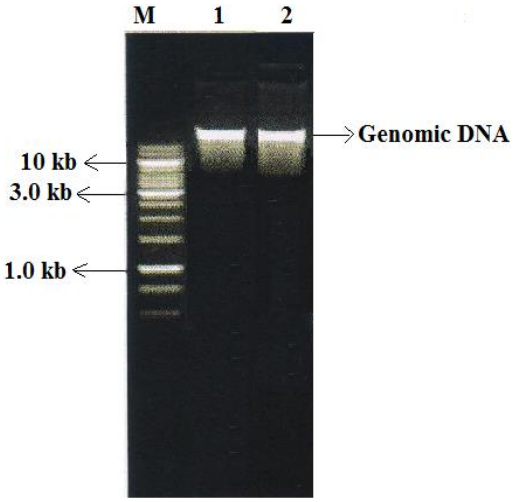

Fig 1: Genomic DNA of the isolates.
Lane 1-LrMK2, Lane 2-CmMK4 and M- Marker

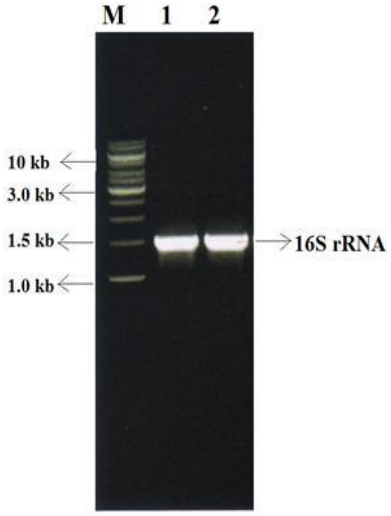

Fig 2: 16SrRNA gene amplification of the isolats.
Lane 1-LrMK2, Lane 2-CmMK4 and M- Marker

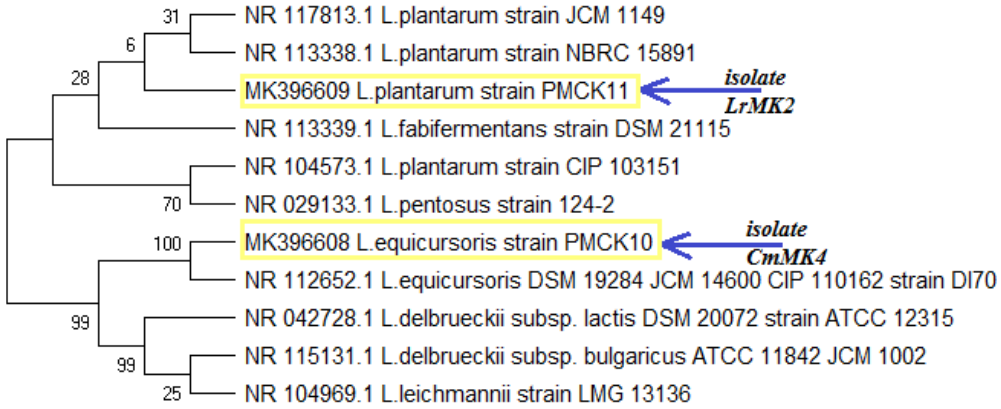

Fig 3 (a): Dendrogram showing phylogenetic relations of the probiotic potential isolates *Lactobacillus plantarum* strain PMCK11 (MK396609) and *Lactobacillus equicursoris* strain PMCK10 (MK396608) shown in arrow marks, with other closely related bacterial strains retrieved from NCBI GenBank. GeneBank accession numbers of the reference strains are shown besides the names. The tree with the highest log likelihood (-3366.49) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The evolutionary history was inferred using the Maximum Likelihood method and Tamura-Nei model [34]. Evolutionary analyses were conducted in MEGA-X [35].

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3.4. Bio-safety assay

After intraperitoneal injections of isolates, LrMK2 and CmMK4 to all the test and control groups of fingerlings *C.catla* and *C.carpio* did not show any pathological symptoms and mortalities as recorded after 21 days trials.

