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

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ABSTRACT

Background: The yogurt consumes in different ways all over the world owing to high nutritional value, which is prominent to identify distinct strains of the local producers with specific characteristics.

Objectives: The purpose for present study is to investigate the presence of probiotic bacteria population 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 was done using the culture method and then isolates were identified by examining their biochemical characteristics and 16S rRNA gene sequence. Finally, the characteristics of sensitivity to acidic conditions, bile salts, antimicrobial functions, survival rate, sensory properties and aroma production for isolates were evaluated.

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

Conclusion: The overall results portrayed ability of isolated strains from yogurt to apply in industry with the technological features and suitable aroma.

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

1. Introduction

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

Probiotics are food supplements that have beneficial effects on 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 and Jeevaratnam, 2018). Probiotic bacteria demonstrate ability to tolerate different pH and bile salts and also adhere to cells of digestive tract wall; as a result, these characteristics are particularly important in present research (Tarrah *et al.*, 2019; Khadivi *et al.*, 2020).

The culture starters are selected microbial strains containing live or inactive cells that affect the organoleptic features of products including its appearance, structure, flavor and aroma (Akpinar *et al.*, 2020). The local yogurts of each region have different microbial flora that causes a unique aroma and taste and also their bacteria are not similar to products prepared with ready-to-use starters (Tian *et al.*, 2020). Isolation and identification of local dairy strains help in creating new products with various aromas and flavors (Vasiee *et al.*, 2014). Generally, *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus bulgaricus* (*L. bulgaricus*) coexist in primary culture starters of common yogurt and due to their balanced growth improving biochemical functions for dairy products (Tarique *et al.*, 2022). The *L. bulgaricus* and *S. thermophilus* have a synbiotic correlation in yogurt starter culture that permit these bacteria to overcome each deficiencies (Liu, 2018). *S thermophilus* lacks some amino acids necessary for acidifying the milk environment, which *L. bulgaricus* replaces (Rao *et al.*, 2015). Enterococci is another common LAB group in yogurt that has become important in food microbiology industry owing to health and microbial attributes (Tarrah *et al.*, 2019). Several studies had reported positive effects of these bacteria on cheese sensory qualities, structure, consistency, texture, taste and color (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 physicochemical features of milk, starter type, temperature and incubation time (Bhardwaj *et al.*, 2008). However, some studies had outlined that a specific lactic acid, acetaldehyde and diacetyl ratio improved final flavor of yogurt (Alighazi *et al.*, 2021; Omar Selim *et al.*, 2023). According to others, acetyl, acetone and ethanol in certain proportions could enhance yogurt flavor (Beyan *et al.*, 2011).

In past studies, 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), probiotic properties of *Enterococcus faecium* (*E. faecium*) and *Enterococcus durans* (*E.*

durans) strains isolated from raw milk and traditional dairy products (Yerlikaya and Akbulut, 2020), equid milk (Kostelac *et al.*, 2021) and probiotic potential of bacteria isolated from local yogurt (Tarique *et al.*, 2022) had been evaluated; but so far, isolation, probiotic investigation and aroma production by LAB of present yogurt have not been assessed.

The aim of present research is to purify and identify by gene sequencing for 16S rRNA LAB isolated from local yogurt and probiotic attributes were investigated in order to introduce them as safe strains that could be used in industrial or products. Finally, the ability to produce aromatic compounds of bacteria isolated from yogurt and their sensory evaluation were performed.

2. Materials and Methods

Materials

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

Isolation of LAB

The 10 g yogurt collected in western region of Iran was transferred to 90 mL 0.1 % peptone water and homogenized (Seaward model, Germany). The surface culture of dilutions prepared on MRS medium was performed in three replicates; in this way, 0.1 mL dilution was poured on each plate and spread with a spreader. It was placed in an anaerobic incubator at 30 and 45 °C for 48 h to make conditions more difficult for undesirable bacteria growth. Colonies were selected with different appearance, colony margin, color and other morphological characteristics from plates including highest dilution; then, all were linearly cultured in a separate plate and after several times of linear medium, single colonies from each isolate were obtained. The isolates were stored in MRS broth containing 15 % glycerol (v/v) at -80 °C to preserve for a long time (Beyan *et al.*, 2011).

Biochemical investigation of isolates

After isolation, morphological tests such as gram staining and biochemical functions including catalase, growth at 10 and 45 °C temperatures, survival from pH 4.4 and also 9.6 in MRS broth, the durham tube experiment to investigate production of CO_2 gas for this environment and viability in 6.5 % salt were conducted. 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) and also 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 MRS culture medium and identification was done based on molecular polyphasic method, which included DNA extraction, 16S rRNA gene amplification, sequencing and finally comparison. In order to DNA isolates, extraction kit was applied and to prepare the initial suspension, each isolate was inoculated in 5 mL MRS broth culture medium and after 24 h at 37 °C, 100 µL suspension was used to continue the work. All steps were performed according to kit instructions; at the end, 50 µL solution containing DNA isolate were obtained for each, which was kept in a freezer at -20 °C for the next stages (Antonsson and Molin, 2003).

