Original Article

Investigation of Probiotic Attributes and Aromatic Components Produced by Lactic Acid Bacteria Isolated From Iranian Traditional Yogurts

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ABSTRACT

Background: Yogurt is consumed in different ways all over the world owing to its high nutritional value, making it important to identify distinct strains from local producers with specific characteristics.

Objectives: The purpose of the present study was to investigate the presence of probiotic bacterial populations in traditional yogurt, as an Iranian dairy product, and their effect on the probiotic specifications of yogurt.

Methods: Initially, the isolation of lactic acid bacteria (LAB) was done using the culture method and then, the isolates were identified by examining their biochemical characteristics and *16S rRNA* gene sequences. Finally, the characteristics of sensitivity to acidic conditions, bile salts, antimicrobial functions, survival rate, sensory properties, and aroma production for the isolates were evaluated.

Results: Twelve isolates were identified from *Lactobacillus* and *Enterococcus* families. In general, the *Lactobacillus plantarum* strain KLDS 1.0725 exhibited the maximum ability to survive under acidic conditions. The *L. plantarum* strain KLDS 1.0725 and *Enterococcus faecium* strain FS019 had the highest survival in 0.3 and 0.5 % bile salts, respectively. *L. plantarum* WCFS1 and *E. faecium* Aus0004 created the maximum and minimum inhibition halos against all pathogens, respectively. The *L. plantarum* strain KLDS 1.0725 indicated enhanced abilities to produce acetaldehyde (25.59 ppm), while *L. delbrueckii subsp. lactis* illustrated the highest diacetyl production (5.96 ppm). The highest acceptability score in the sensory assessment was obtained for the *L. plantarum* strain KLDS 1.0725 and the *E. faecalis* strain V583.

Conclusion: The overall results demonstrated the ability of isolated strains from yogurt to be applied in the industry, exhibiting desirable technological features and suitable aroma.

Keywords: Acetaldehyde, *Enterococcus*, *Lactobacillus*, Probiotic, Yogurt

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Introduction

Yogurt is considered the most popular dairy product owing to its nutritional and health benefits (Yerlikaya & Akbulut, 2020). The milk coagulation and yogurt production processes require fermentation by lactic acid bacteria (LAB) populations (Omar Selim et al., 2023). These species are preferred over other microorganisms in the food industry due to their therapeutic and nutritional attributes (Motamed, 2024). Additionally, biochemical functions and secondary metabolites, such as hydrogen peroxide, diacetyl, and bacteriocin are recognized as unique starter cultures (Nouri et al., 2012; Kamarinou et al., 2022).

Probiotics are food supplements that have beneficial effects on the host by improving intestinal microbial balance (Xu et al., 2020; Faghihi Shahrestani et al., 2020; Soltani et al., 2023). Certain yeasts and bacilli are available probiotics, but LAB and bifidobacteria are the most common microorganisms employed as these strains (Ladha & Jeevaratnam, 2018). Probiotic bacteria demonstrate the ability to tolerate different pH levels and bile salts and also adhere to the cells of the digestive tract wall; as a result, these characteristics are particularly important in the present research (Tarrah et al., 2019; Khadivi et al., 2020).

Culture starters are selected microbial strains containing live or inactive cells that affect the organoleptic features of products, including appearance, structure, flavor, and aroma (Akpinar et al., 2020). The local yogurts of each region have different microbial flora which results in unique aromas and tastes; their bacterial composition is also dissimilar to that of products prepared with ready-to-use starters (Tian et al., 2020). The isolation and identification of local dairy strains aid in creating new products with various aromas and flavors (Vasiee et al., 2014). Generally, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* coexist in primary culture starters of common yogurt, and their balanced growth enhances the biochemical functions of dairy products (Tarique et al., 2022). *L. bulgaricus* and *S. thermophilus* have a synbiotic correlation in yogurt starter culture, allowing these bacteria to compensate for each other's deficiencies (Liu, 2018). *S. thermophilus* lacks some amino acids necessary for acidifying the milk environment, which *L. bulgaricus* provides (Rao et al., 2015). Enterococci is another common LAB group in yogurt that has become important in the food microbiology industry due to their health and microbial attributes (Tarrah et al., 2019). Several studies have reported positive effects of these bacteria on the sensory qualities, structure, consistency, texture, taste, and color of cheese (Margalho et al., 2020). These bacteria have become suitable options for processing dairy products owing to their natural preservatives and aromatic components (Akpinar et al., 2020).

Yogurt flavor results from non-volatile acids, such as lactic, butyric, acetic acids, and aromatic compounds, including diacetyl acetone and acetaldehyde (Alighazi et al., 2021). Acetaldehyde is the primary flavor component in yogurt, which depends on several factors, such as the physicochemical features of milk, the type of starter, temperature, and incubation time (Bhardwaj et al., 2008). However, some studies have outlined that a specific ratio of lactic acid, acetaldehyde, and diacetyl improves the final flavor of yogurt (Alighazi et al., 2021; Omar Selim et al., 2023). According to other research, acetyl, acetone, and ethanol in certain proportions could enhance yogurt flavor (Beyan et al., 2011).

Previous studies have evaluated ewe milk, traditional yogurt, and sour buttermilk in Iran (Motamed, 2024), local Iranian yogurt (Sharifi Yazdi et al., 2017), the isolation of exopolysaccharide-producing LAB in Turkish yogurt (Omar Selim et al., 2023), LAB isolated from dairy products (García-Cano et al., 2019), the probiotic properties of *Enterococcus faecium* and *Enterococcus durans* strains isolated from raw milk and traditional dairy products (Yerlikaya & Akbulut, 2020), equid milk (Kostelac et al., 2021), and the probiotic potential of bacteria isolated from local yogurt (Tarique et al., 2022). However, to date, the isolation, probiotic investigation, and aroma production by LAB in the present yogurt have not been assessed.

The aim of the present research was to purify and identify LAB isolated from local yogurt by gene sequencing of the *16S rRNA*. Probiotic attributes were investigated in order to introduce these strains as safe options for use in industrial products. Finally, the ability of the bacteria isolated from yogurt to produce aromatic compounds and their sensory evaluation were conducted.

