

## ASSESSMENT OF PROBIOTIC CHARACTERISTICS OF *LACTOBACILLUS CURVATUS* AND *LEUCONOSTOC MESENEROIDES* FROM INDIAN FERMENTED NON-DAIRY FOODS

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ABSTRACT

Fermented foods are being broadly explored for different strains of lactic acid cultures which can enhance the organoleptic and nutritional quality of food and promote health of the consumer. Current study emphasizes on the isolation, screening, identification of lactic acid bacteria (LAB) with probiotic properties from non-dairy traditionally fermented Indian foods. In this study, isolation of LAB was performed from fermented cooked whole wheat extract. Total 33 isolates were obtained that displayed Gram positive character, catalase and oxidase negative test and showing  $\gamma$ -hemolysis pattern. The selected isolates were identified based on 16S rRNA sequencing after screening for their probiotic characters. The isolate 'GC03' was identified as *Latilactobacillus curvatus* (100% similarity) and isolate 'GC04' as *Leuconostoc mesenteroides subsp. mesenteroides* (99.35 % similarity) with type strain. Survivability of isolates in gastric environment was evaluated by acid and bile tolerance. Highest percent survivability at pH 2 was seen at 17.16 % and 21.11 % for *Latilactobacillus curvatus* and *Leuconostoc mesenteroides* respectively. Also, *Lat. Curvatus* GC03 showed 35.02 % bile tolerance at 0.3% bile whereas, *Leuconostoc mesenteroides* GC04 28.63 % tolerance. Isolates were tolerant up to 8% NaCl concentration and tolerate temperatures up to 45°C. The significant antibacterial and antibiofilm activity of cell-free supernatants of both the LAB against *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* was observed. The isolates exhibited typical probiotic characters (acid and bile tolerance) along with considerable antibacterial and anti-biofilm activity and could be exploited further for their potential in human endeavour.

**Keywords:** Lactic acid bacteria, whole wheat extract, probiotic

INTRODUCTION

With the growing demand for processed, preserved food and food products across markets, human health is at a greater risk. Food being the sole source of nourishment should provide complete nutritional benefit. A large number of 'good bacteria' are found in food products which can confer additional health benefits to human. Right from our birth we comprise of gut microflora which keeps on altering with changes in food habits. A number of life-style related disorders are linked to dysfunction of gut flora. Fermented foods across the globe like 'kimchi, kombucha, koozu, fermented pickles, idli, dosa, cheeses, yoghurt, kefir, mawe' etc all are categorised as functional foods that confer health beyond its nutritional content (Koirala & Anal, 2021; Syngai et al., 2016).

Probiotic microorganisms have been largely studied. They are found in diverse food samples (dairy and non-dairy). World Health Organisation (WHO) defines probiotic lactic acid bacteria (LAB) as living bacteria, when provided in optimal amounts, confer health benefits on the host (FAO/WHO 2001). Lactic acid bacteria colonising/inhabiting gut can be broadly categorised as autochthonous-ones which reside the gut for a longer time and allochthonous which form the transient flora (Ilango & Antony, 2021). Source and type of food consumed, fermented dairy/non-dairy, cereal/pulses, plant/animal-based; method of preparation and native differences across land decide the diversity in lactic acid bacteria colonising gastro-intestinal tract. Though varied, these organisms share certain features by being Gram positive, catalase and oxidase negative and display tolerance to acid, bile and salt concentration to name a few. These organisms confer numerous health benefits. Regarded as 'GRAS' – generally regarded as safe they provide immunomodulatory effect where they potentially block the settlement of pathogens in intestinal tract. Numerous LAB are identified for their antimicrobial activity against pathogens *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* (Hoxha et al., 2023).

LAB synthesize number of metabolites which show beneficial effect on host. These metabolites are termed as *postbiotic metabolites* (Moradi et al., 2021). Postbiotics include preparations like dead cells, cell free extract/supernatant, lysed-cell extract, specific metabolites produced by LAB like short chain fatty acids, exopolysaccharides, vitamins, antimicrobial substances and proteins. Postbiotic metabolites show potential bioactivities such as anti-inflammatory

effect, antioxidant activity, cytotoxic effect on carcinogenic cell lines (HT-29, CaCo2, HeLa).

Traditionally fermented foods have been consumed for centuries and are known to harbour a diverse range of beneficial microorganisms. This study aimed to isolate and screen lactic acid bacteria (LAB) from non-dairy, conventionally prepared Indian food samples and to assess their distinctive probiotic properties. The isolates were taxonomically identified, and their potential for cholesterol reduction, as well as evaluation of their antibacterial and antibiofilm activities against common pathogens.

