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In vitro Screening and Evaluation of Probiotic Potentials of Lactic Acid Bacteria Isolated from Dairy Products

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ABSTRACT

Background: Lactic acid bacteria (LAB), as probiotics, which play a key role in improving gut health by maintaining microbial balance in the gut and also enhance the food preservation. LAB are widely recognized for their benefit for GIT, including improving intestinal microbial balance. The lactic acid and bioactive compounds produced by these microbes are extensively used in fermented probiotic formulations.

Methods: The LAB are grown in de Man, Rogosa and Sharpe agar (MRS). The morphological and biochemical tests was carried out for Preliminary identification, followed by molecular identification using 16S rRNA gene sequencing. The probiotic properties were assessed based on acid tolerance, bile salt resistance and antimicrobial activity against selected pathogenic bacteria.

Result: Overall, 52 strains of LAB were isolated, from them 11 were selected. They tolerate high acidic pH and bile salts, indicating their ability to survive under gastrointestinal conditions. Because of lactic acid production they shown strong antimicrobial activity against selected pathogens. These results demonstrate the potential of LAB isolates from dairy sources for use in probiotic formulations and food bio preservation. Further studies are recommended to evaluate their efficacy in clinical applications.

Key words: Antimicrobial activity, Gastrointestinal tract, Lactic acid bacteria, Probiotics.

INTRODUCTION

Probiotics are the products which contain good bacteria which helps in the health improvements such as gastrointestinal tract. “This group of bacteria, known as Lactobacilli, possesses unique metabolic features and plays a key role in maintaining beneficial intestinal microflora” (Azadnia and Khannazer, 2009; Bouchibane *et al.,* 2022). The dairy products like yogurt, buttermilk, Kimchi, curd *etc* are rich sources of beneficial lactic acid bacteria (Hadi *et al.,* 2023). LAB is a group of anaerobic, non-motile, Gram-positive bacteria and are non-spore forming (Palomo *et al.,* 2014).

The term LAB is classified in the order *Lactobacillus's*, which includes *Lactobacillus, Lactococcus, Pediococcus, Leuconostoc* and *Streptococcus.* They contribute to enhance the taste and quality of food and they've the capability for bio preservation which increases the food shelf life (Tabanelli *et al.,* 2014; Prabhurajeshwar, 2020). They also produce the bacteriocins which also controls the intestinal infections, lowers the blood ammonia level and furnishing effective resistance against gastric acid (Keresztény *et al.,* 2024).

MATERIALS AND METHODS

Samples collection and enrichment

Different dairy products like curd, butter milk, cheese and yogurt samples from various vendors of Bagalkot city, as well as homemade samples were collected from nearby cities of Bagalkot district from the period of July 2024 to June 2025. The samples were collected in containers aseptically and immediately kept in an icebox until brought to the of laboratory of Biotechnology, department of Davangere University, Davangere, Karnataka, India and stored at 4°C further analysis. The 90 mL sterile normal saline is taken and of 10 mL of sample is suspended in it, later enriched in MRS broth for 18 to 24 hours at 37°C (Zhang *et al.,* 2022).

Isolation and Identification of LAB

The collected samples were serially diluted and were streaked on the plate containing MRS agar medium (Hi-Media, India), which are incubated at the 37°C for 21 to 24 hours, for further analysis. Colonies are selected based on variations in morphology; pigmentation, shape and size are sub cultured on MRS agar. The selected colonies were preserved by streaking and maintained at 4°C on MRS agar slant (Zhao *et al.,* 2024; Nurye, 2023).

Carbohydrate fermentation profile

The sugar fermentation for selected isolated strains was carried out by adding the specific sugar to the basal

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carbohydrate media. Gas is produced in the durham tubes and acid production was identified by a change in color (Bin *et al.,* 2018).

Monitoring growth at different temperature

The LAB are grown at varying temperatures to determine their optimal growth conditions. Utilizing MRS broth, multiple sterile flasks were inoculated with the selected LAB strains, to evaluate bacterial growth, culture tubes were maintained at temperatures of 10, 25, 30, 37 and 45°C for 48 hours and growth was quantified using optical density readings at 600 nm (Asadi *et al.,* 2022).