3.5. Congo red binding assay

Based on Congo red agar method, the selective isolates LrMK2 and CmMK4 were screened and confirmed the biofilm formation. Isolates LrMK2 and CmMK4 showed black colonies with a dry crystalline consistency confirming the biofilm formation. Fig: 3 **(b)**.

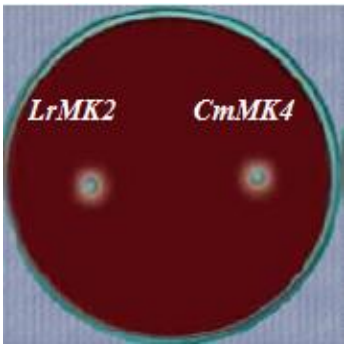

Fig 3 (b): Biofilm formation of isolates on Congo red agar method.

3.6. Physiological characteristics of Selected Isolates

3.6.1 pH tolerance

Isolates LrMK2 and CmMK4 gave promising results to in-vitro selection probiotic criteria such as pH tests. Isolates were studied under the range of pH 2- 9 and measured OD after 2 h incubation. Measurement of optical density at pH 2 - 8 conclude that the isolated strain LrMK2 and CmMK4 are positive results compared to blank and it confirmed that the isolates were a gradual increase in the growth rate up to pH 8 after that pH 9, the OD value was declined compare to pH 8. It proved to the growth rate was decreased and pH 9 to be toxic of both isolates (Fig. 4). This result shows that the highest pH could significantly affect the growth of bacterial strains. Isolates LrMK2 and CmMK4 could able to survive in highly acidic as well as slight alkaline conditions.

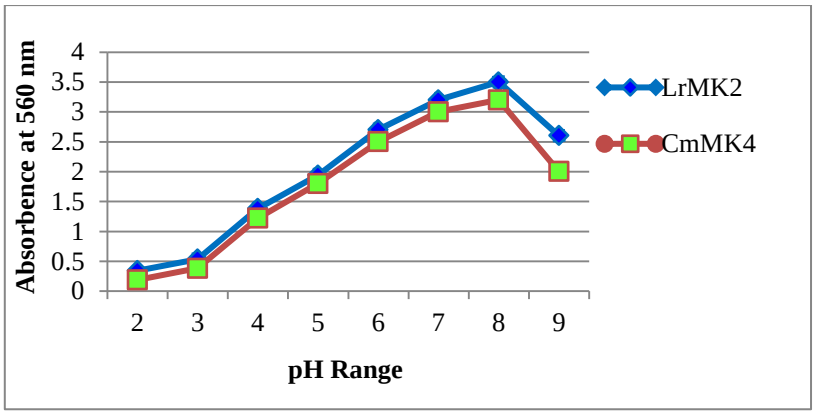

Fig 4: Effect of pH on growth of isolates

3.6.2 Bile Tolerance

Bile salt concentrations were determined in order to survival ability of the isolates LrMK2 and CmMK4 in 0.0, 0.15 and 0.3 % of Ox gall bile salts at 2, 4 and 8 hours incubation. The test isolates were identified as viable form and able to survive at the increasing concentrations of bile salts at 2 and 4 hours incubation. Similarly, the survival percentage of isolated cells significantly decreased in both isolates LrMK2 and CmMK4 after 8 hours incubation of bile salts (Fig.5). In our results shows that both strains were highly significant differences ($P<0.001$) were observed at 2 and 4 hours incubation whereas, proliferation were decreased in the increasing time.

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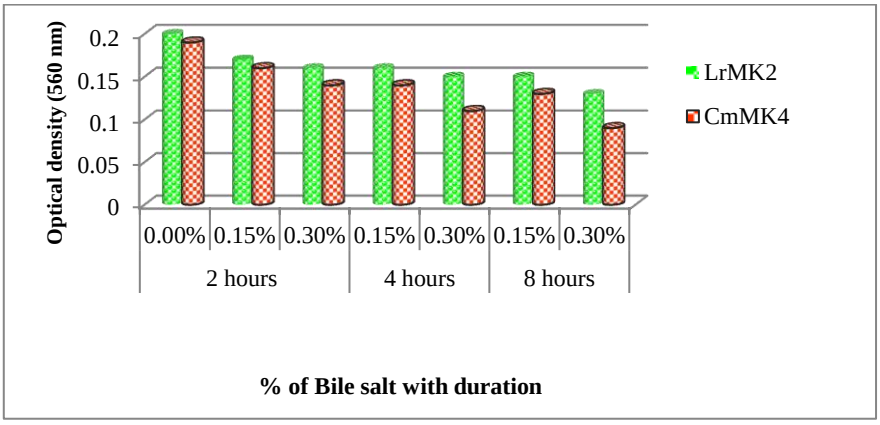