MATERIAL AND METHODS

Sample collection

Fermented foods like cooked fermented wheat extract (traditionally used for Maharashtrian delicacy *kurda*), Anarsae and Jalebi (Indian confectionaries) were collected from Indian households [18.6492° N, 73.7707° E; 18.5089° N, 73.9259° E for Nigdi and Hadapsar respectively in Pune district, Maharashtra State, India]. Fermented cooked wheat extract was prepared where the whole wheat is washed thoroughly, soaked in water for three days, performing daily water change. On the fourth day wheat is ground into a mash, the extract is filtered and cooked until thick consistency is attained. Food sample was collected in sterile eppendorf tubes and subjected for isolation in laboratory. Anarsae and jalebi were prepared traditionally from refined wheat flour.

Media preparation and culture conditions

Lactobacillus MRS (Deman, Rogosa, and Sharpe) broth (HiMedia GM369) supplemented with 2% Agar (Himedia) was used for isolation of LAB. Media was sterilised at 15 lbs pressure, 121°C for 15 minutes. Cultures were grown for 24-48 h at 37°C in microaerophilic conditions. Culture conditions throughout experiments remained constant unless otherwise stated.

Screening and isolation of Lactic acid bacteria (LAB)

For isolation, fermented food sample was diluted (1:10) using sterile saline, vortexed and sample was allowed to settle. The supernatant was then utilised for

isolation of putative LAB. All isolated cultures were streaked separately and maintained on MRS medium. Gram staining and catalase-oxidase test was performed.

#### Acid-Bile tolerance of isolates

MRS medium having pH values of pH 2, pH 3 and pH 4; control was established at pH 6.5, was used for inoculation of  $1 \times 10^8$  CFU/ml culture. The pH of MRS medium was adjusted using 1 N HCl (LOBA Chemicals). Independently, the concentration of bile salts (Ox-Bile, HIMEDIA) was adjusted to 0.1% and 0.3%. For acid and bile tolerance total viable count (TVC) was calculated. Incubation for acid tolerance was 90 minutes and 48 h for bile (Song et al., 2021; Szutowska & Gwiazdowska, 2021). Tolerance to salt (Khushboo et al., 2023) and temperature was examined in the presence of 0.5 to 10 % of sodium chloride (Fischer Scientific) and 25, 35, 45 and 55°C respectively. Tests were repeated thrice in triplicate.

#### Cholesterol reduction ability of LAB

Isolates surviving conditions of acid and bile were tested for bile salt hydrolase (BSH) enzyme. For this qualitative assay LAB were spot inoculated on medium augmented with 0.5 % bile salts and 0.0037% calcium chloride and incubated at 37°C, 48 h. For cholesterol reduction test, MRS broth with 200 µg/ml of cholesterol (Arkray AUTOSPAN® liquid gold cholesterol kit) was inoculated with 24 h old culture of lactic acid bacteria and incubated for 24 h. Next day, tubes were subjected to centrifugation at 10000 rpm, 10 minutes to separate the cells. Residual cholesterol was estimated using the standard enzymatic method using kit according to the manufacturer's instructions. Uninoculated MRS with cholesterol was kept as negative control.

#### Hemolytic assay

Protocol for hemolytic assay was followed according to (Abid et al., 2018). In brief, MRS medium was combined with 5% sheep blood. The cultures were streaked on MRS- Blood Agar plates. The plates were incubated at 35°C and zone around the colony was checked for alpha-, beta- or gamma-hemolysis.

#### Identification of isolates using 16S rRNA gene sequencing method

16S rRNA was carried out at National Centre for Cell Science (NCCS), Pune. Individual isolates plated on MRS agar were submitted. Isolation and extraction of genomic DNA was carried out by the standard phenol/chloroform method. PCR amplification of the 16S rRNA gene was performed using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3']. PCR product was purified by standard PEG-NaCl precipitation and sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per manufacturer's instructions. Sequences were assembled by Laser gene package followed by identification using the EzBioCloud (Chalita et al., 2024)

#### Antibiotic susceptibility of LAB

24 h old bacterial cultures were diluted to approximately  $10^8$  CFU/mL and spread on sterile MRS agar plates. Antibiotic discs (DYNAMICRO® Labs Pvt Ltd – Multidisc ring for Gram positive microorganisms) were placed onto solid media. Growth inhibition was read by measuring the zone diameter after overnight incubation at 37 °C.

#### Antimicrobial assessment of cell-free supernatant of LAB

Preparations of cell-free supernatant (CFS) of probiotic strains was done according to Drumond et al., 2023. Overnight culture was diluted in fresh medium and incubated at 37°C in microaerophilic conditions for 24 h. For this, the falcon tubes were filled to the top with the medium without shaking for 24 h at 37°C. The 24 h culture of the probiotic strains was centrifuged at 10000 rpm, 4°C for 10 minutes, after which the cells were then removed. The supernatant was filter sterilised with a 0.22 µm nylon filter (PRAVEEN STERILETEO, Type- MPNN2502-S) to remove the residue of viable cells and referred to as CFS. CFS was neutralized with 0.1 N NaOH. An aliquot of cell free supernatant of every probiotic strain was plated on MRS agar to ensure the absence of viable cells. For control, uninoculated MRS medium was processed in similar manner and was treated as control. CFS was stored at -20 °C until use.

