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Identification of lactic acid bacteria isolated from traditional cheeses of the Black Sea Region

Identificación de bacterias lácticas aisladas de quesos tradicionales de la Región del Mar Negro

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ABSTRACT

The aim of this study was to isolate and to identify lactic acid bacteria from traditional cheeses of the Black See Region. Artvin Şor, Giresun Tecen, Kargı Tulum, Ordu Kesik and Trabzon Telli cheese were used as cheese samples of the Black Sea Region. The number of lactic acid bacteria in traditional cheese of the Black Sea Region were ranged from 4.62 ± 0.76 and to 7.87 ± 0.64 log cfu·g^{-1}. Gram–positive and catalase–negative colonies were evaluated as lactic acid bacteria based on the morphological and biochemical properties. According to biochemical analysis results, 39 lactic acid bacteria strains were identified by 16S rDNA isolated from cheese samples. Based on the sequence analysis, the indigenous lactic acid bacteria population was identified as *Enterococcus faecium* (35.9%), as *Levilactobacillus brevis* (12.8%), as *Lactiplantibacillus plantarum* (15.3%), as *Pediococcus acidilactici* (7.6%), as *Enterococcus durans* (7.6%), as *Lacticaseibacillus paracasei* (5.1%), as *Lacticaseibacillus casei* (7.6%), as *Leuconostoc mesenteroides* (2.5%), as *Leuconostoc lactis* (2.5%) and as *Weissella cibaria* (2.5%). *Enterococcus* spp. was the dominant lactic acid bacteria in cheese sample. The present findings revealed that lactic acid bacteria populations varied depending on cheese types in terms of cell counts and diversity.

Key words: Cheese; identification, lactic acid bacteria, PCR

RESUMEN

Este estudio tuvo como objetivo aislar e identificar bacterias lácticas a partir de muestras de quesos tradicionales de la región del Mar Negro. Se utilizaron quesos Artvin Şor, Giresun Tecen, Kargı Tulum, Ordu Kesik y Trabzon Telli. El número de bacterias lácticas osciló entre 4,62 ± 0,76 y 7,87 ± 0,64 log ufc·g^{-1}. Las colonias grampositivas y catalasa–negativas se evaluaron como bacterias de ácido láctico en función de las propiedades morfológicas y bioquímicas. Se identificaron 39 cepas de bacterias lácticas mediante 16S rDNA aislado de muestras de queso. Con base en el análisis de secuencia, la población de bacterias ácido lácticas autóctonas se identificó como *Enterococcus faecium* (35,9%), como *Levilactobacillus brevis* (12,8%), como *Lactiplantibacillus plantarum* (15,3%), como *Pediococcus acidilactici* (7,6%), como *Enterococcus durans* (7,6%), como *Lacticaseibacillus paracasei* (5,1%), como *Lacticaseibacillus casei* (7,6%), como *Leuconostoc mesenteroides* (2,5%), como *Leuconostoc lactis* (2,5%) y como *Weissella cibaria* (2,5%), *Enterococcus* spp. fue la bacteria ácido láctica dominante en la muestra de queso. Los presentes hallazgos revelaron que las poblaciones de bacterias ácido lácticas variaban dependiendo de los tipos de queso en términos de recuentos celulares y diversidad.

