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Isolation, Characterization, and Assessment of Potential Probiotic Bacteria in the Gastrointestinal Tract of *Clarias magur* (Hamilton, 1822) for Aquaculture Applications

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ABSTRACT

Probiotic bacteria play an important role in boosting the immune system, aiding enzymatic digestion, maintaining intestinal balance, and inhibiting the growth of harmful microbes in freshwater fish. Probiotics isolated from the intestines of the same host species are generally more effective for colonization than those obtained from unrelated sources. This study evaluated the probiotic potential of gut-derived bacteria from *Clarias magur*. A total of 160 biological samples were collected from multiple locations across four districts in Assam, India. The tested bacterial isolates exhibited significantly different survival rates under various stress conditions. Sixteen isolates survived simulated gastrointestinal conditions (pH 2), with survival rates ranging from 60.90 to 96.30%. In bile tolerance tests, 13 isolates survived in 0.3% bile salt, showing varying tolerance levels. After one hour of exposure to 1% lysozyme (100mg/ L), all 13 isolates demonstrated good survival, with rates ranging from 63.10 to 95.50%. In the presence of 20mM hydrogen peroxide (H_2O_2), 10 isolates remained viable, with survival rates between 43.60 and 72.00%. The 13 isolates exhibited auto-aggregation abilities ranging from 45.13 to 78.70% after 5 hours of incubation, while cell surface hydrophobicity in xylene ranged from 56.55 to 92.00%. Eight isolates displayed varying degrees of antagonistic activity against four fish pathogens: *Aeromonas hydrophila*, *Aeromonas jandaei*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. All lactic acid bacteria (LAB) isolates showed different susceptibility and resistance patterns to clinically relevant antibiotics. Importantly, all strains were γ-hemolytic, exhibiting no hemolytic activity on sheep blood agar.

Molecular sequencing and phylogenetic analysis identified eight LAB isolates as *Lactobacillus reuteri*, *Limosilactobacillus reuteri*, *Enterococcus faecalis*, and *Enterococcus mundtii*. The findings highlight the probiotic potential of LAB isolated from freshwater fish and their possible application as biocontrol agents in aquaculture.

INTRODUCTION

Probiotic bacteria are essential for promoting host health and maintaining nutritional homeostasis by enhancing gastrointestinal function and supporting a balanced

gut microbiota. *Clarias magur*, a high-value freshwater catfish species native to South and Southeast Asia **(Liu *et al.*, 2022; Das *et al.*, 2024)**, relies on its intestinal microbiota for immune defense, nutrient metabolism, growth, and overall health.

Aquaculture has expanded rapidly through intensive farming methods to meet the growing demand for animal protein **(Subasinghe, 2017; Zulkhairi *et al.*, 2020; Borah *et al.*, 2024)**. However, this rapid growth has also led to challenges, particularly disease outbreaks, with bacterial infections being a major cause of mortality in aquaculture **(Giri *et al.*, 2011; Mukherjee & Ghosh, 2016; Banerjee & Ray, 2023)**. Modulating gut microbiota through dietary supplementation offers an innovative strategy to improve intestinal health and overall performance in cultured aquatic species **(Han *et al.*, 2015; Anokyewaa *et al.*, 2021)**. Pathogenic microbes in the gastrointestinal tract can disrupt host immunity, reduce colonization resistance, and impair nutrient digestion, ultimately affecting health and productivity. Therefore, the identification and selection of probiotic strains with antagonistic activity against such pathogens is a critical focus for sustainable aquaculture **(Lazado *et al.*, 2015; Nami *et al.*, 2025)**.

Lactic acid bacteria (LAB) and other beneficial microbes naturally inhabit the intestinal tract and can withstand harsh gastric conditions, including low pH, bile salts, and digestive enzymes, particularly in the upper small intestine, where they exert multiple health-promoting effects **(Alonso *et al.*, 2019; Bordoloi *et al.*, 2025)**. In addition to enhancing immune function, probiotics have been associated with improved growth performance and feed conversion efficiency in fish **(Saba *et al.*, 2024)**.

Probiotics are valued for their broad-spectrum antibacterial activity and potent inhibitory effects against pathogens **(Pereira *et al.*, 2022; Sadeghi *et al.*, 2022; Lee *et al.*, 2024)**. Beyond competitive exclusion and immune modulation, many strains produce digestive enzymes that enhance nutrient assimilation, thereby improving stress resistance and overall health **(Puvanasundram *et al.*, 2021)**. They also exhibit diverse antimicrobial properties, including antibacterial, antiviral, and antifungal activities **(Chauhan & Singh, 2019)**. The use of probiotics in aquaculture has expanded significantly, benefiting a wide range of aquatic animals, including fish and shellfish **(Amoah *et al.*, 2019; Chen *et al.*, 2025)**.

In this study, bacterial isolates obtained from the gastrointestinal tract of *Clarias magur* were identified and evaluated for their probiotic potential.

MATERIALS AND METHODS

Sampling

Healthy *Clarias magur* individuals of consistent size were aseptically collected from the Darrang, Sonitpur, Morigaon, and Nalbari districts of Assam, India. Sampling locations were selected based on habitat characteristics and the proximity of waterbodies, with each population sampled approximately 100km from the nearest other population (Fig. 1). In total, 160 biological samples were obtained from multiple sites across the four districts. Immediately after collection, each fish was placed in a sterilized, clearly labelled

zipper bag and transported to the laboratory in an icebox to maintain sample integrity for subsequent analysis (Fig. 2).

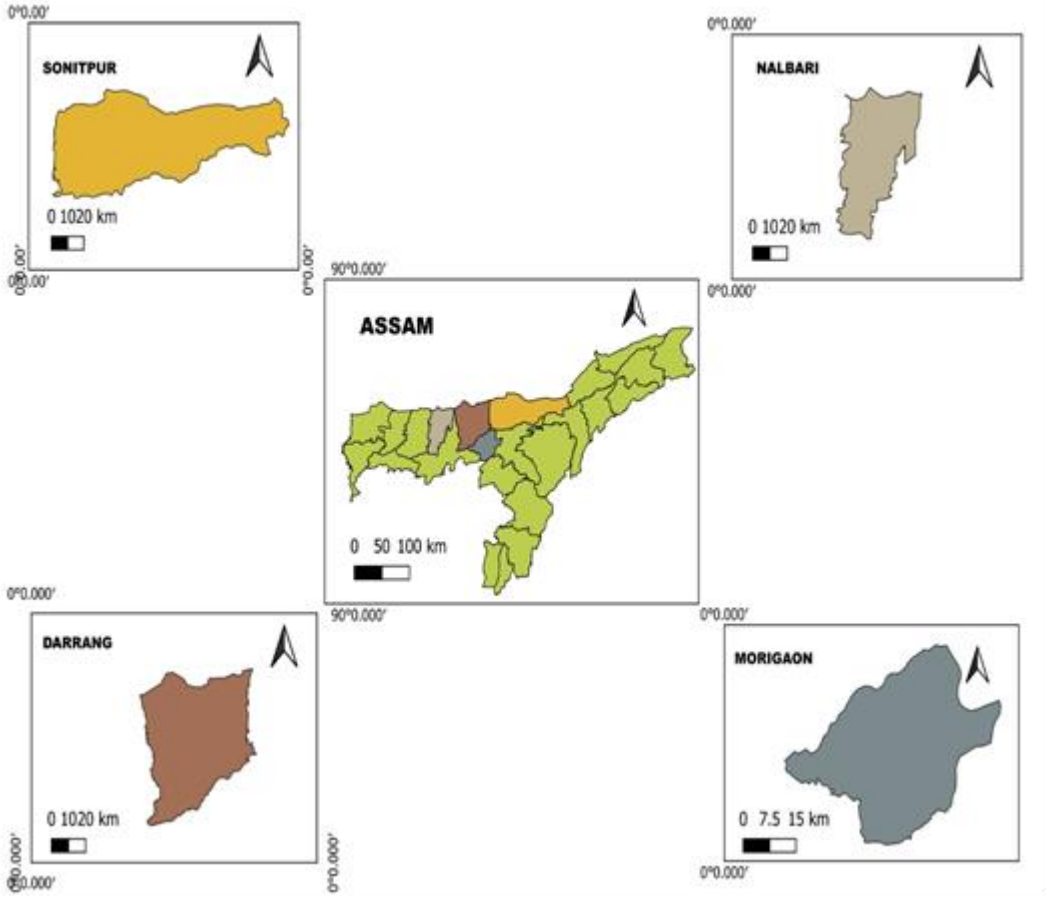

Fig. 1. Locations of sample collection from Sonitpur, Nalbari, Darrang and Morigaon Districts in Assam, India

Inoculation of samples

The specimens were carefully dissected, and the gastrointestinal tracts were aseptically removed (Fig. 3). To isolate lactic acid bacteria (LAB), approximately 4g of intestinal contents from each fish were inoculated into 50mL of de Man, Rogosa, and Sharpe (MRS) broth (HiMedia, GM396-500G) and were incubated aerobically at 37°C for 24 hours to promote bacterial enrichment. Following enrichment, a loopful of the cultured broth was streaked onto MRS agar plates supplemented with L-cysteine and 1% $CaCO_3$. The plates were then incubated anaerobically at 37°C for 24 hours. After visible growth was observed, colonies exhibiting different morphologies were sub-cultured on fresh MRS agar plates and re-streaked four successive times to ensure pure cultures.

