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Phenotypic and molecular identification of the non-pathogenic lactic acid bacteria isolated from the local sheep yogurt

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ABSTRACT

With the growing tendency of people to consume organic dairy products, there is always a risk of pathogenic microbial strains in unhygienic locally prepared products. Local sheep yogurt as an organic product is one of the most valuable dairy products in Iran. The aims of this study were molecular and phenotypic identification of lactic acid bacteria (LAB) isolated from sheep yogurt from Hamadan and investigation of their pathogenic characteristics. The LAB isolated from yogurt samples were identified phenotypically and molecularly by PCR amplification of the 16S rDNA region and subsequent sequencing. The pathogenic and safety characteristics of the isolates including antibiotic resistance, blood hemolysis, amino acid decarboxylase, DNase, and gelatinase activities were then investigated. Forty-seven bacteria were isolated from yogurt samples, and only 22 gram-positive isolates reacted negatively to catalase. Based on the results, the isolates were molecularly characterized as 4 *Lactobacillus helveticus*, 5 *Enterococcus mori*, 2 *Streptococcus salivarius subsp. thermophilus*, 5 *Lactobacillus kalixensis*, and 6 *Lactobacillus delbrueckii subsp. bulgaricus*. Phenotypic identification also confirmed the isolates assigned to *S. thermophilus* and *L. bulgaricus* by molecular identification. The evaluation of the pathogenic characteristics of the species associated with yogurt production, *S. thermophilus* and *L. bulgaricus*, confirmed that two isolates (HL1, HL2) of *L. bulgaricus* are safe as starter culture for yogurt production.

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1- Introduction

Due to the different climatic conditions in Iran, the breeding of domestic animals, including sheep, is widespread and the dairy products obtained from them find many customers in the country. Sheep yogurt is more nutritious than cow yogurt as it has a higher content of fats, calcium and proteins. The unique taste and flavor of sheep yogurt is influenced by various factors, including the type of raw materials, the inherent microbial lactic acid flora, the production methods, and various additives that vary from region to region. Especially for the production of sheep's yogurt, which is mostly done locally and traditionally, the undefined microbial flora of sheep's yogurt is used as native starter culture, whereas in industrial yogurt production from cow's milk, defined safe starter cultures are often used. These starter cultures were previously tested for their non-pathogenic. Therefore, there are always concerns regarding the consumption of local sheep yogurt, as it contains an unknown microbial flora that has not been tested for non-pathogenic and may carry pathogenic genes. Pathogenicity parameters of bacteria include antibiotic resistance, hemolysin, amino acid decarboxylase, DNase and gelatinase activities (1). DNase activity is an indicator of bacterial pathogenicity as it hydrolyzes cellular DNA molecules, and bacterial strains used in fermented products should be free of it (2). Biogenic amines are produced during the decarboxylation of amino acids by microbial activity and can cause health problems such as diarrhea, vomiting, palpitations and headaches in consumers. One of the producers of these biogenic amines in fermented products are lactic acid bacteria (LAB) (3). The enzyme gelatinase is able to hydrolyze gelatin (found in connective tissues) through two reactions, first into polypeptide compounds and then into amino acids. Various bacterial species such *as Staphylococcus aureus*, some Vibrio spp., *Pseudomonas aeruginosa*, and *Bacillus subtilis* are gelatinase-positive. Gelatinase positivity is one of the indicators of bacterial pathogenicity and the microorganisms used in fermentation should be gelatinase-negative (4). The absence of antibiotic-resistant genes in starter cultures of fermented products such as yogurt is also important. This is because the consumption of these products introduces antibiotic-resistant gene-carrying microbial flora into the consumer's body (5). Another important parameter in the use of starter cultures is the absence of hemolytic activity, i.e. the lack of ability to hydrolyze blood cells (6). The isolation and identification of indigenous LAB species from traditional dairy products is necessary to exploit the unique sensory properties of these indigenous strains. In addition to evaluating the technological potential of the strains, their non-pathogenic should also be confirmed. The aim of this study is to isolate and identify the safe LAB found in traditional sheep yogurt in Hamedan.