PCR reaction

Amplification of 16S rRNA gene was performed to sequence and accurately identify isolates by molecular method, which operated based on protected regions for this gene and following general primers were used to conduct PCR reaction:

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

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

Then, microtube containing PCR reagents (5 µL of 10 × PCR buffer, 1.5mM magnesium chloride, 0.2mM dNTPs, 3 pmol for each primer, 1.5 U taq DNA polymerase and 2 µL genomic DNA in 50 µL as a final volume) was placed inside the thermocycler sensquest (Germany) and a temperature program and also specific number of cycles were given to device (Sharifi Yazdi *et al.*, 2017).

PCR product electrophoresis

In this method, 1 % agarose gel was prepared in tris borate EDTA buffer and DNA green viewer was used to observe bands under UV light; then 3 µL PCR were poured into each well. Marker (1 µL) was applied in wells of first and last rows and also terminal one was considered as a negative control. Electrophoresis was done at a voltage of 95 for 45 min; after completion, the desired gel was photographed by document device under ultraviolet rays (Endo *et al.*, 2019).

Sequencing of isolates

After evaluating correctness for PCR reaction by electrophoresis and observing band at 1500 bp position, products were sent to Korea Macrogen Company for sequencing as one-way reading of 27F primer. The obtained sequences were NCBI BLAST database and most strain to desired isolate was determined; therefore, above 97 % was considered as significant similarity (Davoodabadi *et al.*, 2015).

Analysis of probiotic features for identified isolates

Acid resistance test

After the desired isolates were activated, in order to grow colonies better, they were cultured and kept in an incubator for 18 h and also MRS broth environment. Then, centrifugation (4 °C, 5 min and 1000 g) and a washing step with phosphate buffer solution (PBS) sterile at pH=7.2 were performed to purify biomass resulting from bacteria growth and remove MRS broth. After recentrifugation and discarded supernatant, sediment was dissolved in sterile PBS to extent, which

had an absorbance equivalent to 0.5 MacFarland solution. In this step, about 1 % solution prepared in previous stage was added to MRS broth culture with different pH (2, 3 and 7) to analyze acid resistance. It should be mentioned that hydrochloric acid was applied for acidifying culture media. Then, resistance of desired bacteria was checked to distinct pH, samples were taken from culture mediums with different acidic conditions and linear culture was performed on MRS agar culture medium. After 48 h in incubator under 37 °C and anaerobic conditions, counting the grown bacteria illustrated population rate and also resistance to acidic conditions. The viability degrees of strain were calculated by comparing colonies grown on MRS to initial concentration (Vasiee *et al.*, 2014).

Resistance to bile salts

This test was performed on those isolates that had ability to survive in acid resistance system. For this purpose, the desired bacteria were cultured in MRS broth for enrichment and incubated for 24 h at 37 °C. When this time passed and turbidity was created inside, about 0.25 mL poured into pre-sterilized tubes along with PBS solution and 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 autoclave at 121 °C under 15 pressure and absorbance was measured at a wavelength of 600 nm. After second centrifugation and discarding supernatant under sterile conditions, MRS broth culture media with bile salts were poured on sediment inside tubes. Then, they were placed in an incubator at 37 °C about 0, 2 and 4 h, which reflected the retention time of food in small intestine. For this experiment, a surface culture was performed from tube contents at each time interval and after 24 or 48 h, the plates were incubated at 37 °C to check tolerance of desired bacteria to bile salt. Under anaerobic conditions, the total grown colonies were indicative of resistance to bile salt and calculated via comparing the percentage on MRS to initial concentration (Reuben *et al.*, 2020).

Investigation of antibacterial activity

The antibacterial property (against pathogenic bacteria) of isolates was done using the Lawn on spot method. Pathogenic microorganisms included *Staphylococcus aureus* (*S. aureus*), *Bacillus*

cereus (*B. cereus*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were selected as indicators in antibacterial assay.

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

Effect of isolates on sensory evaluation and yogurt aroma

The selected strains of 10^8 CFU/mL *Lactobacillus* and *Enterococcus* were inseminated into pasteurized milk and incubated at 42 °C until clot formation. The following steps were similar to stages 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 was applied to analyze aroma components in sample. Separation was performed by a capillary column for polydimethylsiloxane (PDM) with dimensions of 30 mm× 0.25 mm internal diameter (I.D) silica and 0.25 µm film thickness. Took 1 g prepared material; poured into 1 mL water and shook for two polydimethylsiloxanes types (30 mm ×0.25mm I.D made of silica with a 0.25 mm film thickness). At first, 1 g prepared sample was added to 1 mL water in a vial shake for 2 min and then heated about 20 min at 80 °C, while PDM with solid phase microextraction (SPME) fiber were placed inside it (PDM-80UM); finally, resulting vapors were injected into the device using an SPME syringe (Štoudková and 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 in an optimum incubation temperature about 42 to 44 °C, immediately inoculated with the desired bacterial strains at 10^8 CFU/mL, mixed and incubated at 42 °C for 4 h. After 10 h without clot formation and reaching gel pH to about 4.5 ± 0.02, the yogurt clot was cooled in two stages and temperature was reduced to 5 °C and also stored for 14 days. An expert panel of 15 trained evaluators assessed sensory attributes (total acceptance) for yogurt samples (Sharifi Yazdi *et al.*, 2017).