Fig. 2. Healthy *Clarias magur* collected from Nalbari district, Assam, India

Fig. 3. Gastrointestinal sampling of *Clarias magur* for probiotic characterization

Characterization and identification of isolates

The morphological characteristics of the isolates were assessed by examining bacterial size and shape after Gram staining. Culturing colonies on MRS agar provided additional information on colony shape, size, color, and surface appearance. Preliminary identification was carried out using biochemical tests, including catalase, indole, and carbohydrate fermentation assays. Based on morphological and biochemical characteristics indicative of lactic acid bacteria (LAB), selected isolates were subjected to further analysis.

In vitro assessment of probiotic activity

Isolates exhibiting traits consistent with LAB were evaluated *in vitro* for potential probiotic properties. The assays included acid tolerance, bile tolerance, lysozyme resistance, hydrogen peroxide resistance, auto-aggregation, cell-surface hydrophobicity, antimicrobial activity, antibiotic susceptibility, and hemolytic activity.

Acid tolerance test

Acid tolerance was assessed following the methods described by **Bao *et al*. (2010)** and **Ramos *et al*. (2013)**. The isolates were first cultured in MRS broth and incubated at 37°C for 24 hours. After incubation, cells were harvested by centrifugation at 6,000rpm for 4 minutes at 24°C and washed twice with 0.1% (w/v) sterile peptone water (pH 7.0). The washed cells were adjusted to a concentration of approximately 10^8 cells/mL, then centrifuged again and resuspended either in MRS broth adjusted to pH 2.0 using 1.0 N HCl (treatment) or in unmodified MRS broth (control). Both treatments were incubated at 37°C for 3 hours. Following incubation, a 10^{-2} dilution was prepared, and 50µL of the diluted suspension was spread onto MRS agar plates using sterile cotton swabs. Plates were incubated anaerobically at 37°C for 48 hours, and acid tolerance was determined based on the presence and number of bacterial colonies (Fig. 8A).

$$\% \text{ survivability} = \frac{\text{3 hrs survival count of LAB in pH 2 (Log cfu ml}-1)}{\text{Initial count of LAB (Log cfu ml}-1\text{)}} \times 100$$

Bile tolerance test

The isolates were cultured in MRS broth and incubated at 37°C for 24 hours. Following incubation, cells were harvested by centrifugation at 6,000rpm for 4 minutes at 24°C and washed twice with 0.1% (w/v) sterile peptone water (pH 7.0). The washed cells were adjusted to an approximate concentration of 10^8 cells/mL, centrifuged again, and resuspended in MRS broth containing 0.3% (w/v) bile salt (HiMedia, RM008-500G). For each isolate, a positive control was prepared using standard MRS broth without bile salts.

Both the bile-supplemented and control cultures were incubated at 37°C for 3 hours. After incubation, serial dilutions were prepared to a final dilution factor of 10^{-3}, and 50µL of each diluted suspension was spread onto MRS agar plates using sterile cotton swabs. The plates were incubated anaerobically at 37°C for 24–48 hours. Colony counts were expressed as colony-forming units per milliliter (cfu/mL) **(Mulaw *et al*., 2019)** (Fig. 8B).

$$\% \text{ survivability} = \frac{\text{Log cfu N1}}{\text{Log cfu N0}} \times 100$$

Where, N1 is the viable count of the isolates after incubation at 0.3% w/v bile salt

N0 is the viable count of isolates without bile.

Resistance to lysozyme

Lysozyme resistance was assessed following the method described by **Dias *et al*. (2015)**. The isolates were cultured in MRS broth and incubated at 37°C for 24 hours. After incubation, cells were harvested by centrifugation at 6,000rpm for 4 minutes at 24°C and washed twice with 0.1% (w/v) sterile peptone water (pH 7.0). The washed cells were adjusted to an approximate concentration of 10^8 cells/mL, centrifuged again, and resuspended in MRS broth supplemented with lysozyme at a concentration of 100mg/ L.

For each isolate, a positive control was prepared in standard MRS broth without lysozyme. Both the lysozyme-treated and control cultures were incubated at 37°C for 60

minutes. Following incubation, serial dilutions were prepared to a final dilution factor of 10^{-2}, and 30µL of each diluted suspension was spread onto MRS agar plates using sterile cotton swabs. Plates were incubated anaerobically at 37°C for 48 hours. The survival rate of colonies (Fig. 8C) was calculated using the following equation:

$$\% \text{ survivability} = \frac{\text{60 min sirvival count of LAB (Log cfu ml}-1)}{\text{Initial count of LAB (Log cfu ml}-1\ )} \times 100$$

Resistance to hydrogen peroxide (H_2O_2)

The isolates were cultured in MRS and incubated for 24 hours at 37°C. Subsequent to incubation, cells were collected using centrifugation at 6,000rpm for 4 minutes at 24°C. The bacterial cell pellets underwent two washes with 0.1% (w/v) sterile peptone water (pH 7.0) prior to resuspension. The washed cells were adjusted to an estimated concentration of 10^8 cells/ml. The standardized cell suspensions were centrifuged again and resuspended in MRS broth containing 20mM H_2O_2. A positive control for each of the different cultures was established using regular MRS broth. Both sets were incubated at 37°C for 2 hours. After incubating with a 10^{-2} dilution factor, 50 µl of the diluted suspension was smeared on MRS agar plates with a cotton swab and incubated at 37°C for 48 hours anaerobically. The bacterial colonies were counted, and the percent survival rate was determined (Fig. 8D).

$$\% \text{ survivability} = \frac{\text{2 hrs sirvival count of LAB (Log cfu ml}-1)}{\text{Initial count of LAB (Log cfu ml}-1\ )} \times 100$$

Auto-aggregation

The auto-aggregation ability of the bacterial isolates was investigated using the data in the tudy of **Kos *et al*. (2003)**. The isolates were cultured in MRS and incubated for 24 hours at 37°C. Subsequent to incubation, cells were collected using centrifugation at 6,000rpm for 4 minutes at 24°C. The bacterial cell pellets underwent two washes with 0.1% (w/v) sterile peptone water (pH 7.0) prior to resuspension. The washed cells were adjusted to an estimated concentration of 10^8 cells/ml. The bacterial suspension (4ml) was vortexed for 10 seconds, and auto-aggregation was assessed at hourly intervals over a 5-hour incubation period at room temperature by mixing 0.1ml of upper suspension with 3.9ml of peptone water and measuring absorbance at 600nm.

$$\text{auto aggregation (\%)} = (1 - A_t/A_0) \times 100$$

where, A_t represents the absorbance at time t and A_0 as the absorbance at t = 0.

Determination of cell-surface hydrophobicity

The *in vitro* cell surface hydrophobicity of bacteria was examined as per **Rokana *et al*. (2018)**. The isolates were cultured in MRS broth for 24 hours at 37°C. Cultures grown overnight in MRS broth were centrifuged at 8,000rpm at 4°C for 10 minutes, washed twice with peptone water, and then resuspended in peptone water. Absorbance (A_0) was measured at 600nm. A 3ml volume of cell suspension has been mixed with 1ml of xylene and incubated at 37°C for 1 hour without mixing to facilitate the separation of

the aqueous and organic phases. Subsequently, about 1ml of the aqueous phase was carefully removed, and the absorbance (A_1) was again measured at 600nm.

$$\text{Hydrophobicity (\%)} = (1 - A_1/A_0) \times 100.$$

Where, A_1 represents absorbance after 1hour incubation with xylene.

A_0 represents the initial absorbance at 600nm before adding xylene.

Antimicrobial activity

The antibacterial activity of the lactic acid bacterial (LAB) isolates was determined using the agar well diffusion method **(Piddock *et al*., 1990)**. Each LAB strain was cultured in MRS broth for 24 hours at 37°C, after which cultures were centrifuged at 6,000rpm for 5 minutes. The cell-free supernatant (CFS) was collected and sterilized by filtration through a 0.22µm Millipore membrane filter.

Test pathogens (*Aeromonas jandaei*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) were cultured separately, and 20µL of each suspension was spread evenly onto pre-dried Mueller–Hinton agar (MHA) plates. Wells were aseptically created in the agar, and 80µL of the filtered CFS was added to each well. Plates were kept at 4°C for 30 minutes to allow diffusion, then incubated at 37°C for 24 hours. Antibacterial activity was evaluated by measuring the diameter of the inhibition zones around the wells, with zones ≥ 6mm considered indicative of inhibitory activity **(Xie *et al*., 2015)**.

The bacterial strains used in the inhibition assays were: *A. jandaei* (MTCC 12967), *A. hydrophila* (MTCC 13049), *P. aeruginosa* (GenBank accession no. KC862289), and *S. aureus* (MTCC 96).

