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Isolation of *Lactiplantibacillus pentosus* and *Enterococcus faecium* from Traditional Iranian Koozeh Cheese

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Abstract

Recent studies have directed attention toward isolating lactic acid bacteria (LAB) from fermented foods due to their significant functional and probiotic properties. These bacteria play essential roles in food fermentation, preservation, and potential health benefits, making their characterizat9999999ion from traditional food sources of scientific interest. Two LAB isolates were obtained from traditional Iranian Koozeh cheese and characterized for their potential functional and probiotic properties. The isolates were identified using 16S rRNA gene sequencing as *Lactiplantibacillus pentosus* and *Enterococcus faecium*. Their physiological and probiotic-related properties were evaluated, including tolerance to temperature, pH, and bile salts, as well as cell surface characteristics. The isolates showed survival at 15°C and 37°C, while only *E. faecium* demonstrated growth at 45°C. Hydrophobicity assays revealed values of 29.08 % for *E. faecium* and of 63.57 % for *L. pentosus*, while auto-aggregation was 34.77 % and 38.98 %, respectively. Both isolates showed good tolerance to bile salts and acidic conditions, with strain-dependent differences. *Enterococcus faecium* maintained higher viability than *Lactiplantibacillus pentosus* under increasing bile concentrations and low pH. Overall, both strains demonstrated survival ability under gastrointestinal-like stress conditions, with *E. faecium* showing greater resistance. Additionally, both isolates exhibited co-aggregation ability against *Escherichia coli* (PTCC No. 1330, ATCC 8739) and were non-hemolytic. The co-aggregation percentages were 60.65% for *Enterococcus faecium* and 27.59% for *Lactiplantibacillus pentosus*. Consequently, the results suggest that both strains possess promising functional properties, indicating their potential application in food fermentation and probiotic development.

1. Introduction

Lactic Acid Bacteria (LAB) are a diverse group of Gram-positive, non-spore-forming microorganisms widely used in food fermentation and preservation. They produce lactic acid and other bioactive compounds that drive acidification, flavor development, and textural changes in foods. LAB also plays a key role in inhibiting spoilage organisms and foodborne pathogens. Through these activities, they make a significant contribution to food safety and quality (Akpoghelie *et al.*, 2025). Furthermore, isolating LAB has gained global attention due to its importance in food processing and its beneficial health effects. Distinct LAB strains can modulate gut microbiota, support immune function, regulate inflammation, and produce bioactive compounds. They are commonly found in nature, including within the gastrointestinal tract of humans and animals, as well as in plants and fermented foods (Mahooti *et al.*, 2024; Yahfoufi *et al.*, 2018).

Traditional fermented dairy products are rich sources of LAB, mostly due to spontaneous fermentation by natural microbiota. Iranian Koozeh cheese stands out as a compelling example of artisanal cheese production. The unique micro-ecosystems inherent in its creation, shaped by local raw materials, specific environmental conditions, and traditional processing methods, actively foster the development of distinct, strain-specific LAB populations. The production of Koozeh cheese involves using raw or minimally processed milk, with ripening occurring inside porous clay containers (Koozeh). This traditional maturation method facilitates gradual moisture loss and essential gas exchange, critically influencing the microbial dynamics and sensory characteristics of the final product (Hassan Hassanzadazar *et al.*, 2012). Moreover, this process involves long fermentation times and no defined starter cultures, which promote the growth of autochthonous LAB adapted to harsh conditions such as low pH and high salt. Previous studies regarding Koozeh cheese have reported the presence of various *Lactobacillus* (now reclassified as several genera of *Lactiplantibacillus*), *Lactococcus*, and *Enterococcus* species, some of which exhibit promising probiotic and antimicrobial properties (Edalatian *et al.*, 2012; Mohammadzadeh *et al.*, 2025).

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Lactic acid bacteria isolated from traditional fermented products such as Koozeh cheese show important health potential supported by increasing scientific evidence. These strains can survive harsh gastrointestinal conditions, including gastric acid and bile salts. They also display key probiotic traits such as cell surface hydrophobicity and strong auto-aggregation. These features indicate their ability to pass through the gut and adhere to intestinal epithelial cells, which is essential for their beneficial effects on host health (Hassan Hassanzadazar *et al.*, 2012). Many cheese-derived lactic acid bacteria (LAB) produce antimicrobial compounds, including organic acids, hydrogen peroxide, and bacteriocins. These compounds help hinder foodborne pathogens, facilitating food safety and maintaining a healthy balance of gut microbes. Koozeh cheese, in particular, demonstrates strong antimicrobial activity and promising probiotic properties, highlighting its value as a source of functional LAB (H. Hassanzadazar *et al.*, 2012). Thus, isolation and characterization of LABs from Koozeh cheese have been undertaken and could result in finding novel LAB strains with beneficial properties.