Materials and Methods

Materials

De Man, Rogosa, and Sharp (MRS) agar, as well as mannitol, sorbitol, maltose, fructose, sucrose, galactose, raffinose, glucose, lactose, and glycerol, were purchased from Merck (Germany). The DNA extraction kit, taq DNA polymerase master mix RED, GeneRuler DNA ladder 100 Plus, and polymerase chain reaction (PCR) materials were prepared from Roche (Germany), Amplicon (Denmark), and Fermentase (Canada), respectively.

LAB isolation

A 10 g sample of yogurt collected from the western region of Iran was transferred to 90 mL of 0.1% peptone water and homogenized using a Seaward model homogenizer (Germany). Surface cultures of dilutions prepared on MRS medium were performed in triplicate; specifically, 0.1 mL of each dilution was poured onto each plate and spread with a spreader. The plates were placed in an anaerobic incubator at 30 and 45 ˚C for 48 hours to create conditions that would hinder the growth of undesirable bacteria. Colonies with different appearances, margins, colors, and other morphological characteristics were selected from the plates, particularly from the highest dilution. All selected colonies were then linearly cultured on separate plates and after several rounds of linear culturing, single colonies from each isolate were obtained. The isolates were stored in MRS broth containing 15% glycerol (v/v) at -80 ˚C for long-term preservation (Beyan et al., 2011).

Biochemical investigation of the isolates

After isolation, morphological tests, such as gram staining and biochemical functions, including catalase activity, growth at 10 and 45 ˚C, and survival at pH 4.4 and 9.6 in MRS broth, were conducted. Additionally, the Durham tube experiment was performed to investigate the production of CO_2 gas for this environment, as well as viability in 6.5% salt. Hydrolysis of arginine was performed in MRS broth medium without glucose and meat extract, but containing 0.3 % arginine and 0.2 % sodium citrate instead of ammonium citrate. Grouping of LAB using different sugars (glucose, sucrose, galactose, fructose, lactose, maltose, sorbitol, raffinose, and mannitol) was also performed, along with the use of phenol red broth culture medium (casein peptone+sodium chloride + red phenol) were performed (Bartkiene et al., 2019).

Identification of LAB using *16S rRNA*, PCR molecular method, and DNA extraction

Frozen cultures were activated in an MRS culture medium, and identification was done based on the molecular polyphasic method, which included DNA extraction, *16S rRNA* gene amplification, sequencing, and finally comparison. For DNA extraction, a kit was used, and to prepare the initial suspension, each isolate was inoculated in 5 mL of MRS broth culture medium. After 24 hours at 37 ˚C, 100 µL of the suspension was used for further work. All steps were conducted according to the kit instructions. At the end of the process, 50 µL of a solution containing the DNA isolate was obtained for each sample, which was stored in a freezer at -20 ˚C for subsequent stages (Tarique et al., 2022).

PCR reaction

Amplification of the *16S rRNA* gene was performed to sequence and accurately identify isolates using a molecular method based on protected regions for this gene. The following general primers were used to conduct the PCR reaction:

Forward primer: 27FYM with sequence (5′-AGAGTTTGATYMTGGCTCAG-3′)

Reverse primer: 1492R with sequence (5′ GGTTACCTTGTTACGACTT-3′)

Then, a microtube containing PCR reagents (5 µL of 10× PCR buffer, 1.5 mM magnesium chloride, 0.2 mM dNTPs, 3 pmol of each primer, 1.5 U Taq DNA polymerase, and 2 µL of genomic DNA in a final volume of 50 µL) was placed inside the Thermocycler SensQuest (Germany). A temperature program and a specific number of cycles were set on the device (Sharifi Yazdi et al., 2017).

PCR product electrophoresis

In this method, a 1% agarose gel was prepared in Tris-borate EDTA buffer, and a DNA green viewer was used to observe bands under UV light. Then, 3 µL of PCR product was poured into each well. A marker (1 µL) was applied in the wells of the first and last rows, with the terminal well designated as a negative control. Electrophoresis was done at a voltage of 95 for 45 min. After completion, the gel was photographed by a document device under ultraviolet light (García-Cano et al., 2019).

Sequencing of isolates

After confirming the accuracy of the PCR reaction through electrophoresis and observing a band at the 1500 bp position, the products were sent to Korea Macrogen Company for sequencing, using a one-way reading of the 27F primer. The obtained sequences were compared against the NCBI BLAST database, and the strain most similar to the desired isolate was determined; a similarity of over 97% was considered significant (Kostelac et al., 2021).

Analysis of probiotic features for identified isolates

Acid resistance test

After activating the desired isolates, they were cultured in an MRS broth environment and kept in an incubator for 18 hours to promote better colony growth. Then, centrifugation (4 ˚C, 5 min, and 1000 g) and a washing step with the phosphate buffer solution (PBS) sterile at pH=7.2 were performed to purify the biomass resulting from bacteria growth and to remove MRS broth. After recentrifugation and discarding the supernatant, the sediment was dissolved in sterile PBS to achieve an absorbance equivalent to a 0.5 McFarland solution. In this step, approximately 1% of the solution prepared in the previous stage was added to the MRS broth culture with different pH levels (2, 3, and 7) to analyze acid resistance. It should be mentioned that hydrochloric acid was applied to acidify the culture media. The resistance of the desired bacteria to distinct pH levels was then assessed; samples were taken from the culture media with varying acidic conditions, and a linear culture was performed on an MRS agar culture medium. After 48 h in an incubator at 37 ˚C under anaerobic conditions, the number of grown bacteria was counted to illustrate the population rate and resistance to acidic conditions. The viability of the strains was calculated by comparing the colonies grown on MRS to the initial concentration (Vasiee et al., 2014).