2. Material and methods

1.1. Sampling of yogurt and Isolation of LAB

Three samples of sheep yogurt were collected in rural areas of Hamadan city in Iran under sterile conditions and brought to the laboratory at 4°C (7). The samples were serially diluted to 10^7 in Ringer's solution and surface cultures were established on M17 agar (Merck, Germany) and MRS agar (Merck, Germany). Subsequently, these culture media were incubated anaerobically by anaerocult type A (Merck, Germany) for 48 hours at 39 °C for M17 agar and 45 °C for MRS agar. In the next step, various colonies were purified by streaking on agar plates. The purified isolates were examined by microscopic observation, Gram staining, and catalase test (8-10).

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1.2. Measurement of lactose of milk samples and pH of yogurt samples

The lactose in the milk samples and the pH value in the yogurt samples were measured using a milk analyzer and a digital pH meter respectively.

1.3. Phenotypic identification of isolates at the genus level

The Gram-positive and catalase-negative isolates were screened at the genus level for their growth potential under various conditions, including sodium chloride concentrations of 6.5% and 18%, temperatures of 10°C, 15°C, 30°C, 45°C, pH= 4.4 and pH=9.6, and the ability to produce carbon dioxide gas from glucose. Homofermentative cocci strains that can grow at 30°C and not at 10°C, pH= 9.6, and 6.5% sodium chloride were confirmed as Streptococcus (11). Gram-positive Bacillus strains with negative catalase activity that can grow at 45°C, pH= 4.4 and 6.5% sodium chloride and cannot grow at pH= 9.6, 18% sodium chloride and 15°C and do not produce carbon dioxide gas from glucose were confirmed as homofermentative Lactobacillus (12).

1.4. Phenotypic identification of isolates at the species level

Streptococcus and Lactobacillus spp. confirmed to genus level in the previous phase were further identified to species level using biochemical tests according to the guidelines of the manual (13).

1.5. Molecular identification of isolates at the species level

The DNA of cocci and Bacillus strains was extracted according to Ruiz-Barba, Maldonado-Barragán and Jiménez Díaz (14). In this method, a portion of the colonies of the isolates was dissolved in 100 µL of sterile deionized water and 100 µL of isoamyl alcohol and chloroform (Merck, Germany) solution was added at a ratio of 1:24. The mixture was shaken for 5 seconds and centrifuged at 16000 g for 5 minutes. The upper phase was used for the DNA template. For amplification of the 16S rRNA gene by PCR, 1 µL of each primer (B27F and U1492R) (Bioneer, Korea) at a concentration of 10 picomoles/µl, 12.5 µL of master mix (SinaClon, Iran), 2.5 µL of DNA and 8 µL of sterile deionized water (SinaClon, Iran) were used. After activation at 94°C for 5 min, the PCR reaction consisted of the following temperature-time program for 40 cycles: Denaturation at 94°C for 1 min, annealing at 42°C for 1 min, extension at 72°C for 1 min. Then a final extension step was performed at 72°C for 10 min and a final cooling step at 4°C for 5 min (15). The PCR products were sent for sequencing (Macrogen, Korea), and the sequences obtained were analyzed using the BLAST program on the NCBI website to identify the isolates.

1.6. Assessment of non-pathogenic properties of LAB isolates

In order to evaluate the safety properties of LAB, only bacilli and cocci isolates identified as *L. bulgaricus* and *S. thermophilus* were investigated.

1.6.1. Antibiotic resistance assessment of isolates

Cocci bacteria on M17 broth and Bacillus on MRS broth were activated. After preparing bacterial suspensions with a turbidity of 0.5 McFarland, the cocci isolate on M17 agar and the Bacillus isolates on MRS agar were surface cultured with a sterile swab. Antibiotic disks containing tetracycline, amoxicillin, penicillin, chloramphenicol, erythromycin, vancomycin, and ampicillin were then placed on the agar surface to determine the antibiotic resistance of the isolates. The plates were then incubated at 37°C for 48 hours, and the absence or presence of bacterial growth around the disks indicated

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the sensitivity or resistance of the bacteria to the respective antibiotics (16, 17).

1.6.2. Assessment of hemolytic activity of isolates

According to the manufacturer's instructions (Darvash Co, Iran), each activated isolate was streaked onto blood agar medium containing 5% sheep blood (Darvash Co, Iran) using a sterile loop. The plates were then incubated at 37°C for 48 hours. After incubation, hemolysis around the colonies was examined to determine hemolytic activity.