Fig 5: Bile salt tolerance of isolates

3.6.3 NaCl tolerance and Temperature tolerance of Isolates

Isolates LrMK2 and CmMK4 showed good viability observed in spectrophotometer and growth rates in 1-7% NaCl after 24 h incubation. After that, the growth rate decreased with increasing NaCl concentration (Fig.6). Moreover, the growth rate of isolates LrMK2 and CmMK4 was significantly ($p<0.05$) increased with increasing temperature up to 45^{o}C. There is no growth and viability was observed in 50^{o}C (TABLE.1).

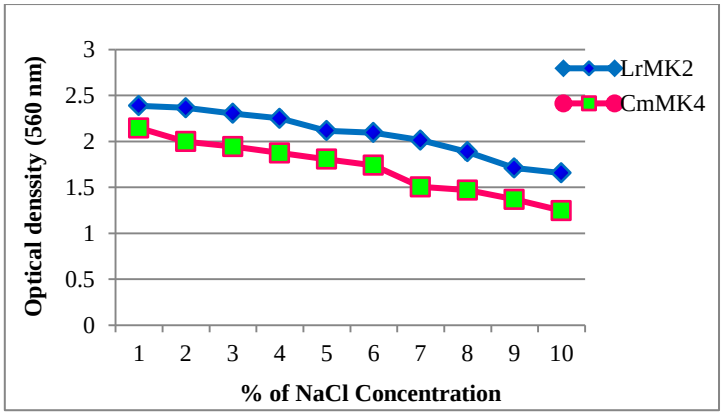

Fig 6: NaCl tolerance of Isolates

3.6.4 Antibacterial activity

The study examined the antibacterial activities of the isolated strains against the test bacteria *Staphylococcus aureus* (KY123795), *Aeromonas veronii* (KY123797) and *Aeromonas hydrophila* (KY123799). Isolate –LrMK2 shows the inhibition zone 21.0 mm, 13.0 mm and 12.0 mm in scale respectively. Isolate CmMK4 shows the inhibition zone 22.5 mm, 11.0 mm, and 14.0 mm in scale respectively (TABLE.3). This result shows that probiotic strains whose safety was taken that are antimicrobial properties against the pathogenic organisms.

TABLE 3: Inhibition Zone produced against different pathogens by isolated potential bacteria

Isolated Strains	*S.aureus*	*Aeromonas veronii*	*Aeromonas hydrophila*
LrMK2	+++	++	++
CmMK4	+++	++	++

Inhibition zone produced in double layer method: + Low (6-10 mm halo diameter);

++ Moderate (11-20 mm halo diameter); +++, high (21- 25 mm halo diameter);

++++, very high (≥26mm halo diameter).

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3.6.5 Antibiotic sensitivity test

The susceptibility and resistance pattern obtained with the isolate LrMK2 and CmMK4 against 9 antibiotics is shown in Fig.7. Susceptibility and resistance testing were studied out to find the antibiotic sensitivity of isolates LrMK2 and CmMK4. Antibiotic susceptibility profiles showed that the strain LrMK2 was sensitive (S) to all antibiotics except Erythromycin, Vancomycin, and Kanamycin, it was Intermediate sensitive (IS), CmMK4 was sensitive to all antibiotics except Methicillin, Chloramphenicol, and Kanamycin.

