RESEARCH ARTICLE

Lactic acid bacteria from flowers and vegetables: A new avenue unlocking non-dairy reservoirs for nutritional and agro-biotechnological applications

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Abstract

Isolation and screening of microbes were the first and important step to explore the diversity of naturally occurring beneficial bacteria. This study hypothesized that lactic acid bacteria (LAB) naturally associated with flowers and vegetables possess unique probiotic traits that could outperform conventional strains in both human and agricultural applications due to their environmental adaptability and enzymatic versatility. The current study is focused on the probiotic potency of LAB associated with flowers and vegetables. A total of 71 strains of bacteria were isolated and 51 were selected based on the LAB characteristics. To ensure probiotic efficacy, a series of *in vitro* tests were conducted to determine the probiotic potential of the isolates including survival in gut conditions (acid, bile and gastric juice tolerance). Seven strains exhibited remarkable tolerance to low pH, bile salt and gastric juice. The safety evaluation (hemolytic, deoxyribonuclease (DNase) and gelatinase activities) of the selected strains proved the non-pathogenic nature and hence generally recognized as safe (GRAS) status, further identified as *Pediococcus pentosaceus* and *Limosilactobacillus fermentum* strains by 16S rRNA sequencing. The carbohydrate utilization pattern coincides with the molecular identification and the enzymatic profiling of 7 isolates showed the ability to produce protease and cellulase, proving their adaptability to the natural habitat. This study shows that naturally occurring LAB from unexplored plant-based niches have dual functions as probiotics for humans and plants. This study is the initial step to establishing low-cost probiotic innovations derived from indigenous microbial resources, improving human health.

Keywords: lactic acid bacteria; *Limosilactobacillus fermentum*; *Pediococcus pentosaceus*; plant; probiotics

Introduction

Bacteria are microorganisms, found almost everywhere on the earth's surface, atmosphere and also upper atmosphere. Although common people usually think, bacteria as a bad thing, but many bacteria live in symbiosis and mutually beneficial relationships with the host. Basically, without microbes, humans wouldn't be alive (1). However, some bacteria are harmful to humans and the environment (*Klebsiella pneumoniae*, *Clostridium tetani*, *Vibrio cholerae,* etc.) (1). But most of them are harmless, they also have some beneficial effects on humans and the environment (nitrifying bacteria, Micrococcus, etc.). Certain bacteria provide health benefits to their host and are known as probiotics. Probiotics are living microorganisms that, when consumed in sufficient amounts, provide beneficial effects on the health of their host (2). Probiotics are used in different fields; accordingly, probiotics are known by many names, such as plant probiotics that enhance plant growth and suppress diseases (*Bacillus*, *Paraburkholderia*, *Acinetobacter,* etc.) and aqua probiotics that improve fish and shrimp survival by modifying the host and ambient microbial community (*Lactobacillus acidophilus*, *Lactobacillus lactic* subsp. *lactis*). Lactic acid bacteria (LAB) and Bifidobacteria are the most important classes of human probiotics. Although lactobacilli are not found in the normal microflora of growing plants, they are present in decayed plants or common in various plant parts, including fruits and vegetables (3).

In nature, plants live in close association with a diversity of macro and microorganisms both within and outside their tissues. Although numerous studies have focused on microbial collections associated with different plant parts, little is known about tripartite interactions between plants, their microbiomes and other multicellular organisms, such as pollinators (4). The sparsely explored sources of LAB, such as flowers, fruits and raw vegetables, constitute a remarkable niche. These raw materials possess high carbohydrate but low protein contents and a slightly acidic pH, providing a suitable niche to several microorganisms. However, the microbial composition in these environments is fluctuating and depends on intrinsic (physical and nutritional conditions) and extrinsic (environmental and harvesting conditions) parameters of the plant matrix (4). It has been claimed that the microorganisms found in these niches may come from the environment, basically, from pollinators. Among the pollinators, honeybees as the most important pollinator insects worldwide and are considered a key species in nature because of their vital role in the maintenance of almost all life on earth. The idea of transmission of these LAB

between flowers and honeybees that visit them has raised an important question: whether the transmission is from the flowers to honeybees or vice versa. Studies suggest that the LAB can affect the chemical compositions of floral nectar and, as a result, attract honeybees towards the nectar (5).

Additionally, a high percentage of these bacteria in the floral nectar is also found in the stomach of honeybees and honey hives. *Lactobacillus plantarum* is commonly found in plant environments, such as nectar, pollen and fermented plant materials, which honeybees regularly forage. The name "*Lactobacillus plantarum*" originates from its ability to ferment plant sugars (lactose and other carbohydrates) into lactic acid, as well as its prevalence in plant-based environments (the genus *Lactobacillus* refers to "milk" or "lactic acid," while *plantarum* reflects its association with plants). This bacterium's adaptability to both plant and gut environments allow it to thrive in the honeybee digestive system, where it supports gut health and prevents pathogen overgrowth (5). The nomadic nature of *L. plantarum*, moving between plants and animal hosts, facilitates its persistence in the bee gut. For instance, strains *L. plantarum* KX519413 and *L. plantarum* KX519414 have been isolated from the gut of the honeybee (*Apis cerana indica*) (6). Although reports on endophyte populations of LAB in plants are scarce, advances in plant-microbe interactions have highlighted their importance as a new class of plant growth-promoting microbes.

Lactic acid bacteria (LAB) has different applications in food, agricultural and environmental fields. In plants, they help to protect the deterioration of fruits and vegetables by colonizing natural micropores and resisting antimicrobial compounds (7). In food systems, LAB and their metabolites are key to preservation and fermentation processes that improve nutrition, flavor and shelf life. Lactic acid bacteria (LAB), also known as the "workhouses" of the fermented food industry, converts various substrates, including dairy, meat, cereals and vegetables into safe, stable and value-added products. In winemaking, *Oenococcus oeni* is the predominant LAB species, for its acid and ethanol tolerance, while other species enhance aroma and flavor (8). Environmentally, LAB supports soil fertility, plant growth and aquaculture health, aids composting and waste reduction and help mitigate volatile organic compounds (VOCs), plant diseases and greenhouse gas emissions (9).

However, LAB from human and animal origins are widely investigated, but LAB associated with plants remain a relatively unexplored area of research. So, this is the main reason behind selecting flowers and vegetables as a source of probiotic bacteria. Not only this reason but also identifying and isolating novel indigenous LAB from these sources is to discover strains that are naturally adapted to plant environments, offering unique advantages for both scientific research and economic production. These novel LAB strains often possess functional traits such as robust resistance to environmental stresses and the ability to produce specialized metabolites that are not typically found in strains isolated from other well-studied niches. Moreover, their dual role as both probiotics for humans and growth-promoting agents for plants bridges the gap between food, agriculture and environmental biotechnology, increasing their competitiveness in multiple sectors.

