Volume 13, Issue 1, Page 111-117, 2024 https://doi.org/10.46810/tdfd.1424945 Research Article

BİNGÖL UNIVERSITY

Türk Doğa ve Fen Dergisi
Turkish Journal of Nature and Science
www.dergipark.gov.tr/tdfd

Isolation, Identification and Molecular Characterization of Lactic Acid Bacteria from Raw Milk Samples Collected from Erzurum Region

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(Received: 24.01.2024, Accepted: 11.03.2024, Online Publication: 26.03.2024)

Keywords
Raw milk, Lactic acid bacteria, Identification, rep-PCR, 16S-rRNA sequence analysis

Abstract: Due to its high-water content, milk is an important source of different microbial contents, especially lactic acid bacteria. This study aims to isolate and identify lactic acid bacteria from raw milk samples collected from Erzurum and its surroundings, and to introduce possible new species, or genera, to the taxonomy. For this purpose, DNAs of pure bacterial cultures obtained from 50 raw milk samples were isolated, isolates that differed from each other were selected by rep-PCR. A total of 11 different species and subspecies [*Corynebacterium casei*, *Enterococcus italicus*, *E. durans*, *Lactococcus lactis*, *Lactococcos lactis* subsp. *lactis*, *Lactococcos lactis* subsp. *hordniae*, *Lactobacillus paracasei*, *Leuconostoc lactis*, *Staphylococcus succinis*, *Streptococcus parauberis* and *S. uberis*] were determined in raw milk samples by 16S rRNA sequence analysis. It was concluded that the $(GTG)_5$-PCR method was more successful than BOX-PCR in the identification of lactic acid bacteria isolated from raw milk samples at a species and subspecies level, and the isolate TS10, which was 98% similar to *S. uberis*, maybe a new species and should be re-examined with advanced diagnostic techniques.

Erzurum Yöresinden Toplanan Çiğ Süt Örneklerinden Laktik Asit Bakterilerinin İzolasyonu, İdentifikasyonu ve Moleküler Karakterizasyonu

Anahtar Kelimeler
Çiğ süt, Laktik asit bakteri, İdentifikasyon, rep-PCR, 16S-rRNA sekans analizi

Öz: Süt, sahip olduğu yüksek su aktivitesi nedeniyle başta laktik asit bakterileri olmak üzere, farklı mikrobiyal içerikler açısından önemli bir kaynaktır. Bu çalışmada Erzurum ve çevresinden toplanan çiğ süt örneklerinden, laktik asit bakterilerinin izolasyonu, identifikasyonu ve olası yeni tür veya cinslerin taksonomiye kazandırılması hedeflenmiştir. Bu amaçla, Erzurum ve ilçelerindeki üreticilerden toplanan 50 adet çiğ süt örneğinden elde edilen saf bakteri kültürlerinin DNA'ları izole edilerek, rep-PCR ile birbirinden farklı olan izolatlar seçilmiş ve 16S rRNA sekans analizi ile çiğ süt örneklerinde 11 farklı tür ve alt-türün [*Corynebacterium casei*, *Enterococcus italicus*, *E. durans*, *Lactococcus lactis*, *Lactococcos lactis* subsp. *lactis*, *Lactococcos lactis* subsp. *hordniae*, *Lactobacillus paracasei*, *Leuconostoc lactis*, *Staphylococcus succinis*, *Streptococcus parauberis* ve *S. uberis*] varlığı tespit edilmiştir. İncelenen çiğ süt örneklerinden izole edilen laktik asit bakterinin tür ve tür altı düzeyde identifikasyonunda $(GTG)_5$-PCR yönteminin, BOX-PCR'a göre daha başarılı olduğu, %98 oranında *S. uberis*'e benzerlik gösteren TS10 kodlu izolatın yeni tür olabileceği ve ileri tanı teknikleri ile yeniden incelenmesi gerektiği sonucuna varılmıştır.