Given this background, the systematic isolation and characterization of LAB from dairy resources such as Koozeh cheese can provide strains with dual technological and health-promoting potential (Ardila, 2025). In vitro evaluation of functional traits such as cell surface hydrophobicity, auto-aggregation, co-aggregation, and tolerance to bile salts and low pH is an essential first step in selecting promising LAB strains. Adequate hydrophobicity and strong auto-aggregation are often associated with improved adhesion to the intestinal mucosa, while co-aggregation with pathogens may help inhibit their colonization. Besides, survival under acidic and bile conditions, which mimic the gastrointestinal environment, is essential for LAB potential assessment (Ouili *et al.*, 2025). This study characterizes LAB isolated from Koozeh cheese using 16S rRNA sequencing, identifying strains such as *Enterococcus faecium* and *Lactiplantibacillus pentosus*. Our primary objective was to isolate and identify LAB from Koozeh cheese collected from different geographical locations and to determine whether these local cheese samples contain LAB, focusing on regional variation rather than on simply reporting LAB presence. Functional properties, including hydrophobicity, aggregation ability, and tolerance to bile salts and pH, were assessed to estimate probiotic potential. The results suggest that Koozeh cheese is a valuable source of functionally significant LAB.

2. Materials and Methods

2.1. Bacterial isolation from Koozeh cheese

Initially, two samples of Koozeh cheeses were collected from the mountain located in the north of Iran (Deylaman, Gilan) during the autumn and were transferred to the lab with proper equipment in ice bags and stored at -20°C for the subsequent experiments. After removal from −20 °C storage, a representative portion of the Koozeh cheese sample was aseptically collected, and 0.1 g was immediately weighed and suspended in 1 mL of saline solution containing 0.9% sodium chloride. The sample was homogenized by vortexing and shaking for 15 minutes to facilitate the isolation of lactic acid bacteria, followed by subsequent bacterial isolation procedures. Following preparation of serial dilution, 0.1 mL of the last four serial dilutions (up to 10^{-9}) was taken and spread onto the surface of the pre-dried de Man Rogosa Sharpe (MRS) agar (Ibersko, Iran) medium. The plates were incubated under anaerobic conditions, followed by inoculation using a candle jar (a sealed glass jar with a lit candle) at 37°C for 48 hours. Morphologically different colonies were then sub-cultivated by streaking on new MRS agar plates after observing and confirming the successful growth of bacteria on MRS agar, and incubated at 37°C for 24-48 hours inside the incubator. Furthermore, the isolated colonies were then cryopreserved with 15% glycerol and stored at -80 °C for future studies (Dosuky *et al.*, 2022).

2.2. Gram Staining

Gram staining was carried out through the standard procedure, and then Gram-positive *bacilli*/*cocci* were chosen for further characterization.

2.3. Catalase Test

Lactic acid bacteria have been tested for their catalase-negative or positive. In general, catalase enzymes break down hydrogen peroxide into oxygen (observed as the formation of bubbles). The catalase test was carried out by directly adding a drop of a 30% solution of hydrogen peroxide to the Petri dish cultured with each isolate. The *E.coli* samples were used to observe catalase-positive.

2.4. Growth rate at different temperatures (Assay of temperature tolerance)

Bacterial growth at different temperatures is commonly used to aid in the screening of lactic acid bacteria, with the typical isolation range for bacilli and cocci being 15–45 °C (Maqsood *et al.*, 2013). Hence, for this test, temperatures of 10 °C, 15 °C, 37 °C, and 45 °C were selected, and after inoculation of 10 mL broth with 0.1 mL of each isolate and incubation for 24-48 h, 0.1 mL inoculum from pre-culture was transferred to MRS plates and incubated at 37°C and 45°C separately for 48 h. The growth of LAB on MRS agar plates was used to designate isolates as temperature-tolerant, as shown in Table 1.