Statistical analysis

Data analysis was performed using SPSS v.20 software package (IBM Corp. NY, USA) and *P*-value less than 0.05 was considered significant.

3. Results

Isolation and identification

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

Lactobacillus and *Enterococcus* bacteria had a special role for aroma production in yogurt; therefore, isolates related to these genera were investigated in the next tests. Table 2 illustrates the results of LAB using carbohydrate fermentation method. The samples (3 isolates) were placed in

group one; which were based on biochemical tests of heterofermentative *Lactobacillus*. The treatments (3 isolates) were in group two and four isolates were in group three; the isolates of both were identified as homofermentative *Lactobacillus*. The isolates that were previously identified as *Enterococcus* based on biochemical tests were placed in group four (2 isolates) and five (1 isolate).

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

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

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

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

According to obtained group by culture-based experiments, a total of 12 isolates from different samples were selected and DNA was extracted. In the next step, amplification for 16S rRNA gene was done using general primers 27FYM and 1492R. Figure 1 outlines the banding profiles for different tested strains, target length is 1500 base pairs. Band location was M100 plus type according to used ladder and demonstrated length of pieces up to 3000 base pairs, which indicates the same and correctness for procedures.

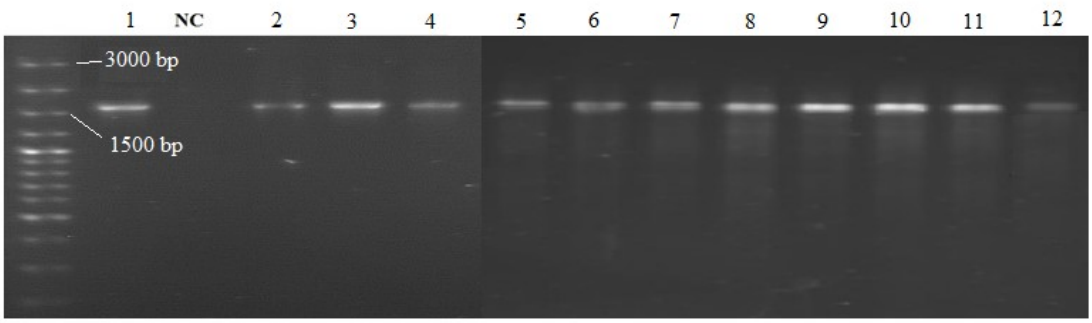


Figure 1. The image of 1500bp amplicons resulting from 16S rRNA PCR reaction in gel electrophoresis (NC: negative control)

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* (*L. plantarum* 5, L1-L5), *Lactobacillus delbrueckii ssp. lactis* (*L. delbrueckii spp. lactis* 2, L6-L7), *Lactobacillus fermentum* (*L. fe*rmentum 1, L8), *Lactobacillus casei* (*L. casei* 1, L9), *E. faecium* 2, E3-E2 and *Enterococcus faecalis* (*E. fecalis* 1, E1).

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

No.	Isolate code	Name of bacteria	Similarity (%)	Accession Number
1	L1	*Lactobacillus plantarum* WCFS1	99	NR_075041.1
2	L2	*Lactobacillus plantarum strain KLDS 1.0725*	100	EU626010.1
3	L3	*Lactobacillus plantarum* PD412	100	AB854180.1