Resistance to bile salts

This test was performed on isolates that demonstrated the ability to ability survive in an acid-resistant environment. For this purpose, the desired bacteria were cultured in MRS broth for enrichment and incubated for 24 h at 37 ˚C. After this period, when turbidity was observed, approximately 0.25 mL was transferred into pre-sterilized tubes along with PBS solution at pH 7.2. Also, MRS broth media with different percentages of bile salts (0.2, 0.3, 0.5, and 1%) were prepared and sterilized by autoclaving at 121 ˚C under 15 psi, and the absorbance was measured at a wavelength of 600 nm. After the second centrifugation and discarding the supernatant under sterile conditions, MRS broth culture media with bile salts were added to the sediment in the tubes. Then, they were placed in an incubator at 37 ˚C for about 0, 2, and 4 h, reflecting the retention time of food in the small intestine. For this experiment, surface cultures were performed from tube contents at each time interval, and after 24 or 48 h, the plates were incubated at 37 ˚C to check the tolerance of desired bacteria to bile salt. Under anaerobic conditions, the total number of grown colonies was indicative of resistance to bile salt and was calculated by comparing the percentage of MRS to the initial concentration (Reuben et al., 2020).

Investigation of antibacterial activity

The antibacterial properties of isolates (against pathogenic bacteria) were assessed using the lawn-on-spot method. The pathogenic microorganisms selected as indicators in the antibacterial assay included *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

Initially, both lactic acid and pathogenic bacteria were activated. After the desired LAB reached the logarithmic phase (approximately 18 hours of enrichment in MRS broth medium), about 5 µL of each was spotted on the surface of brain heart infusion agar (BHI) and incubated at 37 ˚C for 24 hours. After appropriate growth of lactic isolates, the culture medium surface was covered with a soft agar layer (about 10 mL of 0.7% agar + BHI), into which 0.2% of each pathogenic bacterium was inoculated. Then, the plates were placed in an incubator under optimal conditions for the growth of indicator microorganisms. After 8 to 24 hours, their antibacterial properties were observed as a clear halo around the spots inoculated with the desired LAB (Klayraung et al., 2008).

Effect of isolates on sensory evaluation and yogurt aroma

The selected strains of 108 CFU/mL Lactobacillus and Enterococcus were inoculated into pasteurized milk and incubated at 42 ˚C until clot formation occurred. The subsequent steps were similar to those performed for *Lactobacillus* as a single strain starter.

Evaluation of acetaldehyde and diacetyl production by *Lactobacillus* in yogurt

Gas chromatography (GC, Agilent, 7890, USA) equipped with mass spectrometry (Agilent, 5975) and a quadrupole mass spectrometer were applied to analyze aroma components in the sample. Separation was performed by a capillary column made of polydimethylsiloxane (PDM) with dimensions of 30×0.25 mm internal diameter (I.D.) and a film thickness of 0.25 µm. One gram of the prepared material was mixed with 1 mL of water and shaken for two types of polydimethylsiloxane (30×0.25 mm I.D., made of silica with a 0.25 mm film thickness). At first, 1 g of the prepared sample was added to 1 mL of water in a vial, shaken for 2 min, and then heated for about 20 min at 80 ˚C, while the PDM

Table 1. The biochemical assays of isolates in traditional iranian yogurt

Biochemical Assays	Group				
	1	2	3	4	5
Number of isolates	41	12	8	15	11
Growth at 10 ℃	+	+	+	+	+
Growth at 45 ℃	–	±	–	–	+
Growth at pH=4.4	+	–	±	–	+
Growth at pH=9.6	±	+	–	–	+
Growth with 6.5% NaCl	±	–	±	–	+
CO_2 from glucose	–	+	+	–	–
Hydrolysis of arginine	–	+	–	+	+

with solid-phase microextraction (SPME) fiber (PDM-80UM) was placed inside. Finally, the resulting vapors were injected into the device using an SPME syringe (Štoudková & Zemanová, 2007).

Yogurt sensory evaluation

The first step in preparing yogurt samples was to make reconstituted milk using 12% low-fat dry milk powder from Fonterra Company. The obtained pasteurized milk was cooled at an optimum incubation temperature of about 42 to 44 ˚C, then immediately inoculated with the desired bacterial strains at 108 CFU/mL, mixed, and incubated at 42 ˚C for 4 h. After 10 hours, without clot formation and with the gel pH reaching approximately 4.5±0.02, the yogurt clot was cooled in two stages, reducing the temperature to 5 ˚C, and then stored for 14 days. An expert panel of 15 trained evaluators assessed the sensory attributes (total acceptance) of the yogurt samples (Sharifi Yazdi et al., 2017).

Statistical analysis

Data analysis was performed using SPSS software, version 20 (IBM Corp. NY, USA) and a $P<0.05$ was considered significant.

Results

Isolation and identification

According to biochemical results, different isolates were grouped based on common characteristics, and 87 isolates were identified (Table 1). Based on these tests, group one grew at 10 ˚C and pH 4.4, but was unable to hydrolyze arginine and identified as homofermentative *Lactobacillus*. The second group hydrolyzed arginine and grew well at 10 ˚C and pH 9.6, which was considered heterofermentative *Lactobacillus*. In the third group, the isolates were unable to hydrolyze arginine and grew at 10 ˚C, but not at 45 ˚C and pH 9.6; these were recognized as Leuconostoc. The isolates of the *Lactococcus* genus were placed in the fourth group, which had the ability to grow at 10 ˚C and hydrolyze arginine. Finally, the fifth group was able to grow at 10 and 45 ˚C with a 6.5% salt concentration, which was identified as an *Enterococcus* genus.

Lactobacillus and *Enterococcus* bacteria play a special role in aroma production in yogurt; therefore, isolates related to these genera were investigated in the next tests. Table 2 illustrates the results of LAB using the carbohydrate fermentation method. The samples (three isolates) were placed in group one, which was based on biochemical tests of heterofermentative *Lactobacillus*. Three isolates were in group two, and four isolates were in group three; the isolates in both were identified as homofermentative *Lactobacillus*. The isolates that were previously identified as *Enterococcus* based on biochemical tests were placed in groups four (two isolates) and five (one isolate).

According to the groups obtained by culture-based experiments, a total of 12 isolates from different samples were selected and DNA was extracted. In the next step, amplification of the *16S rRNA* gene was done using general primers 27FYM and 1492R. Figure 1 outlines the banding profiles for different tested strains, with a target length of 1500 base pairs. The band location was M100 plus type according to the used ladder, demonstrating piece lengths of up to 3000 base pairs, which indicates the consistency and accuracy of the procedures.

