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# Highly Potent New Probiotic Strains from Traditional Turkish Fermented Foods

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## Abstract

Traditional Turkish fermented foods like boza, pickles, and tarhana are recognized for their nutritional and health benefits, yet the probiotic potential of lactic acid bacteria (LAB) strains isolated from them remains underexplored. Sixty-six LAB strains were isolated from fermented foods using bacterial morphology, Gram staining, and catalase activity. The isolates were differentiated at strain level by RAPD-PCR (Random Amplification of Polymorphic DNA—Polymerase Chain Reaction) and twenty-five strains were selected for further evaluation of acid and bile salt tolerance. Among these, ten strains exhibited high tolerance and were subsequently assessed for adhesion to Caco-2 colorectal carcinoma cells, antimicrobial activity, exopolysaccharide (EPS) production, lysozyme resistance, and hemolytic activity. Using k-means clustering, three strains: *Lactiplantibacillus plantarum* ES-3, *Pediococcus pentosaceus* N-1, and *Enterococcus faecium* N-2 demonstrated superior probiotic characteristics, including significant acid (100% survival at pH3.0) and 0.3% bile salt tolerance (57%, 64%, 67%), strong adhesion to intestinal cells (65%, 88%, 91%), high lysozyme resistance (88%, 88%, 77%), and produced high amounts of EPS. These strains show promising potential as probiotics and warrant further investigation to confirm their functional properties and potential applications.


## Introduction

Probiotics, defined as live microorganisms that confer health benefits to the host when consumed in adequate amounts [1], have been integral to human diets for centuries. Since Metchnikoff's pioneering work on the health benefits of yogurt, the consumption of probiotic-rich foods has gained global popularity [2]. The human microbiota, comprising microorganisms that outnumber human cells by a ratio of 1.3:1, plays a crucial role in maintaining health.

Probiotic microorganisms confer numerous health benefits, particularly within the gastrointestinal tract. They have been implicated in alleviating conditions such as diarrhea, constipation, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), allergies, eczema, lactose intolerance, and *Helicobacter pylori* infections [3]. They are also involved in managing metabolic disorders including diabetes, obesity, and hypercholesterolemia, and in reducing the risks of cancer and cardiovascular diseases [1]. Probiotics exert their beneficial effects through inhibiting the proliferation of harmful bacteria by lowering intestinal pH and secreting antimicrobial substances such as hydrogen peroxide, exopolysaccharides, and bacteriocins [4]. Additionally, some probiotic strains create an anaerobic environment in the gut, which is ideal for the growth of beneficial bacteria [5].

Traditional fermented foods are significant sources of probiotics worldwide. Common probiotic foods include traditional dairy products such as yogurt, kefir, and cheese; fermented vegetables such as sauerkraut, kimchi, and pickles; and various regional specialties such as kombucha (China), boza and tarhana (Türkiye), koumiss (Turkic countries), lassi (India), tempeh (Indonesia), and miso and natto (Japan) [6]. Boza is a traditional, non-alcoholic, viscous Turkish beverage produced by fermenting grains like wheat, millet, and rice flour with yeast and lactic acid bacteria (LAB), resulting in a pale-yellow drink with a sweet–sour taste [7]. Tarhana, another traditional Turkish food, is produced by fermenting a mixture of wheat flour, yogurt, sourdough, herbs, vegetables, and spices, which is then dried for long-term storage; it is highly nutritious and consumed as a snack or soup [8].

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Therefore, the aim of this study is to (i) isolate, identify, and differentiate lactic acid bacteria from underutilized traditional foods, including boza, tarhana, and pickles, and (ii) evaluate their potential as probiotics by assessing their survival in the gastrointestinal tract (GIT), antimicrobial activity, and safety. Our work contributes to the development of new, naturally occurring probiotics with potential applications in functional foods and dietary supplements.

## Materials and Methods

### Materials

A total of sixty-six strains were isolated from traditional foods. Of these, thirty-eight were isolated from Turkish boza (TB), Bulgarian boza (BB), pickled beetroot (PT), white sourdough (EB), and einkorn sourdough (ES). Identification of strains was performed by bacterial morphology, Gram staining, and catalase activity. Twenty-six tarhana isolates (named T, H, and N), previously isolated in an earlier work [8], were included to assess their probiotic potential. All sixty-six isolates (the thirty-eight newly isolated strains and the twenty-eight from previous work) were differentiated using Random Amplification of Polymorphic DNA–Polymerase Chain Reaction (RAPD-PCR), which revealed 25 distinct LAB strains. These 25 distinct strains were then selected for further experiments to analyze their probiotic potential.

Twenty-five LAB strains were grown on MRS and M17 media (Merck KGaA, Germany). *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, and *Proteus mirabilis* ATCC 14153 were grown on Luria Bertani (LB) agar (Merck KGaA, Germany). *Enterococcus faecalis* 7–3 and *Streptococcus pyogenes* ERU-PS strains were kindly provided from Uludağ University (Toğay Lab) and Erciyes University (Sağıroğlu Lab), respectively. These strains were grown in Brain Heart Infusion broth (BHI) (Merck KGaA, Germany). *Caco-2* cells were kindly provided from Middle East Technical University (Banerjee Lab) and grown on Dulbecco's Modified Eagle Medium (DMEM) (Merck KGaA, Germany). Lysozyme from egg yolk (Amresco, MA, USA); fructose, sucrose, and peptone (Sigma Aldrich, MO, USA); Columbia agar (Sigma Aldrich, MO, USA) with 5% sheep blood; 0.1% sterile peptone water, 0.025% Trypsin–EDTA solution (Thermo Fisher Scientific, MA, USA); Bacterial Genomic DNA Miniprep Kit (Axygen, CA, USA) were used in the experiments. Phosphate buffered saline (PBS), HCl, NaOH, KCl, NaCl, $CaCl_2$, $NaHCO_3$, and bile salts purchased from Sigma Aldrich (MO, USA).