1.6.3. Assessment of decarboxylase activity of isolates

According to the manufacturer's instructions (Darvash Co, Iran), each activated isolate was inoculated onto lysine decarboxylase and ornithine decarboxylase media (Darvash Co, Iran) using a sterile loop and incubated at 37°C for 24 hours. The formation of a purple color indicated positive decarboxylase activity, while a yellow color indicated negative results.

1.6.4. Assessment of DNase activity of isolates

According to the manufacturer's instructions (Darvash Co, Iran), each activated isolate was streaked linearly onto DNase agar medium (Darvash Co, Iran) using a sterile loop and incubated at 37°C for 24 hours. After incubation, 3% hydrochloric acid solution was poured over the agar surface. The formation of a halo around the colonies indicated positive DNase activity.

1.6.5. Assessment of gelatinase activity of isolates

According to the manufacturer's instructions (Darvash Co, Iran), each activated isolate was inoculated to a depth of about one centimeter on gelatin agar medium (Darvash Co, Iran) and incubated at 37°C for 24 hours. After incubation, the test tubes were brought to 4°C. The presence of gelatin as a liquid in the tube after 2 to 3 hours indicated positive gelatinase activity.

1.7. Statistical analysis

The lactose of milk samples and the pH value of the yogurt samples and the pathogenic tests including antibiotic resistance, DNase, decarboxylase, gelatinase and hemolytic activities were performed in 3 replicates. The mean diameter of the growth inhibition zone, which was determined from the antibiotic sensitivity of the bacteria, and the standard deviation were calculated.

3. Results and Discussion

1.8. Measurement of lactose and pH

According to the results, the pH of Hamadan sheep yogurt was 4.1±0.13, and the lactose content of raw milk was 4.4 ± 0.2.

1.9. Phenotypic identification of isolates

A total of 47 bacteria were isolated from 3 yogurt samples, 22 of which were gram-positive and catalase-negative. Only the bacteria identified at genus level as Lactobacillus (HL1, HL2, HL3, HL7, HL9, HL10) and Streptococcus (HS1, HS2) were phenotypically identified to species level. The results of phenotypic identification of LAB isolates in yogurt at species level (Tables 1 and 2) showed that *Lactobacillus delbrueckii* subsp. *bulgaricus* is the predominant lactic acid flora in Hamadan sheep yogurt and not Streptococcus. This finding is consistent with the results of Davati and Hesami (18). Several factors may contribute to the reduction of *Streptococcus salivarius* subsp. *thermophilus* in Hamadan yogurt. One of these factors is the presence of autolyzing

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strains of this species in the yogurts of the western regions. Sandholm and Sarimo (19) and Thomas and Crow (20) reported that in yogurts with lower lactose content and lower pH, the rate of autolysis of strains of *S. thermophilus* is higher. There is also the possibility of autolysis of some Streptococcus strains in Hamadan yogurt, which may lead to the loss of these strains and consequently to their non-cultivation and recovery. This is because, according to the results of chemical tests, the lactose content and pH value in Hamadan sheep's yogurt are lower than usual. According to Davati and Hesami (21), the microbial flora of a local doogh (ayran) produced in the western regions of Iran was identified as the following species using 16s metagenomics analysis: *Lactobacillus equicursoris* 24.8%, *Lactobacillus delbrueckii* 51.81%, *Lactobacillus apis* 3.61%, *Lactobacillus ultunensis* 2.79%, *Lactobacillus taiwanensis* 0.86%, *Lactobacillus gigeriorum* 0.85%, *Pediococcus argentinicus* 0.42%, and 13.64% unclassified bacteria. Since doogh itself is a type of product containing salt and yogurt, the absence of Streptococcus species was probably due to their high autolysis rate in the yogurts of the western regions.

Table 1. Phenotypic Diagnosis of *Lactobacillus delbrueckii* subsp. *bulgaricus*

Identified isolate	*Lactobacillus delbrueckii* subsp. *bulgaricus*
Isolate code	HL1, HL2, HL3, HL7, HL9, HL10
Growth at	
15 °C	–
45 °C	+
Acid production from	
Galactose	–
Lactose	+
Maltose	–
Mannitol	–
Mannose	–
Melibiose	–
D-raffinose	–
Sucrose	–
$C0_2$ production from glucose	–

+: 90% or more strains are positive, **-:** 90% or more strains are negative

Table 2. Phenotypic Diagnosis of *Streptococcus salivarius* subsp. *thermophilus*

Identified isolate	*Streptococcus salivarius* subsp. *thermophilus*
Isolate code	HS1, HS2
Growth at	
2% NaCl	–
15 °C	–
30 °C	+
45 °C	–
Acetoin production	+

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Diacetyl production	+
Citrate	–
Acid production from	
Arabinose	–
Galactose	d
Glucose	+
Fructose	+
Mannose	+
Maltose	+
Lactose	+
Sucrose	+
Melibiose	-
Raffinose	d
Rhamnose	–
production from glucose $C0_2$	–

+: 90% or more strains are positive, **-:** 90% or more strains are negative, **d:** 11-89% are positive.