1. INTRODUCTION

People need high-quality foods with high nutritional value to sustain their essential activities. The most preferred and first thing that comes to mind is milk and dairy products. "Raw milk" is defined as milk that is milked from the animal at certain intervals, from which no components are removed, or any other substance is added, and which is not subjected to any prior processing, such as being sent to a factory to be processed [1].

Milk is a nutriment that has a key position in the nutrition process, containing high-quality protein, minerals such as calcium, phosphorus and zinc, and vitamins such as carotenoids, riboflavin, A, D, E, K, B1

(thiamine), B6, B12 and niacin. Since the foods in the milk and dairy products group are rich in calcium, they are important for the healthy development of bones and teeth of individuals, especially in the age groups between adolescence and adulthood; in adults, it has a key role in protecting against cardiological problems, high blood pressure, stroke, osteoporosis, Type 2 diabetes, and colon cancer [2].

Milk allows microorganisms to multiply rapidly under inappropriate milking and storage conditions. Microbial contamination, especially results after milking, increases the acidity in milk and therefore causes coagulation in milk. Medicines used in disease treatment, pesticides, antibiotics, detergent residues, estrus, gestation, and body secretions affect the structure of raw milk. Therefore, in cases where the microorganism load in raw milk is not at an acceptable level, human health will be threatened, and serious milk production losses will grow up [1].

The first diagnosis of lactic acid bacteria as a group was made based on their ability to ferment and coagulate milk with coliform bacteria. Coliform bacteria were separated from the lactic acid bacteria group, with the introduction of microorganisms of the *Lactobacillus* type into taxonomy by Beijerinck in 1901. Lactic acid bacteria are defined as cocci or rod-shaped microorganisms that are gram-positive, endospore, oxidase, catalase-negative, anaerobic, or facultative aerobic, can tolerate acid, can grow at low pH and salt concentrations, are strongly fermentative, produce lactic acid as the final product during sugar fermentation [3,4]. While morphological and physiological tests are widely used in the diagnosis of lactic acid bacteria, now, additional molecular tests (such as API, FAME, SDS-PAGE, PCR-RFLP, rep-PCR and 16S rRNA sequence analysis) are also conducted since merely conventional analyses (such as gram; endospore staining; determination of salt, pH and temperature values at which bacteria develop optimally; motility; catalase; oxidase; and gas production test from glucose) are insufficient for identification [1,5,6].

Most of the lactic acid bacteria found in milk and used as probiotics are classified as GRAS (Generally Recognized as Safe) microorganisms because they are not pathogenic, they produce antimicrobial substances, they are suitable for technological and industrial processes, and they are tolerant to acid and bile. In the last decade, there has been a significant increase in research based on the isolation and identification of lactic acid bacteria from different sources and the determination of their potential to be used as probiotics [1,6]. This study aims to isolate and identify lactic acid bacteria, which are very important for public health and food safety, from raw milk samples collected from Erzurum and its surroundings, and to introduce possible new species or genera into taxonomy.

2. MATERIAL AND METHOD

2.1. Material

50 raw milk samples collected under aseptic conditions from different locations, including Erzurum and its districts (Aziziye, Pasinler, Yakutiye (Dumlu), Tekman, Ispir and Koprukoy) in the summer period, were brought to the laboratory under the cold chain. The analyses and further processing were started immediately.

2.2. Isolation of Lactic Acid Bacteria

For the isolation of test bacteria, a serial dilution tube (10^{-1}-10^{-7}) was prepared using 0.85% sterile physiological water. Subsequently, 0.1 ml of these dilutions was taken and dispersed on MRS (de Man, Rogosa and Sharpe) and M17 Agar and incubated at 35 °C for 48 hours under both aerobic and anaerobic conditions. At the end of this period, isolates that are believed to be different from the developing colonies in terms of cell and colony morphology were selected. Three phase lines were inoculated onto MRS and M17 Agar and incubated at 35 °C. Afterward, colonies were taken from each pure culture with a sterile loop, inoculated into MRS Broth/M17 Broth and kept in a shaking incubator at 35 °C for 24-48 hours. At the end of this period, stock tubes were prepared from the test strains and stored at -86°C until further analysis [7].