#### Agar well-diffusion assay

Similar protocol as (Hoxha et al., 2023) was carried out. Briefly, 24 h old grown cultures of *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* were spread on Nutrient agar plates. CFS of individual isolates were added to wells in 50 µl – 200 µl concentration. 200 µl of MRS alone

was added as a negative control in separate well. The plates were incubated for 24 h at 37°C. Zone diameter was measured.

#### Effect of Cell free supernatant (CFS) on biofilm formation

In order to assess the activity of cell free extract on inhibition of primary cell attachment (Drumond et al., 2023) pathogenic cultures were inoculated and kept overnight. Cell pellet was washed with PBS buffer (0.1 M, pH 7.2) and optical density of culture was adjusted to  $1 \times 10^8$  CFU/ml. 0.1ml of this suspension was added in 96-well microtiter plate and 0.1ml of different LAB CFS were added to every well and plate was incubated at 24 h at 37 °C. Cell suspension with saline and kept as positive control. Uninoculated CFS served as negative control. Biofilm formation was measured by using crystal violet (CV) assay (Elgamoudi & Korolik, 2023). Briefly, the wells were rinsed thrice with distilled water to remove unadhered cells. 0.1 % crystal violet (Fischer scientific CAS NO: 548-62-9) solution was added to wells for 20 minutes. Un-bound stain was removed by wash with distilled water. Bound CV was measured at  $A_{595}$  by addition of 70 % ethanol followed by gentle mixing.

Disruption of preformed biofilm and evaluation of biomass attachment was evaluated as follows. Cell density of pathogen was adjusted to  $1 \times 10^8$  CFU/ml and 0.1 ml of this suspension was added in 96-well microtiter plate along with medium supporting formation of biofilm. Incubation was carried out for 24 h at 37°C to allow cellular attachment and biofilm formation. Following incubation, supernatant of each well was removed, wells were washed with saline to remove unbound bacterial cells followed by addition of 100 µl of cell free supernatant. Untreated MRS was inoculated with pathogenic culture and kept as positive control. Uninoculated CFS served as negative control. The disruption of biofilm was analysed using the Crystal Violet assay as described previously.

#### Statistical analysis

The experiments were performed in triplicate and data was statistically analysed by comparing means (ANOVA), followed by post hoc (Tukey's test) at 95% Confidence level ( $\alpha = 0.05$ ) using Minitab version 17.