1.10. Molecular identification of isolates

The data from 16S rDNA gene sequencing for the LAB isolates of Hamadan yogurt are shown in Table 3 (Figure 1). The length of the sequenced gene was 1500 bp. Based on the results, the isolates were molecularly characterized as 4 *Lactobacillus helveticus*, 5 *Enterococcus mori*, 2 *Streptococcus salivarius subsp. thermophilus*, 5 *Lactobacillus kalixensis*, and 6 *Lactobacillus delbrueckii subsp. bulgaricus*. The diversity of lactic flora in local yogurts from the western regions of Iran has been studied previously. According to Davati (22), the presence of *Pediococcus acidilactici, Enterococcus faecium, Lactobacillus paraplantarum, Enterococcus durans, Lactobacillus delbrueckii, Lactobacillus fermentum*, and *Lactobacillus johnsonii* in yogurt produced by Alvand nomads was confirmed. The diverse lactic flora in Hamadan sheep yogurt is influenced by secondary contamination of the product, different microbial flora and the breed of sheep supplying the milk. Sheep milk can be affected by secondary contamination from sewage-contaminated pastures and animal manure (23).

Table 3. Molecular identification of isolates

Identified species	%Identity in NCBI	Isolate code
Lactobacillus delbrueckii subsp. bulgaricus	100	HL1, HL2, HL3, HL7, HL9, HL10
Streptococcus salivarius subsp. *thermophilus*	99	HS1, HS2
Lactobacillus helveticus	98	HL4, HL5, HL6, HL8
Lactobacillus kalixensis	98	HL11, HL12, HL14, HL16, HL19
Enterobacter mori	99	HE 13, HE 15, HE 17, HE18, HE 20

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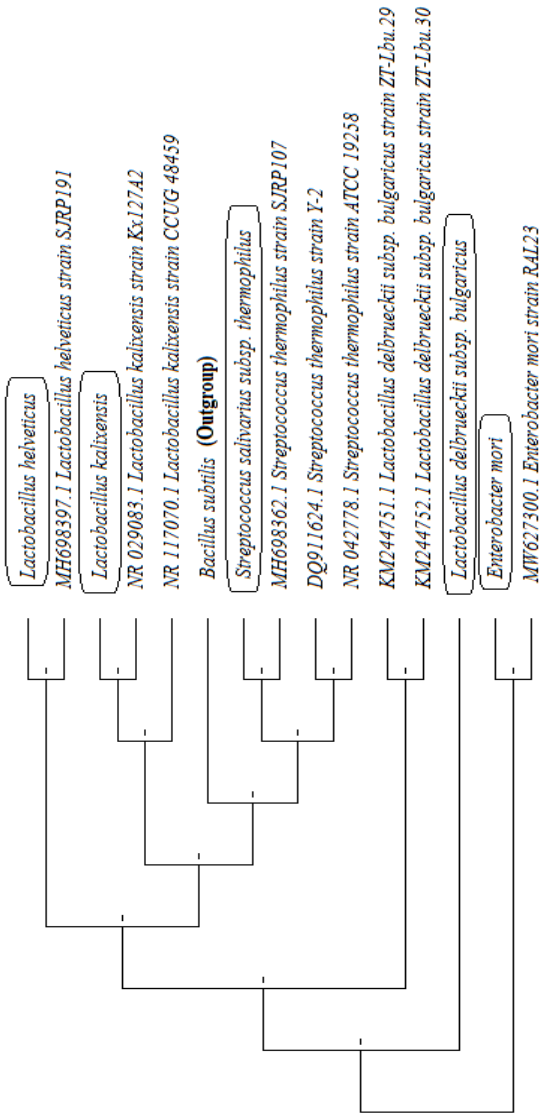

Figure 1. Phylogenetic tree of LAB isolated from sheep's yogurt and comparison with some sequences of same species (Registered in NCBI) and Outgroup