Hemolytic activity

Using a blood agar supplemented with 5% (w/v) sheep blood to test the hemolytic activity of isolated strains and the plates are incubated at 37°C for 48 hours. The hemolytic activity analyzed by examining the zones of lysis around the colonies. The strains which are exhibiting no-hemolytic activity were considered safe, as they did not display the potentially harmful effects associated with hemolytic activity. This ensures the strains are safety for food production or probiotic applications (Zhang *et al.,* 2022).

Characteristics of isolated LAB

Phenotypic characteristics of LAB include morphology, growth rate, acid tolerance, bile salt resistance and metabolic activities. These traits influence the survival and functionality of LAB within the gut environment (Azadnia *et al.,* 2009).

Effect of pH on the growth

From the 1% (v/v) fresh overnight culture, a single isolated colony was inoculated on MRS broth with pH adjusted between 2 to 8 and incubated at 37°C for 24 hours. Optical density at 600 nm was recorded to evaluate bacterial growth at different pH levels, with uninoculated broth used as the control (Dowarah *et al.,* 2018).

Effect of bile salt on LAB growth

To check the effective survival of the LAB stains on host's gut, probiotics must withstand both bile salts and the acidic environment of the GIT. Consequently, probiotic bacteria should be capable of enduring high bile salt concentrations (0.3% w/v) for at least 90 minutes. The isolates were cultured in MRS broth supplemented with bile salts at concentrations ranging from 0.5% to 2.5%. After incubation at 37°C for 18-24 hours, growth was assessed spectrophotometrically at 600 nm, with bile salt-free broth serving as the control (Prabhurajeshwar, 2019; Suez *et al.,* 2019).

Effect of NaCl tolerance on growth of LAB

Selected MRS broth with various concentrations of NaCl (1 to 6%) is used for growth of selected isolates. Each medium containing the isolates were inoculated into broth (10 mL per tube) and incubated at 37°C for 24 hours. Bacterial proliferation was measured by absorbance at 600 nm, with NaCl-free MRS broth included as a negative control (Dowarah *et al.,* 2018).

16s rRNA Based molecular confirmation of LAB

The isolated strains are confirmed by 16S rRNA sequencing which are isolated from different probiotic products (Holt *et al.,* 1994; Shettar *et al.,* 2026). Commercial sequencing services were obtained from the Institute of Barcode Biosciences Pvt. Ltd., Bangalore.

Test of antibiotic susceptibility

The disk diffusion technique, widely used for evaluating antibacterial susceptibility, was carried out in accordance with ISO 10932/IDF 233 standards. The chosen isolates were evenly streaked on agar plates prior to testing. The antibiotic disks of amikacin (10 mg), ciprofloxacin (5 mg), Linezolid (15 mg), clindamycin (30 mg), amoxiclav (10 mg), Tetracycline (30 mg), gentamicin (10 mg), Sulfamethoxazole (10 mg), vancomycin (30 mg). After incubation the inhibition zones of microbes were noted (Pan *et al.,* 2009). As per CLSI data the resistance and sensitivity pattern were interpreted (CLSI, 2023; Prabhurajeshwar, 2017).

Antimicrobial activity

To check the inhibitory compounds such as bacteriocins, organic acids and hydrogen peroxide produced by LAB was evaluated to check their antimicrobial potential using a modified agar well diffusion method. This approach was used to detect the production of inhibitory compounds such as bacteriocins, organic acids, and hydrogen peroxide (García-Cano *et al*., 2014). A cell-free supernatant (25 µL) was introduced into wells of 7 mm diameter prepared in agar plates previously swabbed with 1% (v/v) overnight cultures of the test pathogens. After incubation at 37°C for 24 hours, antimicrobial activity was determined by measuring the diameter of the inhibition zones (Rosenkilde *et al.,* 2024; Jadhav *et al*., 2026).

Test for minimum inhibitory concentration (MIC)

The MIC value of strain's antimicrobial compounds was determined to identify the strain's antimicrobial resistance against specific probiotic LAB *spp.*, The serial two-fold broth dilution (higher and lower) method is used for MIC assay of CFCS (cell-free culture supernatant). The MIC value was confirmed by measuring the absorbance of the pathogen at 600 nm, with LAB-free broth serving as the control (Pan *et al.,* 2009).

Statistical analysis

Data were statistically analyzed and results are expressed as the mean ± standard deviation (SD) of three independent experiments, with each experiment carried out in triplicate.