2.3. Identification of Isolates Using Conventional Methods

Bacteria from the stock were revived using MRS/M17 agar before the test isolates were diagnosed using conventional methods. Subsequently, morphological, physiological, and biochemical characteristics of the isolates were determined [8,9]. Different strains were selected and subjected to molecular characterization.

2.4. Molecular Characterization of Isolates

2.4.1. Genomic DNA isolation and molecular fingerprinting

A single colony was taken from the Petri dishes of each test strain revived on MRS/M17 agar, inoculated into MRS/M17 Broth, and incubated for 48 hours. At the end of this period, the genomic DNA of bacteria was isolated according to the Promega Wizard^{R} genomic DNA purification kit (A2360) protocol [10]. Subsequently, the amount of DNA was adjusted by spectrophotometer to be between A260/A280=1-1.9. To reveal the genomic differences between the test isolates, rep-PCR [BOX-PCR and $(GTG)_5$-PCR] genomic fingerprint analysis was performed using BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') and $(GTG)_5$ primers (5'-GTGGGTGGTGGTGGGTG-3'). To obtain PCR products, 27 µL reaction mixture was prepared as follows: 5 µL Specific Gitschier Buffer, 2.5 µL dimethyl sulfoxide (100%, 20X), 1.25 µL dNTPs (10 mM), 1.25 µL bovine serum albumin (20 gL^{-1}), 3.0 µl primers (5 mM), 0.3 µL Taq polymerase (250 U) and 13.7 µL PCR

grade water. In conclusion, 3 µL of DNA whose concentration was adjusted to 50 ng was added to each PCR tube (excluding the negative). PCR reactions were conducted employing a thermal cycler, with the specified conditions for $(GTG)_5$-PCR as follows: initial denaturation at 94 °C for 7 minutes; 36 cycles at 94 °C for 1 minute, annealing at 53 °C for 1 minute utilizing the $(GTG)_5$ primer, and extension at 65 °C for 8 minutes; followed by a final polymerization at 65 °C for 16 minutes before cooling at 4 °C. In the case of BOX-PCR, the process involves an initial denaturation at 95 °C for 7 minutes, followed by 36 cycles that include 94 °C for 1 minute, annealing at 53 °C for 1 minute using the BOX primer, extension at 65 °C for 8 minutes, and concluding with a final polymerization at 65 °C for 16 minutes in advance of cooling to 4 °C [11-13].

2.4.2. 16S rRNA-PCR

The evolutionarily conserved 16S rRNA gene region is very important in terms of bacterial taxonomy. For this purpose, the 16S rRNA region was amplified using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [14,15]. 13.1 µl ddH_2O, 3 µl 10X PCR buffer, 1.8 µl $MgCl_2$, 1.2 µl DMSO, 0.6 µl dNTP, 3 µl (5 µM) forward primer (27F), 3 µl (5 µM) reverse primer (1492R), 0.3 µl Taq DNA polymerase and 4 µl template DNA (50 ng). The resulting 16S rRNA PCR products were cloned into the pGEM-T Easy Cloning Vector (Promega, Southampton, UK) using *Escherichia coli* JM101 strain according to the manufacturer's instructions. After cloning, colonies that gave positive results were selected; plasmid isolation was performed and sent to Macrogen Company (Netherlands) for sequence analysis. The obtained 16S rRNA sequence results were compared with other bacteria in GenBank® and EzTaxon (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome and http://www.ezbiocloud.net/eztaxon/identify), the similarity ratios between them were determined and their GenBank® No's were achieved [16,17].