Materials and Methods

Sample collection

In this study, 8 types of flowers and 5 types of vegetables were collected locally to isolate LAB. The selected plants included *Moringa oleifera* (muringa flower), *Hibiscus rosa-sinensis* (chembarathi), *Musa acuminata* (banana flower), *Ixora chinensis* (thechi), *Lantana camera* (kongini), *Cassia fistula* (kanikonna), *Rosa rubiginosa* (rose), *Solanum lycopersium* (tomato), *Abelmoschus esculentus* (ladies finger), *Brassica oleracea* var. *Botrytis group* (cauliflower), *Brassica oleracea* var. *Italica* (broccoli) and *Brassica oleracea* var. *Capitate* (cabbage). These plants were selected because their flowers and edible parts provide nutrient-rich, diverse microbial environments that favor LAB growth and diversity, making them ideal for identifying novel probiotic strains with potential food and health applications.

Isolation of lactic acid bacteria

One gram of the sample was cut into small pieces and suspended in a boiling tube containing 10 ml De Man Rogosa Sharpe (MRS) broth. This broth was incubated at 37 °C under anaerobic conditions for 24 hr. The appropriate dilution was plated on MRS agar and incubated under the same conditions as above. Morphologically distinct colonies were further streaked on another MRS agar plate. There are about 71 isolates obtained from both flowers and vegetables. About 51 isolates were selected based on their basic LAB characters, such as catalase negative or slightly positive, oxidase negative, motile or non-motile, non-spore formation and positive milk coagulation test.

Determination of probiotic potential

Acid tolerance test

Overnight cultures of the LAB isolates were inoculated in MRS broth previously adjusted to pH values 2, 3 and 4 with 1N HCl and incubated at 37 °C for 6 hr under 5 % CO_2. The viable counts on MRS agar plates were determined from the initial and final suspensions. Control samples without acidification (pH 6.5) were also prepared. The percentage of survivability was calculated by Eqn. 1 (10).

Percentage of survivability = (log cfu 6th/log cfu 0th) × 100 (Eqn. 1)

Bile tolerance test

Overnight cultures of the LAB isolates were inoculated in MRS broth containing 0.3, 0.5 and 1 % bile salt and incubated at 37 °C for 6 hr under 5 % CO_2. The control consisted of MRS broth without bile. Viable counts on MRS agar plates were determined from the initial and final suspensions. The percentage of survivability was calculated by Eqn. 2 (10).

Percentage of survivability = (log cfu 6th/log cfu 0th) ×100 (Eqn. 2)

Tolerance to simulated gastric juice

Bacterial tolerance to simulated gastric juice was determined with some modifications (11). Simulated gastric juice was prepared by suspending pepsin in saline and adjusting the pH 2 and 3 using 1N HCl. The number of viable cells was determined after 3 hr of incubation. Survivability was determined by using Eqn. 3.

Percentage of survivability = (log cfu 3th/log cfu 0th) ×100 (Eqn. 3)

Carbohydrate fermentation test

Sugar fermentation patterns were detected by using the Hilacto identification kit (KB020-Himedia) as per the manufacturer's instructions.

Molecular identification of isolates

Isolation of genomic DNA

Genomic DNA was isolated from the selected LABs performed as previously described (12). Amplification of 16S rRNA by polymerase chain reaction (PCR) and identification by sequence analysis PCR-based typing method was used for the identification of the isolates using the universal primers. (i) Forward primer- 27 F 5' AGAGTTTGATCMTGGCTCAG 3' and (ii) Reverse primer-1492R 5' AAGGAGGTGWTCCARCC 3'. PCR was carried out in a 50 μL reaction volume containing 50 ng of genomic DNA, 20 pmol of each primer, 1.25 units of Taq DNA polymerase, 200 μM of each dNTP and 1x PCR buffer. PCR was carried out for 35 cycles in a MyCycler TM (Bio-Rad, USA) with the initial denaturation at 94 ℃ for 3 min, cyclic denaturation at 94 ℃ for 30 sec, annealing at 58 ℃ for 30 sec and extension at 72 ℃ for 2 min with a final extension of 7 min at 72 ℃. The PCR product was checked by agarose gel electrophoresis, purified and further subjected to sequencing. The sequence data was checked by blast analysis. The phylogenetic analysis of the 16S rRNA sequences of the isolates obtained in the study was conducted with MEGA 5 using the neighbor-joining method with 500 bootstrap replicates.

Safety parameter study

Anti-hemolytic activity

The selected LAB isolates were streaked on blood agar plates (MRS medium with 5 % fresh blood) and incubated for 24 hr at 37 ℃. Strains that produced green-hued zones around the colonies (α hemolysis) or had no effect on the blood agar plates; hemolysis was classified as non-hemolytic. Strains displacing blood lysis zones around the colonies were considered β hemolysis. The experiment was conducted in triplicate.

DNase activity

To test for deoxyribonuclease enzyme (DNase) production, the LAB isolates were streaked onto a DNase agar medium. After 48 hr (37 ℃), the plates were examined for the appearance of an activity zone. Positive DNase activity was shown by a pronounced zone around the colonies. *Staphylococcus aureus* was used as a reference for quality control (13).

Gelatinase activity test

The gelatin-degrading ability of the LAB was investigated using MRS media containing 3 % (w/v) gelatin. *Pseudomonas aeruginosa* was used as a reference for quality control and was grown on nutrient agar containing 3 % (w/v) gelatin. Gelatin-degrading ability was evaluated by the presence of a clear zone around the bacterial colony (14).

Enzyme profiling of isolates

Amylase activity

For amylase activity, the bacterial strains were inoculated into modified MRS media supplemented with 0.25 % starch. After incubation, the zone of clearance was observed by adding Gram's iodine as a detecting agent.

Lipase activity

Lipase activity was determined by using olive oil (1 %). 50 μL of cell-free supernatant was inoculated in MRS broth containing olive oil (1 %) and arabic gum (1 %). After 48 hr of incubation, the zone was observed.

Protease activity

For the detection of protease activity, 50 μL of cell-free extract was inoculated into skim milk (1 %) agar medium and incubated for 48hr. After incubation, the zone of clearance was observed and measured.

Pectinase activity

All pure colonies from overnight cultured bacteria [freshly activated plates] were transferred to new pectin agar media and incubated at 37 ℃ for 48 hr. At the end of incubation, 0.3 % of Congo red solution was flooded onto the petri dishes and left for 10 min. This solution formed a clear zone around the colonies, which indicates that bacterial isolates can produce pectinase (15).