Table 2. The fermentation results of different carbohydrates by isolates in traditional Iranian yogurt

Carbohydrates	Group				
	1	2	3	4	5
Glucose	+	+	+	+	+
Sucrose	+	+	+	-	+
Galactose	+	+	+	+	+
Fructose	+	+	-	+	+
Lactose	+	-	-	+	+
Maltose	+	+	+	-	+
Sorbitol	-	+	+	-	-
Retene	-	-	+	+	-
Mannitol	-	+	+	-	+

After PCR completion, reactions of products were sent to *Macrogen Korea* for sequencing. Table 3 illustrates that dominant population belongs to *Lactobacillus* genus and rest of *Enterococcus* bacteria. These isolates included *Lactobacillus plantarum* (5, L1-L5), *Lactobacillus delbrueckii subsp. lactis* (2, L6-L7), *Lactobacillus fermentum* (1, L8), *Lactobacillus casei* (1, L9), *E. faecium* 2, E3-E2 and *Enterococcus faecalis* (1, E1).

pH and bile salt resistance

The results related to survival strains at pH levels 2, 3, and 7 are reported in Figure 2a; therefore, the viability of probiotic bacteria declined by pH reduction. Maximum survival level was reported as 100% for all isolates at pH 7; however, for pH levels of 3 and 2, the highest rates were found to be 70% and 46% for the L2 strain, followed by L7 (66% and 41 %) and E1 (61% and 31 %),

Table 3. Identifications of isolates in traditional Iranian yogurt by molecular methods

No.	Isolate Code	Name of Bacteria	Similarity (%)	Accession Number
1	L1	*L. plantarum* WCFS1	99	NR_075041.1
2	L2	*L. plantarum* strain KLDS 1.0725	100	EU626010.1
3	L3	*L. plantarum* PD412	100	AB854180.1
4	L4	*L. plantarum* strain IMAU32489	98	KF149163.1
5	L5	*L. plantarum* strain KLDS 1.0725	98	EU626010.1
6	L6	*L. delbrueckii* spp. lactis	99	AB681888.1
7	L7	*L. delbrueckii* spp. lactis	100	JQ580992.1
8	L8	*L. fermentum* strain KLDS 1.0613	99	EU419592.1
9	L9	*L. casei* strain MRTL3	98	KC568563.1
10	E3	*E. faecium* Aus0004	98	NR_102790.1
11	E2	*E. faecium* strain FS019	100	KC568549.1
12	E1	*E. faecalis* strain V583	98	NR_074637.1

Table 4. Acetaldehyde and diacetyl amounts (ppm) produced by *Lactobacillus* and in combination with *Enterococcus*

Isolates	Acetaldehyde	Diacetyl
L1	2.70±0.01^{i}	4.68±0.05^{c}
L2	4.45±0.03^{e}	0.45±0.03^{j}
L3	2.23±0.03^{j}	4.39±0.05^{d}
L4	3.41±0.01^{g}	2.83±0.01^{f}
L5	25.59±0.05^{a}	0.57±0.05^{i}
L6	2.32±0.0j^{b}	5.96±0.03^{a}
L7	19.2±0.01^{b}	0.42±0.05^{j}
L8	5.50±0.05^{d}	5.50±0.05^{b}
L9	4.17±0.05^{f}	0.8±0.05^{h}
L2E1	13.54±0.03^{c}	0.29±0.05^{k}
L2E2	3.01±0.02^{h}	1.98±0.03^{g}
L2E3	2.23±0.03^{j}	4.01±0.03^{e}

Note: Mean values with different lowercase letters are significantly different (P<0.05).

respectively. The lowest values for the E2 strain were obtained at pH 2 (0 %) and pH 3 (21 %).

As shown in Figure 2b, the viability of probiotic bacteria reduced significantly (P<0.05) with an increase in bile salts. In all concentrations of bile salts, the isolated L2 strain demonstrated the highest survival percentage. The viability rates of the E1 and L2 isolates at the 1 % level were more than 50 %, while others indicated values below this threshold. At a concentration of 0.5%, only L1, L2, and E1 isolates had survival percentages exceeding 50%. However, for all isolates, survival levels of over 50% were reported at a 0.3% bile salt concentration. In general, the isolates of *L. plantarum* strain KLDS 1.0725 (L2) and *E. faecium* strain FS019 (E1) had the highest survival percentage.

Antibacterial attributes

The results of the antimicrobial effect are depicted in Figure 3 for probiotic isolates against selected pathogenic bacteria. The range of inhibition zones measured from 3.8 to 15.6 mm was observed in the isolates, with the highest antimicrobial activity against pathogens demonstrated by *B. cereus*, followed by *S. aureus*, *E. coli*, and *P. aeruginosa*. Among the probiotic isolates, L1 and