3. RESULTS AND DISCUSSION

Lactic acid bacteria have great industrial importance because they are used as starter cultures in the production of various foods, produce antimicrobial substances and included in the formulations of probiotic products. Many dairy products, such as raw milk, kefir and white cheese, are good sources of lactic acid bacteria. Lactic acid bacteria play a very important role in the formation of the unique taste and aroma characteristics of different fermented dairy products. Therefore, the isolation and identification of lactic acid bacteria from natural sources is of great importance [18]. In this study, conventional and molecular characterization of 11 different species and sub-species from raw milk samples collected from Erzurum and its districts were performed.

3.1. Isolation and Identification of Lactic Acid Bacteria

In this study, a total of 150 bacteria were purified (Table 1) isolating from 50 raw milk samples taken from milk producers via cold chain in Erzurum Aziziye, Pasinler, Yakutiye (Dumlu), Tekman, Ispir and Koprukoy districts under aseptic conditions, and stock cultures were prepared.

Table 1. The localities of raw milk samples and Isolated Lactic acid bacteria strains

Location of Isolates	Isolates
Aziziye	TS4,TS19,TS7
Pasinler	TS5,TS6,TS8
Tekman	TS10,TS17
Yakutiye (Dumlu)	TS1,TS2,TS3
Ispir	TS9,TS11
Koprukoy	TS13,TS18

150 pure isolates were subjected to pre-screening in terms of colony morphology, Gram, catalase, and oxidase properties. Consequently, DNA isolations of selected 130 isolates, which were believed to be phenotypically different, were differentiated using rep-PCR [BOX and $(GTG)_5$-PCR], and the presence of 15 different isolates was observed (Figure 1 and Figure 2).

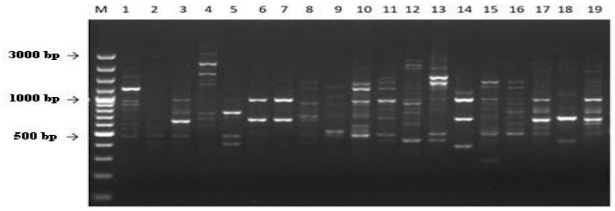

Figure 1. BOX-PCR of test isolates: M [Marker (BioLabs N0550S);100, 200, 300, 400, 500, 600, 700, 800, 900,1000, 1200, 1500, 2000, 3000, 4000, 6000 and 1-19; samples]

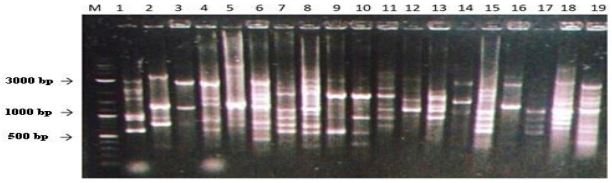

Figure 2. $(GTG)_5$-PCR of test isolates: M [Marker (BioLabs N0550S);100, 200, 300, 400, 500, 600, 700, 800, 900,1000, 1200, 1500, 2000, 3000, 4000, 6000 and 1-19; samples]

In the BOX-PCR analysis performed for this purpose, it was determined that the isolates contained at least two polymorphic bands between 650-3000 bp and a maximum of 11 polymorphic bands between 400-6500 bp. As a result of the later $(GTG)_5$-PCR analysis; it was determined that the test strains gave at least 3 polymorphic bands between 600-3000 bp and a maximum of 14 polymorphic bands between 300-6500 bp. $(GTG)_5$-PCR was more effective in revealing genomic differences between strains than BOX-PCR. Ledina *et al.* [19] performed the isolation and identification of lactic acid bacteria from raw milk and cheese samples in their study, and like our study, they concluded that the $(GTG)_5$ fingerprint analysis method was highly successful in revealing the distinction between strains. The 16S rRNA gene region of 15 test strains which were believed to be different according to

the results of the genomic fingerprint analysis, was amplified with universal primers (27F and 1492R). Afterward, PCR products were run on agarose gel (1%) and it was observed that all isolates gave a single band (1500 bp) (Figure 3). 16S rRNA PCR products were cloned using the appropriate vector system. Colony PCR (Figure 4) was performed by selecting white colonies containing the relevant gene, and plasmid isolation was performed using the isolation kit (A1330) from Promega.