## RESULTS AND DISCUSSION:

#### Isolation of LAB with genotypic identification of selected isolates

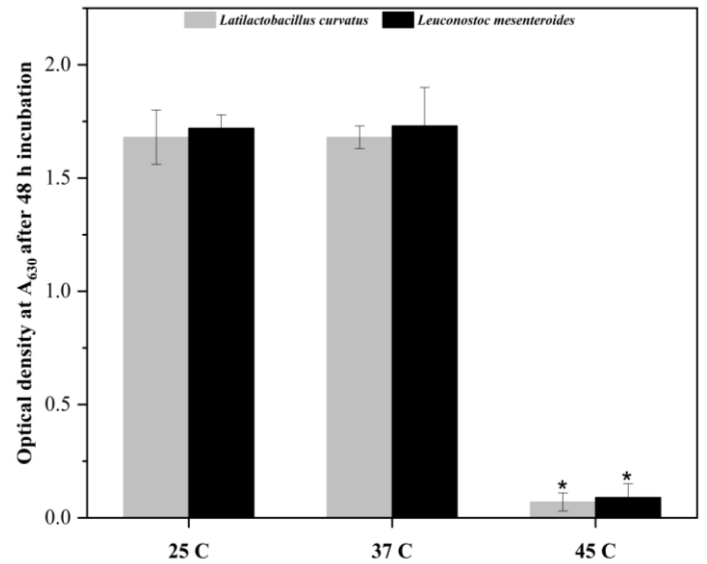
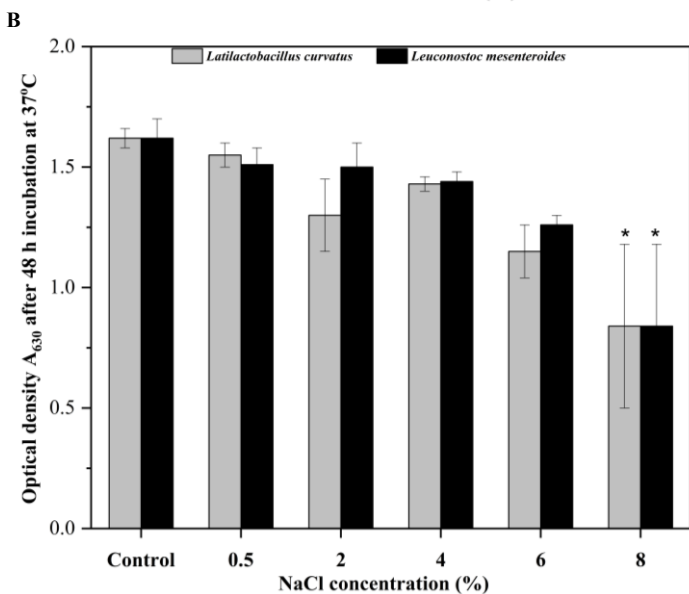
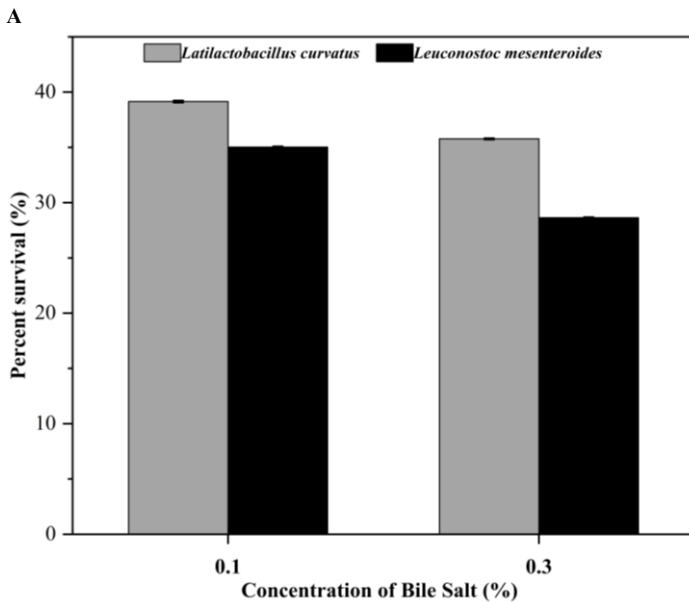
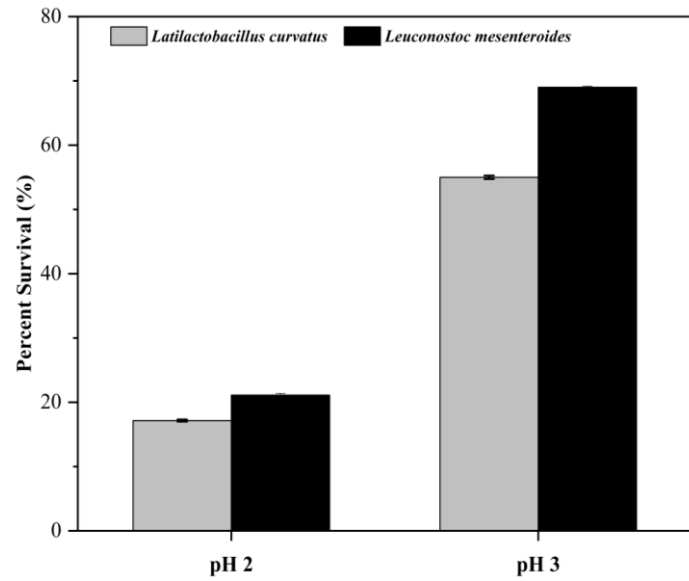
Traditionally cooked whole wheat extract (*Gahu-cheek*), *Anarsae* and *Jalebi* were used for isolation of LAB. These food preparations are native to Maharashtra cuisine. Although considerable literature is available with respect to isolation of probiotic organisms from sourdough and other fermented wheat preparations, *gahu-cheek* has not been extensively used. *Anarsae* and *Jalebi* are also native to Indian culture. A total of thirty-three isolates were obtained after 24-48 h incubation in microaerophilic conditions. Morphologically discrete colonies were selected and preserved in MRS medium. All 33 isolates showed Gram positive character and were catalase-oxidase negative.

Isolate GC03 was identified as *Latilactobacillus curvatus* JCM 1096 (T) [EzBioCloud Accession No. BBBQ01000060] and GC04 as *Leuconostoc mesenteroides subsp. mesenteroides* ATCC 8293 (T) [EzBioCloud Accession No. CP000414]. 16S rRNA gene sequences were compared by BLAST and showed 100 % sequence identity with type strains followed by their submission to Genbank with Accession No. PP832942 and PP832817 for *Latilactobacillus curvatus* and *Leuconostoc mesenteroides* respectively. *L. curvatus* sp. has been reported from sourdough and other wheat preparations (Dertli et al., 2016; Krausova et al., 2019; Minervini et al., 2010; Segli et al., 2021), traditional yoghurt (Heidari et al., 2022), fermented sausages and as starter culture for cheese (Chen et al., 2020). *Leuconostoc mesenteroides* strains are extensively studied and are regarded as probiotic bacteria.