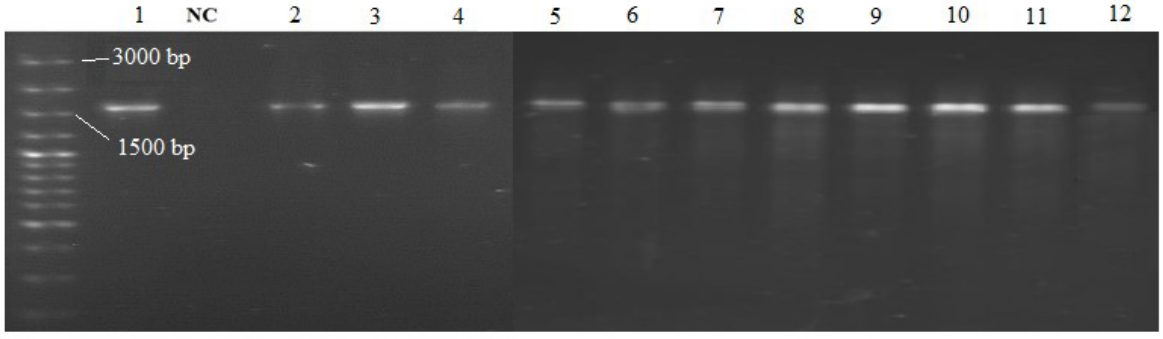
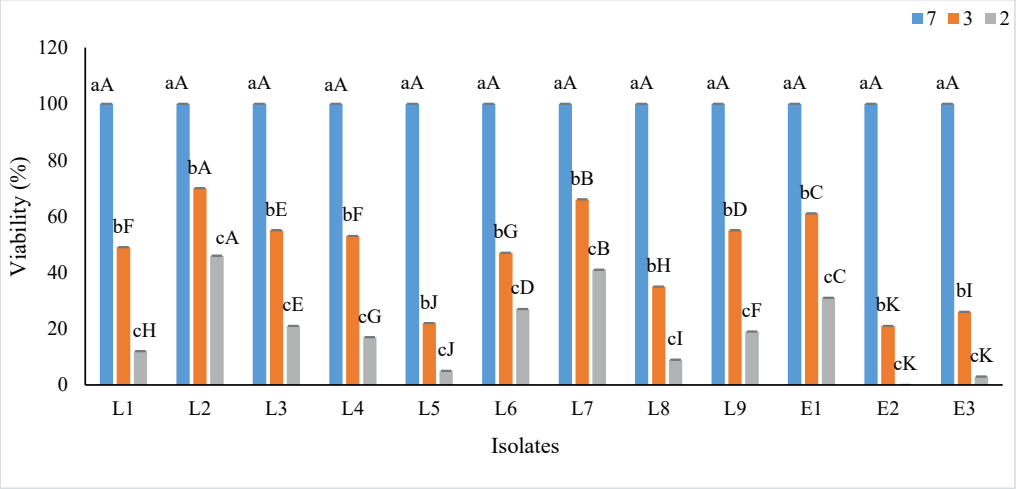


Figure 1. The image of 1500bp amplicons resulting from the 16S rRNA PCR reaction in gel electrophoresis

NC: Negative control.



b

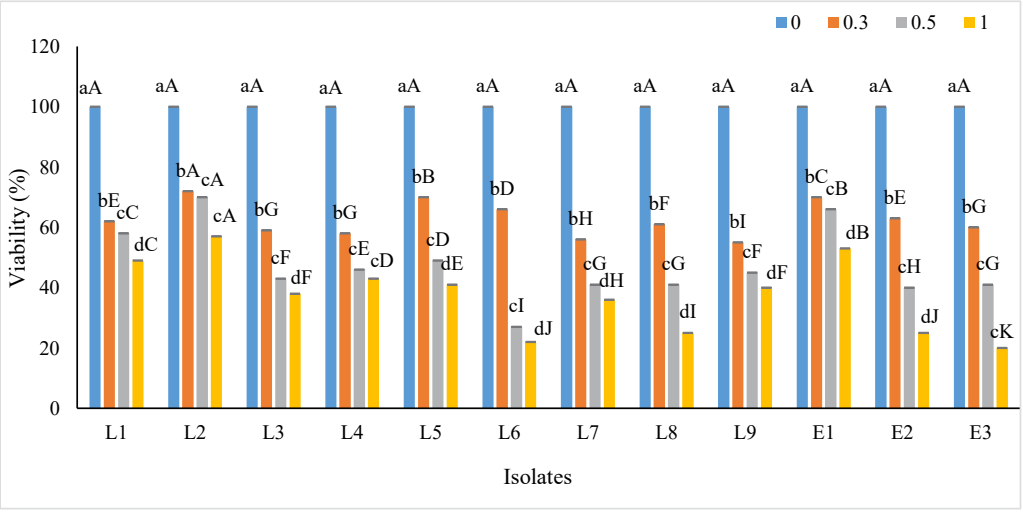


Figure 2. The resistance results for pH (a) and bile salt (b) for isolates in traditional Iranian yogurt

Note: a-d: Significant differences between pathogens in each isolate; A-L: Significant differences between isolates.

E3 created the maximum and minimum inhibition halo against all pathogens, respectively. Different strains of *L. plantarum* exhibited greater antimicrobial activity compared to others, while *E. fecalis* showed less of this feature.

The rate of acetaldehyde and diacetyl production by *Lactobacillus*

The *Lactobacillus* strain mainly produced acetaldehyde, and according to results illustrated in Table 4, Lactobacillus isolates L5 and L7 demonstrated a high ability to produce acetaldehyde, with values of 25.59 ppm and 19.2 ppm, respectively. Strains L6 (5.96 ppm) and L8 (5.50 ppm) formed the maximum diacetyl. Moreover, the results indicated that acetaldehyde levels increased in L2E1, while diacetyl levels reduced compared to L2. In L2E2 and L2E3, acetaldehyde levels declined; however, diacetyl levels enhanced, respectively. This study investigated the production of flavoring compounds such as acetaldehyde and diacetyl in yogurt samples made with *Lactobacillus* as a single-strain starter.

Yogurt sensory evaluation

Figure 4 compares the final scores for the sensory attributes of products containing *Lactobacillus*. Samples with the highest acetaldehyde and diacetyl contents achieved the highest final scores. Samples L5, L7, and L2 obtained higher overall acceptability than other samples due to their higher acetaldehyde content, while sample L6, had a greater diacetyl content. The combination