### Random Amplified Polymorphic DNA-PCR (RAPD-PCR)

The RAPD-PCR technique was used to differentiate the isolates at the strain level and to prevent duplicate studies on the same strain. Bacterial DNA isolations were done as described in Cebeci Aydın et al. [8] using Genomic DNA Miniprep Kit (Axygen, CA, USA). RAPD-PCR experiments were performed using primer M13 (Oligomer, Türkiye) on SimpliAmp Thermal Cycler (Thermo Fisher Scientific, MA, USA). Amplification conditions were as follows; after an initial denaturation at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 42 °C for 35 secs, and extension at 72 °C for 2 min. Analyses of the RAPD-PCR patterns were done with GelComparII (Applied-Maths, Ghent, Belgium). UPGMA analyses were performed and grouping assignments were set arbitrarily at an 80% similarity level [9].

### Identification of Isolates at Species Level by 16S rRNA Sequencing

Following the differentiation of isolates using RAPD-PCR, molecular identification of isolates was conducted using 16S rRNA sequencing. 16S rRNA gene is amplified by using forward (5'-ATCCGAGCTCAGAGTTTGATCCTGGC-3') and reverse primer (5'-TCAGGTCGACGCTACCTTGTTACG AC-3') (Oligomer, Ankara, Türkiye) on SimpliAmp Thermal Cycler (Thermo Fisher Scientific, MA, USA). Validation of amplified fragments was done on agarose gel and sequencing of PCR products was done (Medsantek, İstanbul, Türkiye).

### Tolerance to Acid and Bile Salt

Acid and bile salt tolerance tests were performed to assess the survival of the isolates under GIT conditions. Twenty-five actively growing LAB bacterial cultures were incubated in PBS with pH 2.0 and pH 3.0, bile salt concentrations of 0.3% and 0.5% (w/v) for 1 and 3 h durations at 37 °C [10–12]. The controls were incubated at neutral acidity (pH 7.2) and without bile salts. Following the acid and bile salt tolerance test, samples were diluted and pour-plated into MRS and M17 agar medium and incubated overnight at 37 °C. After the incubation, colonies were counted, and the survival percentages were calculated in comparison with the control group. All experiments were performed in duplicate, including the control group.

### Adhesion to Caco-2 Cells

Adhesion assay was performed according to Xu et al. [13] with slight modifications. Caco-2 cells were cultured at 5% $CO_2$ atmosphere and 37 °C for 16 days to reach

post-confluency. 1 h before the experiment, cultured cells were washed two times with 0.1% sterile peptone water and cultured in non-supplemented DMEM. Then, cultures were washed two times with 0.1% sterile peptone water, added non-supplemented DMEM containing probiotic bacterial cells ($1 \times 10^8$), and incubated for 2 h at 37 °C. Wells were washed, Trypsin–EDTA treated and incubated for 15 min at RT. Lysates were serially diluted and pour-plated on MRS and M17 agar plates in triplicates and incubated for 48 h at 37 °C. Adhesion was calculated as a percentage compared to the control group. All experiments were performed in duplicate, including the control group.

### Antimicrobial Activity

To assess the antimicrobial activity of selected probiotic bacteria, disk diffusion assay was performed [14], and bacteriocin producer *Enterococcus faecalis* 7–3 and kanamycin sulfate (5 mg/ml) (Carl Roth Gmbh, Germany) were used as positive controls [15]. Bacterial suspensions of *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153 were adjusted to 0.5 McFarland standard and planted on Luria Bertani (LB) agar plates using cotton swabs [16, 17]. Each disk was loaded with 50 µl of probiotic bacterial culture supernatant. Empty and MRS broth containing disks were used as negative controls. Agar plates were incubated overnight at 37 °C. After the incubation, the diameters of the zone of inhibition were measured to determine antimicrobial activity. All experiments were performed in duplicates including the control groups.

### Resistance to Lysozyme

Lysozyme resistance of probiotic strains was assessed according to Zago et al. [18]. Overnight grown bacteria precipitated, washed and incubated in Ringer solution (8.5 g/L NaCl, 0.4 g/L KCl and 0.34 g/L $CaCl_2$) for 10 min, added into Sterile Electrolyte Solution (SES; 0.22 g/L $CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L $NaHCO_3$) containing 100 mg/L lysozyme and incubated for 30 min at 37 °C. At the end of the incubation, cells were serially diluted with 0.9% NaCl and pour-plated on MRS and M17 agar plates. After 48 h incubation at 37 °C, colony numbers were counted, and viabilities were calculated. All experiments were performed in duplicates, including the control group.

### Production of Exopolysaccharides (EPS)

To observe the EPS production ability of the probiotics, overnight grown bacteria incubated with fresh MRS broth supplemented with 5 g/L fructose and 40 g/L sucrose at 25 °C for 48 h in anaerobic conditions [19]. At the end of the incubation, produced EPS was detected by the existence of ropy structures in the medium by using an inoculation loop [20]. All experiments were performed in duplicates including the control group.

### Hemolytic Activity

To determine the hemolytic activity of the probiotics, overnight grown bacteria streak plated on Columbia agar containing 5% sheep blood. Plates were incubated at 37 °C for 48 h. *Streptococcus pyogenes* ERU-PS hemolytic bacterial strain is used as positive control. At the end of the incubation, the hemolytic activity of probiotic strains was classified according to zone color around the bacterial colonies. Yellow and green zones were accepted as β-hemolytic and α-hemolytic, respectively, and red zones were accepted as safe (γ-hemolytic) [21]. All experiments were performed in duplicates including the control group.