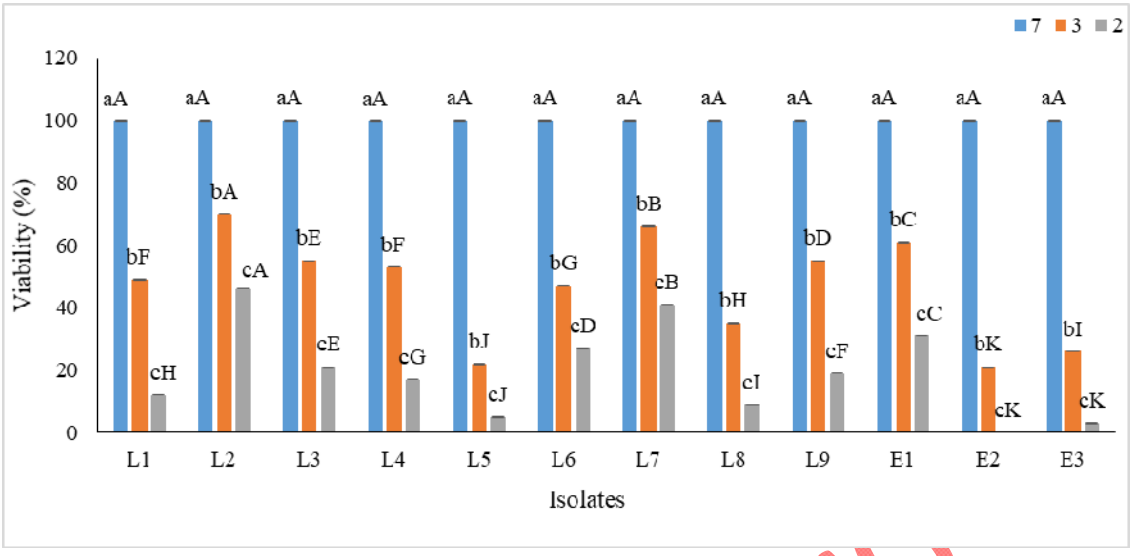
4	L4	*Lactobacillus plantarum strain IMAU32489*	98	KF149163.1
5	L5	*Lactobacillus plantarum* strain KLDS 1.0725	98	EU626010.1
6	L6	*Lactobacillus delbrueckii spp.* lactis	99	AB681888.1
7	L7	*Lactobacillus delbrueckii spp.* lactis	100	JQ580992.1
8	L8	*Lactobacillus fermentum strain KLDS 1.0613*	99	EU419592.1
9	L9	*Lactobacillus casei strain MRTL3*	98	KC568563.1
10	E3	*Enterococcus faecium Aus0004*	98	NR_102790.1
11	E2	*Enterococcus faecium* strain FS019	100	KC568549.1
12	E1	*Enterococcus faecalis strain V583*	98	NR_074637.1

pH and bile salt resistance

The results related to survival strains at pH 2, 3 and 7 are reported in Figure 2a; therefore, viability of probiotic bacteria is declined by pH reduction. Maximum survival level was reported 100 % for all isolates at pH 7; however, for pH 3 and 2, the highest rates were found to be 70 and 46 % for L2 strain followed by L7 (66 and 41 %) and E1 (61 and 31 %), respectively and also the lowest values for E2 strain were obtained at pH 2 (0 %) and 3 (21 %).

As portrayed in Figure 2b, viability of probiotic bacteria reduced significantly ($P < 0.05$) with increase in bile salts. In all concentrations of bile salts, isolated L2 strain demonstrated the highest survival percentage. Viability rates of E1 and L2 isolates at 1 % levels were more than 50 % and others indicated less than mentioned value. At a concentration of 0.5 %, only L1, L2 and E1 isolates had higher than 50 % survival percentages. But for all isolates, further 50 % survival levels had been reported in 0.3 % bile salt concentration. In general, isolates of *L. plantarum strain* KLDS 1.0725 (L2) and *E. faecium strain* FS019 (E1) had the highest survival percentage.

a



b

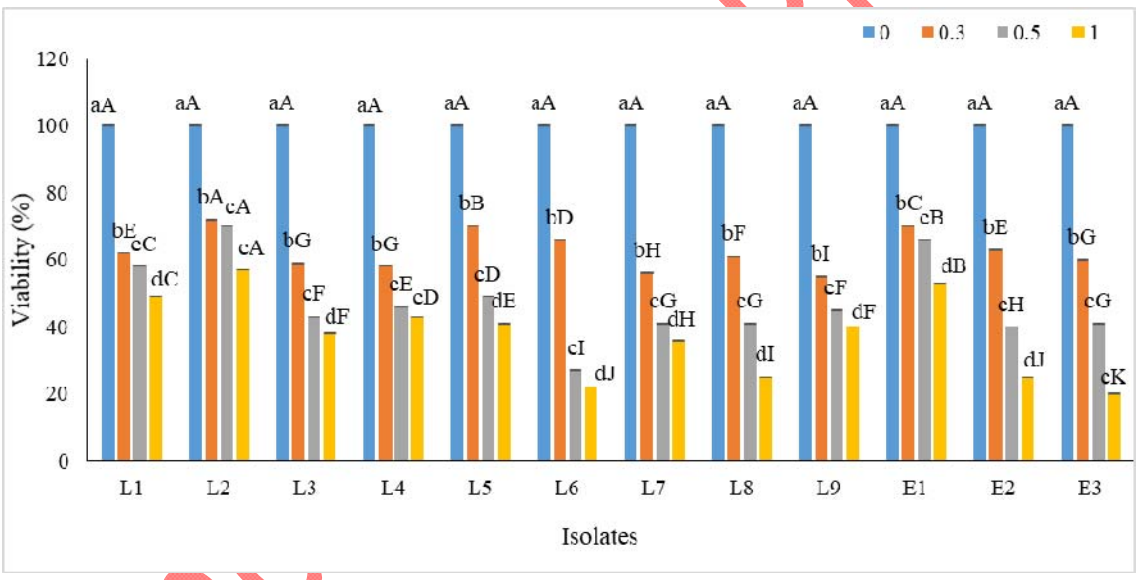


Figure 2. The resistance results of pH (a) and bile salt (b) for isolates in traditional Iranian yogurt