RESULTS AND DISCUSSION

Isolation and screening of lactic acid bacteria

The resulting isolated LAB colonies were labeled as CS-1, CS-2, CS-3 and so on. Out of 52, 11 isolates (CS1-CS11)

were selected and Gram stained. All isolates were observed to be non-motile and lacked endospore formation growth is recorded at 37°C in MRS medium (Table 1). Eleven purified colonies, exhibiting Gram-positive, bacilli and negative catalase activity, were subjected to further identification through microscopic and biochemical analyses in Table 2 and Fig 1.

Carbohydrate fermentation

The sugar fermentation profiles of different LAB isolates were evaluated by using different sugars. The strains ferment sugars, however, variations in sugar utilization were observed among the selected organisms, indicating the unique characteristics of each isolate (Table 2).

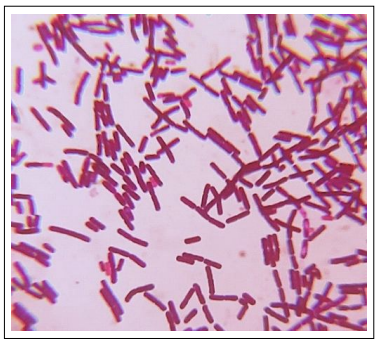


Fig 1: Gram's stain of LAB's isolate.

Table 1: Number of isolates from the probiotic samples.

Source	Total isolates	No. of LAB spp.
Curd	32	17
Yoghurt	2	1
Cheese	7	4
Butter and buttermilk	10	2
Sauerkraut	1	1
Total	52	25 (48.07%)

Preliminary identification of Lactic acid bacterial species

Selected isolate is identified by VITEK 2 Compact system (biome Rieux®). The technique used here is advanced colorimetric, the microbes are identified by biochemical tests. VITEK 2 system database results help in the identifications and classification of microorganisms.

Monitoring growth at different temperatures

The growth at different temperatures such as 10, 21, 37 and 42°C were assessed for selected LAB isolates. Growth at 10°C was observed in 25.78% of the isolates, whereas 44.3% exhibited growth at 37°C. Values were represented in means of three replicates; dissimilar letters show significant difference ($p<0.05$). Most isolates, including CS-2, CS-3, CS-7, CS-8 and CS-11, shows peak growth at Optimal temperature 37°C, suggesting they are well-adapted to human body temperature. All the isolates showed no growth at 10°C, Growth rate decreases at higher temperature of 42°C, though isolates like CS-8 and CS-9 shows moderate growth, suggesting some adaptability to slightly elevated temperatures (Fig 2).

Hemolytic activity

The hemolysis test revealed that most isolates (CS-1, CS-2, CS-3 and CS-11) were non-hemolytic, indicating safety for probiotic use. A few isolates (CS-4, CS-6, CS-7) showed β-hemolysis, suggesting pathogenic potential (Table 3).

Confirmation of isolated strain as LAB by 16S rRNA

The molecular identification is confirmed by 16S rRNA sequencing. It was commercially sequenced at Institute of Barcode Biosciences Pvt. Ltd at Bangalore. Phylogenetic analysis was performed. CS-2 sample was found to be *Lactococcus lactis* showed close similarity based on sequence homology and phylogenetic evaluation (Fig 3). The obtained 16S rRNA sequence was deposited in GenBank and provided with an accession number, PV125035.

Table 2: Morphology of Lactic acid bacteria isolated from the processed samples.

Selected LAB isolates	Gram stain	Shape	Color	Motility	Catalase	Carbohydrate fermentation test					
						Glucose	Fructose	Lactose	Galactose	Sucrose	Mannose
CS-1	Pos	C	Y	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
CS-2	Pos	DC	Cr	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Pos
CS-3	Pos	T	W	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Neg
CS-4	Pos	T	W	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Neg
CS-5	Pos	CC	Gr	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos
CS-6	Pos	CC	Gr	Neg	Neg	Pos	Pos	Neg	Pos	Pos	Pos
CS-7	Pos	C	W	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Pos
CS-8	Pos	DC	Gr	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
CS-9	Pos	DC	Gr	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
CS-10	Pos	C	W	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Pos
CS-11	Pos	Yeast	W	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos

Pos: Positive, Neg: Negative, C- Cocci, DC- Diplococcic, CC- Cocci in chains, Y- Yellow, C- Creamish, Gr- Greyish, T- Tetrad, W- White, Nm- Non motile.

