

Research Article

Probiotic properties of *Limosilactobacillus fermentum* strain TPGMS1 isolated from gut microflora of *Tai-Phake* community of Assam

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Abstract

Probiotics confer health benefits in the host, such as antimicrobial activity, immune system modulation, reduce metabolic disorder and enhance anticancer activity. Most probiotic microorganisms belong to the Lactic Acid Bacteria (LAB) group. Human gut is a WHO/FAO recommended source for LAB. This study aimed to evaluate *in vitro* probiotic properties of LAB isolated from the human gut microflora of *Tai-Phake* community. Among 43 isolated LAB species, 26 were preliminarily selected for their antimicrobial activity. Isolates were evaluated for probiotic characteristics and antimicrobial activities against test microorganisms, *Escherichia coli* ATCC-10536 and *Staphylococcus aureus* ATCC-BAA-1026, using agar well-diffusion method. Eight of these isolates exhibited antagonistic activity against test microorganisms, which is indicated by zone of inhibition (Zoi) of 1.5 cm against *Escherichia coli* ATCC-10536 and Zoi of 2.4 cm against *Staphylococcus aureus* ATCC-BAA-1026. The isolate with the best probiotic properties was identified using 16S rRNA sequencing as *Limosilactobacillus fermentum* strain TPGMS1. It exhibited high tolerance to low acidic condition (pH 2.0) and high bile salt (0.5% and 1%) concentrations, a significant hydrophobicity at 64 % and auto-aggregation of 58%. The results suggest that *Limosilactobacillus fermentum* strain TPGMS1 has high probiotic potential and can be further explored for probiotic formulation.

Keywords: Antimicrobial compounds, Gut microflora, Lactic Acid Bacteria, probiotics, *Tai-Phake* community**INTRODUCTION**

'Probiotics' is a living microbial food supplement that benefits the host by balanced intestinal microbes and could beneficially affect the normal gut flora (Hill *et al.* 2014), modified it further as a living microbe that offers the host various health benefits. Probiotics help to maintain and restore the gut microflora. Many of the bacteria used in probiotic preparations have been isolated from fermented food materials and human fecal samples to improve their chances of survival (Andersson *et al.* 2001, Bukola and Abiodun 2008). Probiotic strains are also being used to improve the health of poultry animals for disease prevention and increased meat production (Kalia *et al.* 2022). The culture conditions for gut microbes require specific nutritional and environmental conditions to support their growth and development. The human gut is heavily colonized by microbes which contribute to a healthy gut by

regulating nutrient metabolism, gut homeostasis and suppressing pathogens (Yadav *et al.*, 2018; Yadav and Chauhan, 2022). Gut bacteria also plays an important role in shaping host's immune system. Gut microflora and their interaction with the surrounding environment are explored to understand their therapeutic potential (Thaiss and Elinav, 2017). These studies highlighted the scope of harnessing the potential of gut microbes as probiotic strains in disease therapeutics (Yadav and Chauhan, 2022). Scientific explorations are required to culture human gut microbes to harness their probiotic potential. Numerous studies show probiotics' potential to prevent cancer initiation and progression by stimulating local and systemic immunity (Samanta, 2022). Probiotics effectively prevent and treat diabetes, hypercholesterolemia, diarrhea, irritable bowel syndrome, and inflammatory bowel disease (Grom *et al.*, 2020). Bannay *et al.*, 2013 reported potential use of some strains of *Lactobacillus* and *Bifidobacterium* to treat

mood disorders and enhance stress tolerance in gut microbiome. LAB plays a very important role in traditional food fermentation and can be found in the Gastro-intestinal tract (GI) of humans and animals (Duar *et al.*, 2017). LAB uses carbohydrates as the only or main source of carbon (George *et al.* 2018). *B. cereus*, *B. clausii*, *B. coagulans*, *B. licheniformis*, *B. polyfermenticus*, *B. pumilus*, and *B. subtilis* are well-characterized commercial probiotic strains (Lee *et al.*, 2015). They produce organic acid from glucose and growth inhibition substances which prevent the proliferation of food spoilage (De Vuyst and Leroy 2007). In addition to contributing to food, flavor, aroma and textures, LAB can also exhibit antimicrobial characteristics and produce peroxidases, organic acids, and bacteriocins (Serek and Oleksy-Wawrzyniak 2021). LAB plays an important role in bio-preservation due to its activities against food-borne pathogens (Ayivi *et al.*, 2020). Based on this background, this study was designed to characterize and evaluate the probiotic potential of indigenous bacterial strains from the gut microflora of the *Tai Phake* community in Assam, India.