2.5. Tolerance to different pH (Assay of acid tolerance)

According to articles conducted beforehand (Mantzourani *et al.*, 2019; Plessas *et al.*, 2017), pH values of 2.5 and 4.5 are utilized. Hence, 10 mL of MRS broth was inoculated with 0.1 mL of the lactic acid bacteria isolates. After that, the cultures were centrifuged, and the pellets were dissolved in different pre-prepared Phosphate Buffered Saline (PBS) with pH adjusted to 2.5 and 4.5 using hydrochloric acid (HCl). Afterwards, serial dilutions were carried out immediately after dissolving pellets in different PBS buffers with pH 2.5 and pH 4.5 (Mantzourani *et al.*, 2019; Maqsood *et al.*, 2013). Then, the PBS-containing pellets were incubated for 2 and 4 h. After incubation, a serial dilution and colony count on MRS agar was carried out to evaluate the corresponding

$\log_{10}$ CFU/ml colony count at the time of 0 was considered as the control.

2.6. Growth rate on bile salt (bile salt tolerance)

Survival rate against bile salt has also been carried out to evaluate the lactic acid bacteria functionality (Gilliland & Walker, 1990; Plessas *et al.*, 2017).

First, fresh broth media of MRS were made and inoculated with 0.1 mL of fresh lactic acid bacteria. Afterward, the 24 h cultured lactic acid bacteria were centrifuged, and the precipitated bacteria were dissolved in pre-prepared 0.2, 0.4, 0.6% bile salt-containing PBS (Mantzourani *et al.*, 2019; Maqsood *et al.*, 2013). Immediately, and again after 3 hours of incubation, serial dilution was followed by plating on MRS agar, and the corresponding concentrations were evaluated as $\log_{10}$ CFU/ml.

2.7. Hydrophobicity

The strain cell surface hydrophobicity was assessed by measuring cell adhesion to hydrocarbon toluene using the method of Valeriano *et al.* (Valeriano *et al.*, 2014). Briefly, an overnight culture was harvested at 10,000 rpm for 15 minutes, washed twice, and then resuspended in PBS to an optical density at 600 nm, corresponding to 10^8 CFU/ml, which was designated as A0.

An aliquot (3 mL) of the cell suspension was mixed with 1 mL of toluene, and the mixture was strenuously mixed for 90 seconds. Then, the mixtures were left to stand at 37 °C for one hour to allow phase separation. Following the toluene phase removal, the OD 600 of the aqueous phase was measured and designated as A1. Each experiment was performed in triplicate from independent cultures. Cell surface hydrophobicity (H) was determined as follows:

$$H(\%) = 1 - \frac{A_1}{A0} \times 100 \quad (1)$$

Where A_1 is the absorbance of the aqueous phase measured at 600 nm after toluene phase removal, reflecting the remaining bacterial cells, while A_0 is the initial absorbance before treatment.

2.8. Auto-aggregation

For Auto-aggregation assessment, 10 ml isolates overnight cultures were harvested by centrifugation at 10,000 rpm for 10 minutes; then the pellets were washed twice with PBS and adjusted to an optical density at 600 nm, corresponding to 10^8 CFU/ml. For the auto-aggregation assay, each bacterial suspension (4 mL) was vortexed ($A_{initial}$) and then incubated for 2 h at 37 °C (A_{2h}). An aliquot was slowly collected from the sample surface, and its absorbance was measured at 600 nm. All aforementioned experiments were repeated three times. Auto-aggregation was determined with the following equation (Zawistowska-Rojek *et al.*, 2022).

$$\text{Auto-aggregation} = 1 - \frac{A_{2h}}{A_{initial}} \times 100 \quad (2)$$

Where $A_{initial}$ and A_{2h} are the absorbances of samples at the time of 0 and after 2 h, respectively.

2.9. Co-aggregation

Following the PBS washing of the overnight culture of isolated strains and *E. coli* (PTCC No: 1330, ATCC 8739), each strain was resuspended in PBS to a final concentration of around 1×10^8 CFU/ml. An equal number of strains and *E. coli* (PTCC No: 1330, ATCC 8739) (1.5 mL) were mixed and then vortexed for 10 s and incubated for 2 h at 37°C.

The absorbance (OD) of the mixed suspension was then measured at 600 nm (OD mix) and compared with tubes containing isolated ($A_{lactobacillus}$) and *E.coli* ($A_{pathogen}$) after incubation. The co-aggregation was measured as the following equation:

$$\text{Coaggregation } (\%) = [1 - \frac{A_{mix}}{(A_{lactobacillus}+A_{pathogen}/2)}] \times 100 \quad (3)$$

In this equation, A mix stands for the absorbance of the mixed suspension of Lactobacillus and pathogen, while $A_{lactobacillus}$ and $A_{pathogen}$ are the absorbances of Lactobacillus and pathogen, respectively.