### Statistical Analysis

Data analysis was performed using GraphPad Prism 10.3.1 Software. One-way analysis of variance (ANOVA) was utilized to determine the differences between treatments, and Tukey's multiple comparison test was applied to detect statistical significances, defined at $P \leq 0.05$. Two-way ANOVA was utilized to determine differences between conditions compared to the control group for each of the selected 10 strains in acid, bile salt, lysozyme tolerance, and adhesion assays. Dunnett's and Bonferroni's multiple comparison tests were applied to the data. Statistical significance was defined at $P \leq 0.05$. All data are expressed with the standard deviation. $P > 0.05$ was considered not significant; $P \leq 0.05$ was considered significant and marked with one asterisk; $P \leq 0.01$ was marked with two asterisks; $P \leq 0.001$ was marked with three asterisks; and $P \leq 0.0001$ was marked with four asterisks. All graphs were generated using GraphPad Prism Software. For generation of heatmaps, all viabilities are normalized to maximum and minimum values for each condition individually, and viabilities over 100% were accepted as 100%.

All data acquired from the experiment were normalized and standardized using IBM SPSS Statistics 29.0.1.0 Software, and all experimental results were checked individually to determine their distribution type for identifying the most promising strains using k-means clustering. Results from bile salt (0.3%, 3 h), pH 3.0 (3 h), adhesion, and lysozyme resistance assays were chosen for further analysis because they had a normal distribution. Then, the data were assessed with a hierarchical clustering algorithm to determine the most suitable cluster number to classify our strains in the most informative way. According to hierarchical clustering

analysis, four clusters were chosen, and the data were analyzed using an unsupervised k-means clustering algorithm, which utilizes kernels to predict the distance among clusters and subjects [22].

## Results and Discussion

### RAPD-PCR

RAPD-PCR was performed to group sixty-six isolates into twenty-five strains. The differentiation of strains was done using the dendrogram (Fig S1), isolates demonstrating < 80% similarity were assigned as different strains and were subjected to further probiotic characterization. The analysis was performed using GelCompar II (Applied-Maths, Belgium).

### Tolerance to Acid and Bile Salts

Probiotics are exposed to high acidity in the stomach and high concentration of bile salts in the duodenum while passing through the gastrointestinal tract. During this passage, bacteria need to survive, reach the colon, and colonize it. In acidic environments, cells lose their ability to maintain homeostasis, leading to protein denaturation, DNA damage, and eventually, cell death [23, 24]. Bacteria and yeasts can cope with acidic environments via various mechanisms, including activation of H + -ATPases, cell membrane remodeling, or intracellular proton consumption [24, 25]. Acid and bile salts can disrupt the structure of bacterial cell membranes, denature proteins, chelate metals such as iron and calcium, and cause DNA damage [26]. Probiotic microorganisms tolerate bile acids and salts by synthesizing protective proteins, altering cell membrane composition, and producing stress response proteins [27, 28].

According to the results from Fig. 1a and Table S1, four out of twenty-five strains showed high viability at pH 2.0 for both 1 and 3 h: MRS ES-7, MRS ES-11, MRS ES-17, and MRS N-1. All strains demonstrated high viability at pH 3.0 for 1 and 3 h, with fourteen strains showing particularly higher viability than the control groups (MRS ES-2, MRS ES-3, MRS ES-7, MRS ES-12, MRS ES-17, MRS PT-2, MRS PT-14, MRS PT-16, MRS N-1, MRS EB-3, MRS T-1, M17 N-2, M17 N-3, M17 N-4) (Table S1). However, thirteen strains exhibited no viability at pH 2.0 after 3 h (MRS ES-1, MRS ES-2, MRS ES-3, MRS PT-1, MRS PT-2, MRS PT-14, MRS PT-16, MRS T-1, MRS T-2, MRS T-7, M17 N-1, M17 N-2, M17 N-3), and five strains showed no viability at pH 2.0 for both 1 and 3 h of incubation (M17 TB-1, M17 TB-2, M17 BB-7, MRS EB-3, M17 N-4) (Table S1).

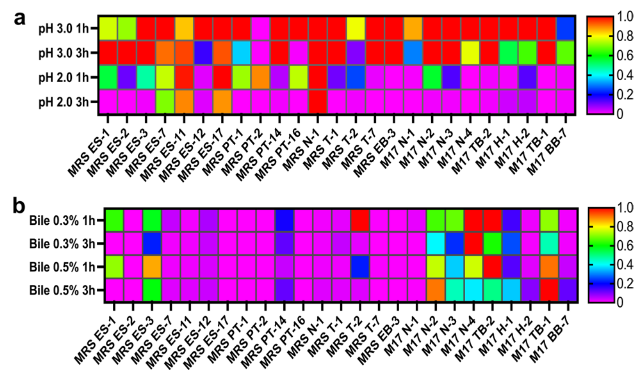


**Fig. 1** Heatmap analysis of isolates in acid (pH 2.0 and pH 3.0) (**a**), and bile salt (0.3% and 0.5%) (**b**). All viabilities were normalized according to maximum and minimum values for each condition individually. Viabilities over 100% accepted as 100%

In the bile salt tolerance test, nine strains maintained viability at 0.3% bile salt for both 1 and 3 h (MRS ES-3, MRS PT-14, MRS T-2, M17 H-1, M17 N-2, M17 N-3, M17 N-4, M17 TB-1, M17 TB-2), and seven strains showed viability at 0.5% bile salt for 1 and 3 h (MRS ES-3, MRS T-2, M17 H-1, M17 N-2, M17 N-3, M17 N-4, M17 TB-1, M17 TB-2) (Fig. 1b) (Table S1). Particularly, M17 N-2,, M17 N-3, M17 N-4 and M17 TB-1, M17 TB-2 strains showed higher viability at both 0.3% and 0.5% bile salt conditions than other strains. In contrast, ten strains showed no viability at either bile salt concentration for 1 and 3 h (MRS ES-2, MRS ES-11, MRS ES-17, MRS PT-1, MRS PT-2, MRS PT-16, MRS T-1, MRS T-7, MRS N-1, and M17 EB-3), while a few strains lost viability only after prolonged incubation (MRS ES-1 and MRS T-2) (Table S1).