Cellulase activity

Bacterial isolates were performed for cellulase production activity by spot inoculation on the cellulose agar media. The use of Congo red (1 %) as an indicator of cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Bacterial isolates showing a clear halo zone on cellulose medium indicated a positive result for cellulose synthesis.

Agarase activity

Screening of agarolytic activity of selected isolates was performed on basal salt solution medium, which was adjusted to pH 7.5 before autoclaving. After the autoclaving process, all the isolated bacteria were inoculated by the streak plate method on the screening medium and incubated at 37 ℃ for 2 days. Lugol's iodine solution was employed to identify agarase enzyme qualitatively; hence, all growth plates were flooded with iodine solution. The presence of a clear zone surrounding the colony shows that it may degrade agar (16).

Chitinase activity

For screening of chitinase-producing microbes, selective media supplemented with 1 % colloidal chitin were used. Selected bacteria were inoculated by the streak plate method on the screening medium and incubated at 37 ℃ for 2 days. The presence of a clear zone surrounding the colony shows that it may degrade chitin.

Statistical analysis

All experiment was carried out in triplicate. Statistical analysis was performed using GraphPad Prism 8.0. Two-way ANOVA was used to determine the level of significant differences ($p<0.05$). All the data were presented as Mean ± SEM from the triplicate analysis.

Results and Discussion

In recent years, researchers have confirmed that the addition of probiotics to the environment is a new era in improving soil, water and air quality. These beneficial microorganisms, including bacteria and fungi in the soil, play a critical role in nutrient cycling, plant growth and disease suppression. In addition, many bacterial species help to control harmful microbes in aquaculture, control algal blooms and certain Bacillus species protect plants from diseases like damping off and root rot while aiding in bioremediation (17). However, to ensure the efficiency and safety of these bacteria, they must be systematically identified and characterized. The purpose of our research was to analyze the potential probiotic characteristics and evaluate the safety of the

LAB isolated from flowers and vegetables. So, this is one of the leading reports to evaluate the probiotic characteristics of the LAB strains isolated from flowers and vegetables.

To isolate functional probiotic bacteria, natural samples (flowers and vegetables) were collected and different morphotypes were identified with their probiotic characteristics. A total of 71 bacterial strains were obtained from 8 flowers and 5 vegetable types collected from the local area. Fifty-one isolates were selected based on LAB characteristics (Gram-positive, catalase-negative, oxidase-negative, milk-coagulating and rod or cocci-shaped). Among these, 36 originated from flowers and 15 from vegetables (Table 1). The presumptive LAB isolates were further characterized for acid, bile and gastric juice tolerances to check their viability under gastrointestinal conditions. The current study points out that natural sources such as flowers and vegetables are a suitable reservoir of LAB.

Probiotic strains tolerate various gastrointestinal barriers, including high concentrations of lysozyme in saliva, stomach acidity, digestive enzymes and bile salts (18). To assess the potential use of LAB as effective probiotics, they need to have the capability to survive under acidic conditions. The exact mechanism is that LAB tolerate acidic environments by actively expelling protons, producing alkaline metabolites and modifying their cell membrane and wall to reduce acid entry and maintain internal pH balance (19). In this study, the survival rate of selected isolates in acidic buffer was examined by the difference in viable cell counts after 0th and 6th hr of incubation. Out of the 51 isolates, 7 isolates survived at pH 2 after 6 hr of exposure, nonetheless with a significant decrease in the viability of isolates. Among them, isolate

no.10 indicated the highest survival rate (101.33 %) under acidic conditions, while isolate no 2 showed the lowest (43.72 %) (Fig. 1).

This study showed similar results to other previous research, where LAB isolates remained viable after exposure to pH levels of 2 and 3. However, a significant decrease in the viability of the isolates was observed after 6 hr of exposure (20). Another study showed similar results, where LAB isolates were viable after exposure to pH 2.5 to 3; nevertheless, they showed more acceptable viability at lower pH levels (21). According to previous studies, all selected LAB isolates (12 strains) exhibited resistance to low pH (both pH 2 and 3), as expressed by a survival rate of >50 % (22). These results are consistent with our results; all isolates showed 50 % above tolerance to pH 2 and 3 (except isolate no. 2 - 43.716 %). Reviews and the findings of this study point out that strain variability may be the reason for survivability in various pH levels.

Bile salt is the most crucial parameter for the selection of probiotics, which are the main components of bile (23). After bacterial exposure to bile salts, disruptions of cellular homeostasis occurred that caused the dissociation of lipid bilayer and integral proteins of their cell membranes, resulting in bacterial leakage and finally the death of the cell. For human use, the recommended concentration of bile salt for the selection of probiotic bacteria is 0.2–0.05 % (23).

In the present study, the ability of the bacterial isolates to tolerate bile was assessed by the growth of bacterial isolates at various concentrations of bile salts ranging from 0.3–1 %. The range of bile concentration chosen was based on physiological concentration in the duodenum or the human gastric juice. Most

Table 1. Preliminary lactic acid bacterial characterisation of 51 selected isolates isolated from flowers and vegetables, 36 isolates from flowers and 15 isolates from vegetables

| Bacterial isolates	Shape	Catalase test	Oxidase test	Endospore formation	Motility test	Milk coagulation test
Flowers	Cocci -9 Rod – 9 Coccobacilli – 7 Short rod - 11	5 isolates are slightly positive 31 isolates are negative	All isolates are Negative	All isolates are non-spore producers	Motile – 21 Non motile – 15	All isolates showed positive milk coagulation
Vegetables	Cocci – 4 Coccobacilli – 6 Short rod - 5	2 isolates are slightly Positive 10 isolates are negative	All isolates are negative	All isolates are nonspore producers	Motile – 2 Non- motile - 13	All isolates showed positive milk coagulation
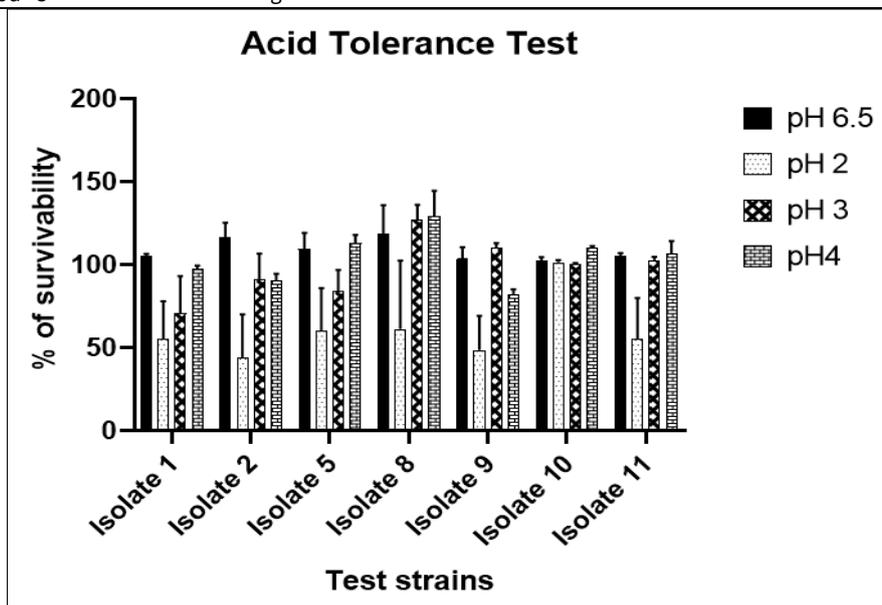
Fig. 1. Percentage of survivability of LAB isolates incubated at different pH values. All isolates (except isolates no. 2 and 9) showed > 50 % survivability.