[a-d]: significant difference between pathogens in each isolate, and [A-L]: significant difference between isolates

Antibacterial attributes

The results of antimicrobial effect are depicted in Figure 3 for probiotic isolates against selected pathogenic bacteria. The range from 3.8 to 15.6 mm was obtained in isolates and antimicrobial activity against pathogens was the highest inhibition halo for *B. cereus* followed by *S. aureus*, *E. coli*. and *P. aeruginosa*. Among probiotic isolates, L1 and E3 created the maximum and

minimum inhibition halo against all pathogens, respectively. Different strains of *L. plantarum* had more antimicrobial functions compared to others and *E. fecalis* had less this feature.

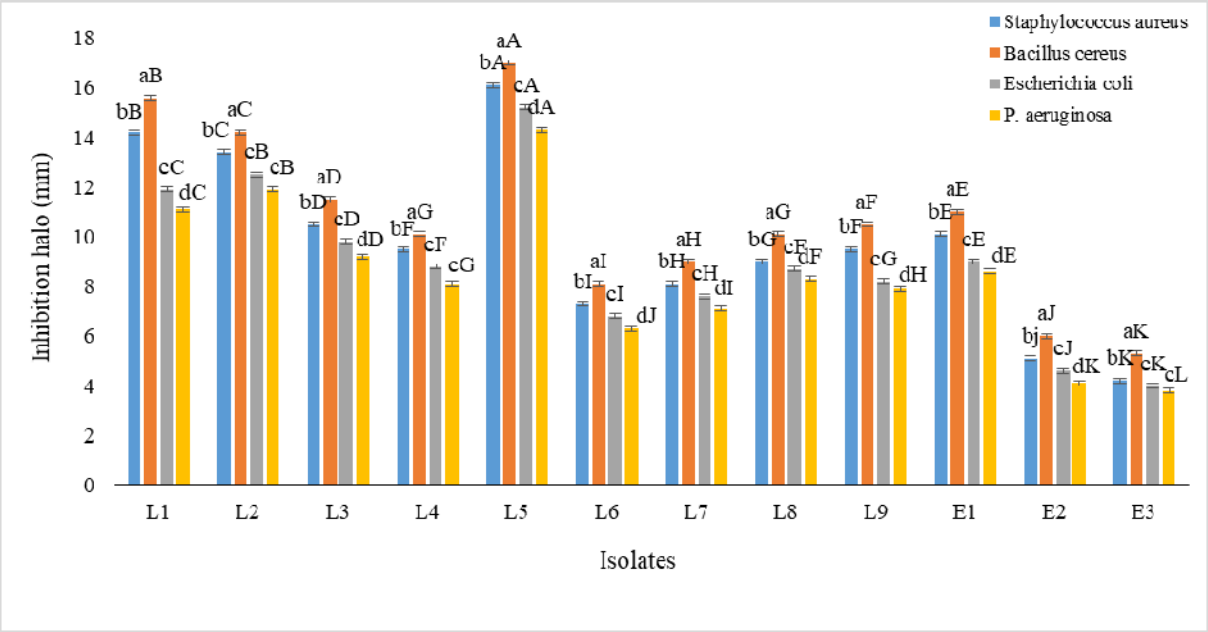


Figure 3. The results of inhibition zone diameter against indicator bacteria

[a-d]: significant difference between pathogens in each isolate and [A-L]: significant difference between isolates

The rate of acetaldehyde and diacetyl production by *Lactobacillus*

The *Lactobacillus* strain mainly accomplished acetaldehyde production and according to results illustrated in Table 4, *Lactobacillus* isolates L5 and L7 had a high ability to produce acetaldehyde, respectively (25.59 and 19.2ppm). L6 (5.96 ppm) and L8 (5.50 ppm) strains formed the maximum diacetyl; moreover, the results indicated that acetaldehyde in L2E1 increased, but diacetyl reduced compared to L2. In L2E2 and L2E3, acetaldehyde declined; however, diacetyl level enhanced, respectively. This study investigated the flavoring compound production such as acetaldehyde and diacetyl in yogurt samples fabricated by *Lactobacillus* as a single strain starter.