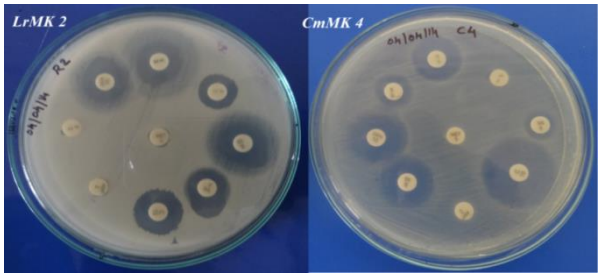

Fig. 7 Antibiotic susceptibility pattern of two bacterial isolates

3.7 Microbial adherence to hydrocarbon (MATH)

Adhesion ability of the isolated strains (LrMK2 and CmMK4) cell surfaces on Xylene, Chloroform and ethyl acetate was tested to assess the results was given in Fig.8. The results show that the adhesion ability of both isolates was significantly higher. Adhesion ability to chloroform and ethyl acetate was analyzed to assess the acid-base characteristics of cell surfaces. Both isolates were a stronger affinity to chloroform (acidic solvent and e- acceptor) than the ethyl acetate (basic solvent e- donor). Percentage of adhesion for LrMK2, 76.42 in Xylene, 72.14 in chloroform and 65.82 in ethyl acetate; for CmMK4, 67.27 in Xylene, in 65.86 in chloroform and 41.08 in ethyl acetate. The adhesion percentage (% of Adhs) of LrMK2 and CmMK4 showed that metabolically the cells are better e- donor and weak e- acceptor.

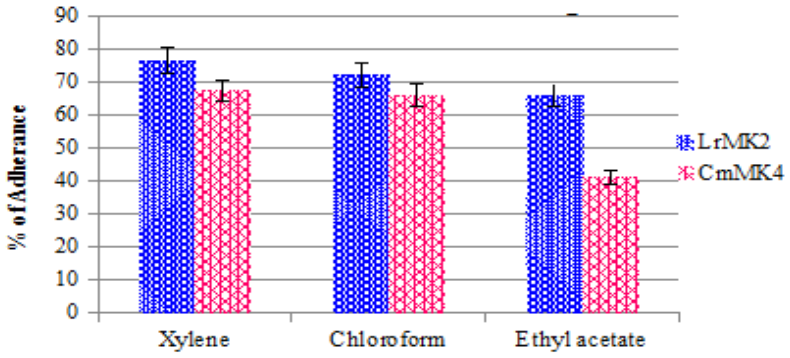

Fig 8: Cell surface adherence of Isolates. Error bar shows that at 5% level of significance

3.9 Autoaggregation assay

The autoaggregation percentage was evaluated by the isolated colonies by sedimentation rates. Results showed that both isolate could autoaggregate increased with increasing incubation period (TABLE.4). Autoaggregation ability isolates LrMK2 and CmMK4 revealed better autoaggregation ability and the percentage was 82.84±0.44% and 77.63±0.99% respectively at 10 hours intervals. The autoaggregation ability at 2 hrs was 27.79±0.68% and 15.96±0.71% respectively. Such a result could indicate a potential capability of the strains adhere to epithelial cells and mucosal surface.

TABLE 4: Percentage of Autoaggregation of bacterial isolates

Strains	% of Autoaggregation on different hours intervals for MRS broth				
	2hrs	4hrs	6 hrs	8 hrs	10 hrs
LrMK2	27.79±0.68	31.24± 1.84	51.07± 0.97	61.29± 0.81	82.84±0.44
CmMK4	15.96±0.71	33.9± 1.37	47.68±0.69	56.84± 0.44	77.63±0.99

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3.10 Co-aggregation assay

Selected strains were able to co-aggregate with the pathogens at varying levels (Fig. 9). Results of Isolate LrMK2 showed that the highest coaggregation percentage with *Staphylococcus aureus* (27.4±5.3%), followed by *Aeromonas veronii* (22.4 ±4.2%) and *Aeromonas veronii* (21.8 ±4.8%). Likewise isolate CmMK4 showed the highest percentage with co-aggregation abilities with the pathogenic isolate *Aeromonas hydrophila* (17.5±3.4%), followed by *Staphylococcus aureus* (16.4±4.4%), *Aeromonas veronii* (12.4±2.8%). Both isolates, LrMK2, CmMK4 showed significant ($P<0.05$) coaggregations with all pathogenic isolates.