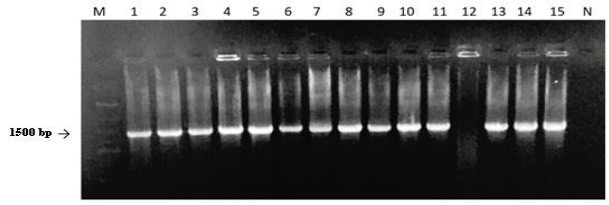

Figure 3. Gel image of 16S rRNAs of various isolates: M [Marker (BioLabs N0550S)];100, 200, 300, 400, 500, 600, 700, 800, 900,1000, 1200, 1500, 2000, 3000, 4000, 6000, N; Negative Control and 1-15; samples]

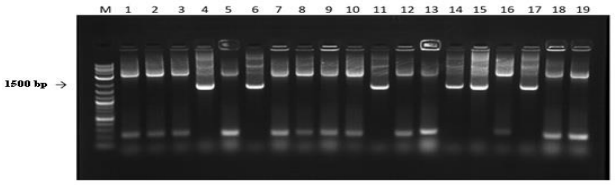

Figure 4. Colony PCR image: M [Marker (BioLabs N0550S);100, 200, 300, 400, 500, 600, 700, 800, 900,1000, 1200, 1500, 2000, 3000, 4000, 6000 and 1-19; samples]

Then, whether the plasmid carried the desired gene region was analysed using the EcoRI rapid cut-off enzyme. Samples that gave positive results and had the optimum concentration were sent to Macrogen (Netherlands) for sequence analysis [20]. After the 16S rRNA sequence data were made significant, they were compared (Blast) with the sequences in Eztaxon and GenBank®, and then GenBank® numbers were obtained from NCBI (Table 2).

Table 2. 16S rRNA sequence analysis results, similarity ratios and GenBank® No's of test isolates

Code of Isolate	Name of Isolate	Similarity Ratio (%)	GenBank® No
TS1	*Enterococcus italicus*	99.43	MW433826
TS2	*Leuconostoc lactis*	99.50	MW433829
TS3	*Enterococcus italicus*	99.23	MW433845
TS4	*Streptococcus parauberis*	99.50	MW433848
TS5	*Enteroccocus durans*	99.43	MW433847
TS6	*Corynebacterium casei*	99.20	MW450777
TS7	*Lactococcus lactis*	99.36	MW450701
TS8	*Lactococcus lactis*	99.50	MW450781
TS9	*Enterococcus italicus*	99.36	MW450782
TS10	***Streptococcus uberis***	**98**	**MW450783**
TS11	*Enterococcus italicus*	99.93	MW450787
TS13	*Staphylococcus succinus*	99.9	MW450820
TS17	*Lactococcus lactis* subsp. *lactis*	99	MW4508551
TS18	*Lactococcus lactis* subsp. *hordniae*	99.85	MW450862
TS19	*Lacticaseibacillus paracasei* subsp. *paracasei*	99.50	MW463461

Using the data obtained within the scope of the study, a phylogenetic tree was drawn with the NJ (Neighbor-Joining) method to reveal the phylogenetic relationship between lactic acid bacteria (Figure 5).