Selection of potential species of LAB by various tests

Determination of optimal growth at different pH

All LAB isolates were exposed to acidic conditions at pH level from 1 to 4 and incubated at 37°C for 24 hours. Isolates CS-2, CS-3, CS-5, CS-6, CS-7 and CS-11 exhibited survival under these conditions. Among them, CS-2, CS-3, CS-5 and CS-7 demonstrated higher tolerance by surviving up to 3 hours in highly acidic environments. The remaining isolates showed decreased survival, failing to tolerate the low pH for longer than 90 minutes (Fig 4).

Table 3: Hemolytic activity of lactic acid bacteria.

Bacterial isolates	Hemolytic
CS-1	Non hemolytic
CS-2	Non hemolytic
CS-3	Non hemolytic
CS-4	β-hemolytic
CS-5	γ-hemolytic
CS-6	β-hemolytic
CS-7	β-hemolytic
CS-8	γ-hemolytic
CS-9	γ-hemolytic
CS-10	γ-hemolytic
CS-11	Non-hemolytic

Tolerance to bile salt

The strains showed good survival and growth across bile salt concentrations of 0.05-2.5%, indicating their tolerance to bile conditions. The survival rates under bile salt at concentration of >0.3% w/v for 11 isolated strains were tested. From the graph (Fig 5), isolates CS-2, CS-5, CS-6 and CS-7 show relatively high tolerance growth than others. This potentiality shows the potentiality of probiotic strains with the ability to persist and remain viable within the GIT.

Tolerance to NaCl

NaCl tolerance test of the 11 isolates showed that they were able to grown in different concentration ranges

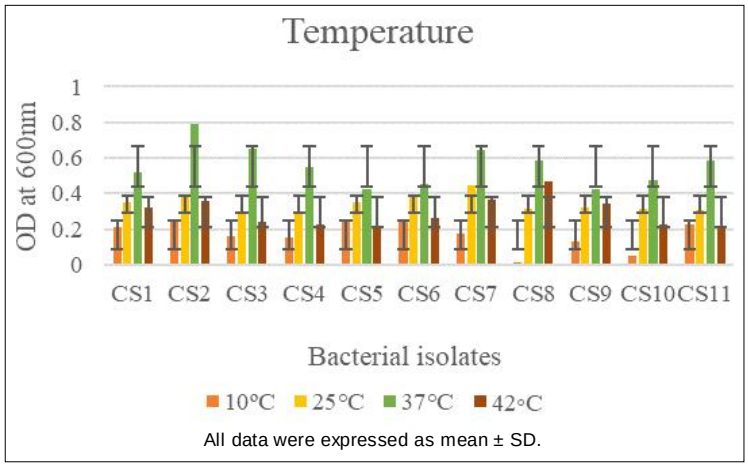


Fig 2: Effect of temperature on the survival rate or growth of bacterial isolates after 24 h of incubation.

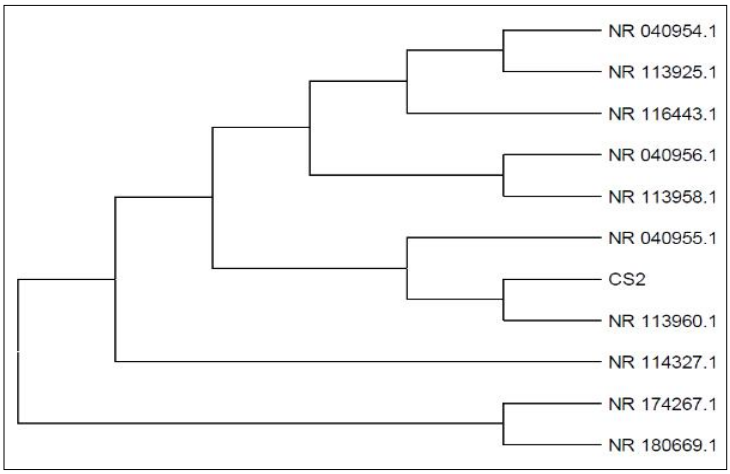


Fig 3: Phylogenetic tree based on neighbor-joining method of 16S rRNA gene sequencing of LAB isolate.

between 1 to 6%. Among these, strains CS-2 and CS-3 exhibited growth comparatively with other isolates (Fig 6).