2.10. Hemolytic Activity Analysis

The isolated strains were cultured in MRS medium for 22–24 h at 37°C. Streak plate methods were performed on sheep blood agar plates (Merck, Germany) to analyze hemolytic activity. Plates were then incubated at 37 °C. The appearance of clear zones around the bacteria colonies was indicated as α-, β-, or γ-hemolysis. If the colony has a grass-green ring around it, it is α-hemolysis; but if a clear ring appears around the colony, it is β-hemolysis, and if we do not observe any changes in the colony, it is γ-hemolysis, which means no hemolysis.

2.11. 16S rDNA sequencing and sequence analysis

After the DNA extraction of strains using a DNA extraction kit (PGA bacterial DNA extraction kit), the quality of the extracted DNA was measured using a micro-UV spectrophotometer (NanoDrop Technologies, United States).

Then, the primer (4f: 5'- TATCGGAGAGTTTGATCCTGG -3' and (1505r: 5'-GATACGGCTACCTTGTTACGA-3)' were used to amplify the 16S rDNA gene. Each polymerase chain reaction (PCR) reaction mixture (25 µl) contained 12.5 µl PCR Master Mix, 9.5 µl nuclease-free H_2O, 1 µl forward primer (1 pmol), 1 µl reverse primer (1 pmol) and 1 µl DNA sample.

The PCR procedure was carried out as follows: pre-denaturation at 95°C for 3 min, followed by 30 cycles (45 s denaturation at 93°C, 60 s annealing at 53°C, and 90 s extension at 72°C), with a final extension at 72°C for 10 min. The PCR products were stored at 4°C, and subsequently analyzed by 2% agarose gel electrophoresis stained with Golden View to confirm the amplification of approximately 1500 bp 16S rDNA fragments. The confirmed PCR products were then sent to STAB VIDA for 16S rDNA sequencing.

The obtained sequences were analyzed using the BLAST program, and homology alignment was performed against bacterial nucleotide sequences available in GenBank for molecular identification of the isolates.

2.12. Statistics

All experiments were carried out in triplicate. Statistical analyses were performed using GraphPad Prism (Version 8.4.3.6). For comparisons between two groups, an unpaired two-tailed Student's t-test was used. A p-value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Identification and assessment of functional properties of isolated strains

In this study, two isolates from Koozeh cheeses were chosen and then underwent several tests. Results showed that both isolates were Gram-positive and catalase-negative, while *Lactiplantibacillus pentosus is* rod-shaped, and *Enterococcus faecium* is a Gram-positive coccus. The isolated strains were identified molecularly through 16S rRNA; the 16S rRNA test accurately identified the strain. Followed by confirmation of 1500-nucleotide fragments of isolates through Electrophoresis. Purified DNA of each isolate has a concentration of 83 ng/µl and 74 ng/µl at a wavelength of 260 nm, respectively. The sequence of the first strain revealed 100% similarity to *Lactiplantibacillus pentosus* (NCBI GenBank accession AZCU01000004). The sequence of the strain itself has been deposited in GenBank under accession number PX247927.1. Additionally, sequence analysis showed that the second strain is 100% identical to *Enterococcus faecium* (GenBank accession LC096216). The strain's sequence has been submitted to GenBank with accession PX249791. Therefore, throughout the article, we mentioned our first isolate as *Lpb. Pentosus* and the second isolate as *E. faecium*. The BLAST result for *Enterococcus faecium* shows a perfect match with 100% identity (1465/1465), full query coverage (100%), and an E-value of 0.0. In contrast, *Lactiplantibacillus plantarum* shows a nearly identical match with ~99.8% identity (1466/1469), also 100% query coverage, and an E-value of 0.0. Both alignments are highly significant, with the first being a perfect match and the second differing by only three bases. The presence of LAB in Koozeh cheese enhances gut health and nutritional value, with species such as *Lactobacillus*, *Enterococcus*, and *Leuconostoc* contributing to its flavor, texture, microbial stability, and health benefits. These isolates are essential for fermentation and flavor development through lactic acid and aroma compound production (Edalatian *et al.*, 2012). Similarly, research on the microbial composition of traditional Koozeh cheese has led to isolate of diverse LABs, including *Lactiplantibacillus pentosus* and *Enterococcus faecium* (Mohammadzadeh *et al.*, 2025; Sonei *et al.*, 2024). These microorganisms contribute to the biochemical characteristics and potential health benefits of the cheese through their metabolic functions and probiotic activities. Isolating *Lactiplantibacillus pentosus* and *Enterococcus faecium* from Koozeh cheese highlights the rich diversity of microbes and the important reservoirs of functional lactic acid bacteria. The harsh environment of fermented dairy, defined by high salinity and low pH, promotes the adaptation and persistence of both *Lactiplantibacillus pentosus* and *Enterococcus faecium* (Maghsoudlou). *L. pentosus* increases microbial safety and texture through acidification and exopolysaccharide production in addition to providing probiotic benefits such as antioxidant activity and survival under gastrointestinal conditions (Behbahani *et al.*, 2024). Furthermore, food-grade traditional sources-derived *E. faecium* isolates like Koozeh cheese contribute to desirable sensory profiles via proteolytic and lipolytic enzymes and bolster safety through the production of bacteriocins, suppressing pathogenic bacteria (Holzapfel *et al.*, 2018). The results demonstrated clear physiological differences between the two isolates, with *Enterococcus faecium* exhibiting growth in 6.5% NaCl and at 45°C, in contrast to *Lactiplantibacillus pentosus*, which did not grow under such conditions. Both strains were catalase- and oxidase-negative, consistent with lactic acid bacteria-related characteristics. Differences in carbohydrate utilization patterns further supported their taxonomic differentiation.