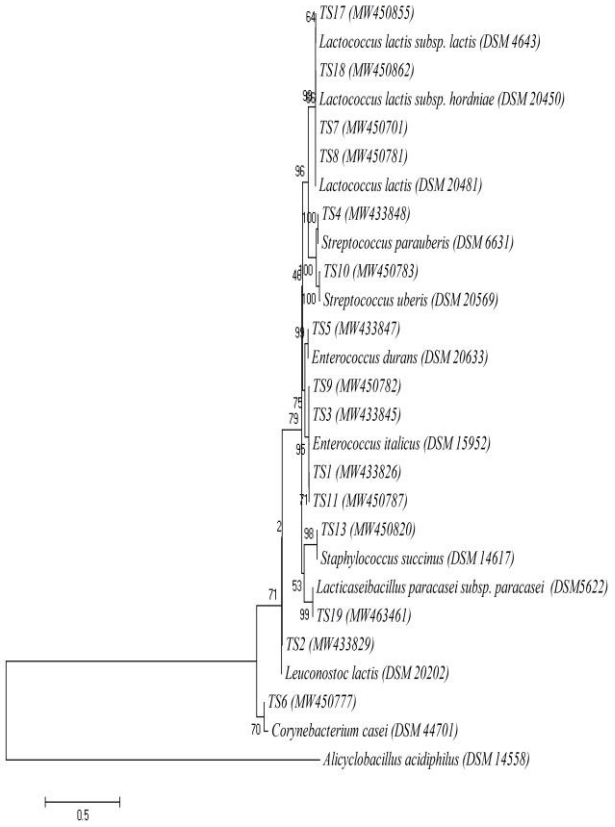

Figure 5. Neighbor-Joining-Phylogenetic tree created with 16S rRNA sequence analysis of lactic acid bacteria and reference strains

In the study conducted by Lusk *et al*. [21], it was concluded that the amplification of the 16S rRNA gene region was highly successful in the identification of the microflora in cheese, especially at the species level. Similar results have been presented by different scientists, and these data are compatible with our results [22,23]. From the test isolates; it was concluded that TS1, TS3, TS9 and TS11 are similar to *Enterococcus italicus*; TS2 is similar to *Leuconostoc lactis*; TS4 is similar to *Streptococcus parauberis*; TS5 is similar to *E. durans*; TS6 is similar to *Corynebacterium casei*; TS7 and TS8 is similar to *Lactococcus lactis*; TS13 is similar to *Staphylococcus succinus*; TS17 is similar to *L. lactis* subsp. *lactis*; TS18 is similar to *L. lactis* subsp. *hordniae*; TS19 is similar to *Lactobacillus paracasei* (≥99%), and the isolate TS10 showed 98% similarity to *Streptococcus uberis*, TS10 which is similar to *S. uberis*, maybe a new species and should be investigated with further diagnostic techniques (Table 2).

In the study conducted by Kirmaci [24], 143 isolates were obtained from raw sheep milk and the species-level identification of the samples was carried out by 16S rRNA sequence analysis. It was determined that 48.95% of 143 isolates are *Enterococcus* spp., 40.55% are *Lactococcus* spp., 9.10% are *Lactobacillus* spp., 0.69% are *Streptococcus* spp. and 0.69% are *Leuconostoc* spp. As a result, it was observed that samples of the

Enterococcus and *Lactococcus* genera constituted the dominant flora. Results like the literature data were obtained in the performed study. Detailed morphological, physiological, and biochemical characteristics of 11 different strains were investigated by randomly selecting one of the isolates belonging to the same species (TS1 from TS1, TS3, TS9 and TS11 isolates; TS7 from TS7 and TS8 strains) that were believed to be different. As a result of this study, it was observed that all the isolates were Gram-positive, endospore negative, nonmotile, with stem cell morphology (excluding TS6 and TS19), catalase (excluding TS6 and TS13) and oxidase (excluding TS4 and TS13) negative, and homofermentative (TS2, TS7 and TS19 are heterofermentative). In their study, Fortina *et al*. [25] revealed that the *Enterococcus italicus* species was gram-positive; it had an endospore, catalase, oxidase negative, homofermentative, nonmotile and stem cell morphology and can grow at 10-45 °C, pH 9 and 6% NaCl concentration. TS1, TS3, TS9 and TS11 isolates which were isolated within the scope of our study, showed 99% similarity to the *Enterococcus italicus* species and also showed characteristics parallel to the literature data.