Antibiotic susceptibility test

The LAB isolates CS-2, CS-3 and CS-5 were tested for antibiotic susceptibility using antibiotics like; amikacin, ciprofloxacin, clindamycin, erythromycin, ceftriaxone, amoxicillin, tetracycline, gentamycin and vancomycin. The isolate CS-2 exhibited the maximum level of sensitivity, with inhibition zones of diameter of 22 mm against amoxicillin and 21 mm against clindamycin. Isolate CS-3 showed an inhibition zone of 20 mm against ceftriaxone, while CS-5 isolate exhibited an zone inhibition of 20 mm against vancomycin. The results are tabulated in Table 4 and Fig 7.

Antimicrobial activity

Selected LAB's antimicrobial activity is examined for isolates by using modified agar well diffusion method. Pathogens such as *E. coli*, *Pseudomonas aeruginosa* and *S. aureus* and *C. albican's* are used for the test of antagonistic effects of LAB. From the isolated 11 LAB, the 3 LAB are selected for the antagonistic activity. The level of antagonistic activity differed among the LAB strains; however, the selected isolates exhibited inhibitory effects against all the tested indicator microorganisms (Table 5). The CS-2 isolate exhibited the highest level of antimicrobial activity (Fig 8).

Test for minimum inhibitory concentration (MIC)

The overall MIC result is concluded as, that all three samples (CS-2, CS-3 and CS-6) exhibit concentration-dependent antimicrobial activity against both Gram-positive and Gram-negative bacteria. Among them, the highest inhibitory effect is shown by CS-2, particularly against *Pseudomonas aeruginosa* and *Escherichia coli*, achieving complete inhibition at lower concentrations. CS-3 displayed

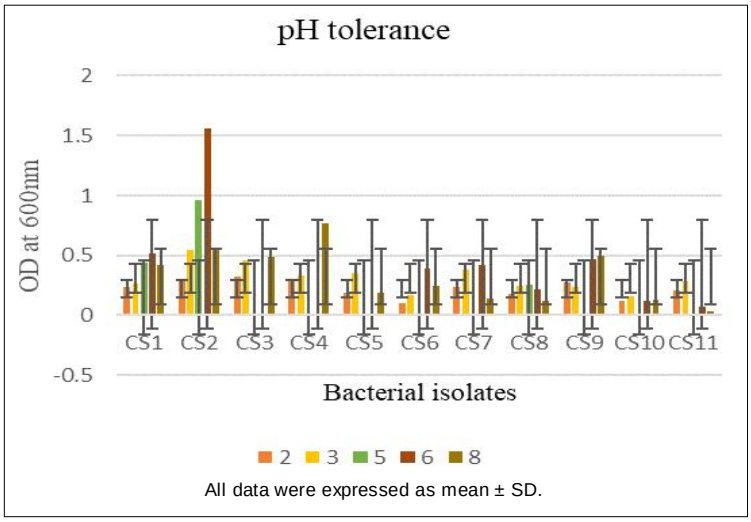


Fig 4: Effect of pH on the growth of LAB isolates after 24 hrs. of incubation at 37°C.

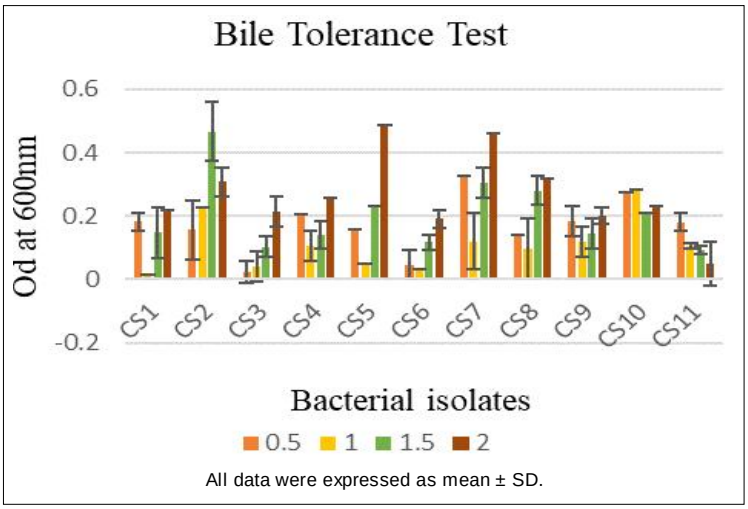


Fig 5: Effect of bile salt on the survival rate of bacterial isolates after 24 hrs of incubation at 37°C.

superior activity against *Enterococcus faecalis*, while CS-6 showed comparatively lower efficacy. *Staphylococcus aureus* appeared more resistant than other organisms. These findings suggest that the tested samples possess significant broad-spectrum antimicrobial properties, with CS-2 being the most potent isolate (Fig 9).