MATERIALS AND METHODS

Collection of stool samples

Stool samples were collected from Nam-Phake village, the largest among the six villages of *Tai Phake* community in Choraideu, Dibrugarh and Tinsukia Districts of Assam. The respondents were from above Adult (18-75 years) age group. The study's objectives were briefly explained to the respondents and they were given a questionnaire. After collection, the samples were immediately transferred to the laboratory. 1 g of each fecal sample was inoculated into 9mL MRS broth and incubated at 37°C for 48 hr. The samples were then plated on MRS agar containing 0.25% cysteine and incubated at 37°C in an anaerobic chamber. In all, 229 isolates were isolated from 87 feces samples from Nam-Phake village. The MRS plates were incubated for 2-3 days at 37° C.

Morphological study of the Isolates

Pure bacterial colonies were analysed for morphological characteristics such as surface characteristics, consistency, opacity, color, size, shape, etc. Biochemical tests viz. Gram-staining, catalase, oxidase, and endospore tests were performed. Gram-positive, stained negative in endospore staining and catalase tests were selected for further analysis.

Study on stress tolerance of Isolates

pH tolerance assay

The viability of potential probiotic isolates was evaluated individually in a pH range of 3,4,6,8 (Broadbent *et al* 2010). The growth was measured at 600 nm at an inter-

val of 3 hr and the viability of cells was confirmed by inoculating (spreading) the MRS agar plates, followed by 24 hrs of incubation. Cell viability was assessed by the colony count method.

NaCl tolerance assay

NaCl tolerance assay was conducted according to the method given by Adnan and Tan (2007). Isolates were grown in MRS broth with different NaCl concentrations ranging from 0.9%, 6% and 8%, and they were incubated at 37°C for 24 hrs. After 24 hrs, the growth rate was calculated by considering the optical density (OD) values, measured at 600 nm. Uninoculated media was taken as a negative control.

Bile salt tolerance assay

Each isolate was inoculated in MRS broth (control) and mixed with 0.5% and 1% (w/v) bile salts (Sodium cholate and Sodium deoxycholate). Absorbance of all the samples was measured at 600 nm to assess their growth in the presence of bile tolerance. The samples were used at 0, 2, and 3 h of incubation and inoculated on MRS agar. All the plates were incubated at 37° C for 48 h and enumerated accordingly (Hoque *et al.* 2010, Liong and Shah 2005, Montet *et al.* 2006).

Hemolysis assay

The isolates' haemolytic activity were evaluated in triplicate, using the method described by Gupta and Malik, 2007. Overnight cultures of Isolates were inoculated in Blood agar plates containing 5% (v/v) defibrinated sheep blood and incubated at 37° C for 2-3 days and observed for the zone of hemolysis around colonies.

Evaluation of antagonistic properties of the Isolates

Antimicrobial activity of isolates against test microorganisms (*Escherichia coli* ATCC-10536 and *Staphylococcus aureus* ATCC-BAA-1026) was assessed using the Agar well diffusion method (Ridwan *et al.*, 2008). Lyophilised cultures of test microorganisms were imported from, MediMerck, Europe by Himedia. The test pathogens (30 µl) were individually inoculated to MRS agar and 30 µl of overnight-grown *isolates* were poured into a well on agar plates. The plates were allowed to dry, incubated for 24-48 h at 37° C and observed for the zone of inhibition.

Hydrophobicity assay

Bacterial cells were harvested by centrifugation at 10,000 × g for 10 min at 4 °C, washed twice with Phosphate Buffer Saline (PBS) of pH 7.2 and, re-suspended in the PBS. Then, the bacterial suspensions were incubated in 1mL aliquots at 37 °C, Absorbance was measured (A_0) and an equal volume of Toluene was added. The 2-phase system was thoroughly mixed for 3 min.

After 1 h of incubation at room temperature, the aqueous phase was removed and its absorbance at 600 nm (A_1) was measured (Lee *et al.* 2016). The percent hydrophobicity of the isolate was determined by the decrease in the level of absorbance and calculated using the following formula:

$$\text{Percentage cell surface hydrophobicity} = (1 - A_1/A_0) \times 100 \quad \text{Eq.1}$$

Where, H= Hydrophobicity, A_0 =Absorbance at time zero, A_1 = Absorbance after 60 min.