Based on studies conducted worldwide, the genus Lactobacillus has shown the highest prevalence (86.96%) among LAB in dairy products (24). In line with these findings, Bhardwaj, Puniya, Sangu, Kumar and Dhewa (25) reported that *Lactobacillus casei* (24.35%) and *Lactobacillus acidophilus* (17.37%) were the dominant microbial flora in Dahi, a traditional dairy product in India. Similarly, in our study, the predominant lactic flora in Hamadan yogurt belonged to the genus Lactobacillus. However, it should be noted that the lactic flora of local dairy products varies depending on the type of product, production method, additives, and climatic conditions. Several studies have been conducted on the microbial flora of local Iranian yogurts. In this context, Bonyadi, Mojarrad Khangah, Qanbarov, Gojezadeh and Dalili Oskuee (26) reported *Lactobacillus delbrueckii* and *Lactobacillus plantarum* as the predominant flora in traditional yogurts from rural areas in East Azerbaijan province, Iran. Jafari, Shariatifar, Khaniki and Abdollahi (27) reported *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Lactobacillus fermentum*, *Lactococcus*

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lactis subsp. *lactis*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, and *Lactobacillus casei* in traditional yogurt in Fars province, Iran. Hajimohammadi Farimani, Habibi Najafi, Fazly Bazzaz, Edalatian, Bahrami, Flórez, et al. (28) isolated *Streptococcus thermophilus* (34) and *Lactobacillus delbrueckii* (the subsp. *bulgaricus* and *lactis*) from Iranian traditional yogurt from different areas of Khorasan-e-Razavi region, Iran.

1.11. Non-pathogenic properties of LAB isolates

One of the most important criteria in the selection of starter cultures for industrial fermentation is high technological potential and safety (lack of pathogenicity). Factors indicating bacterial pathogenicity include antibiotic resistance, DNase, decarboxylase, gelatinase and hemolytic activities. The enzyme DNase hydrolyzes the DNA molecules of organisms (29). In addition, LAB can produce biogenic amines in fermented foods with decarboxylation activity. Consumption of biogenic amines can cause headaches, toxic effects on humans, diarrhea, palpitations, and vomiting (3). Gelatin, one of the most important components of vertebrate connective tissue, is degraded by gelatinases. Gelatinases are extracellular metalloendopeptidases that break down gelatin into polypeptide compounds and then into amino acids (mainly alpha types). In addition to gelatine, gelatinases are also able to hydrolyze fibrinogen, casein, and collagen. Microorganisms used in the fermentation of food should be free of gelatinases (4). Table 4 shows the results of the investigation of the pathogenic and safety properties of strains, including antibiotic resistance, hemolytic, DNase, gelatinase, ornithine decarboxylase, and lysine decarboxylase activities.

Table 4. Pathogenic properties of LAB isolates

Species	Ornithine decarboxylase	Lysine decarboxylase	DNase activity	Antibiotic resistance (diameter (mm) of inhibition zone of microbial growth)	Hemolytic activity	Gelatinase activity
***L. bulgaricus* (HL1)**	Negative	Negative	Negative	Negative 5±0.4	Negative	Negative
***L. bulgaricus* (HL2)**	Negative	Negative	Negative	Negative 4±0.1	Negative	Negative
***L. bulgaricus* (HL3)**	Negative	Negative	Negative	Ampicillin	Negative	Negative
***L. bulgaricus* (HL7)**	Negative	Negative	Negative	Vancomycin	Negative	Negative
***L. bulgaricus* (HL9)**	Negative	Negative	Negative	Vancomycin	Negative	Negative
***L. bulgaricus* (HL10)**	Negative	Negative	Negative	Vancomycin	Negative	Negative
***S. thermophilus* (HS1)**	Positive	Positive	Negative	Negative 7±0.1	Negative	Negative
***S. thermophilus* (HS2)**	Positive	Positive	Negative	Negative 8±0.2	Negative	Negative

According to our results, all strains were free of hemolytic, DNase and gelatinase activities. However, regarding ornithine decarboxylase and lysine decarboxylase activities, only Streptococcus spp. was positive. In addition, *L. bulgaricus* (HL7),