#### Acid, Bile, Sodium chloride and Temperature tolerance

Tolerance to acid, bile and salt are considered inherent properties of probiotic organisms. As per the definition laid by WHO, probiotic organisms should survive the acidic environment of human gastrointestinal tract. The gastric pH can range from 1.5 to 2.0 (Zommiti et al., 2017). In this study, of the thirty-three isolates, five isolates could tolerate pH 2 and pH 3. All isolates could grow abundantly at pH 4 similar to control (MRS broth at pH 6.5). Survivability of isolates was assessed on basis of initial inoculum density. Highest percent survivability at pH 2 was seen at  $21.11 \pm 0.26$  % and  $17.16 \pm 0.16$  % for GC04 and GC03 isolates respectively. Also, GC04 had  $69 \pm 0.18$  % and GC03 had  $55 \pm 0.27$  % survival at pH 3 (Fig 1A). Isolates tolerant to acidic pH suggests their safe passage through the gastric environment. Notably it was observed that survivability of isolate GC03 sharply declined at pH 2 whereas tolerance of isolate GC04 was not significantly affected at the same pH. Different strains of *Lat. curvatus* show 29.26 to 43.58 % survivability at pH 2 after 3 h of incubation (Jin et al., 2023). A strain of *Lactobacillus curvatus* DN317 which was isolated from chicken ceca could easily survive for 30 minutes at pH 2.5 (Zommiti et al., 2017). As for isolate GC04,

different strains of *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 show higher growth at pH 2 compared to *Latilactobacillus curvatus* JCM 1095.



**D** Figure 1 Analysis of probiotic characteristic of isolated LAB. Tolerance of lactic acid cultures to changed concentrations of A) pH, B) Bile salts, C) Salt and D) Temperature; For acid and bile tolerance TVC was calculated and converted to percent survival; for salt and temperature cell density was measured at A<sub>630</sub>. All experiments were performed in triplicate. 95 % confidence level;  $p = 0.05$ .

Acid tolerant bacteria were assessed for their survivability in bile salts. Bile is secreted by small intestine and plays major role in lipid homeostasis and metabolism. Bile is considered cytotoxic to our gut flora owing to amphiphilic nature, hence resistance to bile salts is considered a major criterion for the conferring probiotic status to microorganisms (Cele et al., 2022). Tolerance to bile has been connected to presence of BSH enzyme with cholesterol homeostasis and reduction (Agolino et al., 2024; Ahn et al., 2003). In this study isolate GC03 showed 35.02±0.06 % bile tolerance at 0.3% bile whereas GC04 28.63±0.05 % tolerance (Fig 1B). Multiple strains of *Leuconostoc mesenteroides* sp. suggest 40-60 % survivability in presence of 0.3 % bile salts (Cele et al., 2022). Ability of LAB to tolerate high salt conditions, suggest their possible use as starter cultures in variety of food products. Again, high percent of salt acts as a natural preservative in food industry as this provides long shelf-life of food with low occurrence of spoilage. According to published reports minimum 6 % concentration of NaCl is pre-requisite for food preservation. Both the isolates GC03 and GC04, displayed growth at 8 % NaCl concentration after 48 h incubation at 37 °C (Fig 1C). Growth of lactic acid bacteria significantly reduced at 8% salt concentration, limiting it to 50 % growth compared to control. Lactic acid isolates could tolerate temperature up to 45 °C (Fig 1D).

**Bile salt hydrolase activity and cholesterol reduction test**

In accordance to a recent 2024 report, high blood cholesterol level is considered as major risk factor dealing with rise in cardiovascular diseases/disorder (Goh et al., 2024). Gut microbiota plays significant role in modulating cholesterol levels and until recently few mechanisms have been suggested which are involved in lowering cholesterol levels in host. One of the methods states importance of BSH enzyme which modifies primary bile acids which are then converted to secondary bile acids and are removed via excretory system (Agolino et al., 2024). Reports have indicated presence of bile salt hydrolase enzyme in number of probiotic bacteria viz. *Lactobacillus casei*, *Lactobacillus rhamnosus* GG, *Lactobacillus plantarum* sp (Hernández-Gómez et al., 2021). Both the isolates, *Lat. curvatus* and *Leuconostoc mesenteroides* were positive for the presence of BSH enzyme tested on MRS agar supplemented with bile and calcium chloride. Hydrolysis of bile salt results in lowering of pH due to formation of bile acids which combine with Ca<sup>2+</sup> ions forming a precipitate. The colony morphology of bacteria was granular and white comparable with other lactic acid bacteria strains (Albano et al., 2018). LAB isolated from fermented food have been a potential source of cholesterol-lowering agents (Liong & Shah, 2005). Cholesterol reduction in MRS was detected by an enzymatic method (Walhe et al., 2021). Both the isolates display reduction in cholesterol concentration after 24 h incubation. Amongst the two isolates *Lat. curvatus* showed 22.49 ± 0.001% which is higher than *Leuconostoc mesenteroides* sp. Other lactic acid strains have been reported to reduce cholesterol levels by *Lactobacillus pentosus* (48 %) (Walhe et al., 2021); *Lactobacillus plantarum* CAAS 18008 (21 %) (Ma et al., 2019). Cholesterol lowering ability has also been reported for *Lb. plantarum* VS513 (21%), *Lb. paracasei* ssp. *paracasei* VC213 (18%), *E. faecium* VC223 (17%), and *Lb. casei* VC199 (16%) in fermented food (Albano et al., 2018).