LAB isolates displayed tolerance to bile salt at a higher than 70 % survival rate.

The findings of this study were consistent with previous research, where LAB strains from Neera samples exhibited over 50 % survival at low pH and bile concentrations (24). Similarly, LAB isolated from *Carica papaya* showed more than 90 % survival at 0.3 % and 1 % bile concentrations (25), while another study reported that 12 LAB strains remained resistant to bile salts after 6 hr of exposure (26). This bile tolerance is attributed to strain-specific genes, such as *ula*A and *ula*B in *L. plantarum* S83, which enhance resistance to different salt conditions. Such acid and bile resistance suggests their ability to survive in the gastrointestinal tract (Fig. 2)

In low pH conditions, probiotics must survive the harsh proteolytic ability of pepsin in the stomach. In our experimental results, 7 isolates showed high tolerance towards pepsin and low pH and observed above 60 % tolerance (except isolate no. 2 - 29.5903 % and isolate no. 9 - 26.4942 %). The findings of this study were similar to those of previous research (27). LAB such as *L. paracasei* and *P. pentosaceus* are resistant to pepsin. The main mechanism of survival of LAB in the gastrointestinal tract is producing alkaline compounds within the cytoplasm, modifying their cell envelopes, activating H^+- ATPase and/or actively transporting protons into the cells (28). This finding supports their stability under conditions similar to those of the human stomach, indicating strong potential for probiotic formulations (Fig. 3). The isolates with the highest survival rates in pH, bile salt and gastric juice tests were selected for further investigations. Only 7 out of the 51 isolates had remarkable survival rates after preliminary characterization and these were accordingly selected for further assessment.

Lactic acid bacteria (LABs) are known for their ability to ferment carbohydrates. Each LAB strain ferments sugars differently. This variability is an identification tool. The tested strains were differentiated by their capacity to form acid from sugars through the observation of the color changes (purple to yellow). Test isolates, isolates no. 1, 2, 5, 9, 10 and 11 could utilize xylose, cellobiose, arabinose, maltose, galactose, mannose and trehalose, while they could not ferment melibiose, raffinose and sucrose. The isolate no. 8 could utilize maltose, galactose, melibiose, raffinose, sucrose and trehalose. They could partially utilize xylose, cellobiose, arabinose and mannose. The carbohydrate utilization pattern of the isolated LABs was compared with Berger's manual (Table 2).

According to a previous findings, *P. pentosaceus* isolated from human and animal faeces and different fermented foods were able to utilize carbohydrates such as ribose, maltose, galactose, mannose and fructose (29). The results of the current study of isolate no. 8 were supported by previous research, which observed that *L. fermentum* isolated from dental caries of children was able to ferment some carbohydrates (30).

Table 2. Utilization pattern of different sugars of test isolates as determined with the Hi Lacto identification kit

S. No.	Test	Isolate no 1	Isolate no 2	Isolate no 5	Isolate no 8	Isolate no 9	Isolate no 10	Isolate no 11
1	Esculin hydrolysis	+	+	+	+	+	+	+
2	Catalase	-	-	-	-	-	-	-
3	Xylose	+	+	+	±	+	+	+
4	Cellobiose	+	+	+	±	+	+	+
5	Arabinose	+	+	+	±	+	+	+
6	Maltose	+	+	+	+	+	+	+
7	Galactose	+	+	+	+	+	+	+
8	Mannose	+	+	+	±	+	+	+
9	Melibiose	-	-	-	+	-	-	-
10	Raffinose	-	-	-	+	-	-	-
11	Sucrose	-	-	-	+	-	-	-
12	Trehalose	+	+	+	+	+	+	+

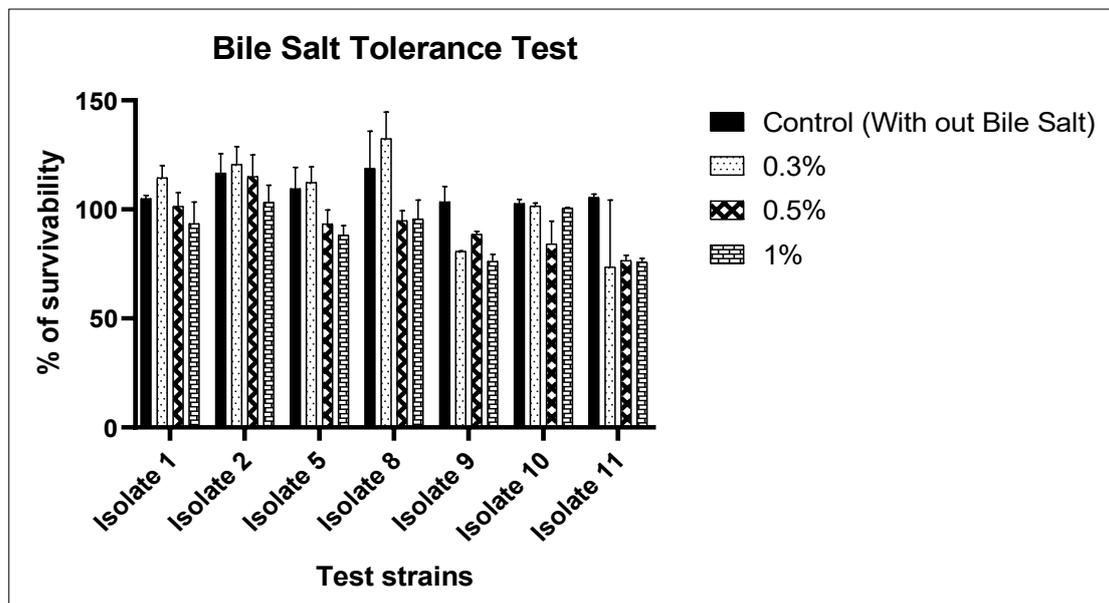+ Positive reaction; ± Weak positive reaction; - Negative reaction