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

Isolates	Acetaldehyde	Diacetyl
L_1	2.70±0.01^{i}	4.68±0.05^{c}
L_2	4.45±0.03^{e}	0.45±0.03^{j}
L_3	2.23±0.03^{j}	4.39±0.05^{d}
L_4	3.41±0.01^{g}	2.83±0.01^{f}
L_5	25.59±0.05^{a}	0.57±0.05^{i}
L_6	2.32±0.0j^{b}	5.96±0.03^{a}
L_7	19.2±0.01^{b}	0.42±0.05^{j}
L_8	5.50±0.05^{d}	5.50±0.05^{b}
L9	4.17±0.05^{f}	0.8±0.05^{h}
L_2E1	13.54±0.03^{c}	0.29±0.05^{k}
L_2E2	3.01±0.02^{h}	1.98±0.03^{g}
L_2E3	2.23±0.03^{j}	4.01±0.03^{e}

Mean values with different lower case letters are significantly different ($P < 0.05$)

Yogurt sensory evaluation

Figure 4 compares the final scores for *Lactobacillus* containing products sensory attributes, those 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 having more acetaldehyde and sample L6, which had more diacetyl. The combination for *Lactobacillus* and *E. faecium*in L2E1 treatment had a positive effect on the sensory properties of final products compared to single *Lactobacillus* and obtained the most score.

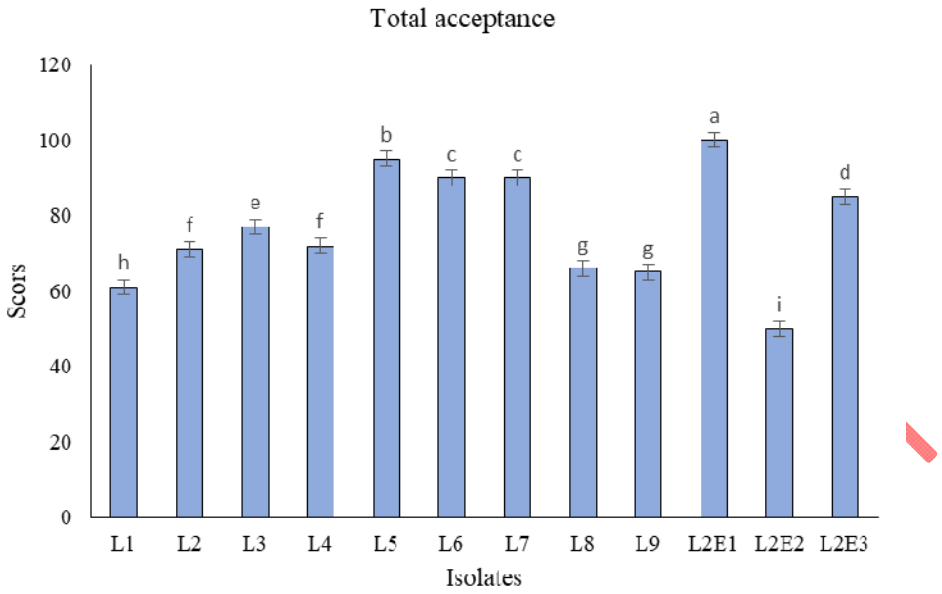


Figure 4. The results of total acceptance for sensory evaluation in traditional Iranian yogurt

Mean values with different lower case letters [a-g] are significantly different ($p < 0.05$)

4. Discussions

Purified isolates were first subjected to tests, which gram-positive and catalase negative selected as strains that had potential to be included in group of LAB (Bartkiene *et al.*, 2019). Four LAB were isolated from local yogurt based on 16S rDNA sequencing, which were named *S. thermophilus*, *L. delbrueckii*, *Lacticaseibacillus rhamnosus* (*L. rhamnosus*) and *E. faecium* (Tarique *et al.*, 2022). From the sample of local yogurt, 21 exopolysaccharide producing bacteria strains including *L.delbrueckii* subsp. bulgaricus, *S. thermophilus*, *Leuconostoc mesenteroides* and *L. plantarum* had been isolated (Omar Selim *et al.*, 2023). LAB were isolated from whey protein, milk protein concentrate, buttermilk powder, yogurt, mozzarella and gouda chesses including *L. casei*, *Lactobacillus paracasei*, *Pediococcus acidilactici* (*P. 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 pickle and identified based on biochemical and molecular assays (Yu *et al.*, 2023). The 80 fructose strains were isolated from fermented cocoa bean and sequences as *P. acidilactici* (n = 52), *L. plantarum* (n = 10), *Pediococcus pentosaceus* (*P. pentosaceus*, n = 10), *Bacillus subtilis* (n =