**Hemolytic test**

According to the reference directed by the National Institute of Health, National Library of Medicine, probiotic microorganisms belong to 'GRAS' category and is considered safe to use for human purpose (Joint FAO/WHO guidelines for evaluation of probiotics in food, 2002). Non hemolytic nature suggests the non-virulent nature of LAB (Khushboo et al., 2023). Isolates were evaluated for hemolytic activity. Both the isolates displayed  $\gamma$ -hemolysis, showing no zone of erythrocyte hemolysis around the streak lines. *Pseudomonas aeruginosa* ATCC 27853 served as positive control showing  $\alpha$ -hemolysis.

**Antibiotic susceptibility of lactic acid isolates**

LAB strains were assessed for their susceptibility against twelve antibiotics (Table1). *Lat. curvatus* and *Leuconostoc mesenteroides* were tested for their susceptibility against antibiotics. All cultures showed resistance against Amikacin 30 mcg and Gentamicin 10 mcg (Aminoglycoside); Sparfloxacin 5 mcg (Fluoroquinone), Cefadroxil 30 mcg, Cefuroxime 30 mcg and Cefoperazone 75 mcg (Cephalosporin); Ampiclox 20 mcg ( $\beta$ -lactam). *L. mesenteroides* was sensitive for Ciprofloxacin 5 mcg while *Lat. curvatus* isolate was resistant. Isolates showed sensitivity towards Clarithromycin 15 mcg and Roxithromycin 15 mcg (macrolides) with zone of inhibition  $\geq 25$  mm. *L. curvatus* and *Leuconostoc mesenteroides* showed intermediate sensitivity against Azithromycin (14-17 mm) (Macrolide). All lactic acid isolates show resistance to Vancomycin and Kanomycin (Szutowka & Gwiazdowska, 2021). Isolates *Lat. curvatus* and *Leuconostoc mesenteroides* also displayed resistance to Vancomycin. The standards used to determine sensitivity, intermediate sensitivity and resistance of isolates towards antibiotics were referred from the data received with the antibiotic disc, which is adapted from CLSI standards. Continued consumption of antibiotics naturally affect our gut flora which leads to reducing the diversity of microbiome and gut dysbiosis and leads to adverse effect on digestion and absorption of nutrients from food (Lathakumari et al., 2024). From our study it is revealed that these lactic acid bacteria can be promising probiotic candidates which may not wash out by the use of common antibiotics yet being sensitive to broad spectrum to macrolides like Clarithromycin and Roxithromycin which can inhibit the protein synthesis and inhibit LAB.

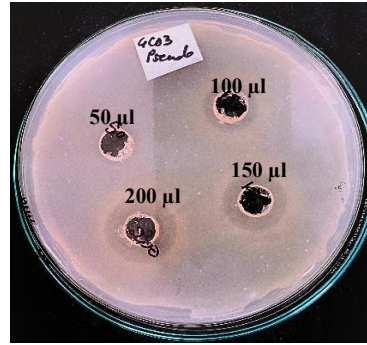
**Table 1** Antibiotic sensitivity of Lactic acid bacteria isolated from fermented whole wheat extract

Sr no	Antibiotic + Disc content (mcg)	<i>Latilactobacillus curvatus</i>	<i>Leuconostoc mesenteroides</i>	CLSI zone of inhibition
1	Amikacin AN 30	R	R	
2	Ciprofloxacin CIP 5	R	S (30 mm)	$\geq 21$ mm (S)
3	Clarithromycin CLR 15	S (30 mm)	S (30 mm)	$\geq 18$ mm (S)
4	Cefotaxime CF 30	R	R	
5	Sparfloxacin SF 5	R	R	
6	Cefuroxime CR 30	R	R	
7	Cefoperazone CFP 75	R	R	
8	Ampiclox ACX 20	R	R	
9	Cefadroxil CD 30	R	R	
10	Roxithromycin RX 15	S (30 mm)	S(30 mm)	$\geq 18$ mm (S)
11	Gentamycin G 10	R	R	
12	Azithromycin AZ 15	I (16 mm)	I (16 mm)	$\geq 18$ mm (S) 14-17 mm (I)