Palabras clave: Queso; identificación; bacterias lácticas; PCR

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INTRODUCTION

Cheese is a food product with high nutritional value, obtained by pre–treating milk and usually fermenting it, and consumed with pleasure all over the world. In addition, cheese is one of the oldest fermented food products made by human [1]. France, Netherlands, and Italy were famous for cheeses. Also, Turkey is a very rich country in terms of cheese diversity [2]. Nowadays, 4,000 different variety of cheese are present in the world. Turkey manufactures approximately 200 variety of cheese [3]. Although the production methods of these cheese varieties are similar to each other, their chemical, physical and sensory properties vary depending on the milk, microflora or starter culture, environmental factors such as climate and region. The Black Sea Region is one of the regions of Turkey with rich cheese varieties. More than 30 types of cheese are manufactured in this region. The most well–known cheese types in the Black Sea Region are Civil Cheese, Kargi Tulum Cheese, Imansiz Cheese, Kurchi Cheese, Minzi Cheese and Kolot Cheese [4]. Kargı Tulum Cheese is a traditional cheese originating from the Kargı district in Çorum province, Turkey. It is crafted using a variety of milks, such as cow, sheep, or buffalo milk, depending on local availability and preferences. It is typically made from milk collected during the autumn season, which contributes to its rich, high–fat content. Telli cheese is a traditional cheese from the central districts of Trabzon and Artvin, as well as the Sürmene and Akçaabat administrative districts of Trabzon in Turkey. It is made primarily from cow's milk. Telli cheese has thicker threads compared to the thinner threads of Civil Cheese, which is also sourer and salt–free in flavor. Artvin Şor cheese is made in the Savsat district of Artvin and its vicinity. Its name derives from the word Şor which means "bitter" in the region. This cheese is of a dark yellow color, is very salty and has a bitter taste [4].

In recent years, due to the increasing demand of consumers for natural products, interest in local cheese varieties has increased [5]. It is possible that local cheese varieties will be produced industrially and delivered much more and subsequently become a demanded product in the world market [6]. However, cheese industrially produced with starter cultures is different from traditional cheese with spontaneous lactic microflora (i.e. nonstarter lactic acid bacteria) regarding sensory quality properties. Starter lactic acid bacteria (LAB) are deliberately added to the milk for the production of acid during cheese processing. However, nonstarter lactic acid bacteria or indigenous microflora are inherently present in raw milk and play an important role in the developing of cheese flavor [3, 7].

The presence of numerous enzymes and indigenous lactic acid bacteria in milk improves quality properties of traditional cheeses. Native LAB develop texture and flavour properties of cheeses with microbiological and biochemical changes [7]. In industrial cheese production, the main lactic acid bacteria utilized as starter cultures are *Lactobacilus casei*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [8]. In order to produce traditional cheese industrially, it is essential to determine the microflora of the local cheese. Additionally, the characterization of the dominant microflora provides whether it is possible to use as a starter culture [9]. Recently, molecular methods have been utilized to identify lactic acid bacteria. Especially, the sequences of the gene 16S of the ribosomal RNA is highly effective method to describe the phylogenetic degree of relatedness among bacteria. Hence, 16S rRNA sequence analysis has been progressively performed to characterize the bacterial diversity of various cheeses [10].

The dairy industry works with the limited variety of starter cultures. However, there is a need for indigenous LAB strains as starters to produce more flavorful cheeses [11]. A few studies have reported to determine indigenous lactic acid bacteria in the traditional cheese of the black sea region. This research aims to identify lactic acid bacteria in the traditional cheese of the black sea region by using PCR based molecular techniques.

MATERIALS AND METHODS

Cheese samples

Cheese samples were obtained from Black Sea Region in Turkey. Traditional Turkish cheeses of Black Sea Region used in this study are Telli cheese (TP) from Tonya/Trabzon, Şor cheese (AŞ) from Artvin, Tecen cheese (TC) from Giresun, Kargı Tulum cheese (KT) from Kargı/Çorum and Kesik cheese (OK) from Ordu.

Isolation and enumeration of lactic acid bacteria in traditional cheeses

Cheese sample (25 g) was diluted with 225 mL of peptone water (Sigma) and then homogenized with stomacher for 2 min at 10 strokes per second. Serial dilution method is used for the enumeration of lactic acid bacteria. Using a 1/10 dilution rate, the homogenate was diluted to a dilution level of 10^7. By taking 0.1 mL from the appropriate dilutions, the homogenate was cultivated in petri dishes containing the selective medium. The two selective media were utilized for enumeration and isolation of bacteria. LAB with mainly lactococci are grown on M17 agar acc. to TERZAGHI (M17 Agar, Merck, Germany) at 30°C for 48–72 h under anaerobic conditions; LAB with mainly lactobacilli are grown on de MAN, ROGOSA and SHARPE (MRS) Agar (Merck, Germany) at 37°C for 48–72 h under anaerobic conditions. For anaerobic conditions, anaerobic jars and anaerocult A were applied. Following incubation, the colonies grown on M17 and MRS plates were enumerated. From each cheese sample, approx. Ten colonies with distinct morphological characteristics were selected from MRS and M17 agar plates and subsequently transferred to fresh plates for further purification. The assessment of Gram–positive and catalase–negative colonies was performed in a medium containing 6.5% and 18% NaCl and at temperatures of 10 and 45°C, at pH 4.4 and pH 9.6. Furthermore, CO_2 production as a result of glucose metabolism of the isolates and arrangement of cells (tetrad form, chain form) was assessed. Gram–positive and catalase negative pure isolates were selected from all cheese samples, and they were stored at -20°C (Arçelik, 4252EY, Türkiye) with glycerol (Merck, Darmstadt, Germany) stocks [7, 12, 13].