3.2. Acid and bile tolerance

To evaluate whether lactic acid offers biological benefits, it is necessary to first establish a strain's ability to withstand acidic and bile salt conditions in the gastrointestinal tract. The survival rate of isolated strains at pH 4.5 and 2.5, and the amount of different bile salt concentrations, including 0.2, 0.4, and 0.6%, were tested and shown in Figures 1A and 1B, respectively. *Lpb. Pentosus* and *E. faecium* showed 100% survival at pH 4.5, as this is the natural pH for them. At a more acidic condition, pH 2.5, time 0 (T0), the $\log_{10}$ CFU/mL of the isolates *Lpb.Pentosus* and *E. faecium* were 7.793 ± 0.111 and 8.377 ± 0.226, respectively. Afterward, at time 2 (T2), *Lpb.Pentosus* and *E. faecium* showed more reduction where *Lpb. Pentosus* $\log_{10}$ CFU/mL was 7.34 ± 0.114 and *E. faecium* was 7.4 ± 0.05. Regarding 0.2, 0.4, and 0.6% bile salt concentration, for 0.2 bile salt concentration *Lpb. Pentosus* and *E. faecium* $\log_{10}$ CFU/mL dropped from 9.023 ± 0.124 and 9.553 ± 0.071 at (T0) to 7.670 ± 0180 and 8.987 ± 0.122 at (T3). At a concentration of 0.4% bile salt, the survivability of *Lpb. pentosus* remained relatively stable, exhibiting no significant change from 8.99 ± 0.161 at T0 to 9.017 ± 0.07 at T3, whereas *E. faecium* demonstrated a reduction in survivability, decreasing from 9.79 ± 0.20 at T0 to 9.197 ± 0.186 at T3.

At a harsher condition, when we increased the bile salt concentration to 0.6%, both strains could not withstand the condition, where at (T0) *Lpb. Pentosus* and *E. faecium* showed 7.743 ± 0.068 and 7.847 ± 0.060 survival rates, respectively, and after three hours the survivability of both strains decreased slightly to 6.483 ± 0.042 and 7.170 ± 0.066, respectively. Invistigations showed that at pH 4.5, both *L. pentosus* and *E. faecium* exhibited similar survivability, indicating that this mildly acidic environment does not significantly differentiate their tolerance or growth capacity. This observation aligns with a study in 2018, where the authors reported that species maintained stable populations under moderately acidic conditions, suggesting their adaptability in environments such as fermented foods or the gastrointestinal tract, where pH approximates this range. This similarity may be due to the fact that pH 4.5 is within the acid-adaptation range of both species, allowing them to maintain cellular homeostasis through common protective mechanisms,