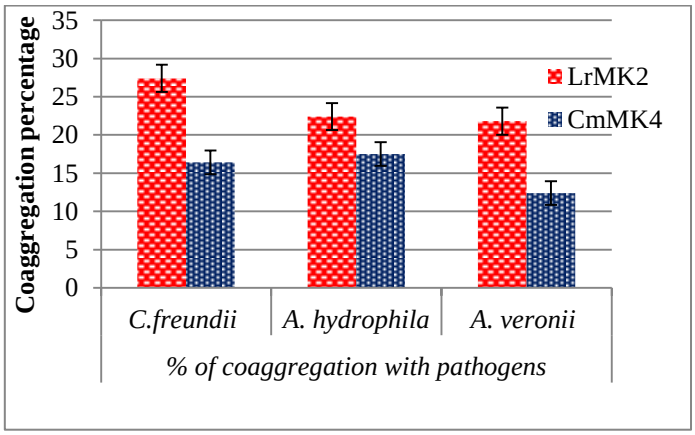

Fig 9: Coaggregation with pathgens

3.11 Hemolytic activity

The isolates LrMK2 and CmMK4 did not exhibit clear transparent or green-hued zone on the blood agar plates, surrounding the colonies, and thus found non-hemolytic or γ-hemolytic colonies, Fig. 10.

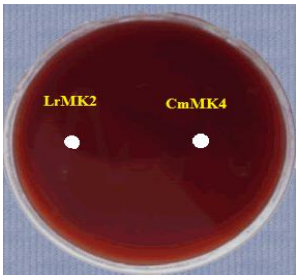

Fig 10: Hemolytic activity of freshwater fish intestinal isolates

4. DISCUSSION

C.catla, *L.rohita*, *C.mrigala*, and *C.carpio* were collected from Aaliyar dam and Thirumoorthi dam, Tamilnadu. The intestine suspected and cultured for *Lactobacillus* species. The intestine of fish is a very complex ecosystem, in which several hundreds of bacterial species are present [36]. Detection of *Lactobacillus* species will always involve the elimination of most of the other group bacteria. The biochemical characterization used for identification of *Lactobacillus spp*. may suggest that some clarity in relation to the occurrence of the bacterial strains in nature. Our biochemical results have been found to be similar to the findings of earlier workers [37], [6].We find out so different isolates from the four fish species on the basis of morphology and biochemical characterization. From this study, we targeted dominant *Lactobacillus* colonies were identified and its potentiality were analyzed. The isolated *Lactobacillus* species (LrMK2) were relatively similar to the species described [38], [39], [6], and the other isolate CmMK4 were rare one previously, a researcher not yet isolated in fish sources. Fishes at all growth stages may expose to the bacteria from the aquatic habitat. Some of them are harmful others are beneficial. The isolated fish intestinal *Lactobacilli* in our study have been designated as *L. plantarum* (LrMK2) and *L. equicursoris* (CmMK4). Recent studies are analyzed for control of pathogens in the fish farms should be improved by studying the beneficial bacteria [40], [41]. As *Lactobacillus* has documented health effects and is naturally present in the intestinal tract of all fishes [15] and other animal groups, knowledge on the presence of *Lactobacillus sp*. as natural flora in fish may lead to feeding supplementation to improve fish health [42].

Selective two *Lactobacillus* isolates (LrMK2 and CmMK4) are strong extracellular enzyme producers were considered as the primary criteria for the potential probiotic candidates in view of inducing improved nutrient utilization to support growth. Isolates of this study depicted that heterotrophic forms within the intestinal tract of Inland fishes were represented by different bacteria capable of producing digestive enzymes and anti-nutritional factors degrading enzymes, which were compliant with the previous observations recorded from the intestinal tract of the *C. catla, L.beta, L. rohita* and *Puntius javanicus* [43], *L.beta*, [44], *C.catla*, and *C. mrigala*, [45] *C. cirrhosus* [16], Indian major carps, [46].

Molecular identification of the *Lactobacillus* isolates LrMK2 revealed 99 % identity with *L.plantarum* which has been isolated and reported from the gut of freshwater fishes [8], and isolate CmMK4 revealed 99% identity with *L.equicursoris* which has been reported from the faeces of a thoroughbred racehorse [37]. These isolates have gram-positive facultative anaerobic *Lactobacillus* species is used as potential probiotics.