The present study, selective MRS agar proved for the isolation and functional attributes of LAB from commercially available probiotic supplements, as it facilitated efficient LAB isolation by promoting their growth while suppressing competing microbiota. This observation is considered with earlier findings Yadav *et al.* (2024) confirming the suitability of MRS for LAB growth.

Table 4: Antibiotic susceptibility test for selected LAB isolates.

Antibiotics	CS2	CS2	CS5
Amikacin	18±0.01	17±0.01	10±0.01
Ciprofloxacin	20±0.03	19±0.03	14±0.03
LE	18±0.02	18±0.02	18±0.02
Clindamycin	21±0.04	18±0.04	16±0.04
Erythromycin	14±0.02	16±0.02	19±0.02
Ceftriaxone	20±0.03	20±0.03	18±0.03
Amoxicillin	22±0.04	16±0.04	19±0.04
Tetracycline	18±0.05	19±0.05	14±0.05
Gentamycin	19±0.02	15±0.02	19±0.02
Sulfamethoxazole	20±0.02	18±0.02	13±0.02
Vancomycin	18±0.01	19±0.01	20±0.01

A Considerable variation in LAB diversity and viable counts was observed among the analyzed supplements, highlighting the lack of uniform standardization in commercial probiotic formulations and also reported by Hill *et al.* (2014). Such differences in strain composition and population density are significant, as probiotic efficacy is both strain-specific and dose-dependent (FAO/WHO, 2002; Sanders *et al.,* 2019).

Phenotypic, biochemical and 16S rRNA gene sequencing revealed a diverse LAB community, including *Lactococcus, Lactobacillus, Pediococcus, Leuconostoc* and *Bifidobacterium*. The findings are similar with previous studies on commercial probiotics (Ouwehand *et al.,* 2002). Additionally, the consideration of species of *Enterococcus* and *Streptococcus* indicates the expanding diversity of probiotic strains. However, their use warrants careful safety evaluation due to potential opportunistic pathogenicity (Hill *et al.,* 2014; Sanders *et al.,* 2019).

Functional characterization showed that most isolates tolerated acidic conditions (pH 2.0-4.0) and bile salts, indicating their ability to survive gastrointestinal transit, this observation aligns with probiotic selection criteria (FAO/WHO, 2002). Several isolates exhibited antibacterial and antifungal activity against common pathogens, due to the production of organic acids, hydrogen peroxide and bacteriocins similar observations are considered by Havenaar and Huis (1992); Ouwehand *et al.* (2002).

Table 5: Antagonistic activity of LAB isolates against test pathogens.

LAB	*E. coli*	*P. aeruginosa*	*S. aureus*	*C. albicans*	*Mucor ssp*
Control	21 mm	15 mm	19 mm	21 mm	9 mm
CS-2	20 mm	9 mm	20 mm	19 mm	11 mm
CS-3	29 mm	8 mm	18 mm	21 mm	10 mm
CS-7	00 mm	0 mm	18 mm	17 mm	6 mm

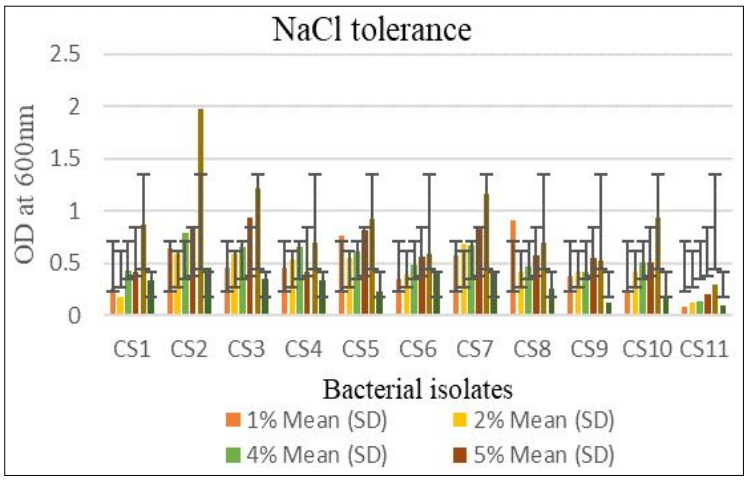


Fig 6: NaCl % on the survival rate of bacterial isolates after 24 hours of incubation.