including proton pumping, membrane adaptation, and stress-response activation (Pérez Montoro *et al.*, 2018). Under highly acidic conditions (pH 2.5), both isolates showed a decrease in viability over time. *Lpb. pentosus* declined from 7.793 ± 0.111 to 7.34 ± 0.114 log CFU/ml, while *E. faecium* decreased from 8.377 ± 0.226 to 7.40 ± 0.05 log CFU/ml after 2 hours. Despite this reduction, both strains maintained relatively high survival, indicating strong acid tolerance. This agrees with previous reports showing that *Lpb. pentosus* can survive simulated gastric conditions at low pH for several hours (Montoro *et al.*, 2016). Furthermore, Zhang *et al.* assessed the probiotic potential of *Enterococcus faecium* strain 9N-2 and reported that the strain maintained its viability under low pH conditions, demonstrating notable acid tolerance, probably because of proper acid-resistance mechanisms. These findings support the ability of certain *E. faecium* strains to withstand acidic stress, consistent with the moderate reduction observed in our study at pH 2.5 (Neslihan Dikbaş *et al.*, 2024). Bile salt resistance is a necessary criterion for LAB survival, as bile can damage bacterial membranes. In this study, both *Lpb. pentosus* and *E. faecium* exhibited concentration-dependent tolerance to bile salts, with relatively high survival at 0.2% and 0.4%, particularly for *E. faecium*. However, at 0.6%, a significant decline in viability was observed, mainly in *Lpb. pentosus*, showing increased stress at higher concentrations. These findings are consistent with earlier reports demonstrating that *Lpb. pentosus* from fermented olives can tolerate up to ~0.3% bile salts, with reduced survival at higher levels (Montoro *et al.*, 2016). Moreover, similar findings have been described for *Lactobacillus plantarum*, where survival declined significantly at bile concentrations ≥0.5%, demonstrating a concentration-dependent inhibitory effect within the physiological range.

E. faecium's better tolerance may be due to its better mechanisms for managing bile-induced stress, such as superior efflux pump activity and a more stable cell membrane (Gökmen *et al.*, 2024). Viable counts above 6 log CFU/ml, even at 0.6% bile, exhibit considerable bile resistance in both strains. Since physiological bile levels are around 0.2–0.3%, their strong survival offers a good possibility for gastrointestinal persistence.

3.3. Temperature survival

Bacterial isolates were tested for growth at 10 °C, 15 °C, 37 °C, and 45 °C; although the typical range for isolating bacilli and cocci is 15–45 °C, 10 °C was included as a minimum cold check to screen strains with lower-temperature tolerance. In this study, they were tested at 10°C, 15°C, 37°C, and 45°C. As shown in Table 1, both strains were able to survive at 15°C and 37°C. No growth was observed at 10°C for either strain. At 45°C, *E. faecium* showed growth, whereas *Lpb. pentosus* did not grow.

After isolation, the biochemical characterization of the isolated strains was carried out to confirm their phenotypic identity. The results demonstrated clear physiological differences between the two isolates, with *Enterococcus faecium* exhibiting growth in 6.5% NaCl and at 45°C, in contrast to *Lactiplantibacillus pentosus*, which did not grow under such conditions. Both strains were catalase- and oxidase-negative, consistent with lactic acid bacteria-related characteristics. Differences in carbohydrate utilization patterns further supported their taxonomic differentiation.

3.4. Hydrophobicity

The cell surface hydrophobicity was evaluated by affinity to the organic solvent (Toluene) of the lactic acid bacteria strains and is a measure of their intestinal colonization, that is, adhesion and persistence once they have entered the intestinal cavity, *Lpb. Pentosus* hydrophobicity was a mean of 63.57% and *E. faecium* showed 29.08% of hydrophobicity.