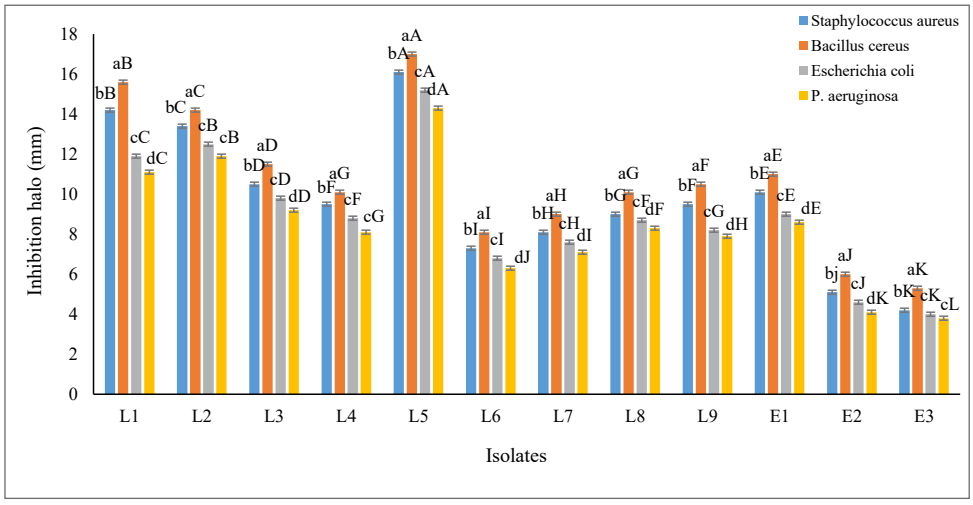


Figure 3. The results of inhibition zone diameter against indicator bacteria

Note: a-d: Significant differences between pathogens in each isolate; A-L: Significant differences between isolates.

of *Lactobacillus* and *E. faeciumin* in the L2E1 treatment positively affected the sensory properties of the final products compared to a single *Lactobacillus*, resulting in the highest score.

Discussion

Purified isolates were first subjected to tests, in which gram-positive and catalase-negative strains were selected as those with the potential to be included in the group of LAB (Bartkiene et al., 2019). Four LAB were isolated from local yogurt based on 16S rDNA sequencing, including *S. thermophilus*, *L. delbrueckii*, *Lacticaseibacillus rhamnosus*, and *E. faecium* (Tarique et al., 2022). From the sample of local yogurt, 21 exopolysaccharide-producing bacterial strains, including *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *Leuconostoc mesenteroides*, and *L. plantarum* have been isolated (Omar Selim et al., 2023). LAB were isolated from whey protein, milk protein concentrate, buttermilk powder, yogurt, and mozzarella and Gouda chesse, including *L. casei*, *Lactobacillus paracasei*, *Pediococcus acidilactici* and *L. plantarum* (García-Cano et al., 2019). The strains of *L. fermentum* FM 8, *Lactobacillus*. sp FM 10, and *L. plantarum* FM 17 were separated from pickles and identified based on biochemical and molecular assays (Yu et al., 2023). Eighty fructose strains were isolated from fermented cocoa beans and identified as *P. acidilactici*

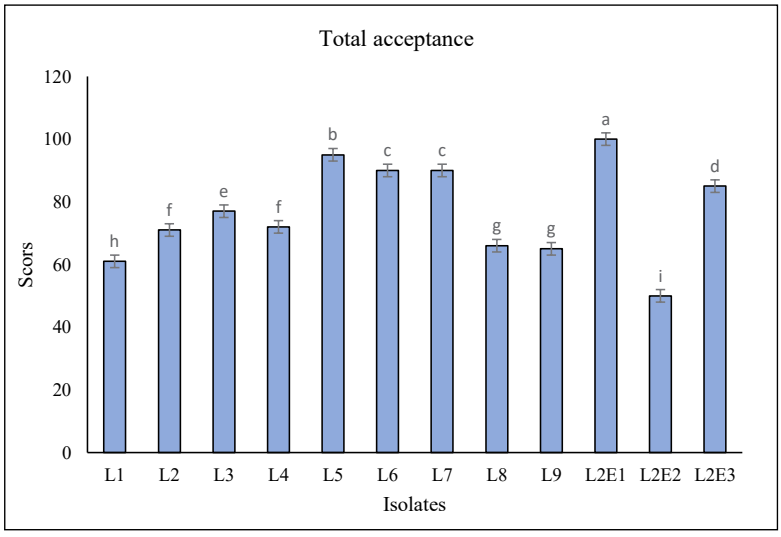


Figure 4. The results of total acceptance for sensory evaluation in traditional Iranian yogurt

Note: Mean values with different lowercase letters (a-g) are significantly different (P<0.05).

(n=52), *L. plantarum* (n=10), *Pediococcus pentosaceus* (n=10), *Bacillus subtilis* (n=4) and *Leuconostoc pseudomesenteroides* (n=4) (Viesser et al., 2020). The strains isolated from Teff injera dough, Ergo, and Kocho products included *L. plantarum* strain CIP 103151, *L. paracasei* subsp. tolerant strain NBRC 15906, *L. paracasei* strain NBRC 15889, and *L. plantarum* strain JCM 1149 (Mulaw et al., 2019). The probiotic potential of local Iranian yogurt was investigated, and six probiotic isolates were detected, belonging to *P. acidilactici*, *L. plantarum*, *Lactobacillus brevis*, *Lactobacillus kefiri*, and *L. fermentum* (Sharifi Yazdi et al., 2017).

Environmental conditions, such as low pH can prevent metabolism and reduce the growth and survival of lactic acid isolates (Yu et al., 2023). Some acids, such as hydrochloric acids in the human stomach can destroy biomolecules, such as proteins, fatty acids, vitamins, and nucleic acids (Vasiee et al., 2014). Every day, about 2 L of gastric juice with a pH close to 1.5 is secreted from the lining cells, creating difficult conditions for microorganism survival. The pH of gastric juice typically ranges from 3.0, with a level of 2.0 often used to simulate stomach conditions (Xu et al., 2020). Therefore, resistance to acidic conditions is one of the important factors for accepting microorganisms as probiotics (Ladha & Jeevaratnam, 2018). These strains act as a buffer after consumption, aided by carrier matrices and molecules that protect against extreme pH levels in the stomach (Xu et al., 2020). It is necessary to evaluate their resistance to bile salts to assess the potential of LAB and their introduction as probiotic strains (Yerlikaya & Akbulut, 2020). Oxal is a natural component related to cows, which includes both conjugated and unconjugated bile salts (Kostelac et al., 2021). Isolates that can resist high concentrations of bile salts can survive and grow in normal concentrations within the human gastrointestinal system (Yu et al., 2023). The secretion of bile extract into the duodenum directly disrupts the growth of probiotic bacteria, and bile acids have antimicrobial activity, acting as detergents that can disrupt biological membranes due to their bipolarity (Tarique et al., 2022).