Overall, *L plantarum* strains MRS ES-3, MRS ES-7, and *P. pentosaceus* strain MRS N-1 demonstrated the highest resistance to acidity, consistent with the literature [29–31]. *L plantarum* PT-14 showed increased acid tolerance upon prolonged exposure (165% at 1 h to 215% at 3 h). Contrary to the findings of Succi et al., *L. plantarum* strains were not universally resistant to bile salts [32], with only strains MRS ES-3 and *P. pentosaceus* MRS N-1 showing high tolerance to both bile salts and acidity. *Levilactobacillus brevis* MRS ES-11 exhibited high acid tolerance but was not resistant to bile salts. To the best of our knowledge, this is the first study to report an *Enterococcus dispar* strain (MRS T-2) having high acid and bile salt tolerance.

As a result of evaluating the acid and bile tolerances of twenty-five strains, ten strains (*L. plantarum* MRS ES-3, MRS ES-7, MRS PT-14; L. brevis MRS ES-11; *P. pentosaceus* MRS N-1; *E. dispar* MRS T-2; *E. faecium* M17 BB-7, M17 N-2, M17 N-3; and *E. durans* M17 TB-2) were

selected for further probiotic analyses due to their high viability under acidic and bile salt conditions (Table 1).

## Identification of Isolates at Species Level by 16S rRNA Sequencing

Among the ten selected strains, four of them (MRS N-1, MRS T-2, M17 N-2, M17 N-3) were identified in a previous study conducted in our laboratory [8]. The remaining six isolates (MRS ES-3, MRS ES-7, MRS ES-11, MRS PT-14, M17 BB-7, M17 TB-2) were identified by 16S rRNA sequencing (see Table 1) and submitted to NCBI GenBank database (PP024635-PP024640).

**Table 1** List of strains selected for further studies and the selection criteria

| Isolate | Identified Species | Reason of Selection |
|---|---|---|
| MRS ES-3 | *L. plantarum* | High tolerance to acid and bile salts |
| MRS ES-7 | *L. plantarum* | High tolerance to acidity |
| MRS ES-11 | *L. brevis* | High tolerance to acidity |
| MRS PT-14 | *L. plantarum* | High tolerance to acidity |
| MRS N-1 | *P. pentosaceus* | High acidity tolerance |
| MRS T-2 | *E. dispar* | High tolerance to bile salts |
| M17 BB-7 | *E. faecium* | Increased viability at prolonged acidity |
| M17 N-2 | *E. faecium* | High tolerance to acid and bile salts |
| M17 N-3 | *E. faecium* | High tolerance to acid and bile salts |
| M17 TB-2 | *E. durans* | High tolerance to acid and bile salts |

## Adhesion to Caco-2 Cells

Colonization resistance is important for gut health because only adherent cells can effectively grow and colonize the gut. They show beneficial effects as long as they colonize the mucosa. They can hinder infections by preventing the adhesion and colonization of pathogenic bacteria on the mucosa through competitive adhesion.

According to the results, MRS ES-3, MRS N-1, MRS T-2, M17 BB-7, M17 N-2, and M17 N-3 showed high adhesion to Caco-2 cells (> 37%). Particularly, MRS N-1 and M17 N-2 showed over 87% adhesion to Caco-2 cells (Fig. 2).

Even though LAB isolates were reported to have a wide range of adhesion (0.6–90%) [33, 34], all lactic acid bacterial isolates obtained in our study displayed significant adhesion to Caco-2 cell line (Fig. 2). Out of the ten selected strains, *L. plantarum* MRS ES-3, *P. pentosaceus* MRS N-1, and *E. faecium* M17 N-2 showed superior adhesion to Caco-2 cells (> 60%): This corroborates the literature, where several *L. plantarum* strains have been reported to have good adhesion properties [34, 35]. Surprisingly, *P. pentosaceus* MRS N-1 displayed exceptional adhesion (> 85%) to Caco-2 cells since *Pediococcus* strains are not known for their strong adhesion properties (reportedly between 4 and 16%) [36]. *L. plantarum* MRS ES-3, MRS ES-7, and MRS PT-14 strains exhibited varying degrees of adhesion to Caco-2 cells (65%, 22.5%, and 37.6%, respectively). The different rates of adhesion between strains may result from different adhesion mechanisms, cell-wall components (peptidoglycans, teichoic acid, or polysaccharides), and/or cell-wall proteins. *Enterococcus faecium* M17 BB-7, M17 N-2, and M17 N-3 strains showed varying degrees of adhesion (58.6%, 90.9%, and 37.4%) to Caco-2 cells, corroborating