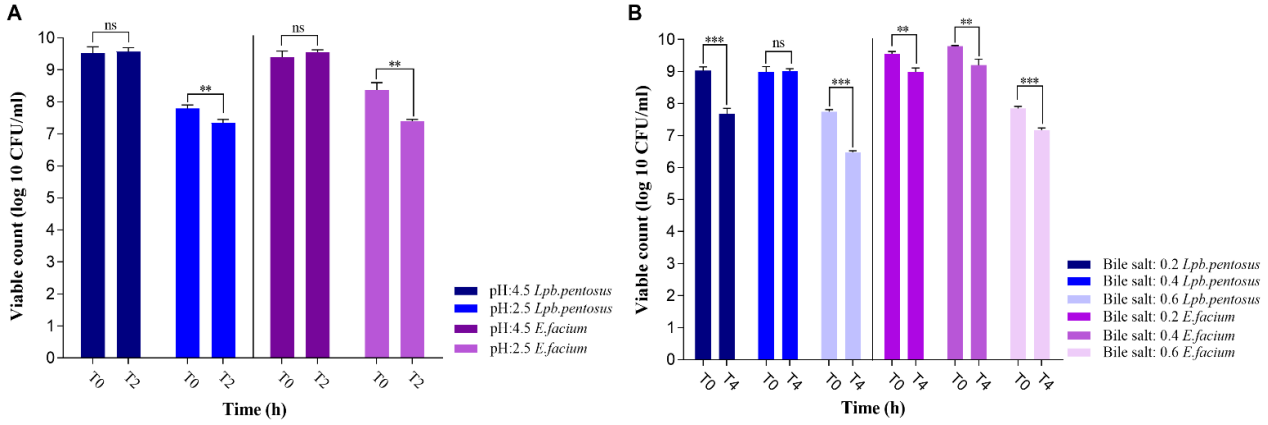

Figure 1. Evaluation of the survival rates of *Lpb. pentosus* and *E. faecium* after conducting acid and bile tolerance assays. A) Acid tolerance; B) Bile tolerance.Note. The data are shown as the mean ± standard deviation (SD) of three independent experiments. Statistical analysis for each dataset was performed using an unpaired two-tailed Student's t-test of three replicates. Symbols *, **, and *** represent $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively, while ns indicates no significant difference.

Table 1. The Effect of Different Incubation Temperatures on the Growth and Viability Lpb. pentosus and E. faecium

Strain	10 ˚C	15 ˚C	37 ˚C	45 ˚C
Lpb.Pentosus	-	+	+	-
E. faecium	-	+	+	+

The data is shown in Figure 2. Worth mentioning, cell surface hydrophobicity is a vital indicator of bacterial adhesion and potential intestinal colonization.

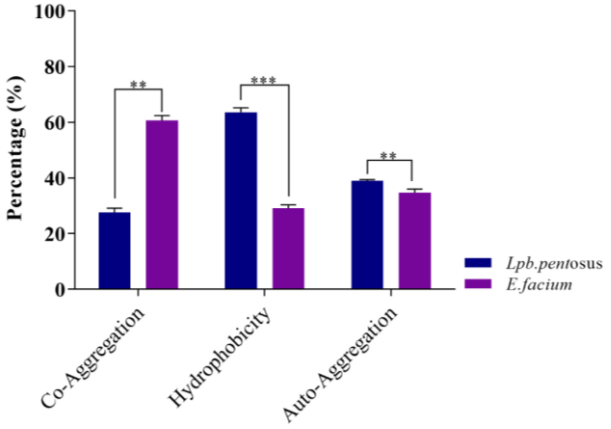

Figure 2. Comprehensive evaluation of functional properties of lactic acid isolates *Lpb. pentosus* and *E. faecium*, including co-aggregation, hydrophobicity, and auto-aggregation. Note. Statistical differences between strains for each assay were determined using unpaired two-tailed t-tests of three independent experiments. Symbols *, **, and *** demonstrate the p value 0f ≤ 0.05, ≤ 0.01, and ≤ 0.001 respectively.

The data is shown in Figure 2. Worth mentioning, cell surface hydrophobicity is a vital indicator of bacterial adhesion and potential intestinal colonization. In this study, *Lpb. Pentosus* showed significantly higher hydrophobicity (63.57%) than *E. faecium* (29.08%), presenting a stronger affinity for epithelial surfaces and potentially greater gastrointestinal persistence.

These outcomes align with previous reports on *Lactiplantibacillus pentosus* isolated from fermented olives, in which hydrophobicity values of 50–70% were associated with enhanced adhesion capacity (Montoro *et al.*, 2016).

Moreover, lower hydrophobicity values, similar to those observed for *Enterococcus faecium*, have also been reported, with hydrophobicity ranging from 20–40%, reflecting strain-specific surface characteristics (Neslihan Dikbaş *et al.*, 2024).