Antibiotic susceptibility assay

Antibiotic susceptibility was assessed using the Kirby–Bauer disc diffusion method **(Bauer *et al*., 1966)**, following the procedure of **Mulaw *et al*. (2019)**. The antibiotics tested were tetracycline (10µg), vancomycin (10µg), co-trimoxazole (25µg), chloramphenicol (10µg), streptomycin (25µg), rifampicin (15µg), ciprofloxacin (5µg), and gentamicin (10µg).

Isolates were cultured in MRS broth at 37°C for 24 hours, and 100µL of actively grown culture was spread onto MHA plates using a sterile cotton swab. Antibiotic discs were aseptically placed on the solidified agar, and plates were held at 4°C for 30 minutes to allow diffusion before anaerobic incubation at 37°C for 24–48 hours. The results were interpreted according to HiMedia guidelines: sensitive (S) ≥ 21mm, intermediate (I) 16–20mm, and resistant (R) ≤ 15mm.

Hemolytic activity

Hemolytic activity was evaluated by streaking the isolates onto agar plates enriched with 5% sheep blood **(Gouthami *et al*., 2014)**. Plates were incubated at 37°C for

48 hours. Hemolysis was determined based on the appearance of zones around colonies: α-hemolysis (greenish zone), β-hemolysis (clear zone), or γ-hemolysis (no zone) **(Kuebutornye *et al*., 2020)**.

Molecular identification of isolates

Genomic DNA was extracted from selected isolates using the phenol–chloroform method **(Han *et al*., 2018)**. DNA integrity was assessed by 1% agarose gel electrophoresis, and purity and concentration were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Nanodrop-1000). DNA purity was confirmed by a 260/280 ratio of approximately 1.8.

The 16S rRNA gene was amplified using previously reported primers and PCR conditions **(Deka *et al*., 2023)**. PCR products were visualized by agarose gel electrophoresis under a gel documentation system (Gel Doc XR+, Bio-Rad).

Purification and sequencing

Amplified PCR products were purified using a PCR cleanup kit (Nucleopore PCR Clean-up Gel Extraction, NP 36105) and sequenced bidirectionally using an ABI 3500 capillary sequencer (Eurofins Genomics India Pvt. Ltd., Bangalore). Sequence similarity was determined using the NCBI BLASTn tool, and partial 16S rRNA sequences were submitted to GenBank after verification.

Phylogenetic analysis

Partial 16S rRNA gene sequences from the selected isolates were compared to GenBank entries using NCBI BLAST to identify closely related taxa. Additional relevant 16S rRNA sequences from putative probiotic bacteria were retrieved for phylogenetic analysis. A phylogenetic tree was constructed using the maximum likelihood method with the Kimura 2-parameter model in MEGA11 **(Tamura *et al*., 2021)**, and bootstrap analysis was performed with 1,000 replicates to assess branch support .

RESULTS

Characterization and identification of the isolates

The cultural, morphological, and biochemical characteristics of the probable lactic acid bacterial isolates were used to identify them.

Cultural characteristics of suspected lactic acid bacterial isolates

The suspected lactic acid bacterial colonies were identified as white, convex, well-defined, round-shaped, raised, soft, and shiny, showing a clear zone around their colonies on MRS-cysteine medium (Fig. 4). A total of 66 isolates out of 160 isolates from *Clarias magur* specimens exhibited characteristics of lactic acid bacteria based on culture analysis.

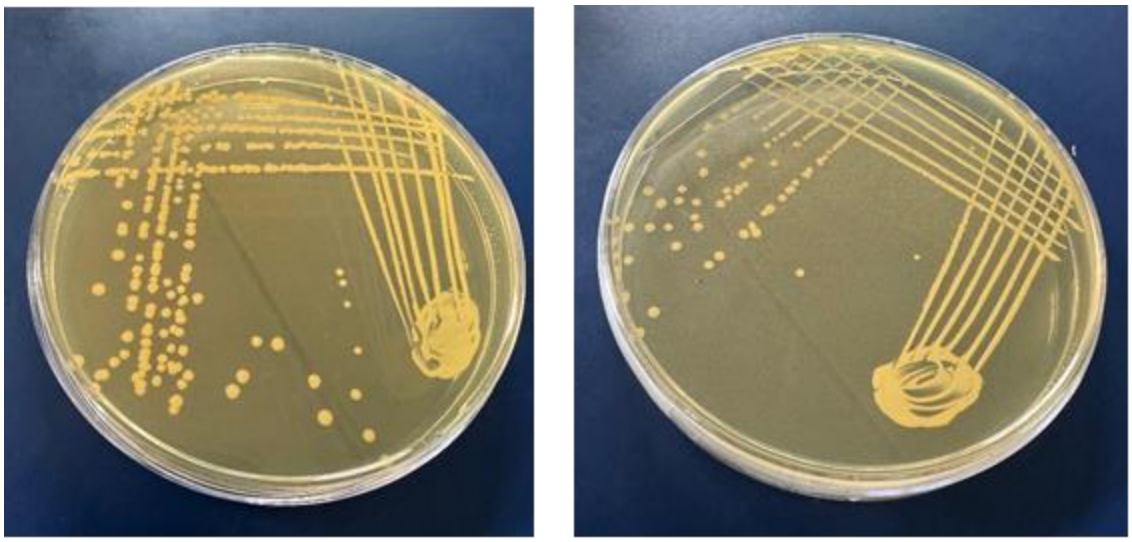

Fig. 4. Streak plates showing pure culture of gut-derived bacterial isolate for probiotic evaluation

Morphological characteristics of suspected lactic acid bacterial isolates

The morphological properties of the probable *Lactobacillus* isolates were investigated after staining the culture smears using Gram's staining procedure, and the isolates were observed to be rods/tick-shaped, cocci, and Gram-positive, which was suggestive of lactic acid bacteria (LAB) spp. All the total of 66 isolates from *Clarias magur* suspected to be lactic acid bacteria (LAB) spp. based on cultural characteristics were further confirmed as such after Gram's staining (Fig. 5).

Biochemical characteristics of suspected lactic acid bacterial isolates

All 66 isolates from *Clarias magur* were subjected to a catalase test along with other biochemical tests, including indole tests and carbohydrate fermentation. Catalase and indole tests were negative for all isolates, and all the isolates fermented three sugars, namely lactose, D-fructose, and maltose.

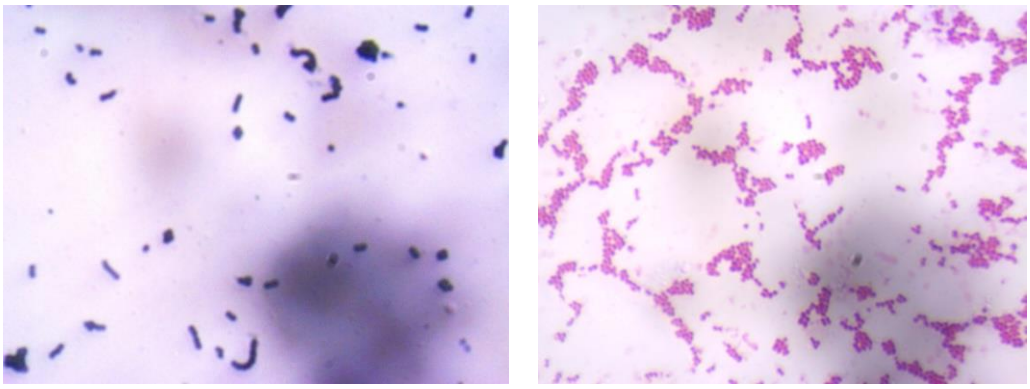

Fig. 5. Gram-stained microscopic images of lactic acid bacteria showing rod and cocci shaped morphology

In vitro assessment of the isolates for probiotic activity

Acid tolerance

To assess acid tolerance, all 66 isolates—identified based on their cultural, morphological, and biochemical characteristics—were incubated in MRS broth adjusted to pH 2.0 for 3 hours at 37°C **(Maurer *et al*., 2015)**. Of these, only 16 lactic acid bacterial (LAB) isolates survived under these conditions. The survival rates of the selected isolates ranged from 60.90% to 96.30% (Table 1; Fig. 6). The highest acid tolerance was observed in isolates CM02, CM08, CM01, and CM06, with survival rates of 96.30, 95.70, 95.40, and 94.40%, respectively. In contrast, isolates CM16, CM09, and CM12 exhibited the lowest viability, with survival rates of 60.90, 61.70, and 64.40%, respectively, after 3 hours of incubation.

Table 1. Acid tolerance of lactic acid bacterial isolates with their colony counts and percent of survivability at pH 2.0.