Legends: 'R' Resistant; 'S' Sensitive; 'I' Intermediate. Zone diameters (mm) were assigned 'R/S/I' according to CLSI standards

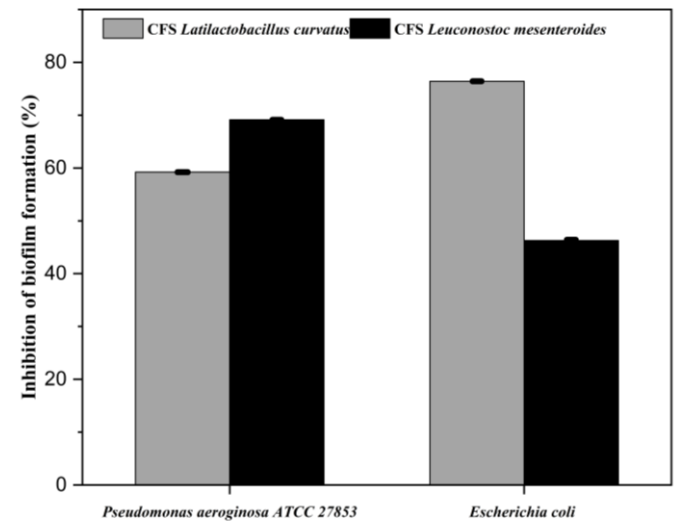
**Antimicrobial and anti-biofilm activity of CFS of lactic acid isolates**

Cell free supernatants of LAB are known to exhibit antimicrobial activity against pathogens. Two Gram negative and one Gram positive pathogen, namely *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* were used for this experiment. Agar well diffusion assay was carried out using different concentrations of undiluted CFS from overnight grown culture.

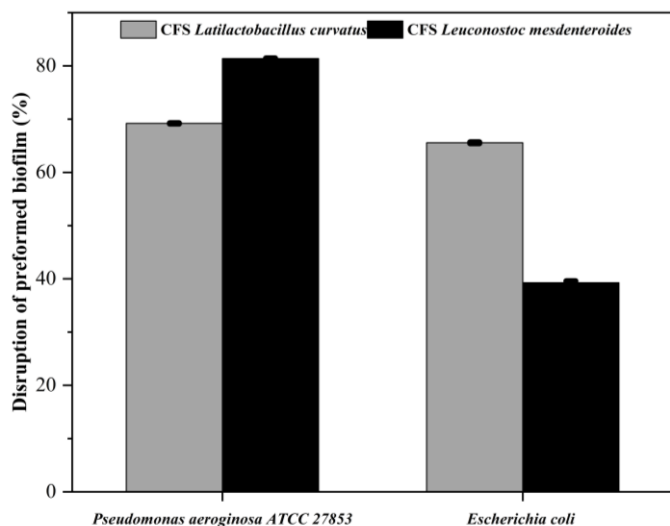


**Figure 2** Antimicrobial activity of *Latilactobacillus curvatus* GC03 CFS against *Pseudomonas* sp.

CFS of *Leuconostoc mesenteroides* and *Lat. curvatus* displayed antibacterial activity against all pathogens except *Staphylococcus aureus*. Zone of inhibition observed for CFS of GC03 against *Pseudomonas aeruginosa* ATCC 27853 was  $13.60 \pm 0.534$  mm (Fig 2) and for *E. coli* was  $12.00 \pm 1.00$  %. Whereas CFS of GC04 displayed  $11.87 \pm 0.37$  mm zone against *Pseudomonas aeruginosa* ATCC 27853 and  $11.83 \pm 2.13$  mm zone for *E. coli*. Again, CFS of both lactic acid bacteria showed no zone of resistance for *Staphylococcus aureus* ATCC 25923. Different strains of *Lat. curvatus* isolated from *jangajji* samples (Korean fermented food) show weak inhibitory effect against *Pseudomonas* sp and other (Jin et al., 2023). Also *Lat. curvatus* LAB 3H displayed antimicrobial activity against common pathogens namely *Staphylococcus aureus* PTCC1917 and *Escherichia coli* PTCC1276 (Heidari et al., 2022). *Latilactobacillus curvatus* Z12 and Z19 displayed antimicrobial activity against *Escherichia coli* (ATCC25922) and *Staphylococcus aureus* (ATCC25923) (Zhang et al., 2021). This suggests mixed degree of microbial inhibition across different strains of same genus. Several probiotic *Leuconostoc* sp have been reported to show good antimicrobial activity against *Pseudomonas* sp (Toushik et al., 2022). This property of lactic bacteria isolated from traditional Indian foods could have added effect on host. With appreciable salt tolerance at 8% NaCl concentration and antibacterial activity these probiotic lactic acid bacteria could serve as capable starter culture candidates. Again, CFS of LAB contain bacteriocins and antimicrobial substances which prevent the colonization of potential pathogen in the GI tract which might help maintaining optimal gut microflora.