3.5. Auto-aggregation

Following 2 hours, *Lpb. Pentosus* showed a 38.98% auto-aggregation rate while *E. faecium* showed a 34.77 % auto-aggregation rate. This test is usually carried out to predict the strain adhesion ability. Often, each strain with this ability, as well as co-aggression ability, can be a candidate for pathogen inhibition (Tuo *et al.*, 2013). The data is shown in Figure 2. Clearly, auto-aggregation ability, relating to the capacity of bacterial cells to adhere to each other and form clusters, was moderate in both strains after 2 h, with *Lpb. pentosus* (38.98%) slightly exceeding *E. faecium* (34.77%). These values indicate a favorable potential for colonization and biofilm formation, as aggregation enables bacteria to persist in the gut environment. A similar study has reported desirable auto-aggregation percentages for various strains isolated from wheat that have documented probiotic potential (Gao *et al.*, 2026).

3.6. Co-aggregation with *E.coli*

This test is of importance as the co-aggregation rate can be related to the isolate's competition ability with the pathogen, and the strain with this potential can be regarded as an option for bacterial therapy. As shown in Figure 2, *Lpb. pentosus* exhibited an aggregation rate of approximately 27.59% with *E. coli*, whereas *E. faecium* showed a higher rate of 60.65%. This suggests that *E. faecium* likely has stronger specific surface interactions with *E. coli*, resulting in enhanced co-aggregation. Co-aggregation results revealed a contrasting pattern between the two strains.

While *Lpb. pentosus* demonstrated moderate co-aggregation with *Escherichia coli* (27.59%), *E. faecium* exhibited a substantially higher co-aggregation rate (60.65%). High co-aggregation ability is considered beneficial for competitive exclusion, as probiotic strains can bind to pathogens and limit their attachment to intestinal epithelial cells. Similar strong co-aggregation capacities have been reported for selected *E. faecium* probiotic candidates, suggesting potential application in pathogen inhibition strategies (N. Dikbaş *et al.*, 2024). Co-aggregation results revealed a contrasting pattern between the two strains. While *Lpb. pentosus* demonstrated moderate co-aggregation with *Escherichia coli* (27.59%), *E. faecium* exhibited a substantially higher co-aggregation rate (60.65%). High co-aggregation ability is considered beneficial for competitive exclusion, as probiotic strains can bind to pathogens and limit their attachment to intestinal epithelial cells. Altogether, the combined hydrophobicity, auto-aggregation, and co-aggregation profiles suggest that *Lpb. pentosus* may have stronger adhesion potential, while *E. faecium* may display better pathogen-interaction ability, suggesting complementary probiotic characteristics between the two strains

3.7. Hemolytic Activity Analysis

Both isolates showed no hemolytic activity in comparison to the control *Staphylococcus aureus*, which showed the typical β-hemolytic activity. Overall, Koozeh cheese-isolated *Lactiplantibacillus pentosus* and *Enterococcus faecium* showed acid and bile tolerance, hydrophobicity, and aggregation-related properties in the applied assays. *L. pentosus* showed higher adhesion-related characteristics, whereas *E. faecium* showed stronger stress tolerance and co-aggregation ability. These results outline the main observed probiotic-related properties of the isolates

4. Conclusion

This study evaluated the functional properties of *Lactiplantibacillus pentosus* and *Enterococcus faecium* isolated from traditional Koozeh cheese, focusing on hydrophobicity, auto-aggregation, co-aggregation, acid resistance, and bile salt tolerance. Both strains demonstrated considerable functional potential, including strong adhesion-related traits and good survival under gastrointestinal-like conditions, suggesting their suitability as candidate probiotic LAB. Nonetheless, their co-aggregation ability also indicated possible antimicrobial interactions against pathogens, supporting their functional relevance. While these isolates demonstrated promising in

vitro properties, the safety of *E. faecium* requires strict evaluation due to potential virulence factors and antibiotic resistance genes in certain strains. Given that this study is limited to preliminary phenotypic characterization, comprehensive strain-level assessments, including virulence gene screening, antibiotic susceptibility profiling, hemolytic/cytotoxicity assays, and molecular identification, are vital to ensure safety and functional viability. Thus, future genomic characterization and in vivo confirmation are critical before employing these strains in functional food or probiotic products.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Authors' Contributions

Project supervision, Administration and Conceptualization were performed by Davood Zare and Maliheh Safavi. Methodology was conducted by Mehran Mahooti and Esmaeil Mortaz. Data curation, Data analysis, Interpretation of results were performed by Mehran Mahooti and Saeed Mirdamadi. The first draft of the manuscript was written by Mehran Mahooti. All authors have read and approved the final version of the manuscript.

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