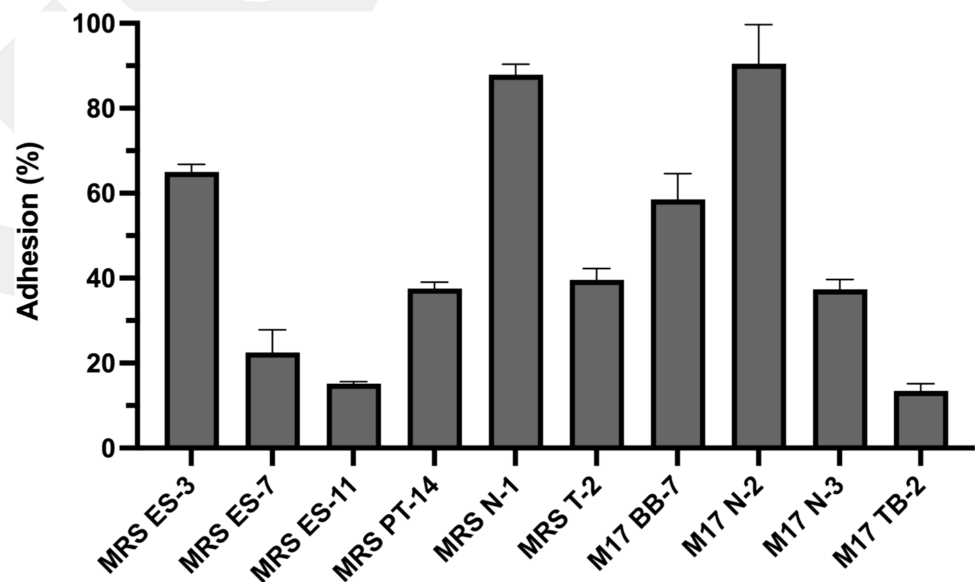


**Fig. 2** Adhesion of isolates to Caco-2 cells

with other reports [37]. *E. durans* M17 TB-2 strain showed only 13.5% adhesion to Caco-2 cells.

## Antimicrobial Activity

LAB produce two main groups of substances with antimicrobial properties. The first group is non-bacteriocin structures (organic acids, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, and 3-hydroxy fatty acids) [4]. The second group is peptide or protein-structured bacteriocins, produced and secreted by bacteria [35]. For the antimicrobial activity assays, the 10 selected strains were evaluated. Kanamycin sulphate (5 mg/ml) was used as a positive control, and *E. faecalis* 7–3 cell-free supernatant was used as a positive control for *S. aureus* ATCC 6538. According to the results of the antimicrobial assays, only kanamycin showed strong antimicrobial activity against all pathogens. Additionally, *E. faecalis* 7–3 showed a moderate effect on *S. aureus* ATCC 6538 (Table 2) [15]. Of the tested strains, only *L. plantarum* MRS ES-3 showed a limited effect against *S. aureus* ATCC 6538 and *K. pneumoniae* ATCC 4352. The remaining strains were considered ineffective against these pathogens according to the criteria of the Clinical & Laboratory Standards Institute (CLSI) [38].

## Hemolytic Activity

The absence of hemolytic activity is an important safety criterion when selecting probiotic bacterial candidates. Microorganisms with hemolytic activity cause hemolytic anemia by breaking down red blood cells. As a result, oxygen cannot be transported to the cells, leading to decreased energy production [39]. Indeed, none of our strains possess hemolytic activity. Hence, they can be considered as safe in this respect (Table 3).

**Table 3** Lysozyme tolerance, EPS production and hemolytic activity of strains

| Strain | Lysozyme tolerance (%) | EPS production | Hemolytic activity |
|---|---|---|---|
| MRS ES-3 | 87,82 ± 5,77 | ++ | None |
| MRS ES-7 | 175,53 ± 8,87 | ++ | None |
| MRS ES-11 | 264,98 ± 18,89 | ++ | None |
| MRS PT-14 | 125,53 ± 13,48 | ++ | None |
| MRS N-1 | 87,98 ± 3,07 | ++ | None |
| MRS T-2 | 51,06 ± 4,26 | ++ | None |
| M17 BB-7 | 38,98 ± 1,97 | + | None |
| M17 N-2 | 76,56 ± 7,03 | + | None |
| M17 N-3 | 87,08 ± 6,18 | + | None |
| M17 TB-2 | 56,98 ± 3,40 | + | None |

## Resistance to Lysozyme

Resistance to lysozyme in saliva is a significant factor in the selection of probiotic bacteria. Lysozyme causes degradation of peptidoglycan; therefore, resistance to lysozyme is crucial for bacteria to reach the stomach and intestines without damage. Lysozyme resistance testing revealed that *L. plantarum* and *L. brevis* strains MRS ES-7, MRS ES-11, and MRS PT-14 had the highest resistance (175%, 265%, and 125% viability, respectively). MRS ES-3, MRS N-1, M17 N-2, and M17 N-3 strains showed viability between 76 and 87% and MRS T-2, M17 BB-7, and M17 TB-2 strains showed viability between 38 and 56% (Table 3).