Auto-aggregation test

Overnight grown bacterial cells were harvested at $5000 \times g$ for 10 min, washed with PBS, and resuspended in PBS to 10^8 CFU/mL (Del Re *et al.*, 2000). The level of absorbance (A_{600}) has been adjusted to a value of 0.25 ± 0.05 to standardize the number of bacteria (10^7 – 10^8 CFU/mL). 3 mL of each bacterial suspension was vortex for 10 seconds and incubated at 37°C for 2 h. The absorbance of the supernatant was measured at 600 nm using a spectrophotometer. The % auto-aggregation was measured using the formula:

$$\text{Auto-aggregation (\%)} = [1 - (A_{\text{time}}/A_0) \times 100] \quad \text{Eq.2}$$

Where, A_{time} represents the absorbance at a particular time and A_0 represents the absorbance at time 0 h (Zommiti *et al.* 2017)

Molecular level identification

The most potent isolate among the selected isolates was identified at the molecular level by 16S rDNA sequencing. Molecular identification (16S rDNA analyses) of selected isolate was carried out through Genetic Analyzer (ABI 3130) at Biokart, Bangalore. 16S rDNA fragment of the isolate G-21 was amplified by using forward (5'- GGATGAGCCCCGCGCCTA-3') and reverse (5'- CGGTGTGTACAAGGCCCGG -3') primers and 1066 bp long consensus sequence was generated using aligner software. The 16S rDNA sequence of the isolate was used to carry out BLAST with the 16S rDNA sequence database of NCBI Genbank.

Statistical analysis

All the results were from three independent observations (Mean $\pm$ SD). Tukey's multiple comparison tests were used with significance determined at p value = 0.05. Software SPSS version 17.0 was used for the statistical analysis.

RESULTS

Two hundred and twenty nine isolates were isolated from eighty-six fecal samples. Only 119 isolates were

Table 1. Colony morphology of Isolates, isolated from gut microflora of Tai-Phake community of Assam

Isolates	Form	Elevation	Margin	Colour	Gram's reaction	Catalase	Endospore staining
G1	Circular	Convex	Entire smooth	White	+ve	-ve	-ve
G2	Circular	Flat	Entire smooth	Creamy white	+ve	-ve	-ve
G3	Circular	Raised	Undulated	White creamy	+ve	-ve	-ve
G4	Circular	Convex	Entire smooth	White	+ve	-ve	-ve
G5	Circular	Convex	Entire smooth	White	+ve	-ve	-ve
G6	Circular	Raised	Entire smooth	Yellowish white	+ve	-ve	-ve
G7	Circular	Convex	Ropy	Yellowish white	+ve	-ve	-ve
G8	Circular	Convex	Entire smooth	Dull	+ve	-ve	-ve
G9	Circular	Slightly flat	Undulated smooth	Yellowish white	+ve	-ve	-ve
G15	Round	concave	Irregular	Whitish	+ve	-ve	-ve
G20	Round	Convex	Ropy smooth	Creamy white	+ve	-ve	-ve
G21	Round	Convex	smooth	White	+ve	-ve	-ve
G29	Circular	Convex	Entire smooth	White	+ve	-ve	-ve
G30	Circular	Flat	Entire smooth	Creamy white	+ve	-ve	-ve
G31	Circular	Raised	Undulated smooth	White	+ve	-ve	-ve
G32	Circular	Convex	Entire smooth	White	+ve	-ve	-ve
G33	Circular	Convex	Entire smooth	White	+ve	-ve	-ve
G34	Circular	Raised	Ropy	Yellowish white	+ve	-ve	-ve
G35	Circular	Convex	Entire smooth	Yellowish white	+ve	-ve	-ve
G36	Circular	Convex	Entire smooth	Dull	+ve	-ve	-ve

G-1 to G-36 are the isolates, isolated from gut microflora of Tai-Phake community, which showed probiotic properties

selected for further analysis based on morphological characteristics (Table 1).

Screening of low pH tolerance

Acid tolerance is considered an essential assessment criterion for evaluating probiotic bacterial species. In this assay, the survivability of the 119 isolates, examined in MRS broth adjusted to pH3, pH4, pH6 and pH8 with 4N HCl or 4N NaOH, showed that CFU/mL of Isolates G-1, G-2, G-7, G-16, G-17, G-22, G-26, G-29, G-52, G-56, G-60, G-66, G-67, G-71, G-72, G-75, G-76, G-78, G-80, G-83, G-89, G-95, G-96, G-100, G-104, G-105, G-118, G-120, G-122, G-130, G-132, G-139, G-140, G-159, G-160, G-173, G-174, G-178, G-184, G-186, G-190, G-193, G-194, G-196, G-212, G-214, G-217 were zero at pH 3 after 24 hours of incubation. Out of the 119 isolates, 72 isolates namely G-3, G-9, G-12, G-16, G-18, G-20, G-21, G-31, G-33, G-37, G-38, G-40, G-47, G-48, G-50, G-52, G-53, G-56, G-58, G-68, G-70, G-73, G-74, G-77, G-84, G-85, G-90, G-92, G-93, G-94, G-99, G-103, G-106, G-107, G-113, G-114, G-116, G-118, G-121, G-123, G-126, G-127, G-133, G-135, G-145, G-149, G-154, G-161, G-165, G-166, G-167, G-171, G-175, G-183, G-185, G-188, G-199, G-200, G-202, G-206, G-207, G-208, G-209, G-210, G-215, G-216, G-218, G-221, G-226 were able to survive at pH 3. The graphical representation of the inhibitory activities is presented in Fig. 1.