Isolate No.	Colony count after 3 hours at Normal MRS (control)	Coloney count after 3 hours at pH 2	CFU/ml after 3 hours at Normal MRS (control)	CFU/ml after 3 hours at pH 2	Percentage of survivability
CM01	268	257	5.36×10^5	5.14×10^5	95.40%
CM10	298	201	5.96×10^5	4.02×10^5	67.45%
CM02	>300	289	6.00×105	5.78×105	96.30%
CM03	215	184	4.30×10^5	3.68×10^5	85.50%
CM11	274	182	5.48×10^5	3.64×10^5	66.50%
CM04	>300	265	6.00×10^5	5.30×10^5	88.30%
CM12	278	179	5.56×10^5	3.58×10^5	64.39%
CM09	230	142	4.60×10^5	2.84×10^5	61.70%
CM05	268	241	5.36×105	4.82×105	89.90%
CM14	>300	224	6.00×105	4.48×10^5	74.66%
CM06	284	268	5.68×105	5.36×105	94.40%
CM13	221	144	4.42×105	2.88×105	65.10%
CM7	296	273	5.92×105	5.46×105	92.20%
CM15	196	146	3.92×105	2.92×105	74.50%
CM08	>300	287	6.00×105	5.74×105	95.70%
CM16	259	158	5.18×105	3.16×105	60.90%

Bile tolerance

The 16 isolates exhibiting high acid tolerance were further evaluated for bile salt resistance by incubating them in MRS broth supplemented with 0.3% (w/v) bile salts for 3 hours at 37°C, simulating intestinal transit conditions **(Maurer *et al.*, 2015)**. Thirteen isolates demonstrated bile salt tolerance, although their viability varied considerably. Survival rates ranged from 46.90% to 95.40% (Table 2 & Fig. 6). The highest tolerance was observed in isolate CM01 (95.40%), followed by CM06, CM07, and CM05, each with a survival rate of 94.30%. In contrast, isolates CM13, CM09, and CM12 showed the lowest survival under bile stress, with rates of 46.90, 44.40, and 57.60%, respectively.

Table 2. Tolerance of lactic acid bacterial isolates to 0.3% OX bile with their colony count and % survivability

Isolate No.	Colony count after 3 hours at Normal MRS (control)	Coloney count after 3 hours at ox Bile	CFU/ml after 3 hours Normal MRS (control)	CFU/ml after 3 hours at ox Bile	Percent survivability
CM01	>300	279	1.5×10^{7}	1.395×10^{7}	93.3%
CM10	276	217	1.38×107	1.085×107	77.8%
CM02	201	179	1.005×107	8.95×106	89.1%
CM03	>300	245	1.5×107	1.225×107	81.4%
CM11	289	204	1.445×107	1.02×107	70.7%
CM04	237	216	1.185×107	1.08×107	91.2%
CM12	297	172	1.485×107	8.6×106	57.6%
CM05	>300	281	1.5×107	1.405×107	94.3%
CM06	287	273	1.435×107	1.365×107	95.4%
CM09	279	121	1.395×107	6.05×106	44.4%
CM07	228	216	1.14×107	1.08×107	94.3%
CM08	264	241	1.32×107	1.205×107	91.2%
CM13	211	97	1.055×107	4.85×10^{6}	46.9%

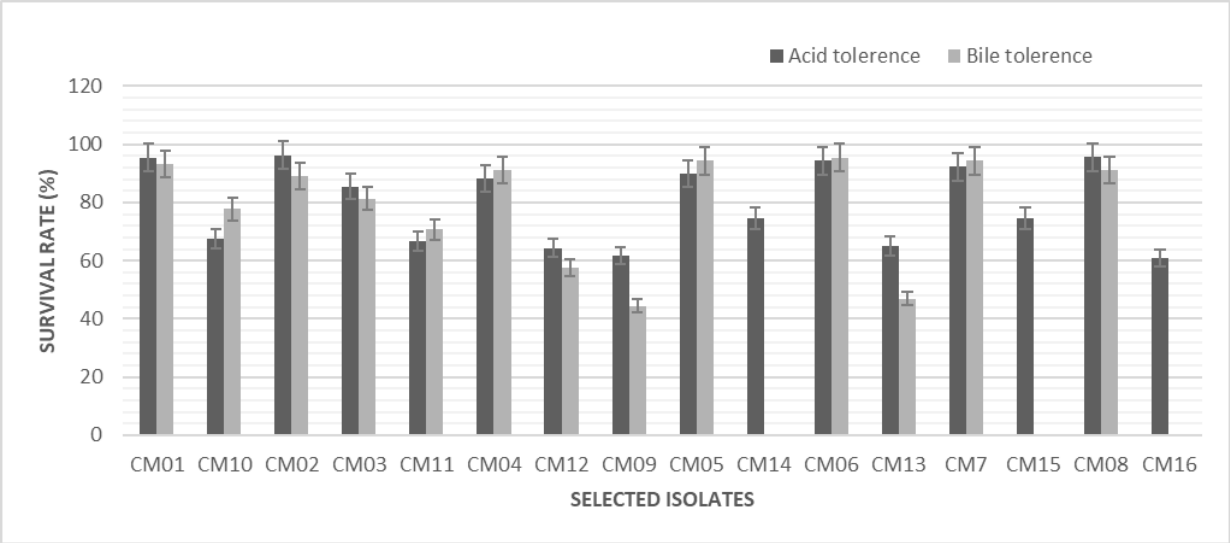

Fig. 6. Tolerance of lactic acid bacterial isolates to low pH and high bile salt concentrations

Lysozyme tolerance

To determine the isolates' ability to resist enzymatic challenges in the gastrointestinal tract, those that survived both acidic and bile conditions were treated with 1% lysozyme (100mg/ L). The percentage survival rates of the investigated isolates ranged from 63.10 to 95.50%. Isolate CM07 showed maximum tolerance, followed by isolates CM04, CM06, and CM05, which had survival rates of 95.50, 95.10, 93.30, and 91.60%, respectively. Isolates CM09, CM10, and CM13 had the lowest survivability rates, at 63.10, 69.20, and 72.40%, respectively (Table 3 & Fig. 7).

Table 3. Lysozyme tolerance test of lactic acid bacterial with their colony counted and percentage of survivability at 1% lysozyme

Isolate No.	Colony count after 1 hours in normal MRS broth (control)	Colony count after 1 hours of lysozyme treatment	CFU/ml after 1 hours in normal MRS broth (control)	CFU/ml after 1 hours of lysozyme treatment	Percent survivability
CM01	284	247	9.47×10^5	8.23×10^5	87.20%
CM10	271	191	9.03×10^5	6.37×10^5	69.20%
CM02	310	276	1.03×10^6	9.20×10^5	89.10%
CM03	258	221	8.60×10^5	7.37×10^5	87.20%
CM11	290	240	9.67×10^5	8.00×10^5	81.30%
CM04	>300	284	1.00×10^6	9.47×10^5	95.10%
CM12	273	219	9.10×10^5	7.30×10^5	79.40%
CM05	190	173	6.33×10^5	5.77×10^5	91.60%
CM06	269	247	8.97×105	8.23×105	93.30%

CM09	174	108	5.80×105	3.60×105	63.10%
CM07	>300	289	1.00×10^6	9.63×10^5	95.50%
CM08	201	177	6.70×105	5.90×105	87.1%
CM13	215	153	7.17×10^5	5.10×10^5	72.4%

Resistance to hydrogen peroxide (H_2O_2)

All isolates that demonstrated tolerance to acid, bile, and lysozyme were further assessed for oxidative stress resistance by exposure to 20mM hydrogen peroxide (H_2O_2). Of the 13 isolates tested, 10 retained viability under these conditions, with survival rates ranging from 43.60% to 72.00% (Table 4 & Fig. 7). The highest resistance was observed in isolates CM03, CM05, and CM04, each with survival rates of 72.00, 72.00, and 68.40%, respectively. In contrast, isolates DR36, CM10, and CM06 showed the lowest survivability, with rates of 43.60, 45.70, and 51.20%, respectively.

Table 4. Tolerance of lactic acid bacterial (LAB) isolates in 20 mM concentration of Hydrogen peroxide with their colony counts and survivability percentage

Isolate No.	Colony count after 2 hours in normal MRS broth (control)	Colony count after 2 hours of 20 mM H_2O_2 treatment	CFU/ml after 3 hours in normal MRS broth (control)	CFU/ml after2 hours of 20 mM H_2O_2 treatment	Percent survivability
CM01	246	165	4.92×105	3.30×105	68.0%
CM10	224	102	4.48×10^5	2.04×10^5	45.7%
CM02	221	137	4.42×10^5	2.74×10^5	63.1%
CM03	292	215	5.84×105	4.30×105	72.0%
CM04	264	179	5.28×105	3.58×105	68.4%
CM05	279	209	5.58×105	4.18×105	72.0%
CM06	221	113	4.42×105	2.26×105	51.2%
CM09	234	103	4.68×105	2.06×105	43.6%
CM07	157	99	3.14×105	1.98×105	63.1%
CM08	248	150	4.96×105	3.00×105	60.0%