To establish the phylogenetic analysis of the isolates, the 16S rRNA genes of the two isolates (LrMK2 and CmMK4) sequences was analysed. The obtained phenotypic data were aligned Mega X software and construct in neighbour – join tree. Here the important point is the phenotypic data achieved by different methods sometime generate different results [47]. Isolates LrMK2 on its phenotypic and phylogenetic uniqueness, it is considered that the similar isolate studied previously by Maji and Mohanty, [8], hence we propose a similar type of species name *L.plantarum*. Isolates CmMK4 considered on its phenotypic and phylogenetic distinctiveness, it is considered that the isolates studied represent a new species in fish intestinal tissue origin of the genus *Lactobacillus*, for which we propose neighbor-joining species name *L.equicursories*.

Safety evaluation of the isolates should be beneficial to the host, which is an important precondition towards consideration of it as a probiotic [48]. In our present study, the in-vivo trial was carried out for safety evaluation of identified isolates (LrMK2 and CmMK4) and it was found the absence of mortality or any other pathological signs on fingerlings of *C.catla* and *C.carpio*. The present observation was strongly confirmed that the isolated *Lactobacillus* strains could be considered as safe strains in aquaculture practices. Few reports have supported that the *Lactobacillus sp.* are safe to be as beneficial for aquaculture application [49], [50], [51]. If the beneficial isolates might also have the ability to survive and colonize the fish intestinal tract; and also the ability of beneficial isolates to form biofilms helps to survive in the intestinal tract of host and is considered to be responsible for persistent or chronic infections. From the biofilm formation of isolates by Congo red agar method, both isolates (LrMK2 and CmMK4) displayed black colonies with dry crystalline consistency suggesting that is biofilm forming strains [52].

Isolates have been demonstrated that for potential ability of *Lactobacillus*, acid tolerance is an important criterion, and the pH 3 has been considered standard for the viability of isolates, [53]. In this study, both isolated *Lactobacillus* strains (LrMK2 and CmMK4) have shown viability at the level of pH 2 - 9. Our results suggested that both strains can be potential probiotics as it can survive even at low pH environment [54], [55]. Jose et al., [56] documented that the *Lactobacilli* tolerated and survived in MRS broth (pH 3), while the reduction in viability has been seen at pH 2. The survivability and growth of the isolate in the high concentration of bile in the stomach passage time and adherence on the fish intestinal region are important characteristics to be noted. In this study identified isolates (LrMK2 and CmMK4) were able to survive up to 0.3% of Ox gall bile salt at 2, 4 and 8 hour incubation, similar reports obtained earlier [17]**,** who found that in an environment of 0.3% bile salt concentration as similar strain of *L.plantarum.* Ahmad et al., [57] have stated that *L.plantarum* was able to survive transit through the intestinal environment where it could activate effectively. The survivability of the isolates in the presence of NaCl (1-7%), indicating their high level of NaCl tolerance and survival of temperature tolerance up to 45^{o}C was observed by Jeronymo-Ceneviva, [58]. The present results revealed that *Lactobacillus sp.* isolated from freshwater fishes was able to tolerate 1-7% of NaCl and excellent growth was perceived at 1-6% NaCl, whereas moderate growth was observed at 7% concentration of NaCl, then the concentration increases the survivability of isolates were decreased. Temperature tolerance was obtained up to 45^{o}C. A similar type of results has been confirmed to Pratiksha et al., [59], who found that some commercial Lactobacillus spp. and Ferrando et al., [60] for *L.plantarum* strains which showed delayed growth obtained at 4% and 6% NaCl concentration.