4) and *Leuconostoc pseudomesenteroides* (n = 4) were identified (Viesser *et al.*, 2020). The strains isolated from Teff injera dough, Ergo and Kocho products were *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 had been investigated and detected six probiotic isolates belonged to *P. acidilactici*, *L. plantarum*, *Lactobacillus brevis* (*L. brevis*), *Lactobacillus kefiri* and *L. fermentum* (Sharifi Yazdi *et al.*, 2017). Environmental condition such as low pH can prevent metabolism, reduce growth and survival of lactic acid isolates (Yu *et al.*, 2023). There are some acids such as hydrochloric acids in human stomach that destroy biomolecules such as proteins, fatty acids, vitamins and nucleic acids (Vasiee *et al.*, 2014). Every day about 2 L gastric juice with a pH close to 1.5 is secreted from lining cells and provides difficult conditions for microorganism survival and pH for gastric juice is typically 3.0 and 2.0 level is often used to simulate stomach conditions (Xu *et al.*, 2020). Therefore, resistance to acidic status is one of the important factors for accepting microorganisms as probiotics (Ladha and Jeevaratnam, 2018). These strains become a buffer after consumption with the help of carrier matrix and molecules, which protect against extreme pH in stomach (Xu *et al.*, 2020). It is necessary to check their resistance about bile salts for evaluating potential of LAB and introducing as probiotic strains (Yerlikaya and Akbulut, 2020). Oxal is a natural components related to cow, which includes conjugated and unconjugated bile salts (Kostelac *et al.*, 2021). Those isolates that resist high concentrations of bile salts can survive and grow in the normal concentration in human gastrointestinal system (Yu *et al.*, 2023). The secretion of bile extract into duodenum directly disrupts the growth for probiotic bacteria and bile acids have antimicrobial activity that act as a detergent that can disrupt biological membranes due to bipolarity (Tarique *et al.*, 2022).

In a study, strains isolated from traditional yogurt were exposed to different bile salts (cholic, oxgall and taurocholic acid) and their growth percentages were studied and results generally exhibited that in presence of cholic, isolates indicated the lowest growth compared to oxgall and taurocholic acids and also *S. thermophilus* isolates had more resistance ability to bile salts than *L. rhamnosus*, *L. delbrueckii* and *E. faecium* (Tarique *et al.*, 2022). Similar to present results, resistance to bile acids

had been reported in selected isolates from dairy and fermented products (Yerlikaya and Akbulut, 2020). Among of *L. plantarum* KO9 and *L. plantarum* M2 isolated from equid milk at pH 3.0, there were no statistical differences in target bacterium population compared with control and lowest survival rates were observed at pH 1.5 (76 % towards to control); therefore, *L. plantarum* KO9 showed no significant difference in survival feature 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 resistance investigation to low pH and bile salts on isolates obtained from fermented grains showed that none of them observed at pH 2, but *L. plantarum* was able to grow in pH 3 and also 0.6 and 0.3 % bile salts had an effect on their population, but *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, which four (4.44 %) isolates showed 45.35 to 97.11 % and 38.40 to 90.49 % survival rates at pH values (2, 2.5 and 3) for 3 and 6 h; in that order, four acid-tolerant isolates were found in 0.3 % bile salt during 24 h with 91.37 to 97.22 % survival rate, 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 log 10 (CFU/mL) at pH 2.5 for 2 h (Bartkiene *et al.*, 2019). Isolate of *Lactobacillus sakei* ADM14 obtained from kimchi was able to survive in strong pH from 2 to 3 and 1.0 % bile salts (Won *et al.*, 2020). In line with present result, *E. faecium* strains did not grow in pH 2.0 but with combination of *E. durans* had resistance to 0.3 and 0.5 % bile salts and maintained their viability (Yerlikaya and Akbulut, 2020). The strains isolated from several sources and specific species had different resistance to bile acids (Abdalla *et al.*, 2021). Two factors help microorganisms to grow in high concentrations of bile salts; first is protective effect for food matrix and second considers production hydrolyzing enzyme, which can break down bile salts into amino acids and cholesterol and also reduce their toxic influence on bacteria (Turgay and 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 including *E. coli* and *P. aeruginosa* were resistant to probiotic bacteria compared to gram-positive such as *S. aureus* and *B. cereus*; generally, gram-negative bacteria are more resistant to antimicrobial agents than gram-positive due to presence of an outer membrane around cell wall that limits diffusion of hydrophobic compounds through lipopolysaccharide and *L. plantarum* 445 exhibited the highest antagonistic features against *E. coli*, *S. aureus* and *Listeria monocytogenes* (*L. monocytogenes*) EGD-e with activities of 3.65, 2.43 and 3.89 log CFU/mL, respectively (Xu *et al.*, 2020). Average zones of inhibition by which crude extracts inhibited growth for food-borne pathogens (*S. aureus* ATCC 25923, *L. monocytogenes*, *E. coli* ATCC 25922 and *Salmonella enterica*) were ranged 17 to 21 mm (Mulaw *et al.*, 2019). Inhibition percentage of *L. plantarum* M2 neutralised supernatant was 68.18 % and 57.23 % against *Salmonella Typhimurium* (*S. Typhimurium*) and *S. aureus*, respectively (Kostelac *et al.*, 2021). The isolated *L. plantarum* No. 122, *L. casei* No. 210, *L. 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 also *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 with zones of inhibition ranging from 12.5 ± 0.71 to 20 ± 0 mm (Reuben *et al.*, 2019). In consistent with present result, inhibitory activities of LAB illustrated in previous researches (Xu *et al.*, 2020). It was reported that some strains of Enterococcus including *E. faecalis* and *E. faecium* had ability to produce bacteriocins with inhibitory effect on *Clostridium botulinum*, *S. aureus*, *Vibrio cholera*, *L. monocytogenes* and *Clostridium perfringens* and also similar to results of present study, several *Enterococcus* strains exhibited weak activity against *B. cereus* (Yerlikaya and Akbulut, 2020).