Screening of bile-tolerant lactic acid bacteria

Bile tolerance is considered one of the essential properties required for lactic acid bacteria to survive in the small intestine (Ibrahim and Benzkorovainy, 1993). The isolates whose optical density (O.D.) was recorded as

zero at pH 3 were excluded from the bile tolerance assay. The effects of bile salt on the growth of the LAB isolates, after 24 hours of incubation at 37°C indicated that all the isolates grew well at 0.05% bile. The variability of growth of the isolates were observed in 0.5 % and 1% Oxgall concentrations as represented in Fig. 2 and Fig. 3. Isolates G-9, G-3, G-15, G-20, G-31, G-47, G-73, G-92, G-94, G-99, G-106, G-117, G-133, G-135, G-145, G-149, G-161, G-199, G-200, G-202, G-206, G-215 were more resistant than other isolates and exhibited resistance at 1% bile. The isolates were grown in bile esculine agar (4% Oxbile), to evaluate their capacity to hydrolyse bile esculin (Fig. 2). Isolates G-9, G-3, G-15, G-20, G-21, G-31, G-47, G-73, G-92, G-94, G-99, G-106, G-117, G-133, G-135, G-145, G-149, G-161, G-199, G-200, G-202, G-206, G-215 could hydrolyse bile esculin agar during 24 hours of incubation period (Fig. 3).

Hemolytic test

Non-hemolytic bacteria are not virulent, and lack hemolysin, which ensures that opportunistic virulence will not appear among these strains (Peres *et al.* 2014). When inoculated on Blood Agar Plates, all the Isolates displayed no hemolysis (γ -hemolysis). Indicator organism *Staphylococcus aureus*, exhibited β haemolysis and the isolates G-1, G-3, G-9, G-15, G-20, G-21, G-31, G-47, G-73, G-92, G-94, G-99, G-106, G-117, G-133, G-135, G-145, G-161, G-165, G-166, G-185, G-199, G-200, G-202, G-206, G-215 exhibited α haemolysis which indicates their non-pathogenic and safe character. Other Isolates G-2, G-7, G-16, G-17, G-22, G-26, G-29, G-52, G-56, G-60, G-66, G-67, G-71, G-72, G-75, G-76, G-78, G-80, G-83, G-