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L. bulgaricus (HL9) and *L. bulgaricus* (HL10) were resistant to vancomycin, while *L. bulgaricus* (HL3) was resistant to ampicillin. Several studies have reported the resistance of Lactobacillus to antibiotics, especially vancomycin, and the decarboxylase activity of LAB. Bernardeau, Vernoux, Henri-Dubernet and Guéguen (30) reported Lactobacillus species with decarboxylase activity and resistance to antibiotics, especially vancomycin. Perin, Miranda, Todorov, de Melo Franco and Nero (31) reported positive antibiotic resistance, DNase, decarboxylase, gelatinase, and hemolytic activities in many LAB strains in goat milk. Domingos-Lopes, Stanton, Ross, Dapkevicius and Silva (32) showed that most LAB strains of artisanal Pico cheese reacted positively to resistance to aminoglycoside antibiotics and nalidixic acid, while they reacted negatively to histamine and DNase production. They also reported strains with gelatinase and alpha-hemolytic activities. Omafuvbe and Enyioha (33) reported Lactobacillus species including *L. acidophilus*, *L. fermentum*, and *L. casei* with amino acid decarboxylase activity that can produce ornithine, lysine, and tyrosine amines. Therefore, in the present study, most LAB strains isolated from Hamadan yogurt were safe compared to other studies that have investigated the presence of unsafe LAB strains in various dairy products around the world.

6. Conclusion

The presence of unsafe indigenous LAB strains in local dairy products is due to the diversity of the genome of the microbial flora in these products. Local dairy products from different regions have inherent bacterial strains that are specific to the geographical area and may differ from other similar species in terms of pathogenic parameters. Based on our study results and similar research, the probable presence of autolytic Streptococcus spp. in local yogurt from western Iran is suggested. The results of phenotypic identification were approximately consistent with molecular identification. However, there may be unrecovered LAB spp. in Hamadan yogurt that were damaged due to environmental stressors and cannot be cultured. When investigating the non-pathogenic of LAB isolated from Hamadan yogurt, *L. bulgaricus* (HL1, HL2) are recommended as safe starter cultures for industrial yogurt production.

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شناسایی فنوتیپی و مولکولی باکتری‌های اسید لاکتیک غیربیماری‌زای جدا شده از ماست گوسفندی محلی

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چکیده

با افزایش تمایل مردم به مصرف محصولات لبنی ارگانیک، همواره خطر سویه‌های میکروبی بیماری‌زا در محصولات محلی وجود دارد. ماست گوسفندی محلی به عنوان یک محصول ارگانیک از باارزش‌ترین محصولات لبنی در ایران است. هدف از این مطالعه تشخیص فنوتیپی و مولکولی باکتری‌های اسید لاکتیک جدا شده از ماست گوسفندی همدان و بررسی خواص بیماری‌زای آن‌ها است. جدایه‌های لاکتیکی از نمونه‌های ماست از نظر فنوتیپی و مولکولی با تکثیر ناحیه 16S rDNA توسط واکنش PCR و به دنبال آن توالی‌یابی شناسایی شدند. خصوصیات بیماریزایی و ایمنی جدایه‌ها شامل مقاومت به آنتی‌بیوتیک، همولیز سلول‌های خونی، فعالیت‌های آمینواسید دکربوکسیلازی، DNase، و ژلاتینازی بررسی شدند. ۴۷ باکتری از نمونه‌های ماست جدا شدند و تنها ۲۲ جدایه گرم مثبت و کاتالاز منفی بودند. براساس نتایج، جدایه‌ها از نظر مولکولی به عنوان ۴ *لاکتوباسیلوس هلوتیکوس*، ۵ *انتروکوکوس موری*، ۲ *استرپتوکوکوس سالیواریوس* زیرگونه *ترموفیلوس*، ۵ *لاکتوباسیلوس هلوتیکوس* و ۶ *لاکتوباسیلوس دلبروکی* زیرگونه *بولگاریکوس* تشخیص داده شدند. شناسایی فنوتیپی همچنین حضور جدایه‌هایی که متعلق به تولید ماست بودند را تایید کرد. ارزیابی خواص بیماری‌زایی گونه‌های مرتبط با تولید ماست، *استرپتوکوکوس سالیواریوس زیرگونه ترموفیلوس* و *لاکتوباسیلوس دلبروکی زیرگونه بولگاریکوس*، تایید کرد که تنها دو جدایه از *لاکتوباسیلوس دلبروکی زیرگونه بولگاریکوس* (HL1, HL2) به عنوان کشت آغازگر برای تولید ماست ایمن هستند.

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