Extraction of bacterial DNA from pure isolates of cheese samples

Bacterial DNA from pure isolates was extracted using method described in previous studies [7, 14, 15]. 2 mL overnight culture were obtained for total DNA extraction. Microbial cells were centrifugated (Hermle Z326K, Germany) for 5 min at 4,000 g at 4°C. Supernatant was poured and then the pellets were resuspended in 500 µL of the lysis buffer. After incubation (Nuve ES 120 cooled incubator, Türkiye) for 10 min at 65°C, 150 µL potassium acetate was added. The mixture

was centrifugated for 5 min at 4°C and at 12,000 g and supernatant was transferred into sterile Eppendorf tube by adding isopropyl alcohol at equal volume. The mixture was centrifugated (Hermle Z326K, Germany) for 2 min at 4°C and at 12,000 g and then supernatant was discarded. The pellets were suspended in ethanol (70%, V/V) and this mixture was centrifugated (Hermle Z326K, Germany) for 1 min at 4°C and at 12,000 g. The pellet washed in ice–cold 70% ethyl alcohol. DNA was dried by vacuum centrifugation and resuspended in 50 µL ultrapure water. The PCR products were electrophoresed (MS Major Science, Mini 300, Taiwan) on a 1.5% agarose gel to examine the excepted size and to separate amplification products. DNA was stored at -20°C (Arçelik, 4252EY, Türkiye) for following trials.

16S rDNA sequence analysis

This research utilized two universal primers: 27F (5′–CCGCGGCTGCTGGCACGTA–3′) and 1492R (5′–GTGCGGGCCCCCGTCAATT–3′). For reaction, the mixture of 2 µL dNTP (10 pM), 2 µL primer (10 pM), 3 µL $MgCl_2$, 10 µL10X tampon, 0.25 µL Taq polymerase (Go Taq Hot Start Polymerase) was suspended with distilled pure water at 50 µL total volume. After an initial denaturation was performed at 94°C for 5 min., the products were amplified (PCR–BioRad T100 Thermal Cycler, USA) through 35 cycles. Each cycle was denaturation at 94°C for 30 s, annealing at 52°C for 40 s, elongation at 72°C for 90 s. Lastly, the final extension step was performed at 72°C for 5 min. The resulting PCR products were then sent to BMLabosis (Ankara, Türkiye) in Ankara for the process of sequencing. The sequences were compared with those in the NCBI GenBank database utilizing the BLAST tool [7, 14, 15].

Statistical analysis

Statistical analyses were conducted utilizing Windows SPPS 20.0 software statistical package program (SPSS Inc., Chicago, IL, USA) according to the randomized block experimental plan. The data were evaluated using one–way analysis of variance and significance differences were determined according to Duncan's multiple comparison test.

RESULTS AND DISCUSSION

Number of lactic acid bacteria in traditional cheeses

TABLE I gives the mean quantities of LAB in cheese samples. The numbers of LAB on MRS and M17 plates varied from 4.62 and 7.87, and from 4.89 to 8.80 log cfu·g^{-1}, respectively.