Fig. 2. Percentage of survivability of LAB isolates at different bile salt concentrations. All isolates showed > 70 % of survivability in different bile salt concentrations.
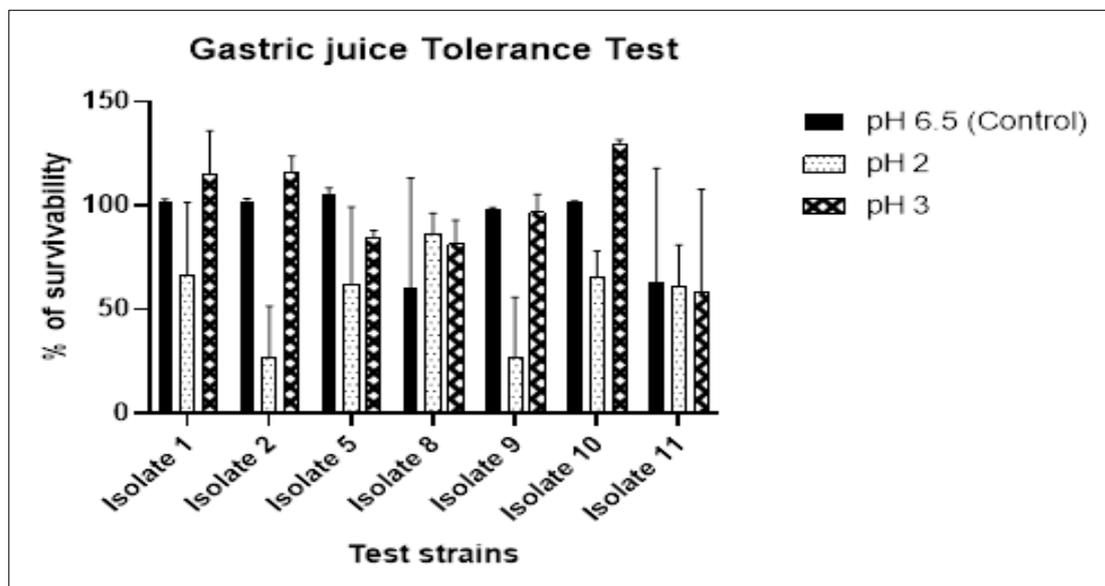
Fig. 3. Percentage of survivability of LAB isolates in simulated gastric juice at different pH conditions. All isolates (except isolates no. 2 and 9) show > 60 % survivability in pepsin and low pH.

Based on Food and Agriculture Organization (FAO)/ World Health Organization (WHO) guidelines (2), analyzing and identifying probiotic microorganisms with 16S rRNA patterns can be considered an internationally accepted method. Identification of probiotic potential LAB by 16S rRNA has been referred to as a very reliable method for species-level identification of probiotic microorganisms. Several studies have supported this approach for identifying probiotic LAB (31, 32). The 16S rRNA gene sequencing identified 6 isolates named as MBTUSKKTRF01, MBTUSKKTRF02, MBTUSKKTRF05, MBTUSKKTRF09, MBTUSVKTRV010 and MBTUSVKTRV011 as *P. pentosaceus* strains and MBTUSKKTRF08 was identified as *L. fermentum* strain (Table 3; Fig. 4). This study showed similar results to other previous research, *P. pentosaceus* JBCC 106 isolated from five traditional Korean fermented foods, Jangajji (33) and *L. fermentum* from edible sources (beet root and mango) (34). Identification of isolates by carbohydrate utilization pattern coincided with molecular identification and their 16S rRNA nucleotide sequences were deposited in GenBank. The above research indicates that flowers and vegetables are natural habitats of LAB, especially *P. pentosaceus* (35, 36). The congruence between biochemical and molecular identification supports their reliability.

The safety assessment of potential probiotic strains is a critical prerequisite for their application in the food and health sectors. The absence of hemolytic activity is primarily a condition for the selection of probiotics. In this study, 7 isolates did not cause any lysis of the erythrocytes in blood agar and they showed no hemolysis (Fig. 5). The results align with previous findings, which reported that the two LAB isolates, *L. paracasei* 11 W and *L. plantarum* 40C, show no hemolytic activity (26). Similarly earlier studies suggested that LAB from Chinese spontaneously fermented non-dairy food products also showed no hemolytic activity (37). DNase activity indicates the capacity to hydrolyze the

DNA molecules (presence of deoxyribonucleases). The DNase enzymes contribute to pathogenicity by degrading host DNA and facilitating evasion from neutrophil extracellular traps (38). In this study, all LAB isolates showed the absence of DNase activity (Fig. 6). Their absence supports the non-virulent nature of the isolates.

Gelatinase is a zinc metalloproteinase secreted by pathogenic bacteria. It breaks down gelatin (a structural protein found in connective tissues), enabling the bacteria to invade the host by degrading tissue protein components and thereby promoting their spread. This type of bacteria has a higher likelihood of translocating from the gut to the liver, spleen, heart and mesenteric lymph nodes (39). In the present study, we tested whether any of the LAB could hydrolyze gelatin. There is no gelatinase activity observed in any of the LAB isolates (Fig. 7). In prior studies, it has been demonstrated that *L. paracasei* (40) and *L. plantarum* (41) are gelatinase-free and can be used as potential probiotics. These results indicate the safety of the strains for use in industrial applications.

Enzymatic screening showed the absence of amylase, lipase, pectinase, agarase and chitinase activities, whereas all isolates were positive for protease and cellulase production. Proteolytic enzymes, including protease and peptidase, catalyze protein and polypeptide fragment hydrolysis, with extracellular protease primarily assisting in the absorption of large polypeptides into smaller molecules (42). In this study, 7 isolates were observed precipitated zone on MRS agar with skim milk media (Fig. 8). So, strongly proteolytic bacteria can further break down the precipitate to soluble components with the formation of an inner transparent zone. Similar findings were observed in the previous study that *L. lactis* subsp. *lactis* BRM3 showed higher proteolytic activities produced during the fermentation of milk (43).