Isolated two *Lactobacillus* strains showed promising results and could be developed as a potential probiotic species. The isolated strains LrMK2 and CmMK4 showed good zone of inhibition against *Staphylococcus aureus* (KY123795), *Aeromonas veronii* (KY123797) and *Aeromonas hydrophila* (KY123799) respectively. These results were in support of a study undertaken to establish the levels of sensitivity of *Lactobacillus spp*. to various antimicrobial activity [39], [8]; [61],

[62]. The antibiotic sensitivity against Streptomycin, Penicillin, Methicillin, Chloramphenicol, Gentamicin, and Ampicillin for LrMK2 strains are highly sensitive and strain CmMK4 sensitive to Vancomycin, Streptomycin, Erythromycin, Penicillin, Gentamicin, and Ampicillin. Salminen et al. [63] reported too, the Vancomycin resistance as an intrinsic property of *Lactobacilli*, while the *L. plantarum* isolates had been reported to be sensitive to most of the antibiotics tested, such as penicillin G, Ampicillin, chloramphenicol and ciprofloxacin, and Vancomycin, [64]. Many of the *Lactobacillus* strains are naturally resistant to Vancomycin raising concerns regarding the transferability of such resistance to other pathogenic bacteria.

Another criterion for selected isolates with probiotic potential was an investigation of hydrophobicity, as the isolates were studied using chloroform, xylene and ethyl acetate and it was revealed that both strains exhibited hydrophobicity. As the results, two isolates LrMK2 and CmMK4 were highly hydrophobic at 10-hour interval, and this was confirmed to indicate that the isolates can adhere to the fish intestine. The adhesion capacity of this isolates was much higher when compared to the previous report [7]. The adhesion ability of isolates is too high, epithelial cells and mucosal surface of fish, pathogenic colonization was prevented [39]. Kos et al.,[30] recorded maximum hydrophobicity in chloroform when the strains *L.acidophilus* M92, *L.plantarum* L4, and *Enterococcus faecium* L3 were tested against xylene, chloroform, and ethyl acetate.

Autoaggregation ability isolates is the key factors that determine the ability of the probiotics to adhere the intestinal tract and coaggregation ability helps to form a barrier that reduced or prevent the colonization of pathogens [65]. Selective isolates with autoaggregation ability and hydrophobic cell surface could have a high chance for adhesion to host intestinal cells; it was supported by Abdulla et al. [66]. Kos et al., [30] reported that the isolated cell surface proteins influenced autoaggregation ability and adhesiveness of *Lactobacillus sp*. In our results are in general agreements with the results of Abdulla et al. [66]. In the current study, isolates with more autoaggregation were associated with lower coaggregation (i.e. aggregation between genetically different strains) was obtained, which is similar to the previous results of Sahoo et al., [9]. They suggested that in the intestinal tract of animals, coaggregation of *Lactobacillus spp.* with pathogens is an important host defense mechanism.

Hemolysis analysis is one of the pathogenic properties of bacteria, as it facilitates infection by a microbial entry into the small lesions in the skin and mucous [67]. According to FAO/WHO [68], it is important as a safety prerequisite since many organisms are able to synthesis exotoxins that induce partial or total lysis of human or animal cells. In our present study confirmed that the isolated *Lactobacillus* strains did not show any hemolytic activity and hence it can be used with food ingredients for better health. The nonpathogenic property of *Lactobacillus* isolates was confirmed by hemolytic activity on a blood agar plate which showed a negative result [42]. Some beneficial *Bacillus* species have to prove the non-hemolytic activity [7] has confirmed that *Bacillus spp.*, have shown the non-hemolytic activity. Likewise (LrMK2 and CmMK4) discarded hemolytic isolates to avoid injuries in tissues of fish *Channa striatus.*

5. CONCLUSION

We conclude that two *Lactobacillus* species from the intestinal tract of *C.catla L.rohita, C.mrigal and C.carpio*, has been isolated and evaluated for its probiotic potential with the resistance to low pH, bile salts (0.3%). Both isolates are increasingly regarded as an integral component of the host due to significant role in the modulation of the immune system and the proliferation of the intestinal tract and the regulation of the dietary intake. The susceptibility to antibiotics, cell aggregation and non-hemolytic ability revealed its safe status for commercial purposes. Understanding the factors the influence the composition of these microbial communities is essential to host health management, and the application of aquatic animals still requires basic investigation. We suggest that *L.equicusoris* PMCK10 and *L.plantarum* PMCK11 as a potential probiotic species with different functional properties and novel compounds.

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