A



**B** **Figure 3** Anti-biofilm activity of cell free supernatant (CFS) of lactic acid cultures against known pathogens, A) Inhibition of biofilm formation; B) disruption of preformed biofilm. Assay was carried out in 96-well plate. Biofilm formation was estimated by CV staining (A<sub>595</sub>). Saline served as control against CFS. Assay was performed in triplicate. Statistical analysis using Minitab Version. 17. One-way Anova Tukey’s test was performed. *p* = 0.05. Percent inhibition of biofilm formation and disruption are significant when compared to control which is considered 100 percent growth.

**Table 2** Summary of key probiotic traits of isolates GC03 and GC04

Sr No	Probiotic property	<i>Latilactobacillus curvatus</i> GC03	<i>Leuconostoc mesenteroides</i> GC04
1	<b>Tolerance to intestinal environment</b>	Percent Survival	
	Acidic pH (pH 2)	17.16 ±0.16	21.11±0.26
	Bile Salt concentration (0.3 %)	35.02±0.06	28.63±0.05
2	<b>Tolerance to stressed environment</b>		
	Salt concentration (8%)	+	+
	Temperature (45 °C)	+	+
3	<b>Hypocholesterolemic activity</b>		
	BSH enzyme production	+	+
	Cholesterol reduction	22.49±0.001%	7.5±0.001%
4	<b>GRAS status</b>		
	Hemolytic assay	γ hemolysis	γ hemolysis
	Antibiotic susceptibility	S (3)	S (4)
5	<b>Antagonistic activity <i>Pseudomonas aeruginosa</i> ATCC 27853, <i>Staphylococcus aureus</i> ATCC 25923 and <i>Escherichia coli</i></b>		
	Antibacterial activity	+	+
	Antibiofilm activity	+	+

Legends: “+” indicate positive response for given trait;  
S indicate susceptibility of isolate to number of antibiotics represented in brackets  
“±” indicates standard deviation

Anti-biofilm activity of CFS of LAB was assessed in 96-well microtiter plate using the crystal violet absorbance assay. All isolates showed significant anti-biofilm activity against *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli*. Biofilm formed without the CFS were considered as 100%. CFS of *Lat. curvatus* and *Leuconostoc mesenteroides* showed 59.22±0.04% and 69.12±0.04% biofilm inhibition against *Pseudomonas aeruginosa* ATCC 27853 respectively (Fig 3A). Disruption of preformed biofilm was performed for all three pathogenic cultures. CFS of *Lat. curvatus* displayed 69.21±0.07% disruption for *Pseudomonas sp* and 65.57±0.12% disruption of biofilm formed by *E. coli*. Again, CFS of *Leuconostoc sp* disrupted preformed biofilm of *Pseudomonas sp* by 69.21±0.07% and *E. coli* by 65.57 ±0.12% (Fig 3B). Surprisingly, CFS of *Lat. curvatus* and *Leuconostoc mesenteroides* displayed no antibacterial and anti-biofilm activity and biofilm disruption against *Staphylococcus aureus*. These results suggest lactic acid bacteria

synthesize and release bioactive compounds such as bacteriocins and peptides which have potent anti-biofilm activity. Such beneficial components of the lactic acid bacteria are labelled as ‘postbiotic’ compound and can be either dead cells, cell lysate, metabolites (SCFA, EPS) or the cell free supernatant (Wegh et al., 2019). The summary of all the probiotic properties of isolates has been compiled in Table 2.

**CONCLUSION**

The study reports a total of thirty-three isolates obtained from traditionally fermented native/local food samples, five isolates displayed significant tolerance to acid (pH 2, pH 3) and bile salts (0.3 %). Notably, the selected isolates demonstrated the ability to survive and grow in high salt concentrations (up to 8%) and elevated temperatures (up to 45 °C). Molecular identification of selected bacterial isolates revealed two LAB i.e. *Latilactobacillus curvatus* GC03 and *Leuconostoc mesenteroides* GC04. Both the isolates displayed γ-hemolysis on blood agar, indicating their safety for human use. The cell-free supernatants (CFS) of these isolates showed significant antibacterial and antibiofilm activities against common pathogenic bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli*) supporting their probiotic potential. *Lat. curvatus* is known for its bacteriocin production, suggesting its possible application in preventing food spoilage, particularly in refined flour-based products. Similarly, *Leuconostoc mesenteroides* species have been traditionally used in the preservation of meat products. Furthermore, these isolates can be assessed for exopolysaccharide (EPS) production - an additional beneficial trait of lactic acid bacteria (LAB). Collectively, the physiological and functional characteristics of indicate their potential use as a starter culture or in probiotic formulations for alleviation of mild hypercholesterolemia.

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