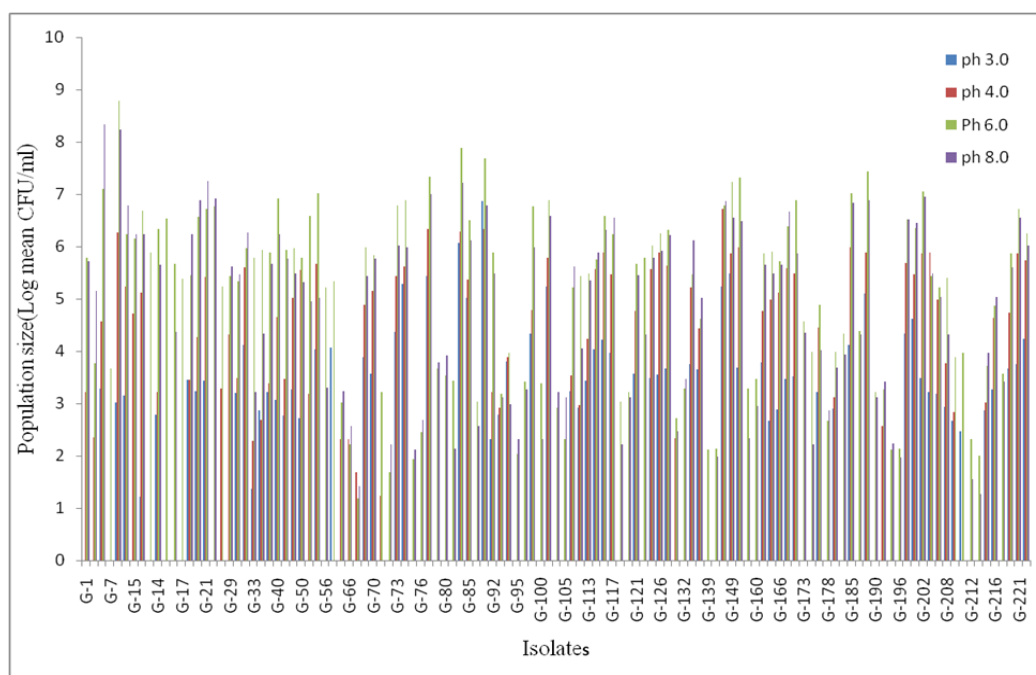


Fig. 1. Effect of different pH on population size of different isolates

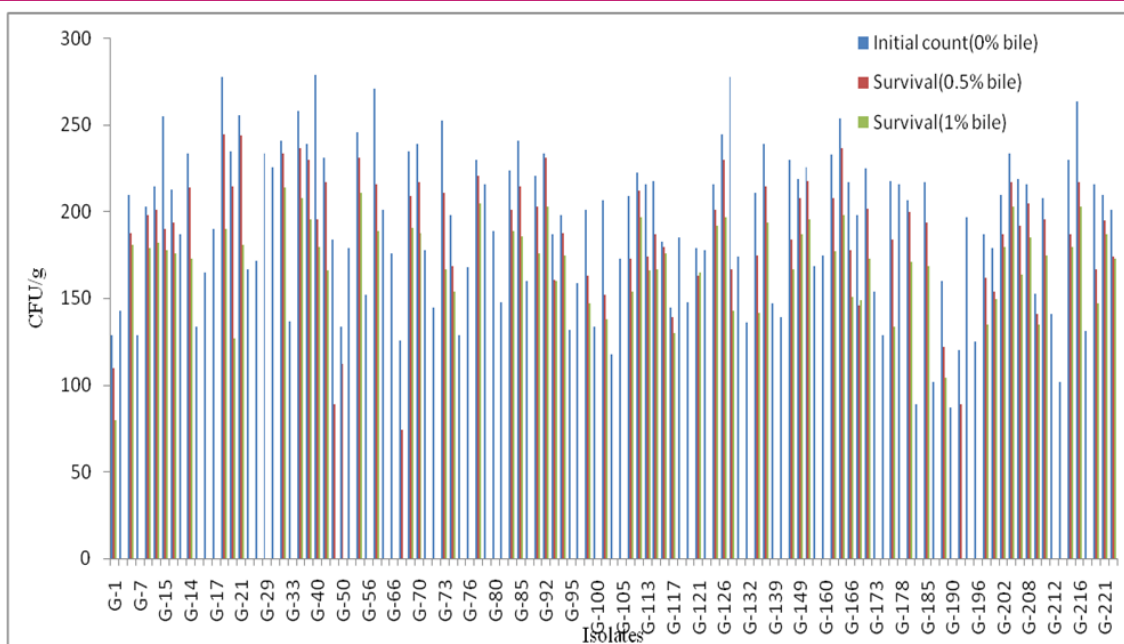


Fig. 2. Bile tolerance exhibited by Isolates

89,G-95,G-96,G-100,G-104,G-105,G-118,G-120,G-122,G-130,G-132,G-139,G-140,G-159,G-160,G-173,G-174,G-178,G-184,G-186,G-190,G-193,G-194,G-196,G-212,G-214,G-217 showed β haemolysis.

Antibacterial activity

Twenty six Acid- bile tolerant probiotic LAB Isolates demonstrated significant antimicrobial activity against test pathogens *Escherichia coli* ATCC-10536 and *Staphylococcus aureus* ATCC-BAA-1026. Zone of inhibition ranged from 0.8 cm to 1.6 cm with G-9 showing the highest ZOI of 1.5 cm against *Staphylococcus aureus* ATCC-BAA-1026 and ZOI of 2.4 cm against *E. coli* ATCC-10536 (Fig. 4).

Auto-aggregation assay

All isolates showed high degree of auto-aggregation. Isolate G-15 showed the highest auto-aggregation as 69.61%, G-3 showed 68.07%, G-21 showed 64 %, G-200 showed 65.04%. Isolate G-185 showed minimum auto-aggregation at 41.51%. In the present study, all tested strains showed high auto-aggregation ability after 24 h of incubation (Fig. 5).

Hydrophobicity test

All isolates showed high degree of cell surface hydrophobicity. Isolate G-15 and G-199 showed the highest cell surface hydrophobicity whereas lowest hydrophobicity was displayed by G-99 and G-215. Isolate G-21 showed 58% of hydrophobicity.