In a study, strains isolated from traditional yogurt were exposed to different bile salts (cholic, ox gall, and taurocholic acid), and their growth percentages were studied. The results showed that in the presence of cholic, isolates indicated the lowest growth compared to ox gall and taurocholic acids, and also *S. thermophilus* isolates had more resistance to bile salts than *L. rhamnosus*, *L. delbrueckii*, and *E. faecium* (Tarique et al., 2022). Similar to the present results, resistance to bile acids has been reported in selected isolates from dairy and fermented products (Yerlikaya & Akbulut, 2020). Among *L. plantarum* KO9 and *L. plantarum* M2 isolated from equid milk at pH 3.0, there were no statistical differences in the target bacterium population compared to the control, and the lowest survival rates were observed at pH 1.5 (76 % compared to the control). Therefore, *L. plantarum* KO9 showed no significant difference in survival at all three concentrations of bile salts, while *L. plantarum* M2 exhibited a reduction trend at 1.5 mg/mL (2.8 %) and 3.0 mg/mL (5.7 %) levels (Kostelac et al., 2021).

The investigation of resistance to low pH and bile salts in isolates obtained from fermented grains showed that none of them were observed at pH 2, but *L. plantarum* was able to grow at pH 3. Also, 0.6% and 0.3 % bile salts affected their population; however, *L. plantarum* grew in both concentrations (Xu et al., 2020). Among strains isolated from Teff injera dough, Ergo, and Kocho products, a total of 90 LAB were isolated, of which four (4.44%) isolates exhibited survival rates of 45.35% to 97.11% and 38.40% to 90.49% at pH values of 2, 2.5, and 3 for 3 and 6 hours, respectively (Mulaw et al., 2019). L. paracasei No. 244, *L. casei* No. 210, L. brevis No. 173, *Lactobacillus farraginis* No. 206, *P. pentosaceus* No. 183, *Lactobacillus uvarum* No. 245, and *L. plantarum* No. 135 strains isolated from sour dough indicated viable counts higher than 7 log10 (CFU/mL) at pH 2.5 for 2 hours (Bartkiene et al., 2019). The isolate of Lactobacillus sakei ADM14 obtained from kimchi was able to survive at strong pH levels ranging from 2 to 3 and in 1.0 % bile salts (Won et al., 2020). In line with the present result, *E. faecium* strains did not grow at pH 2.0; however, in combination with *E. durans*, they exhibited resistance to 0.3% and 0.5% bile salts and maintained their viability (Yerlikaya & Akbulut, 2020). The strains isolated from several sources and specific species had different resistance to bile acids (Abdalla et al., 2021). Two factors contribute to microorganisms' ability to grow in high concentrations of bile salts: The first is the protective effect of the food matrix, and the second involves the production of hydrolyzing enzymes, which can break down bile salts into amino acids and cholesterol, thereby reducing their toxic influence on bacteria (Turgay & Erbilir, 2006).

Probiotics release antimicrobial metabolites, such as organic acids, hydrogen peroxide, diacetyl, ethanol, phenols, and bacteriocins into their environment to kill pathogenic bacteria through a competitive elimination mechanism (Rao et al., 2015; Tarrah et al., 2019). Gram-negative bacteria, including *E. coli* and *P. aeruginosa* were resistant to probiotic bacteria compared to gram-positive ones, such as *S. aureus* and *B. cereus*. Generally, gram-negative bacteria are more resistant to antimicrobial

agents than gram-positive bacteria due to the presence of an outer membrane around the cell wall, which limits the diffusion of hydrophobic compounds through lipopolysaccharides. and *L. plantarum* 445 exhibited the highest antagonistic features against *E. coli*, *S. aureus*, and *Listeria monocytogenes* EGD-e, with activities of 3.65, 2.43, and 3.89 log CFU/mL, respectively (Xu et al., 2020). The average zones of inhibition for crude extracts that inhibited the growth of food-borne pathogens (*S. aureus* ATCC 25923, *L. monocytogenes*, *E. coli* ATCC 25922, and *Salmonella enterica*) ranged from 17 to 21 mm (Mulaw et al., 2019). The inhibition percentage of *L. plantarum* M2 neutralized supernatant was 68.18 % and 57.23 % against *Salmonella Typhimurium* and *S. aureus*, respectively (Kostelac et al., 2021). The isolated *L. plantarum* No. 122, *L. casei* No. 210, *Lactobacillus curvatus* No. 51, *L. paracasei* No. 244 and *L. coryniformis* No. 71 inhibited pathogenic growth (Bartkiene et al., 2019). The *L. reuteri* I2, *P. acidilactici* I5, I8 and c3, *P. pentosaceus* I13, and *E. faecium* c14 isolated from broiler chickens inhibited *E. coli* ATCC 10536, E. coli O157: H7 ATCC 43894, *E. faecalis* ATCC 51299, *S. typhimurium* ATCC 14028, *Salmonella enteritidis* ATCC 13098 and *L. monocytogenes* ATCC 19113, with the pathogens tested showing zones of inhibition ranging from 12.5±0.71 to 20±0 mm (Reuben et al., 2019). Consistent with the present results, the inhibitory activities of LAB have been illustrated in previous research (Xu et al., 2020). It was reported that some strains of *Enterococcus*, including *E. faecalis* and *E. faecium* can produce bacteriocins with an inhibitory effect on *Clostridium botulinum*, *S. aureus*, *Vibrio cholera*, *L. monocytogenes* and *Clostridium perfringens*, Similar to the results of present study, several Enterococcus strains exhibited weak activity against *B. cereus* (Yerlikaya & Akbulut, 2020).