Nine out of ten strains exhibited over 50% viability, and six strains showed over 85% viability in the lysozyme assay. *L. plantarum* strains are known to have higher resistance against lysozyme and even to exhibit increased viability after 30 and 120 min of incubation at different concentrations

**Table 2** Zone of inhibition diameters against pathogens

| Strain | Zone of Inhibition (ZOI) Diameters (mm) | | | | |
|---|---|---|---|---|---|
| | *S. aureus* ATCC 6538 | *E. coli* ATCC 25922 | *K. pneumoniae* ATCC 4352 | *P. mirabilis* ATCC 14153 | *S. epidermidis* ATCC 12228 |
| MRS ES-3 | 9,5 ± 0,5 | 7,5 ± 0,5 | 9,5 ± 0,5 | 9 ± 1 | 8 ± 0 |
| MRS ES-7 | 7 ± 0 | 7 ± 0 | 8 ± 0 | 8 ± 0 | 8 ± 0 |
| MRS ES-11 | 7 ± 0 | 7 ± 0 | 7 ± 0 | 8 ± 0 | 8 ± 0 |
| MRS PT-14 | 7 ± 0 | 7 ± 0 | 7 ± 0 | 7,5 ± 0,5 | 8 ± 0 |
| MRS N-1 | 7 ± 0 | 7 ± 0 | 7,5 ± 0,5 | 7 ± 0 | 8,5 ± 0,5 |
| MRS T-2 | 7 ± 0 | 8 ± 0 | 7,5 ± 0,5 | 7 ± 0 | 9 ± 0 |
| M17 BB-7 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| M17 N-2 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| M17 N-3 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| M17 TB-2 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Kanamycin | 15,5 ± 0,5 | 18 ± 0 | 18,5 ± 0,5 | 17 ± 1 | 23 ± 0 |
| EF 7–3 | 14 ± 0 | 8 ± 0 | 11 ± 1 | 10 ± 0 | 9 ± 0 |

[40]. In addition, *P. pentosaceus,* and *E. faecium* strains have been reported to have good viability against lysozyme [41, 42].

### Production of Exopolysaccharides

EPS are complex glycan structures produced by bacteria, providing protection against environmental stressors such as pH, temperature, and toxins. They also aid in bacterial adhesion and biofilm formation [20]. EPS production has a positive effect on adhesion to intestinal cells and colonization [20]. EPS help bacteria form biofilms and prevent pathogens from forming biofilms. Additionally, EPS produced by LAB have anti-inflammatory, anti-viral, anti-bacterial, and anti-biofilm activities [43]. Moreover, heteropolysaccharides (HePS) can be used as biothickeners to develop the flavor and stability of dairy products.

EPS production by LAB isolates was detected by visual observation of the ropiness of the bacterial pellet. All of the isolates were determined to produce EPS (Table 3). Particularly, strains *L. plantarum* MRS ES-3, MRS ES-7, MRS PT-14; *L. brevis* MRS ES-11; *P. pentosaceus* MRS N-1; and *E. dispar* MRS T-2 produced noticeably more EPS than the remaining strains that grow on M17 medium. Several LAB strains, including *L. plantarum, E. faecium, L. brevis, P. pentosaceus* are reported as EPS producers [20, 43, 44].

### Selecting for Best Performing Strains

The unsupervised k-means clustering algorithm was applied to classify the probiotic strains based on their characteristics, such as acid and bile salt tolerance, lysozyme resistance, and adhesion to Caco-2 cells [45] (Tables 4 and S3). This statistical approach allowed us to group the strains into four distinct clusters, each representing different levels of probiotic potential. The k-means analysis was crucial in identifying the strains that exhibited the most desirable probiotic properties, as it enabled the differentiation of strains based on their overall performance across multiple tests.

**Table 4** Clusters of each bacterial strain according to k-means clustering algorithm ($P \leq 0.05$)

| Strain | Cluster | Distance |
|---|---|---|
| MRS ES3 | 3 | 0,693 |
| MRS ES7 | 2 | 0,992 |
| MRS ES11 | 2 | 0,707 |
| MRS PT14 | 2 | 0,611 |
| MRS N1 | 3 | 1,073 |
| MRS T2 | 1 | 0,696 |
| M17 BB7 | 1 | 0,696 |
| M17 N2 | 3 | 1,235 |
| M17 N3 | 2 | 1,134 |
| M17 TB2 | 4 | 0,000 |

Strains assigned to cluster 3 (*L. plantarum* MRS ES-3, *P. pentosaceus* MRS N-1, and *E. faecium* M17 N-2) showed significantly higher acid and bile salt tolerance, stronger adhesion to Caco-2 cells, and better lysozyme resistance than strains in other clusters. The clustering highlighted that these three strains had a balanced combination of essential probiotic traits, setting them apart from others. For example, the strains in cluster 3 demonstrated consistently high viability under acidic conditions (pH 3.0 for 3 h) and exhibited superior adhesion to Caco-2 cells, suggesting their strong potential to survive and colonize the gastrointestinal tract.

In contrast, other clusters contained strains that performed well in some tests but lacked overall probiotic robustness. By using k-means clustering, we objectively identified *L. plantarum* MRS ES-3, *P. pentosaceus* MRS N-1, and *E. faecium* M17 N-2 as the most promising probiotic candidates based on their multiple strengths and demonstrated that this statistical method was useful in the selection process.

## Conclusion

This study successfully identified three promising probiotic strains: *L. plantarum* MRS ES-3, *P. pentosaceus* MRS N-1, and *E. faecium* M17 N-2 from traditional Turkish fermented foods, demonstrating their strong acid and bile salt tolerance, lysozyme resistance, adhesion to intestinal cells, and exopolysaccharide (EPS) production. Notably, *E. dispar* MRS T-2 was identified for the first time as having high acid and bile salt tolerance, making it an important addition to the range of potential probiotic strains. The discovery of *E. dispar* as a candidate expands the diversity of probiotic strains and opens up new possibilities for its application in functional foods and dietary supplements.

These findings highlight the valuable role of traditional Turkish fermented foods as a source of novel and robust probiotic strains with potential health benefits. The identified strains, particularly the three most promising candidates, exhibit characteristics that could make them excellent contributors to the development of next-generation probiotic products. Further research is necessary to explore their full potential, including their effects on gut health, immune modulation, and other health-related benefits.

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## Declarations

**Conflict of interest** None of the authors of this study have any financial interest or conflict with industries or parties.