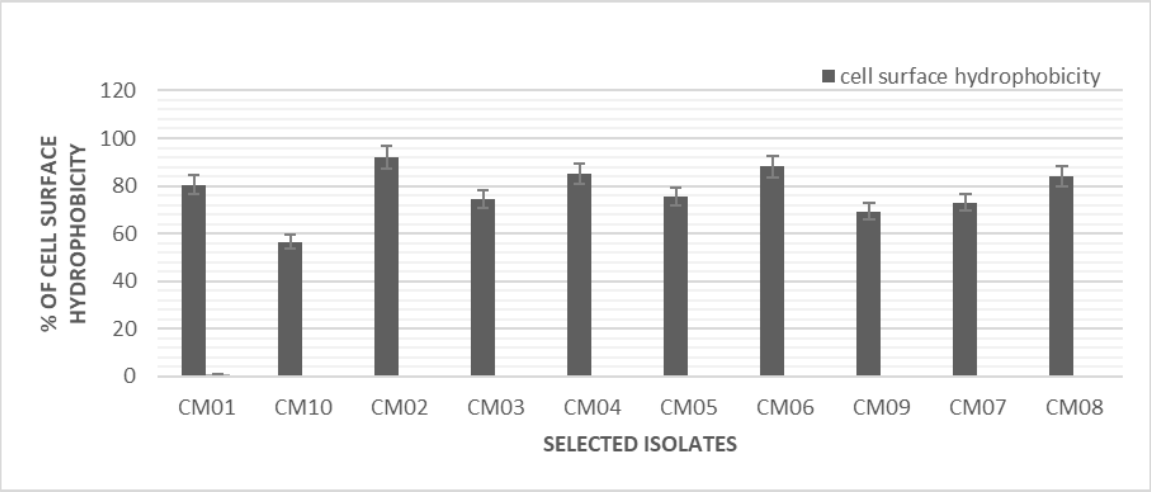

Fig. 7. Percentage of cell surface hydrophobicity shown by the selected lactic acid bacterial isolates

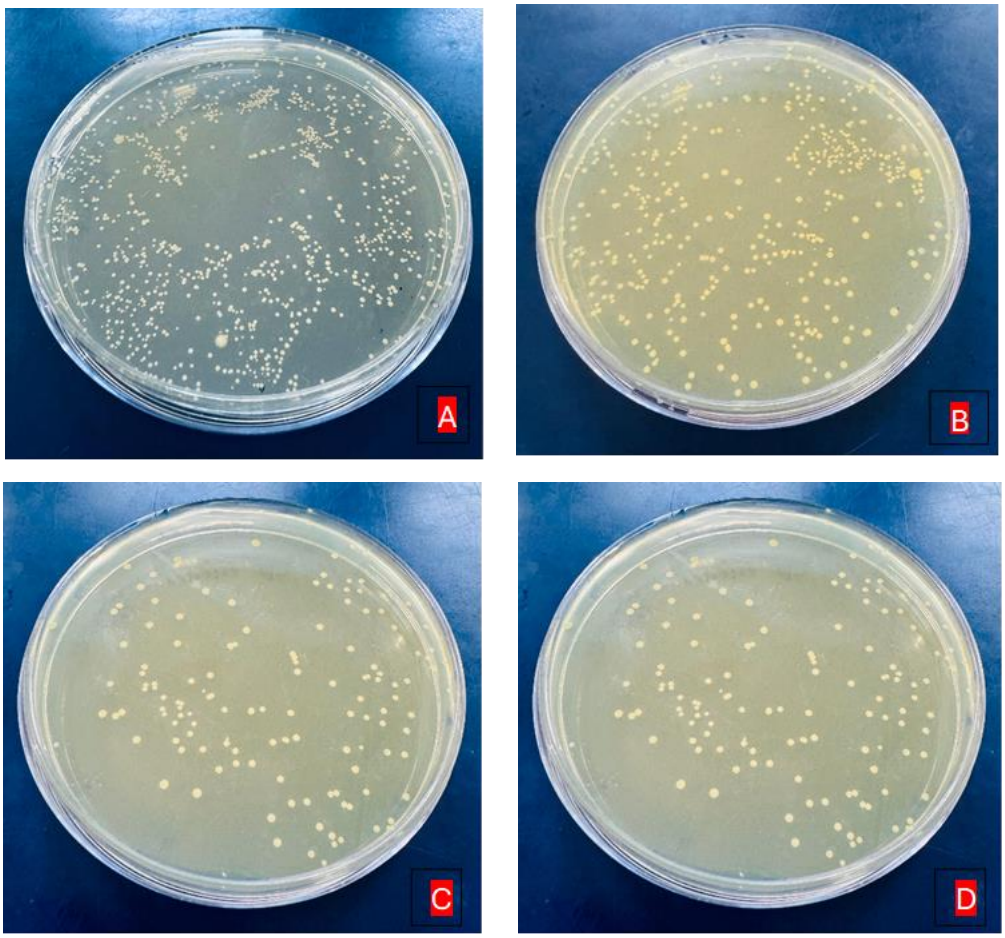

Fig. 8. Growth of lactic acid bacterial isolates (**A.** pH 2; **B.** 0.3% bile salt; **C.** 1% lysozyme; **D.** 20 mM concentration of hydrogen peroxide)

Auto-aggregation assay

All ten isolates demonstrated the capacity for auto-aggregation. The percent auto aggregation of these isolates is presented in Table (5). The values of auto aggregation vary between 45.13 and 78.70% following 5 hours of incubation. The maximum auto-aggregation value was recorded for isolate CM03 at 78.70%, whereas the minimum value was noted for isolate CM05 at 45.13%.

Table 5. Auto-aggregation properties of selected lactic acid producing bacterial isolates

Isolate No.	O.D at 600 Nm						% of auto aggregation				
	Initial/ 0hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
CM01	0.2087	0.1543	0.0896	0.0767	0.0621	0.0547	26.07	57.07	63.25	70.24	73.79
CM10	0.1896	0.1639	0.1325	0.1152	0.0923	0.0825	13.55	30.12	39.24	51.32	56.49
CM02	0.1890	0.1643	0.1225	0.0983	0.0734	0.0623	13.07	35.13	47.99	61.16	67.05
CM03	0.1967	0.1432	0.1213	0.0823	0.0524	0.0419	27.20	38.33	58.16	73.36	78.70
CM04	0.2116	0.1744	0.1325	0.0832	0.0623	0.0512	17.58	37.38	60.68	70.56	75.80
CM05	0.2167	0.1990	0.1784	0.1663	0.1352	0.1189	8.17	17.67	23.26	37.61	45.13
CM06	0.1898	0.1645	0.1432	0.1218	0.0832	0.0745	13.33	24.55	35.83	56.16	60.75
CM09	0.2012	0.1642	0.1433	0.1287	0.1164	0.0989	18.39	28.78	36.03	42.15	50.84
CM07	0.2012	0.1850	0.1432	0.1012	0.0765	0.0674	8.05	28.83	49.80	61.98	66.50
CM08	0.1987	0.1656	0.1421	0.0982	0.0789	0.0521	16.66	28.49	50.58	60.29	73.78

Cell surface hydrophobicity assay

The 10 isolates percentages of cell surface hydrophobicity varied from 56.55 to 92.00% (Table 6 & Fig. 9). When tested with xylene, isolate CM02 had the maximum hydrophobicity, with a percentage of 92.00%, while isolate CM10 displayed the lowest value, at 56.55%. These results indicated that lactic acid bacteria typically have hydrophilic surface characteristics and that cell surface hydrophobicity is a strain-dependent property.

Table 6. Percentage of cell surface hydrophobicity shown by the selected lactic acid bacterial isolates

Isolate No.	O.D at 600 Nm		% of cell surface hydrophobicity
	Initial/ 0hr.	1 hr.	
CM01	0.1905	0.0376	80.33%
CM10	0.2065	0.0897	56.55%
CM02	0.2311	0.0185	92.00%

CM03	0.1804	0.0461	74.43%
CM04	0.2134	0.0321	85.02%
CM05	0.1876	0.0459	75.52%
CM06	0.2321	0.0278	88.02%
CM09	0.1989	0.0611	69.35%
CM07	.2121	0.0572	73.05%
CM08	0.2208	0.0354	84.02%

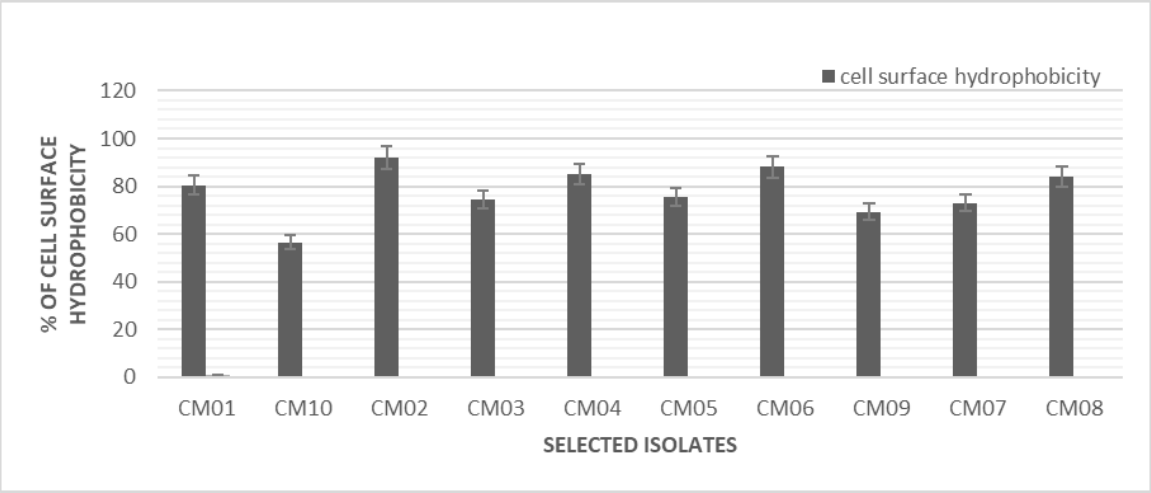

Fig. 9. Percentage of cell surface hydrophobicity shown by the selected lactic acid bacterial isolates

Antimicrobial activity

Table (7) summarizes the antibacterial activity of the selected lactic acid bacterial (LAB) isolates against four major aquatic pathogens: *Aeromonas jandaei*, *Aeromonas hydrophila*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Two isolates, CM09 and CM10, exhibited no inhibitory effect against any of the tested pathogens.