Molecular identification

The partial 16S-rDNA sequences of the isolate, G-21, after BLASTn analyses at NCBI, were observed in sep-

arate clades in respective phylogenetic analyses (Fig. 6). The strain was found to be a novel strain of *Limosilactobacillus fermentum* strain and the accession number PP916573 was assigned after submission to GenBank Database.

Ethical approval

All experimental protocols were approved by the Institutional Ethical Committee, Gauhati University, Guwahati, File no-GUIEC-61/2021 dated 25.03.2021)

Statistical analysis

All the results are from three independent observations (Mean $\pm$ SD). Tukey's multiple comparison tests were used with significance determined at p-value = 0.05. Software SPSS version 17.0 was used for the statistical analysis.

DISCUSSION

As microorganisms obtained from the human intestine have a wide range of applications in scientific research and industry, researchers continuously explore human gut microbes as a potential pool for novel microbial strains. Gut microflora of the *Tai Phake* community of Assam are almost unexplored. The present study identified 26 potential probiotic LABs from gut microflora of this community. Out of those, 1 isolate, G-21, was screened as the most promising candidate based on its acid-bile tolerance, pH tolerance, antimicrobial activity, and was identified using 16S rRNA sequence. The isolate showed acid tolerance (Fig. 1), bile tolerance (Fig. 2) and grew at 0.9, 6, and 9 % NaCl concentrations. The findings on salt tolerance of LAB isolates

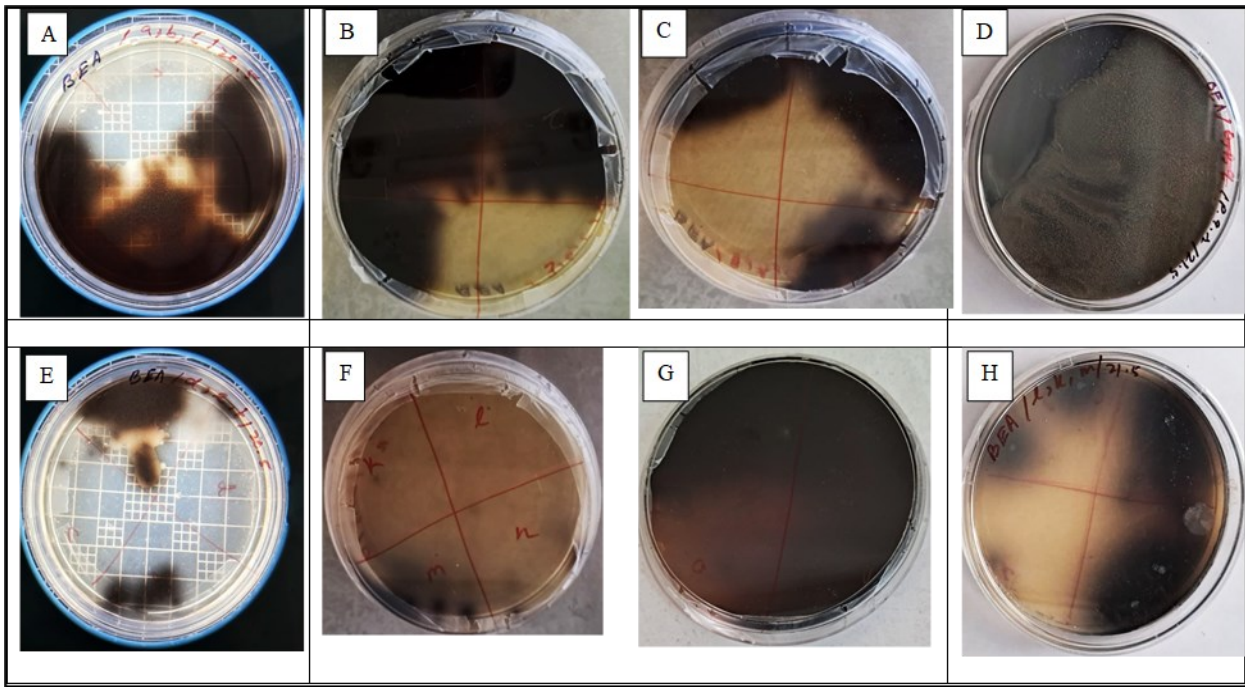


Fig. 3. Showing hydrolysis of bile esculentin agar by isolates. A-Control (C) and Isolate G-3, G-9, G-15, B-Control, Isolate G-20, G-21, G-31, C-Control and Isolate G-47, G-73, G-92, D-Isolate-99, G-106, G-117, G-133, E- Control, Isolate G-135, G-145, G-149, F-Isolate G-169, G-199, G-200, G- Isolate G-3, G-9, G-21, G-202, H-Isolate-G-21, G-206, G-215, G-9