The differences in aromatic compounds of yogurt versus milk are most likely due to metabolic functions for LAB such as proteolytic and lipolytic activity (Lubbers *et al.*, 2004). Yogurt aroma

417 created by LAB is a complex mixture of aromatic components including volatile substances in milk
418 (Yerlikaya and Akbulut, 2020). The most effective ingredients in creating flavor and aroma help
419 manufacturers to make uniform products more welcomed by consumers (Cheng, 2010). Carbonyl
420 constituents including acetaldehyde and diacetyl are the main substances in yogurt, which cause the
421 most yogurt flavor and aroma (Pourahmad and Assadi, 2005). In present research, the yogurt taste
422 was constantly changing during production and storage, which caused by bacteria enzymes eventually
423 led to formation or conversion of other compounds and their loss due to volatility (Cheng, 2010). This
424 study evaluated the presence of desired volatile components in prepared yogurt samples after 14 days
425 at 5 °C. So far, more than 90 flavoring substances had been identified among which volatile acids and
426 carbonyls including acetaldehyde and also diacetyl indicated the most significant impact on yogurt
427 flavor (Lubbers *et al.*, 2004). The easy growth conditions, adaptability to different situations and heat
428 resistance of *E. faecium* caused to presence of pathogen in many specimens; therefore, it could be
429 considered a natural microflora (Yerlikaya and Akbulut, 2020). These ordinary dairy products exhibit
430 amazing aromas and flavors owing to their unique biochemical functions such as proteolysis, lipolysis
431 and citrate breakdown, which had been reported that better flavor resulted only when greater than 8.0
432 mg/kg acetaldehyde was produced in yogurt (Chen *et al.*, 2017). The typical concentrations of
433 diacetyl were reported in range from 0.2 to 3 mg/kg for yogurt (Cheng, 2010). Optimal ratio of
434 diacetyl and acetaldehyde was determined to be 4 and 16 mg/L in yogurt (Tian *et al.*, 2020). Four
435 types of pickles (without treatment, inoculated using *L. fermentum* FM 8, *Lactobacillus* spp. FM 10
436 and *L. plantarum* FM 17) were fermented at 25 °C for 15 days and 40 volatile compounds of free
437 amino acids were detected (Yu *et al.*, 2023). The study of coffee fermentation had demonstrated that
438 among different isolates, *L. plantarum* LPBF 35 indicated a special role in aroma and produced a
439 wide range of influencing compounds (acetaldehyde, ethyl acetate, nonanal and octanoic acid) in
440 cacao fermentation (Viesser *et al.*, 2020).

441 In study conducted on fermented pickles, sample containing *L. plantarum* FM 17 as a starter
442 obtained the highest sensory evaluation score in terms of overall acceptance (Yu *et al.*, 2023).
443 Acetaldehyde imparts a fresh and green flavor, which is considered to be the most important

contributor to typical yogurt aroma (Tian *et al.*, 2020). Fermentation of coffee beans by LAB demonstrated production of a wide range for aroma compounds by *L. plantarum* (Viesser *et al.*, 2020).

5. Conclusion

It seems that 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 preserve microbial and genetic reserves. In this research, the 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 potential to produce an adequate aroma, which could be applied in industry.

Ethical Considerations

Compliance with ethical guidelines

No ethical considerations were represented in present study.

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Author contributions

All authors equally contributed to preparing this research.

Conflict of Interest

The authors report no conflicts of interest.

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

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چکیده

زمینه: 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.

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

روش‌ها: ابتدا جداسازی باکتری‌های اسید لاکتیک با روش کشت انجام شد، سپس سویه‌های جداسازی شده با بررسی ویژگی‌های بیوشیمیایی و توالی ژن S rRNA16 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: 12 سویه جداسازی شده از خانواده لاکتوباسیلوس و انتروکوک شناسایی شدند، به طور کلی، *لاکتوباسیلوس پلانتاروم* گونه KLDS 1.0725 حداکثر توانایی جهت زنده ماندن طی شرایط اسیدی را نشان داد. همچنین، سویه‌های *لاکتوباسیلوس پلانتاروم* گونه KLDS 1.0725 و *انتروکوکوس فاسیوم* گونه FS019 بیشترین زنده مانی را در 0/3 و 0/5 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 1.0725 توانایی بیشتری جهت تولید استالدئید (25/59 ppm) و *لاکتوباسیلوس دلبروکی* گونه لاکتیس حداکثر دی استیل (5/96 ppm) را نشان داد. بیشترین امتیاز پذیرش در ارزیابی حسی برای سویه *لاکتوباسیلوس پلانتاروم* گونه KLDS 1.0725 و *انتروکوکوس فاسیوم* گونه V583 به دست آمد.

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

واژگان کلیدی: استالدئید، انتروکوکوس، لاکتوباسیلوس، پروبیوتیک، ماست