Table 3. Identification of test isolates and their GenBank accession numbers

| S. No.	Test isolates	Strain name	NCBI accession number
1	Isolate no: 1	*Pediococcus pentosaceus* MBTUSKKTRF01	ON931481
2	Isolates no: 2	*Pediococcus pentosaceus* MBTUSKKTRF02	ON931482
3	Isolates no: 5	*Pediococcus pentosaceus* MBTUSKKTRF05	ON931483
4	Isolates no: 8	*Limosilactobacillus fermentum* MBTUSKKTRF08	ON892030
5	Isolates no: 9	*Pediococcus pentosaceus* MBTUSKKTRF09	ON908585
6	Isolates no: 10	*Pediococcus pentosaceus* MBTUSVKTRV010	OP492077
7	Isolates no: 11	*Pediococcus pentosaceus* MBTUSVKTRV011	OP492087
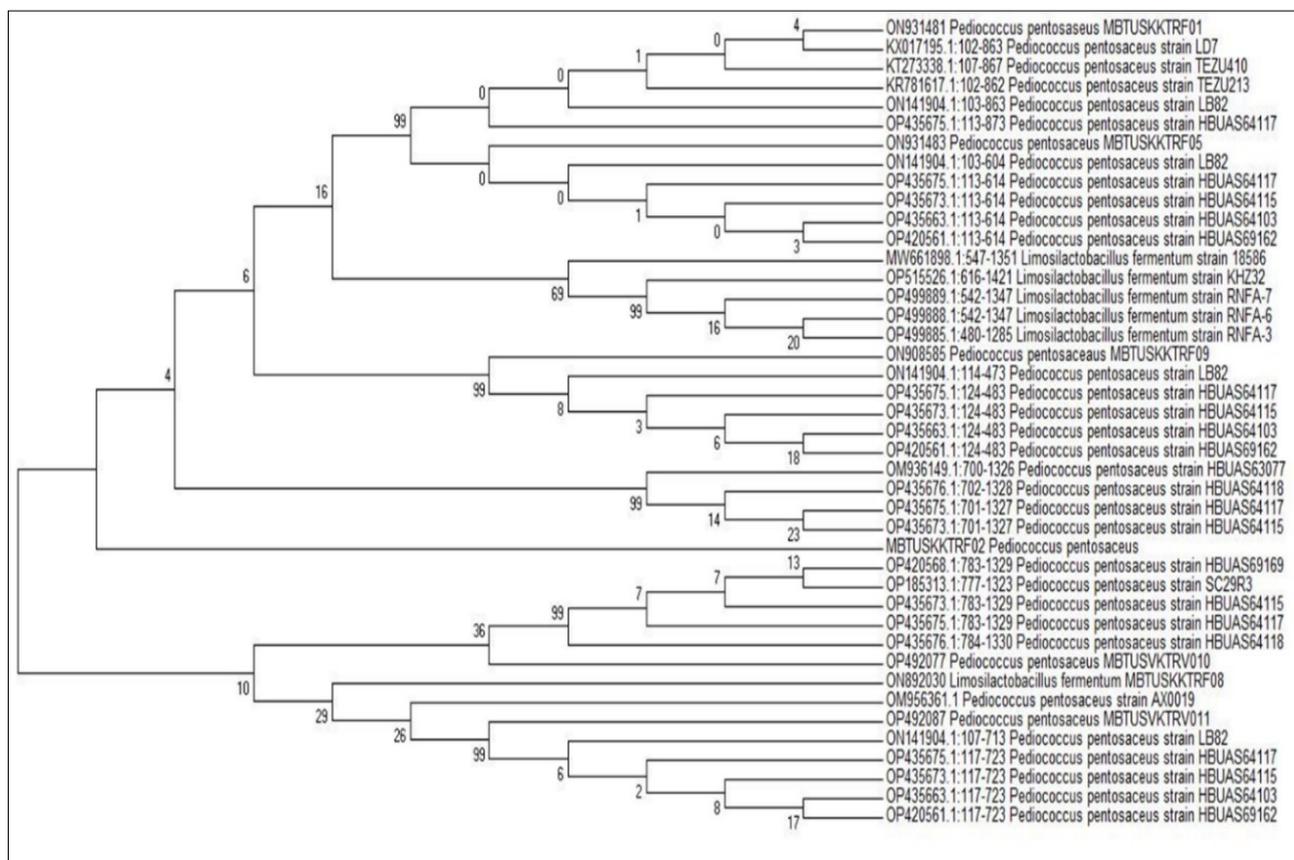
Fig. 4. Phylogenetic tree of seven isolates obtained by a neighbour-joining analysis of BLAST sequences. Numbers at nodes indicate the percentage of bootstrap values from 500 replicates.
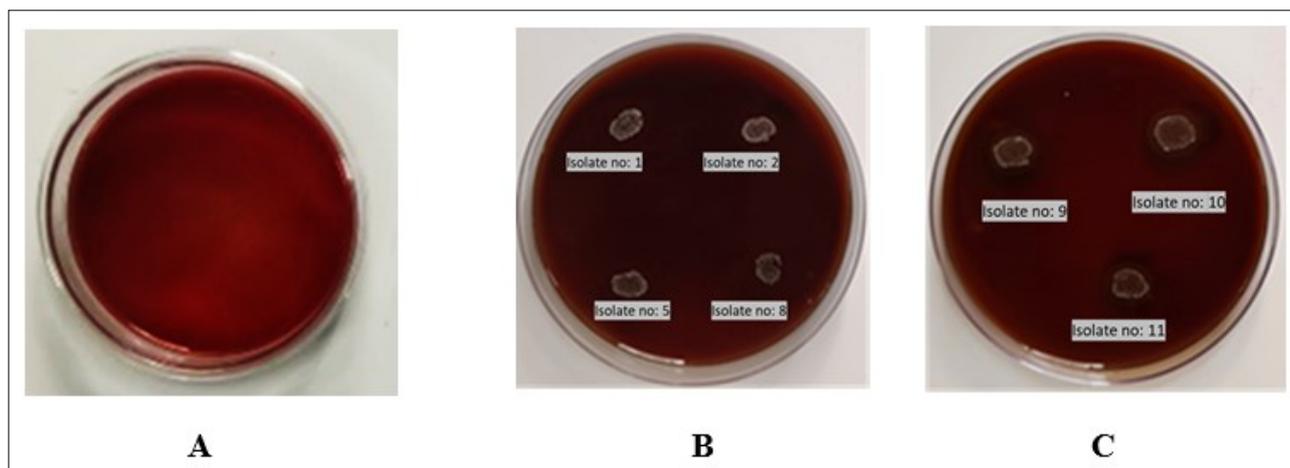