follow the findings of Raisagar *et al.* (2022), in which LAB cultures (*Lactobacillus casei* and *Bifidobacterium bifidum*) exhibited tolerance against bile (0.5% to 2.0%), NaCl (1-7%) and grew well in a pH range 3-8. The viability of LAB isolates at low pH in this study is similar to that of Baloch *et al.* (2019), in which *Lactiplantibacillus plantarum* Lp-1, which could survive at pH 3 for 4 h of incubation. G-21 isolate grew well in a pH range of 2-8 in our study (Fig. 1). Khalil *et al.* (2018) reported that seven *Lactobacillus* strains showed the ability to survive under conditions of pH 3 in 3 h of incubation with strain *L. plantarum* (DUR8) achieving the highest survival rate of 90.24%. The viability of the potential probiotics in bile salt media is similar to the study of Zhang *et al.* (2020).

Bile salt tolerance is considered an important selection criterion for probiotic isolates to survive the conditions

in the small intestine. In this study, all isolates demonstrated good tolerances at 0.5% bile salt, (Fig. 2). Their survival rate ranged from 82.30 to 99.20%, consistent with the findings of Mulaw *et al.* (2019). G-20 and G-21 isolates survived in the presence of bile salts (0.5% and 1%) for 3 h, which was confirmed by plating after incubation. Indicator organism *Staphylococcus aureus*, exhibited β haemolysis and the isolates G-1, G-3, G-9, G-15, G-20, G-21, G-31, G-47, G-73, G-92, G-94, G-99, G-106, G-117, G-133, G-135, G-145, G-161, G-165, G-166, G-185, G-199, G-200, G-202, G-206, G-215 exhibited α haemolysis which indicates their non-pathogenic and safe character. Probiotic isolates exhibit variation in antagonistic activity, indicating they are pathogen-specific. Edalati *et al.* (2018) reported that LAB isolated from raw camel's milk showed antagonistic activity against *E. coli* and *B. cereus*. In our study, all 20 iso-



Fig. 4. Antimicrobial activity by *Limosilactobacillus fermentum* strain TPGMS1 against test pathogen. A- ZOI of Isolates *Limosilactobacillus fermentum* strain TPGMS1 against *Staphylococcus aureus* ; B- ZOI against *Escherichia coli* by *Limosilactobacillus fermentum* strain TPGMS1