Isolates CM01 and CM04 did not inhibit *A. jandaei* but displayed moderate to strong antibacterial activity against *A. hydrophila*, *P. aeruginosa*, and *S. aureus*, with inhibition zones ranging from 19 to 22mm. In contrast, isolates CM02, CM05, and CM07 demonstrated broad-spectrum antibacterial activity, effectively inhibiting all four pathogens, with inhibition zones between 18 and 22mm.

The neutralized, cell-free supernatants of CM07 and CM08 also exhibited strong antibacterial activity, suppressing both Gram-negative (*P. aeruginosa* and *A. hydrophila*) and Gram-positive (*S. aureus*) pathogens. However, *A. jandaei* was not inhibited by CM08. Isolate CM06 displayed a narrower spectrum of activity, inhibiting *A. jandaei*, *A. hydrophila*, and *S. aureus*, but showing reduced efficacy against *P. aeruginosa*, with a minimal inhibition zone of approximately 13mm (Fig. 10).

Table 7. Inhibitory activity of the lactic acid bacterial isolates against selected pathogen

Isolates	Zone of inhibition shown against different pathogens tested			
	Aeromonas jandaei	***Aeromonas hydrophila***	***Pseudomonas aeruginosa***	***Staphylococcus aureus***
CM01	-	22	19	20
CM02	19	19	21	19
CM03	19	-	21	22
CM04	-	21	19	21
CM05	20	-	20	22
CM06	21	20	13	12
CM07	18	20	19	19
CM08	-	20	19	20

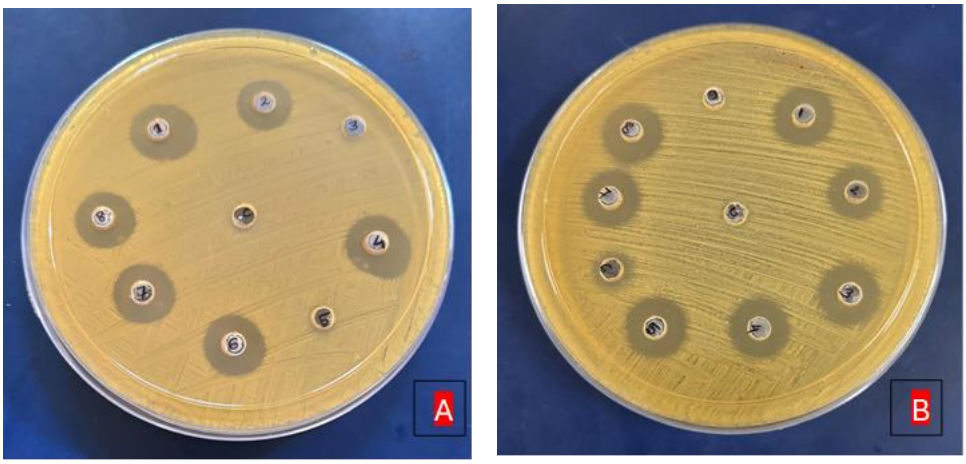

Fig. 10. Isolates showing inhibitory effect against A. *Aeromonas hydrophila*, B. *Staphylococcus aureus*

Antibiotic susceptibility

The selected isolates were tested for susceptibility to vancomycin (10µg), tetracycline (10µg), chloramphenicol (10µg), streptomycin (25µg), rifampicin (15µg), co-trimoxazole (25µg), ciprofloxacin (5µg), and gentamicin (10µg).

As shown in Table (8) and Fig. (11), none of the isolates exhibited complete resistance to all antibiotics. Isolate CM08 displayed the broadest susceptibility profile, being sensitive to vancomycin and gentamicin and showing intermediate sensitivity to several others, including streptomycin and ciprofloxacin. In contrast, isolate CM03 showed resistance to most antibiotics, with susceptibility limited to chloramphenicol and rifampicin. Similarly, isolates CM02 and CM07 exhibited narrow susceptibility, responding only to chloramphenicol and rifampicin while showing resistance to most other agents.

Ciprofloxacin and co-trimoxazole showed the highest resistance rates, with 62.5 and 75% of isolates resistant, respectively. Gentamicin also demonstrated reduced efficacy, with multiple isolates displaying intermediate or resistant profiles. In comparison, chloramphenicol and rifampicin were the most effective, with the majority of isolates showing susceptibility. Tetracycline displayed moderate activity, although resistance was recorded in isolates CM03, CM05, CM07, and CM08.

Table 8. Susceptibility of the lactic acid bacterial isolates to different antimicrobial agents

Isolate	Susceptibility of the isolates to different antimicrobial agents															
	Vancomycin (VA)		tetracycline (TE)		Chloramphenicol (C)		Streptomycin (S)		Rifampicin (RIF)		Co-Trimoxazole (COT)		Ciprofloxacin (CIP)		Gentamycin (GEN)	
	ZOI (mm)	Inference	ZOI (mm)	Inference	ZOI (mm)	Inference	ZOI (mm)	Inference	ZOI (mm)	Inference	ZOI (mm)	Inference	ZOI (mm)	Inference	ZOI (mm)	Inference
CM01	13	R	25	S	26	S	14	R	41	S	16	I	-	R	21	S
CM02	-	R	22	S	28	S	11	R	34	S	-	R	-	R	18	I
CM03	-	R	-	R	25	S	10	R	33	S	-	R	12	R	15	R
CM04	22	S	14	R	22	S	16	I	29	S	14	I	-	R	22	S
CM05	16	I	10	R	19	I	14	I	17	I	-	R	16	I	20	I
CM06	13	R	23	S	21	S	15	R	27	S	22	S	-	R	18	I
CM07	-	R	-	R	24	S	10	R	38	S	-	R	14	R	16	I
CM08	23	S	15	R	16	I	17	I	18	I	19	I	17	I	21	S

Hemolytic activity

On sheep blood agar, all eight lactic acid bacterial (LAB) isolates exhibited γ-hemolytic activity, indicating no hemolysis. The absence of hemolytic activity suggests that these isolates are non-virulent, supporting their suitability as potential probiotic candidates.

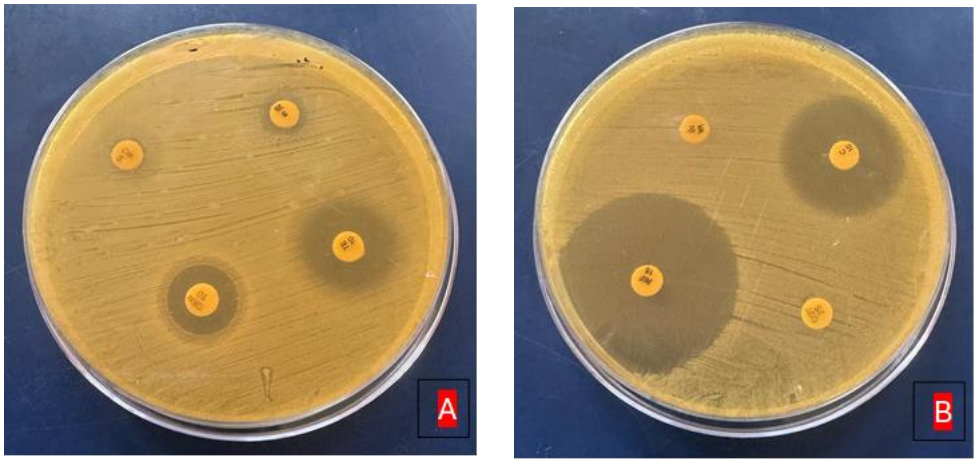

Fig. 11. Antimicrobial susceptibility of the lactic acid bacterial isolates. The clear zones surrounding the antibiotic discs indicate the sensitivity against antimicrobial agents.

Molecular characterization of the isolates

DNA extraction and quantification

The purity of extracted DNA was considered acceptable when the 260/ 280nm OD ratio was approximately 1.8. The DNA extracts from the eight selected potential probiotic isolates showed OD ratios ranging from 1.79 to 1.85 and concentrations between 60 and 150ng/ μL.