Fig. 5. Non-hemolytic nature of test isolates. (A) MRS control showing absence of a clear zone; (B) isolates 1, 2, 5, and 8; (C) isolates 9, 10, and 11, all showing absence of a clear zone, indicating the non-pathogenic nature of the test isolates.
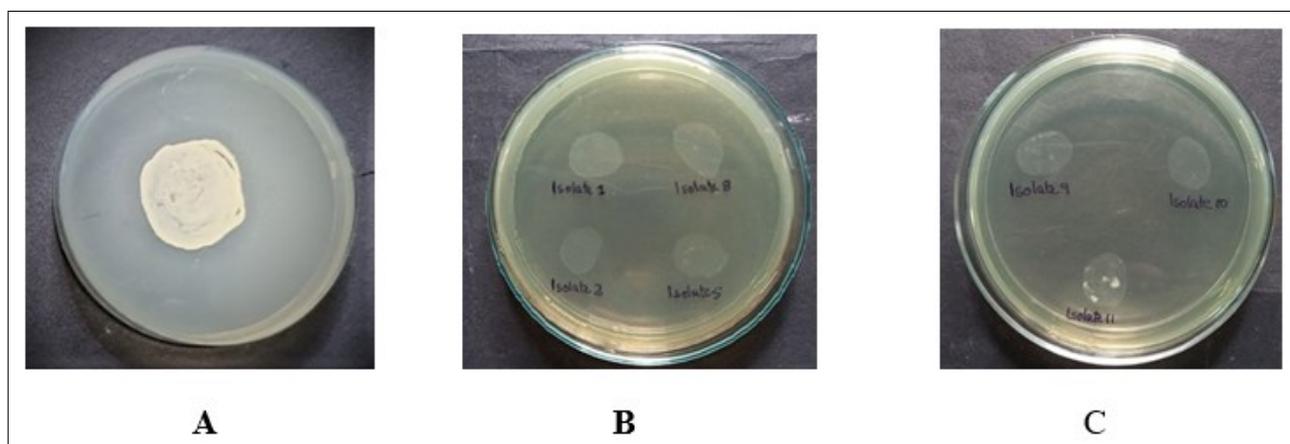

Fig. 6. Absence of DNase activity in test isolates. (A) Positive control (*Staphylococcus aureus*) showing a clear zone around the colony, indicating DNase activity; (B, C) absence of a clear zone, indicating lack of DNase activity in the test isolates.
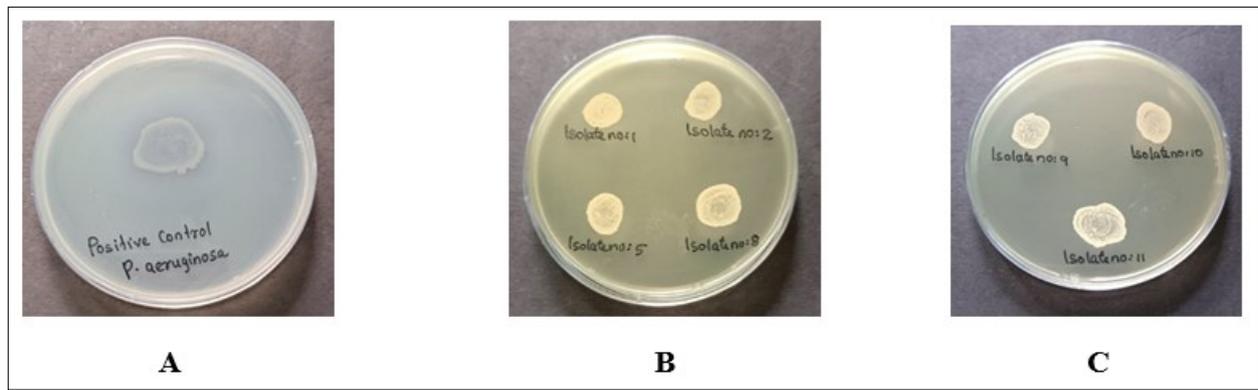
Fig. 7. Gelatin-degrading ability of test isolates. Bacteria with gelatinase activity show a clear zone around the colony. A: Positive control (*P. aeruginosa*) indicates the presence of gelatinase activity; B-C: show the absence of a clear zone around the colonies, indicating the absence of gelatinase activity in the test isolates.
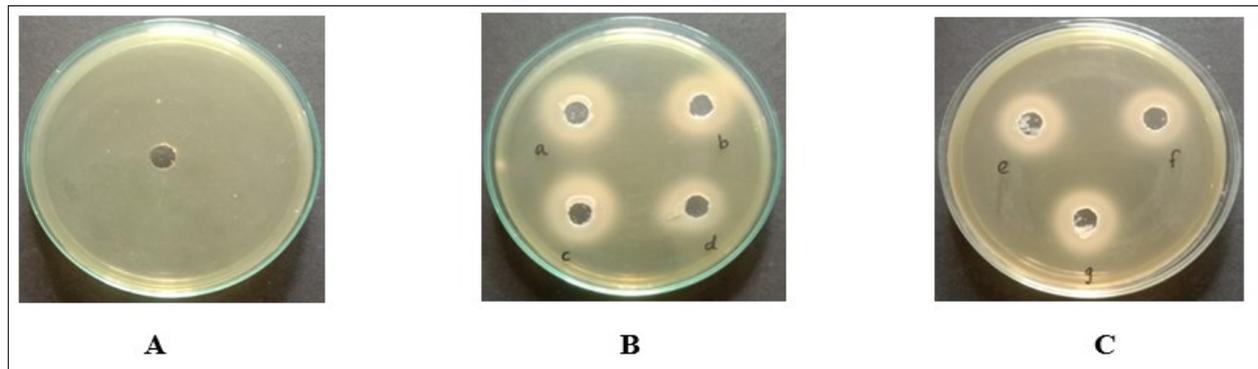

Fig. 8. Protease enzyme activity of test isolates. Bacteria with protease activity show a precipitated zone around the colony. A: Control MRS broth; B-C: test isolates (B) a: MBTUSKKTRF01, b: MBTUSKKTRF02, c: MBTUSKKTRF05, d: MBTUSKKTRF08, (C) e: MBTUSKKTRF09, f: MBTUSVKTRV010, g: MBTUSVKTRV011).

Bacteria with extracellular cellulase enzyme activity can hydrolyze carboxymethylcellulose (CMC) in media, forming clear zones that degrade polysaccharides into shorter saccharides. Then they cannot adsorb Congo red dye. CMC is the best substrate for cellulase production because it can induce the bacteria to produce cellulase enzymes (42). The present study reveals those 7 isolates of LAB produce cellulase enzymes (Fig. 9). Previous studies observed that about 28 bacterial isolates from peat soil from Ogan Komering Ilir, South Sumatra, Indonesia showed cellulase activity in CMC medium (44). The presence of both protease and cellulase enzymes suggests potential applications in agriculture, human health and animal health.