## References


1. Markowiak P, Ślizewska K (2017) Effects of probiotics, prebiotics, and synbiotics on human health. Nutrients. https://doi.org/10.3390/NU9091021
2. Vasiljevic T, Shah NP (2008) Probiotics—from Metchnikoff to bioactives. Int Dairy J 18:714–728. https://doi.org/10.1016/J.IDAIRYJ.2008.03.004
3. Ayivi RD, Gyawali R, Krastanov A et al (2020) Lactic acid bacteria: food safety and human health applications. Dairy 1:202–232. https://doi.org/10.3390/DAIRY1030015
4. Arena MP, Capozzi V, Russo P et al (2018) Immunobiosis and probiosis: antimicrobial activity of lactic acid bacteria with a focus on their antiviral and antifungal properties. Appl Microbiol Biotechnol 102:9949–9958. https://doi.org/10.1007/S00253-018-9403-9/FIGURES/1
5. Jonkers DMAE (2016) Microbial perturbations and modulation in conditions associated with malnutrition and malabsorption. Best Pract Res Clin Gastroenterol 30:161–172. https://doi.org/10.1016/J.BPG.2016.02.006
6. Vera-Santander VE, Hernández-Figueroa RH, Jiménez-Munguía MT et al (2023) Health benefits of consuming foods with bacterial probiotics, postbiotics, and their metabolites: a review. Molecules. https://doi.org/10.3390/MOLECULES28031230
7. Akpinar-Bayizit A, Yilmaz-Ersan L, Ozcan T (2010) Determination of Boza's organic acid composition as it is affected by raw material and fermentation. Int J Food Prop 13:648–656. https://doi.org/10.1080/10942911003604194
8. Cebeci Aydın A, Polat MF, Çalış B (2020) Isolation of lactic acid bacteria from Tarhana. ÇOMÜ Ziraat Fakültesi Dergisi 8:51–59. https://doi.org/10.33202/COMUAGRI.624715
9. Cebeci A, Gürakan GC (2008) Molecular methods for identification of Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus using methionine biosynthesis and 16S rRNA genes. J Dairy Res 75:392–398. https://doi.org/10.1017/S0022029908003543
10. Argyri AA, Zoumpopoulou G, Karatzas KAG et al (2013) Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. Food Microbiol 33:282–291. https://doi.org/10.1016/J.FM.2012.10.005
11. Maragkoudakis PA, Zoumpopoulou G, Miaris C et al (2006) Probiotic potential of Lactobacillus strains isolated from dairy products. Int Dairy J 16:189–199. https://doi.org/10.1016/J.IDAIRYJ.2005.02.009
12. Abid S, Farid A, Abid R et al (2022) Identification, biochemical characterization, and safety attributes of locally isolated Lactobacillus fermentum from Bubalus bubalis (buffalo) milk as a probiotic. Microorganisms. https://doi.org/10.3390/MICROORGANISMS10050954
13. Xu H, Jeong HS, Lee HY, Ahn J (2009) Assessment of cell surface properties and adhesion potential of selected probiotic strains. Lett Appl Microbiol 49:434–442. https://doi.org/10.1111/J.1472-765X.2009.02684.X
14. Balouiri M, Sadiki M, Ibnsouda SK (2016) Methods for in vitro evaluating antimicrobial activity: a review. J Pharm Anal 6:71. https://doi.org/10.1016/J.JPHA.2015.11.005
15. Karaalioğlu O, Özmen-Toğay S, Ay M et al (2019) Çiğ balık örneklerinden izole edilen Enterococcus faecium ve Enterococcus faecalis suşlarının gıda güvenliği yönünden bazı özelliklerinin değerlendirilmesi. Turk Hijyen ve Deneysel Biyoloji Dergisi 76:341–352. https://doi.org/10.5505/TURKHIJYEN.2019.97268
16. Heller AA, Spence DM (2019) A rapid method for post-antibiotic bacterial susceptibility testing. PLoS One 14:e0210534. https://doi.org/10.1371/JOURNAL.PONE.0210534
17. Humphries RM, Ambler J, Mitchell SL et al (2018) CLSI methods development and standardization working group best practices for evaluation of antimicrobial susceptibility tests. J Clin Microbiol. https://doi.org/10.1128/JCM.01934-17
18. Zago M, Fornasari ME, Carminati D et al (2011) Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. Food Microbiol 28:1033–1040. https://doi.org/10.1016/J.FM.2011.02.009
19. Garai-Ibabe G, Areizaga J, Aznar R et al (2010) Screening and selection of 2-Branched (1,3)-β-d-glucan producing lactic acid bacteria and Exopolysaccharide characterization. J Agric Food Chem 58:6149–6156. https://doi.org/10.1021/JF904529Q
20. Jurášková D, Ribeiro SC, Silva CCG (2022) Exopolysaccharides produced by lactic acid bacteria: from biosynthesis to health-promoting properties. Foods. https://doi.org/10.3390/FOODS11020156
21. Yasmin I, Saeed M, Khan WA et al (2020) In Vitro probiotic potential and safety evaluation (hemolytic, cytotoxic activity) of bifidobacterium strains isolated from raw camel milk. Microorganisms. https://doi.org/10.3390/MICROORGANISMS8030354
22. Zhang L, Qu H, Liu X et al (2022) Comparison and selection of probiotic Lactobacillus from human intestinal tract and traditional fermented food in vitro via PCA, unsupervised clustering algorithm, and heat-map analysis. Food Sci Nutr 10:4247–4257. https://doi.org/10.1002/FSN3.3018
23. Bearson S, Bearson B, Foster JW (1997) Acid stress responses in enterobacteria. FEMS Microbiol Lett 147:173–180. https://doi.org/10.1111/J.1574-6968.1997.TB10238.X
24. Cotter PD, Hill C (2003) Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiol Mol Biol Rev 67:429–453
25. Wang C, Cui Y, Qu X (2018) Mechanisms and improvement of acid resistance in lactic acid bacteria. Arch Microbiol 200:195–201. https://doi.org/10.1007/S00203-017-1446-2/FIGURES/1
26. Begley M, Hill C, Gahan CGM (2006) Bile salt hydrolase activity in probiotics. Appl Environ Microbiol 72:1729–1738
27. Bustos AY, Font de Valdez G, Fadda S, Taranto MP (2018) New insights into bacterial bile resistance mechanisms: the role of bile salt hydrolase and its impact on human health. Food Res Int 112:250–262. https://doi.org/10.1016/J.FOODRES.2018.06.035
28. Horáčková Š, Plocková M, Demnerová K (2018) Importance of microbial defence systems to bile salts and mechanisms of serum cholesterol reduction. Biotechnol Adv 36:682–690. https://doi.org/10.1016/J.BIOTECHADV.2017.12.005
29. Śliżewska K, Chlebicz-Wójcik A (2020) Growth kinetics of probiotic Lactobacillus strains in the alternative, cost-efficient semi-solid fermentation medium. Biology. https://doi.org/10.3390/BIOLOGY9120423
30. Çakır E, Arıcı M, Durak MZ, Karasu S (2020) The molecular and technological characterization of lactic acid bacteria in einkorn sourdough: effect on bread quality. J Food Measurement Charact 14:1646–1655. https://doi.org/10.1007/S11694-020-00412-5/FIGURES/2
31. Tokatlı M, Gülgör G, Bağder Elmac S et al (2015) In vitro properties of potential probiotic indigenous Lactic acid bacteria