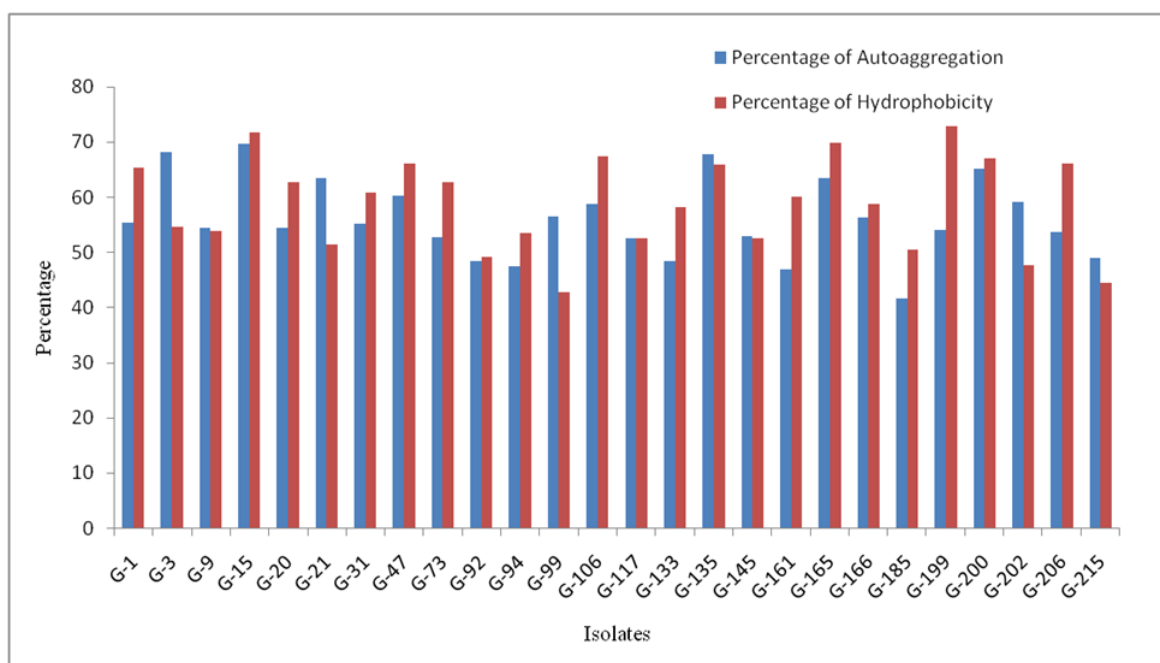


Fig. 5. Hydrophobicity and Autoaggregation Analysis Test

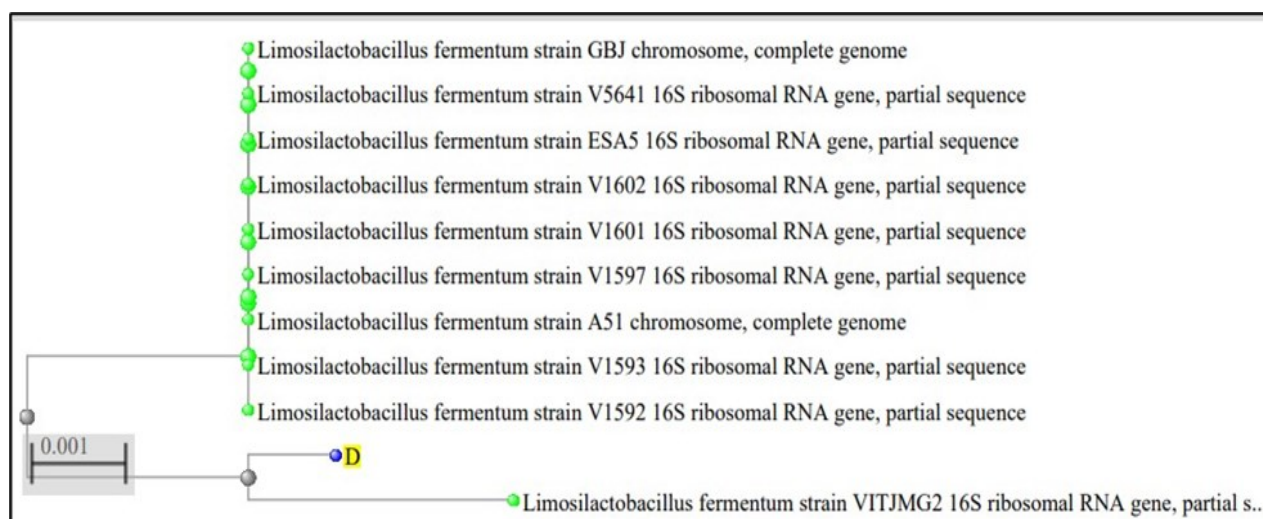


Fig. 6. Phylogenetic analysis of *Limosilactobacillus fermentum* strainTPGMS1

lates showed various degrees of antagonistic activity by forming Zone of inhibition (Zoi), ranging from 0.8 cm to 1.6 cm against *Staphylococcus aureus* ATCC-BAA-1026 and Zoi of 2.4 cm against *E. coli* ATCC-10536. G-21 showed the highest Zoi of 1.5 cm against *Staphylococcus aureus* ATCC-BAA-1026 and Zoi of 2.4 cm against *E. coli* ATCC-10536. Kumari *et al.* (2024) reported that partially purified cell-free bacteriocin-like inhibitory substance from *B.borstelensis* showed very good antagonistic activity against *Bacillus subtilis* (KK01), *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* (HCS36), and *Escherichia coli* (O22). The outcome of 16S rDNA sequencing of one isolate was interesting because the isolate may be a novel strain of *Limosilactobacillus fermentum* strainTPGMS1, which is evident from the phylogenetic tree (Fig. 6),

although further sequencing of the whole genome is required to confirm its novelty.

Conclusion

As the Isolates from our study showed favorable probiotic attributes, we can conclude that gut microflora is a suitable and potential sources for isolating novel probiotics strains of human origin. Isolate G-21 was identified as *Limosilactobacillus fermentum* strainTPGMS1 by 16s RNA sequencing, showed antagonistic activity against test pathogens, survived in harsh conditions, showed α hemolysis and good degree of hydrophobicity as well as auto-aggregation. Therefore, this strain can be considered promising "next generation" probiotic candidates, which may be useful to the pharma-

ceutical industry. As indigenous microbes are specific to a population, hence, this strain from gut microflora of *Tai-Phake* community may pave the way for its use in the formulation of future probiotics.

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Conflict of interest

The authors declare that they have no conflict of interest.

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