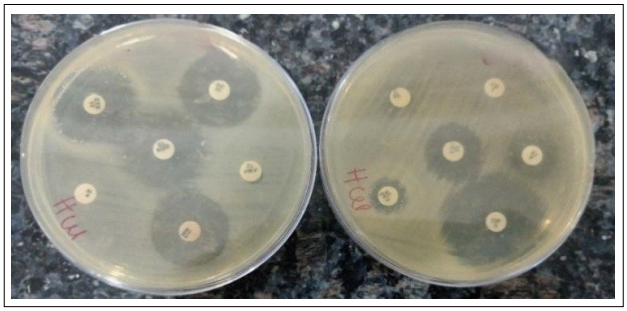
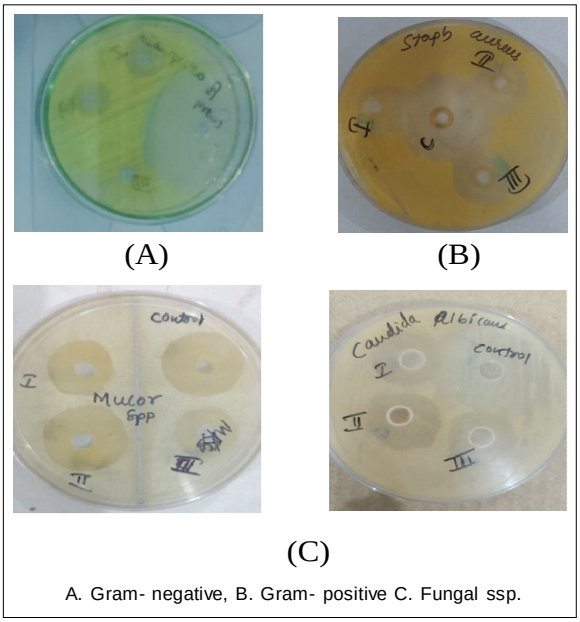


Fig 7: Antibiotic susceptibility test for isolated organisms.



A. Gram- negative, B. Gram- positive C. Fungal ssp.

Fig 8: Antagonistic activity of LAB against test pathogens.

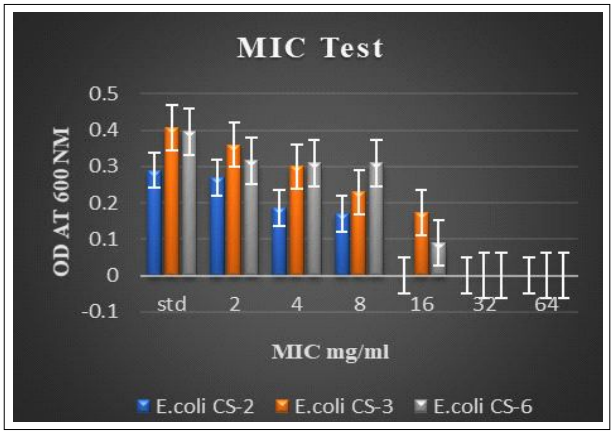


Fig 9: The MIC test by double dilution method.

Notably, strains CS-2 and CS-3 demonstrated strong, strain-specific antimicrobial efficacy, underscoring the functional diversity of probiotic LAB. The findings further highlight the importance of strain evaluation for probiotic efficacy (Sanders *et al.,* 2019).

CONCLUSION

Isolation and screening of lactic acid bacteria (LAB) from various commercial probiotic supplements revealed a wide variety of LAB species exhibiting important probiotic properties, including tolerance to acidic and bile environments, antimicrobial activity and surface adhesion capabilities. These results offer valuable insights into the functional potential of LAB in probiotic products and emphasize the importance of further *in vivo* studies to assess their efficacy and safety. Future research should involve comprehensive functional analyses of LAB strains in clinical settings to validate their therapeutic benefits.

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Disclaimers

The views and conclusions expressed in this article are solely of the authors and do not necessarily represent the views of their affiliated institutions.

Informed consent

Not applicable.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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