PCR amplification, sequencing of the 16S rRNA gene, and molecular phylogeny

PCR amplification of the 16S rRNA gene from all isolates produced amplicons of 1,400– 1,600bp (Table 9 & Fig. 12). BLASTn analysis identified the isolates as *Lactobacillus reuteri*, *Limosilactobacillus reuteri*, *Enterococcus faecalis*, and *Enterococcus mundtii*.

Phylogenetic analysis was performed on 22 nucleotide sequences, with 1,461 positions in the final dataset, using the maximum likelihood (ML) method. The sequences obtained in this study, submitted to GenBank under accession numbers PV973010–PV973017, formed distinct clades with corresponding reference sequences of *L. reuteri*, *L. reuteri* (Limosilactobacillus), *E. faecalis*, and *E. mundtii* from the GenBank. These associations were strongly supported by bootstrap values of 95– 100% (Fig. 13).

Table 9. Identified potential probiotic isolates by 16S rRNA gene sequencing and their Genbank accession numbers

Sl.no	Sample ID.	Location	Date of collection	Length(bp)	Probiotic bacterial species identified	GenBank accession no.
1	**CM01**	Darrang	28/12/2023	1445	*Lactobacillus reuteri* isolate	PV973010

2	**CM02**	Darrang	22/12/2023	1410	*Limosilactobacilus reuteri* isolate	PV973011
3	**CM03**	Morigaon	12/01/2024	1437	*Limosilactobacilus reuteri* isolate	PV973012
4	**CM04**	Nalbari	06/04/2024	1423	*Enterococcus faecalis* isolate	PV973013
5	**CM05**	Morigaon	28/01/2024	1500	*Lactobacillus reuteri* isolate	PV973014
6	**CM06**	Darrang	28/12/2023	1441	*Limosilactobacilus reuteri* isolate	PV973015
7	**CM07**	Nalbari	06/04/2024	1448	*Lactobacillus reuteri* isolate	PV973016
8	**CM08**	Morigaon	12/01/2024	1583	Enterococcus *mundtii* isolate	PV973017

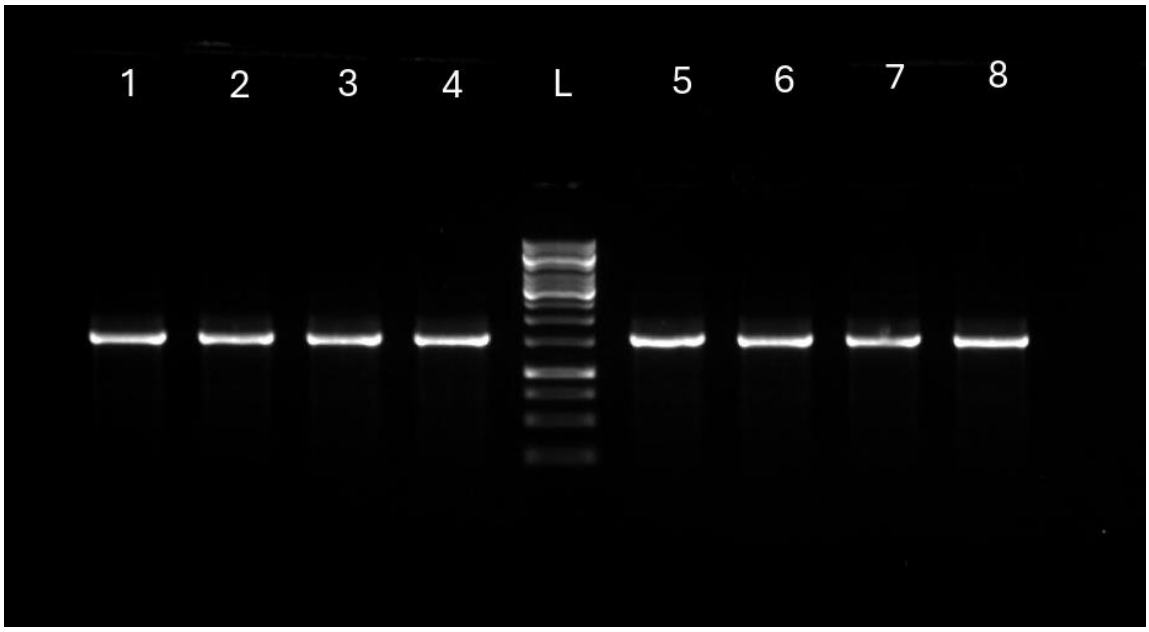

Fig. 12. Identification of lactic acid bacterial *16S rRNA* gene of the isolates by PCR. Lane 1-4- Samples, Lane 5- 1 kb ladder (Himedia, MBT051-200LN). Lane 6-8- Isolated sample

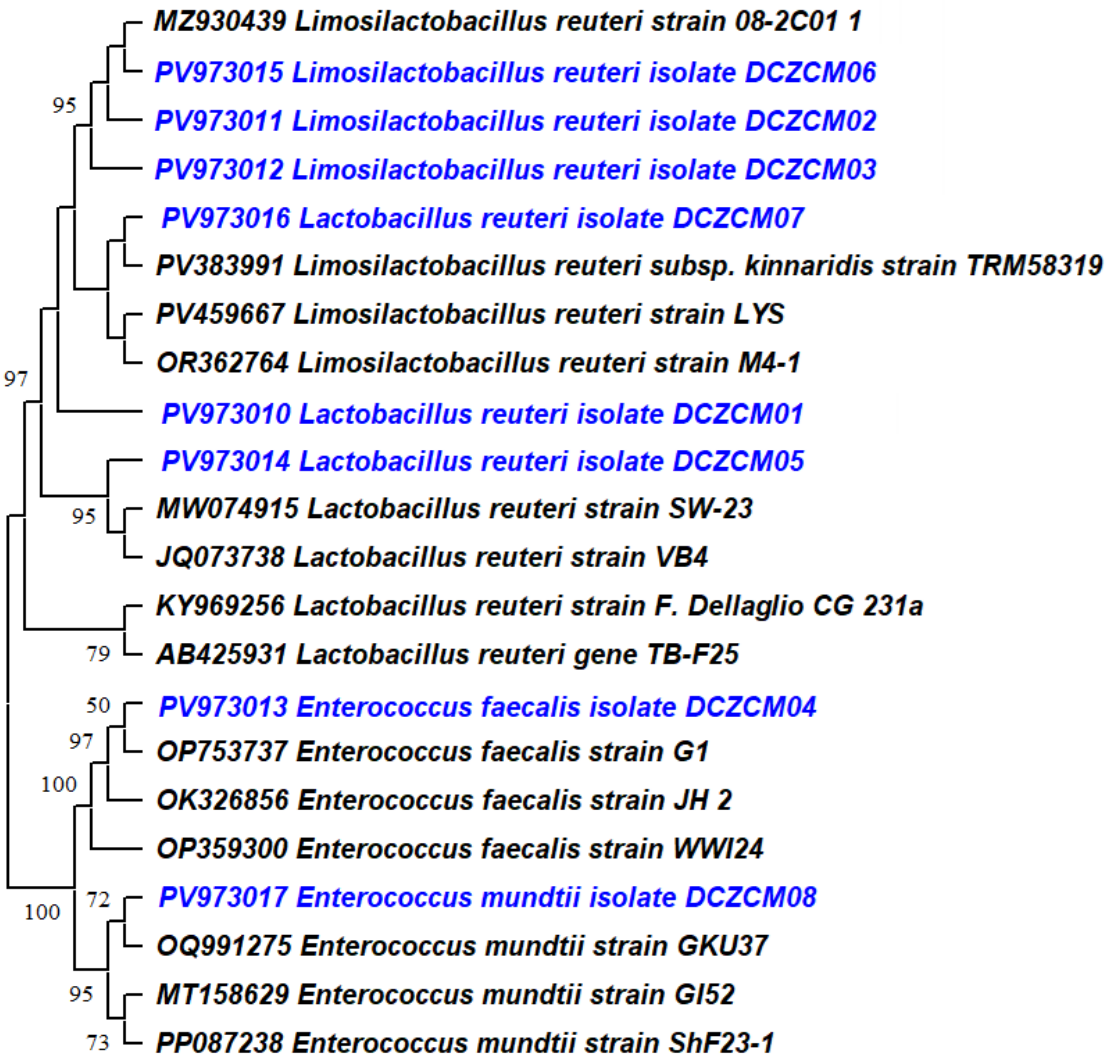

Fig. 13. Maximum likelihood phylogeny inferred by using method and Kimura 2-Parameter model, as implemented in MEGA11 **(Tamura *et al*., 2021)**. The bootstrap consensus tree was derived from 1,000 replicates.

DISCUSSION

For a bacterial strain to be classified as a probiotic, it must meet specific scientific criteria **(Byakika *et al*., 2019; Binda *et al*., 2020)**. The capacity to withstand low pH and high bile salt concentrations is one of the most important characteristics **(Puvanasundram *et al*., 2021; Amenu & Bacha, 2023; El Far *et al*., 2024)**. To pass through the stomach, probiotics must overcome both physical and chemical barriers. They require high bile salt concentrations to thrive in the small intestine **(Ruiz-Ramírez *et al*., 2023)**. Before colonizing GIT epithelial cells, lactic acid bacteria (LAB) must first transit

through the upper GIT, which can be as acidic as pH 2–4 for up to 3 hours at 37°C **(Thakkar *et al.*, 2015; Ruiz-Gonzalez *et al.*, 2024)**.