It was determined that the isolate TS10, which has a 98% similarity to *Streptococcus uberis* was gram-positive, catalase-negative, and grown in the 0-6% salt concentration range, at 15-50 °C. In the study conducted by Odierno *et al*. [26], the same bacterial species were isolated from raw milk, identification was performed, and similar results were obtained. Unlike others, the genomic similarity ratio of the isolate we obtained to the strains in GenBank® is 98% instead of 99%.

As a result of the studies carried out to determine the salt tolerance (0-12%) of the isolated lactic acid bacteria: it was determined that test isolates TS1, TS2, TS6, TS7, TS12, TS13 and TS18 were grown at 0-8%; the samples TS4, TS5 and TS10 were grown in the 0-6% salt range, and the TS17 was grown in the 0-10% salt range. In the study conducted to determine the pH ranges in which test strains can grow: it was observed that isolates TS1, TS2, TS5 and TS18 were grown in the pH range of 5-11; isolates TS4, TS7, TS17 and TS19 were grown in the pH range of 5-9; isolates coded TS6, TS10 and TS13 were grown in the pH range of 3-11. Furthermore, it was determined that all the strains grew at 20-50 °C (except for isolates TS7, TS17 and TS19, these strains can grow at the highest temperature of 45 °C), while isolates TS10 and TS13 could also grow at low temperatures such as 15 °C.

In the study conducted by Tanguler [27], it was determined that *Lactobacillus*, *Pediococus* and *Leuconostoc* genera isolated from turnip juice grew at pH 4 but could not grow at pH 9.6. It was determined that other bacteria, except *L. buchneri*, from the species *Lactobacillus plantarum*, *L. buchneri*, *L. brevis*, *L. fermentum* and *L. delbrueckii*, which were used as controls for the same study, grew at pH 4.4. It was determined that the TS2 determined as *Leuconostoc lactis* grew in the pH range of 5-11, unlike the literature.

Kuikui *et al*. [28] isolated lactic acid bacteria from rice silage and identified those using phenotypic and genotypic methods. Because of the phenotypic characterization, it was observed that most of the isolates did not show any growth at low pH and temperature values such as pH 3 at 45 °C and could only grow at a maximum salt concentration of 6.5%. In our study, contrary to the literature data of the isolates; it has been determined that most of the isolates can grow at 20-50 °C, in the pH range of 3-11, and at 0-8% salt concentration. This reveals that the physiological characteristics of isolates may vary depending on the location and the difference in isolated sources.

In this study conducted by Brennan *et al*. [29], it was determined that *Corynebacterium casei* LMG S-19264^T standard strain obtained from cheese is a catalase-positive, oxidase negative, non-motile and non-spore-forming bacterium. In parallel with the literature data, it was determined that the isolate TS6 (*C. casei*) obtained from raw milk was catalase positive, oxidase negative, non-motile, grew at 0-8% salt concentration, in the pH range of 3-11 at 20-50 °C.

In the study conducted by Sánchez *et al*. [30], it was determined that the optimum pH at which the isolate CRL264, which is like *Lactococcus lactis*, could grow was 6.6 and the temperature was 30 °C. In parallel with the literature data, it was observed that the TS7 obtained in our study showed growth at pH 5-9 and 20-45 °C.

4. CONCLUSION

As a result of the research, it was concluded that raw milk samples collected from Erzurum and its districts have a very wide microflora most of the species belonging to the genera *Lactococcus* and *Enterococcus* constitute the dominant flora compared to other species according to conventional test results that must be supported by molecular methods. Among the rep-PCR methods, $(GTG)_5$-PCR is more effective in the identification of lactic acid bacteria at the species and sub-species level than the BOX-PCR method, in view of the fact that isolates survive a wide range of pH, temperature and salt concentrations. Some differences between the physiological characteristics of the bacterial strains isolated within the scope of the study and the literature data may depend on the source from which the strains were isolated, ambient conditions and the isolated habitat. These isolated and identified strains are applicable in biotechnological processes, and also according to the 16S rRNA gene analysis results, the TS10 showed 98% similarity to the *Streptococcus uberis*, maybe a new species and must be analysed with further diagnostic techniques.

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