All *in vitro* experiments in this study were performed in triplicate to ensure reliable and reproducible data. However, large-scale production poses a higher risk of unexpected errors due to complex and dynamic factors such as temperature, pH and nutrient availability, often requiring real-time troubleshooting and process adjustments during ongoing production. In the agricultural industry, various bacteria (*Pseudomonas* and *Bacillus*) based products are already well established in the market. However, LAB offer several advantages over these conventional bacterial strains, particularly in terms of safety and specialized functions such as biocontrol, stress tolerance and soil remediation. Their long history in food science has earned them a "generally recognized as safe" (GRAS) status, which further supports their use in sustainable agriculture and the reduction of chemical inputs.
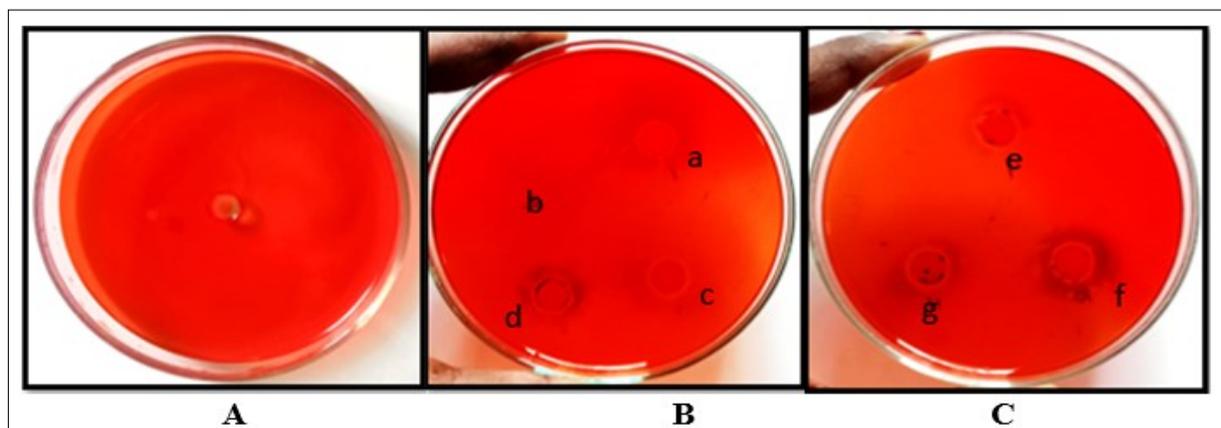

Fig. 9. Cellulase enzyme activity of test isolates. Bacteria exhibiting cellulase activity show a clear zone around the colony. A: Control MRS broth showing absence of a clear zone, indicating lack of cellulase activity; B–C test isolates: (B) a: MBTUSKKTRF01, b: MBTUSKKTRF02, c: MBTUSKKTRF05, d: MBTUSKKTRF08; (C) e: MBTUSKKTRF09, f: MBTUSVKTRV010, g: MBTUSVKTRV011, showing clear zones around the colonies after addition of 1% Congo red, indicating cellulase activity.

The LAB isolates investigated here demonstrate promising potential in the expanding probiotic market; however, achieving economic viability and competitiveness will depend on addressing key challenges, including production scale-up, clinical validation and differentiation from well-established commercial probiotic strains. It is important to note that this research represents only preliminary work and extensive future research is needed to optimize fermentation parameters, assess stability and safety and conduct thorough efficacy evaluations to establish these isolates as commercially viable probiotic candidates. Such advancements will strengthen product formulation, enhance manufacturing scalability and support their successful introduction into both agricultural and probiotic markets.

Future studies mainly focus on elucidating molecular mechanisms of LAB-host interactions, optimizing LAB strains for specific crop and human microbiome needs and assessing their synergistic effects with novel agricultural amendments like silica nanoparticles and biochar to enhance plant and soil health; integrating these probiotics into crop cultivation aligns with sustainable food security innovations such as silica nanoparticles improving germination and biochar reducing nitrous oxide (N_2O) emissions, ultimately maximizing LABs' multifunctional roles as biofertilizers, growth promoters and health protectants through cutting-edge plant-microbe and soil-microbe engineering strategies (45, 46).

Today, artificial intelligence (AI) is present in all fields and advanced research on probiotics must integrate AI technologies. One of the best approaches involves using digital twins and the industrial metaverse. The future work could integrate the digital twin and industrial metaverse frameworks in probiotic biotechnology by developing virtual replicas of fermentation systems. A digital twin can simulate the physiological responses of LAB under varying pH, bile concentration and nutrient conditions to optimize growth and production before large-scale cultivation and significantly reduce experimental cost and resource consumption. In an industrial metaverse environment, researchers and engineers can work together in a virtual smart probiotic factory using 3D digital models of fermenters, sensors and microbial cultures in real time. The addition of AI, IoT (Internet of Things) based data acquisition and predictive analytics allows continuous monitoring, adaptive control and techno-economic forecasting of probiotic manufacturing processes. So, this digital ecosystem accelerates the design and scale-up of probiotic strains and also aligns with the principles of Industry 5.0 by promoting both economic and environmental sustainability. In the present study, the digital models of the 7 LAB isolates could be a part of a virtual database, which predicts modeling of probiotic performance and supports global collaboration in strain improvement and industrial application development (47–50).

Conclusion

The present study confirms the hypothesis that non-dairy plant reservoirs, particularly flowers and vegetables, serve as rich and underexplored sources of probiotic diversity, revealing a wide spectrum of LAB with promising multifunctional attributes. The identified isolates, especially *P. pentosaceus* and *L. fermentum*, demonstrated notable acid and bile tolerance, enzymatic activity and safety characteristics, indicating their strong potential for plant-based, eco-friendly and economically sustainable probiotic applications. From an industrial perspective, this investigation represents the first step toward large-scale product development, creating opportunities to diversify probiotic manufacturing beyond conventional dairy systems. The thorough characterization of these locally isolated LAB strains establishes a scientific foundation for their industrial utilization in developing functional foods, nutraceuticals and bio-preservatives derived from plant-based sources.

Future studies, the integration of the digital twin system accelerates the transition from laboratory discovery to commercial production, ensuring both environmental and economic sustainability. Furthermore, genomic, metabolomic and process engineering analyses will be crucial for unlocking the full biotechnological potential of these isolates, ultimately paving the way for innovative, large-scale probiotic product development within the next generation of smart, data-driven biomanufacturing systems.

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Authors' contributions

SK carried out the investigation, methodology, analysis and writing manuscript. KTR carried out validation, supervision and editing the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

Declaration of AI assisted technologies in the writing process

During the preparation of this work, the author(s) used GraphPad Prism 8 and MEGA in order to draw a graph, statistical analysis and phylogenetic tree construction respectively. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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Additional information