originating from traditional pickles. Biomed Res Int. https://doi.org/10.1155/2015/315819
32. Succi M, Pannella G, Tremonte P et al (2017) Sub-optimal pH preadaptation improves the survival of Lactobacillus plantarum strains and the malic acid consumption in wine-like medium. Front Microbiol 8:251445. https://doi.org/10.3389/FMICB.2017.00470/BIBTEX
33. Fonseca HC, de Sousa MD, Ramos CL et al (2021) Probiotic properties of Lactobacilli and their ability to inhibit the adhesion of enteropathogenic bacteria to Caco-2 and HT-29 Cells. Probiotics Antimicrob Proteins 13:102–112. https://doi.org/10.1007/S12602-020-09659-2
34. Leska A, Nowak A, Czarnecka-Chrebelska KH (2022) Adhesion and anti-adhesion abilities of potentially probiotic lactic acid bacteria and biofilm eradication of honeybee (*Apis mellifera* L.) pathogens. Molecules 27:8945. https://doi.org/10.3390/MOLECULES27248945/S1
35. Choi M, Lee Y, Lee NK et al (2019) Suitability of Lactobacillus plantarum SPC-SNU 72–2 as a probiotic starter for sourdough fermentation. J Microbiol Biotechnol 29:1729–1738. https://doi.org/10.4014/JMB.1907.07039
36. Rao Y, Tao Y, Li Y et al (2019) Characterization of a probiotic starter culture with anti-Candida activity for Chinese pickle fermentation. Food Funct 10:6936–6944. https://doi.org/10.1039/C9FO01191A
37. Wang J, Da R, Tuo X et al (2020) Probiotic and safety properties screening of Enterococcus faecalis from healthy Chinese infants. Probiotics Antimicrob Proteins 12:1115–1125. https://doi.org/10.1007/S12602-019-09625-7/FIGURES/4
38. CLSI (2020) Performance standards for antimicrobial susceptibility testing-30th Edition
39. Azman WNW, Omar J, Koon TS, Ismail TST (2019) Hemolyzed specimens: major challenge for identifying and rejecting specimens in clinical laboratories. Oman Med J 34:94. https://doi.org/10.5001/OMJ.2019.19
40. Turchi B, Mancini S, Fratini F et al (2013) Preliminary evaluation of probiotic potential of *Lactobacillus plantarum* strains isolated from Italian food products. World J Microbiol Biotechnol 29:1913–1922. https://doi.org/10.1007/S11274-013-1356-7/TABLES/8
41. García-Ruiz A, González de Llano D, Esteban-Fernández A et al (2014) Assessment of probiotic properties in lactic acid bacteria isolated from wine. Food Microbiol 44:220–225. https://doi.org/10.1016/J.FM.2014.06.015
42. Sarkar SL, Hossain MI, Monika SA et al (2020) Probiotic potential of Pediococcus acidilactici and *Enterococcus faecium* Isolated from Indigenous Yogurt and Raw Goat Milk. Microbiol Biotechnol Lett 48:276–286. https://doi.org/10.4014/MBL.1912.12009
43. Prete R, Alam MK, Perpetuini G et al (2021) Lactic acid bacteria exopolysaccharides producers: a sustainable tool for functional foods. Foods. https://doi.org/10.3390/FOODS10071653
44. Werning ML, Hernández-Alcántara AM, Ruiz MJ et al (2022) Biological functions of exopolysaccharides from lactic acid bacteria and their potential benefits for humans and farmed animals. Foods. https://doi.org/10.3390/foods11091284
45. Nami Y, Panahi B, Mohammadzadeh Jalaly H et al (2020) Application of unsupervised clustering algorithm and heat-map analysis for selection of lactic acid bacteria isolated from dairy samples based on desired probiotic properties. LWT 118:108839. https://doi.org/10.1016/J.LWT.2019.108839

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