In this investigation, the isolates tested showed varying levels of viability under acidic conditions (pH 2) and 0.3% bile. At pH 2 and 0.3% bile salt, isolates CM01, CM06, CM07, and CM08 had more than 90% viability. High survival rates of LAB, particularly *Lactobacillus reuteri*, have been observed at a stomach pH of 2.0 and a bile concentration of 0.3% **(Liu & Bangash, 2024)**.

The 13 samples found to be acid- and bile-tolerant were subjected to lysozyme treatment (100mg/ L) for 1 hour. All isolates showed good lysozyme tolerance, indicating a high level of lysozyme resistance. This suggests that the isolates could survive in saliva, where the maximum lysozyme concentration used to simulate *in vivo* conditions is 10mg/L **(Elshaghabee *et al.*, 2017)**. *Lactobacillus reuteri* isolates (CM01 and CM05) showed survival rates of 87.20 and 91.60%, respectively. *Limosilactobacillus reuteri* isolates (CM02, CM03, CM06, and CM07) demonstrated survival rates between 87.10 and 93.30%. *Enterococcus faecalis* isolate (CM04) and *Enterococcus mundtii* isolate (CM08) showed survival rates of 95.10 and 87.10%, respectively. These findings are consistent with those of **Rajoka *et al.* (2017)**, who reported that LAB isolates had survival rates exceeding 91% in 1% lysozyme.

Hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) can accumulate under high oxygen conditions. These substances can cause oxidative stress, damage biological components, and disrupt normal physiological processes **(Amaretti *et al.*, 2013; Jomova *et al.*, 2023)**. LAB strains have been shown to possess strong antioxidant properties, making them promising candidates as natural, high-quality antioxidants **(Mishra *et al.*, 2015; Bryukhanov *et al.*, 2022)**. These probiotics may help eliminate H_2O_2 and other ROS from the host's stomach, reducing oxidative damage to host cells and potentially lowering the risk of some chronic diseases **(Amaretti *et al.*, 2013)**.

In the present study, *L. reuteri* isolates (CM01 and CM05) showed survival rates of 68.00 and 72.00%, respectively. *L. reuteri* isolates (CM02, CM03, CM06, and CM07) demonstrated survival rates between 51.20 and 72.00%, while *E. faecalis* isolate (CM04) and *E. mundtii* isolate (CM08) showed survival rates of 68.40 and 60.20%, respectively, after 2 hours of incubation with 20 mM H_2O_2. These findings agree with **Zhou *et al.* (2022)**, who found that *Lactobacillus* isolates had survival rates of 85.30 and 24.74% after 2 hours of incubation with 10 mM and 20 mM H_2O_2, respectively.

Auto-aggregation is a key trait of probiotic bacteria, promoting adhesion to intestinal epithelial cells and aiding effective colonization of the gastrointestinal tract **(Juntarachot *et al.*, 2023)**. The ability of probiotics to co-aggregate with pathogenic bacteria can also help form a physical barrier on the mucosal surface, limiting pathogen attachment and invasion. These interactions are critical for competitive exclusion of harmful microorganisms and maintenance of intestinal homeostasis **(Hameed *et al.*, 2023; Dabous *et al.*, 2024)**. In this investigation, *L. reuteri* strain CM03 showed a high

auto-aggregation capacity of 78.70%. **Divisekera *et al*. (2019)** reported that isolate RV19 (*L. lactis*) achieved the highest auto-aggregation rate (68.6%) after 5 hours of incubation. **Somashekaraiah *et al*. (2019**) reported an auto-aggregation rate of 78.95%, while **Zeng *et al*. (2020)** found rates between 85.20 and 88.01% after 4 hours.

Cell surface hydrophobicity influences bacterial adhesion to host surfaces and may improve persistence in the gastrointestinal tract, enhancing potential health benefits **(Okochi *et al*., 2017)**. In this study, *L. reuteri* (CM02) exhibited a total cell surface hydrophobicity of 92.00%, indicating strong adhesion potential. These results align with prior findings that certain probiotic *Bacillus* strains display cell surface hydrophobicity often exceeding 50% **(Mladenović *et al*., 2020; Nwagu *et al*., 2020; Pełka *et al*., 2025)**. **Jena *et al*. (2013)** reported that *L. helveticus* PJA had a hydrophobicity of 78.3%, while **Vincetha *et al*. (2016)** and **Divisekera *et al*. (2019)** found values ranging from 76% to those as low as 45.2%, depending on the strain tested.

Probiotic microorganisms produce bioactive compounds—such as organic acids, hydrogen peroxide, and bacteriocins—that inhibit pathogen growth **(İspirli *et al*., 2015)**. LAB are particularly noted for their strong antagonistic effects against a wide range of gastrointestinal pathogens **(Latif *et al*., 2023)**. Using probiotic strains with well-characterized antimicrobial profiles is a strategic way to boost host immunity and promote microbial balance in the gut **(Sachdeva *et al*., 2025)**. In this study, the selected LAB showed varied antagonistic effects against four fish pathogens. CM02 and CM05 exhibited broad-spectrum antibacterial activity, with inhibition zones of 18– 22mm against most pathogens. CM01 and CM04 were ineffective against *Aeromonas jandaei* but active against other pathogens. CM08 also showed no activity against *A. jandaei* but inhibited the others, while CM06 had reduced activity against *Pseudomonas aeruginosa*. These findings highlight CM02, CM05, and CM07 as promising candidates with strong antibacterial potential. The results are consistent with previous studies on *L. lactis* from *Oncorhynchus mykiss* **(Pérez-Sánchez *et al*., 2011)** and *Oreochromis niloticus* **(Kaktcham *et al*., 2017)**, as well as research on *E. faecalis*, *L. lactis*, and *L. fermentum* from *O. niloticus* **(Reda *et al*., 2018)**. LAB's antagonistic effects are linked to production of bacteriocins, organic acids, hydrogen peroxide, and enzymes **(Gonçalves *et al*., 1997; Lin *et al*., 2013)**.

LAB isolated from fish guts displayed varied antibiotic resistance, suggesting potential intrinsic or adaptive mechanisms **(Sharma *et al*., 2014)**. Antibiotic tolerance in probiotics is undesirable because of the risk of transferring resistance genes to pathogens. Safe use in aquaculture therefore requires phenotypic and molecular testing to confirm the absence of transferable resistance **(Ashraf & Shah, 2011)**. In this study, LAB isolates showed the highest susceptibility to chloramphenicol (87.50%) and rifampicin (87.50%), followed by tetracycline (50%) and gentamycin (50%). Moderate susceptibility was observed for streptomycin (37.50%), vancomycin (37.50%), and co-trimoxazole (25%), with the lowest rate for ciprofloxacin (12.50%). All isolates were resistant to at least one

antimicrobial agent, highlighting the need for careful screening to prevent resistance transfer **(Jian *et al.*, 2021; Mujawar *et al.*, 2021)**. Many LAB species, especially *Lactobacillus*, possess intrinsic resistance to vancomycin, which distinguishes them from other Gram-positive bacteria **(Gupta *et al.*, 2021)**.

The European Food Safety Authority (EFSA) recommends assessing hemolytic activity in bacterial isolates intended for food use. GRAS (Generally Recognized as Safe) or QPS (Qualified Presumption of Safety) strains should undergo this evaluation **(FAO/WHO, 2006; Subasinghe *et al.*, 2017)**. In this study, hemolytic activity of eight isolates was tested on 5% sheep blood agar. None showed α- or β-hemolysis; all were γ-hemolytic, indicating no hemolytic activity. **Oh and Jung (2015)** reported γ-hemolysis in *Lactobacillus* from traditionally fermented millet-based alcoholic beverages, consistent with these findings. Similarly, **Wang *et al.* (2018)** observed minimal hemolytic activity in *Lactobacillus* from spontaneously fermented Chinese non-dairy foods.

CONCLUSION

This study successfully isolated and characterized indigenous bacterial strains from the gastrointestinal tract of *Clarias magur* (Hamilton, 1822), identifying four candidates with promising probiotic potential: *Lactobacillus reuteri* (CM01, CM05, CM07), *Limosilactobacillus reuteri* (CM02, CM03, CM06), *Enterococcus faecalis* (CM04), and *Enterococcus mundtii* (CM08). These isolates exhibited key functional traits, including acid and bile tolerance, lysozyme resistance, hydrogen peroxide resistance, auto-aggregation capacity, cell-surface hydrophobicity, antimicrobial activity, antibiotic susceptibility profiles, and absence of hemolytic activity. Collectively, these attributes indicate their potential to colonize the host gut, aid nutrient digestion, and enhance disease resistance. The findings underscore the value of host-associated microbiota as a source for developing species-specific probiotics in aquaculture. Consequently, these four bacterial strains—either individually or in combination for enhanced efficacy against aquatic pathogens—represent suitable candidates for probiotic development in aquaculture.

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