The differences in aromatic compounds between yogurt and milk are most likely due to the metabolic functions of LAB, such as proteolytic and lipolytic activity (Lubbers et al., 2004). The aroma of yogurt created by LAB is a complex mixture of aromatic components, including volatile substances found in milk (Yerlikaya & Akbulut, 2020). The most effective ingredients in creating flavor and aroma assist manufacturers in producing uniform products that are more appealing to consumers (Cheng, 2010). Carbonyl constituents, including acetaldehyde and diacetyl, are the main substances in yogurt, that contribute to its flavor and aroma (Pourahmad & Assadi, 2005). In the present research, the yogurt taste was constantly changing during production and storage, which was caused by bacterial enzymes that eventually led to the formation or conversion of other compounds and their loss due to volatility (Cheng, 2010). This study evaluated the presence of desired volatile components in prepared yogurt samples after 14 days at 5 ˚C. So far, more than 90 flavoring substances have been identified, among which volatile acids and carbonyls, including acetaldehyde and also diacetyl, indicated the most significant impact on yogurt flavor (Lubbers et al., 2004). The easy growth conditions, adaptability to different situations, and heat resistance of *E. faecium* have resulted in its presence in many specimens; therefore, it could be considered a natural microflora (Yerlikaya & Akbulut, 2020). These ordinary dairy products exhibit amazing aromas and flavors owing to their unique biochemical functions, such as proteolysis, lipolysis, and citrate breakdown. It has been reported that better flavor results only when more than 8.0 mg/kg of acetaldehyde is produced in yogurt (Chen et al., 2017). The typical concentrations of diacetyl have been reported to range from 0.2 to 3 mg/kg in yogurt (Cheng, 2010). optimal ratio of diacetyl and acetaldehyde was determined to be 4 and 16 mg/L in yogurt (Tian et al., 2020). Four types of pickles (without treatment, inoculated using *L. fermentum* FM 8, *Lactobacillus* spp. FM 10, and *L. plantarum* FM 17) were fermented at 25 ˚C for 15 days, during which 40 volatile compounds of free amino acids were detected (Yu et al., 2023). The study of coffee fermentation demonstrated that among different isolates, *L. plantarum* LPBF 35 indicated a special role in aroma and produced a wide range of influencing compounds (acetaldehyde, ethyl acetate, nonanal, and octanoic acid) during cacao fermentation (Viesser et al., 2020).

In a study conducted on fermented pickles, the sample containing *L. plantarum* FM 17 as a starter received the highest sensory evaluation score in terms of overall acceptance (Yu et al., 2023). Acetaldehyde imparts a fresh and green flavor, which is considered to be the most important contributor to typical yogurt aroma (Tian et al., 2020). The fermentation of coffee beans by LAB demonstrated the production of a wide range of aroma compounds by *L. plantarum* (Viesser et al., 2020).

Conclusion

It seems that the process of collecting and identifying local strains from traditional fermented products can provide useful information for scientific and commercial applications, in addition to examining characteristics that preserve microbial and genetic reserves. In this research, 12 strains of *Lactobacillus* and *Enterococcus* bacteria were detected, which had probiotic properties (resistance to acid and bile salts) in traditional yogurt. According to tests, the identified strains indicated probiotic features and the potential to produce an adequate aroma, which could be applied in industry.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

All authors equally contributed to preparing this research.

Conflict of interest

The authors declared no conflict of interest.

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مقاله پژوهشی

بررسی ویژگی‌های باکتری‌های پروبیوتیک و تولید ترکیبات معطر توسط باکتری‌های اسید لاکتیک جدا شده از ماست‌های سنتی ایرانی

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چکیده

زمینه مطالعه: ماست به دلیل ارزش تغذیه‌ای بالا در سراسر جهان به روش‌های مختلفی مصرف می‌شود که جهت شناسایی سویه‌های متمایز تولیدکنندگان محلی، ماست با ویژگی‌های خاص بسیار مورد توجه قرار گرفته است.

هدف: هدف پژوهش حاضر بررسی وجود باکتری‌های پروبیوتیک در ماست سنتی به عنوان فرآورده لبنی ایرانی است.

روش کار: ابتدا جداسازی باکتری‌های اسید لاکتیک با روش کشت انجام شد، سپس سویه‌های جداسازی شده با بررسی ویژگی‌های بیوشیمیایی و توالی ژن 16S rRNA شناسایی شدند. نهایتا، خصوصیات حساسیت به شرایط اسیدی، نمک‌های صفراوی، عملکردهای ضد میکروبی، زنده مانی، حسی و تولید عطر و طعم سویه‌ها مورد ارزیابی قرار گرفت.

نتایج: ۱۲ سویه جداسازی شده از خانواده لاکتوباسیلوس و انتروکوک شناسایی شدند، به طور کلی، لاکتوباسیلوس پلانتاروم گونه KLDS ۱/۰۷۲۵ حداکثر توانایی جهت زنده ماندن طی شرایط اسیدی را نشان داد. همچنین، سویه‌های لاکتوباسیلوس پلانتاروم گونه KLDS ۱/۰۷۲۵ و انتروکوکوس فاسیوم گونه FS۰۱۹ بیشترین زنده مانی را در ۰/۳ و ۰/۵ درصد نمک‌های صفراوی داشتند. لاکتوباسیلوس پلانتاروم گونه WCFS1 و و انتروکوکوس فاسیوم گونه Aus0004 به ترتیب بیشترین و کمترین هاله بازدارندگی را در برابر تمام عوامل بیماری زا ایجاد کردند. سویه لاکتوباسیلوس پلانتاروم گونه KLDS ۱،۰۷۲۵ توانایی بیشتری جهت تولید استالدئید (۲۵/۵۹ ppm) و لاکتوباسیلوس دلبروکی گونه لاکتیس حداکثر دی استیل (۵/۹۶ ppm) را نشان داد. بیشترین امتیاز پذیرش در ارزیابی حسی برای سویه لاکتوباسیلوس پلانتاروم گونه KLDS ۱،۰۷۲۵ و انتروکوکوس فاسیوم گونه V583 به دست آمد.

نتیجه‌گیری نهایی: نتایج کلی، توانایی سویه‌های جدا شده از ماست را برای کاربرد در صنعت با ویژگی‌های تکنولوژیکی و عطر مناسب نشان داد.

کلیدواژه‌ها: استالدئید، انتروکوکوس، لاکتوباسیلوس، پروبیوتیک، ماست

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