TABLE I
The numbers of LAB in cheese samples (log CFU·g^{-1})

Samples	MRS agar	M17 agar.
TP	7.87 ± 0.64^{c}	8.80 ± 0.59^{d}
AŞ	6.28 ± 0.25^{b}	7.95 ± 2.93^{c}
TC	7.14 ± 0.12^{bc}	7.35 ± 0.61^{c}
KT	6.38 ± 0.21^{b}	5.72 ± 0.03^{b}
OK	4.62 ± 0.76^{a}	4.89 ± 0.40^{a}

$^{a–b}$: Different superscripts in the same column indicate a significant difference at $P<0.05$., TP: Telli cheese from Tonya/Trabzon, AŞ: Şor cheese from Artvin, TC: Tecen cheese from Giresun, KT: Kargı Tulum cheese from Kargı/Çorum and OK: Kesik cheese from Ordu

The lowest LAB counts belong to Ordu Kesik cheese samples, while the highest LAB counts belong to Trabzon Telli cheese samples. In general, LAB counts in Kesik cheese and Telli cheese was statistically different ($P<0.05$) from other cheese while difference among LAB counts in Şor cheese, Tecen cheese and Kargı Tulum cheese was found to be insignificant ($P>0.05$) except Kargı Tulum cheese sample in M17 plate.

In line with the present results, previous researchers stated that traditional cheeses contain lactic acid bacteria counts at wide range from 4 and to 9 log cfu·g^{-1} [3, 7, 16]. Various researchers highlighted those differences in the number of LAB may result from production methods, the type of milk, storage period, and regional factors [2, 16, 17]. For example, the number of LAB in Telli cheese is found quite high in this study since Trabzon Telli cheese is primarily produced in farms under primitive conditions and usually based on spontaneous fermentation [18]. In brief, spontaneous fermentation, a long period of ripening period and traditional production techniques are responsible for numerous LAB in cheese [18].

Isolation and identification of lactic acid bacteria in traditional cheeses

A total of 147 strains were isolated, with 75 strains from MRS agar and 72 strains from M17 agar. These strains were subjected to morphological and biochemical tests. Lactic acid bacteria isolates were morphologically examined using a light microscope following gram–staining and catalase tests.

The morphological and biochemical properties of lactic acid bacteria colonies were examined before DNA extraction. The Gram–positive and catalase negative colonies were divided into three groups consisting of cocci, bacilli or coccobacilli. In the M17 plates, the cocci form (61.90%), bacilli form (28.58%) and coccobacilli form (9.52%) were observed at different proportions. In the MRS plates, bacilli and cocci colonies were 55.56% and 38.88% of the total lactic acid bacteria colonies, respectively while coccobacilli represented 5.56% of all isolates. The cocci mesophilic colonies were significantly higher in Tecen and Şor cheese according to other cheese samples and bacilli colonies were higher in Kargı Tulum cheese.

According to biochemical test results, total 39 strains including 8 strains from Kesik cheese, 5 strains from Şor cheese, 10 strains from Tecen cheese, 8 strains from Kargı Tulum cheese and 8 strains from Telli cheese were selected for further identification. These 39 isolates were defined as lactic acid bacteria according to the results of PCR. FIG. 1 presents the gel images obtained after the PCR analysis of the samples. These 39 strains include 14 *Enterococcus faecium* (35.9%), 5 *Levilactobacillus brevis* (12.8%), 6 *Lactiplantibacillus plantarum* (15.3%), 3 *Pediococcus acidilactici* (7.6%), 3 *Enterococcus durans* (7.6%), 2 *Lacticaseibacillus paracasei* (5.1%), 3 *Lacticaseibacillus casei* (7.6%), 1 *Leuconostoc mesenteroides* (2.5%), 1 *Leuconostoc lactis* (2.5%) and 1 *Weissella cibaria* (2.5%).

TABLE II displays identification of LAB isolates from cheese samples. As a result of sequence analysis, the autochthonous LAB strains was defined as *Enterococcus* spp. (43.58%), *Lactiplantibacillus* spp. (15.38%), *Lacticaseibacillus* spp.

FIGURE 1. Gel images of LAB isolates by16S rDNA PCR

(12.82%), *Levilactobacillus* spp. (12.82%), and *Pediococcus* spp. (7.69%), *Leuconostoc mesenteroides* (5.12%), as *Weissella cibaria* (2.56%). Out of the 17 *Enteroccus* spp., 14 were classified as *Enterococcus faecium*, and 3 as *Enterococcus durans*. Out of the 5 *Lacticaseibacillus* spp., 3 were classified as *Lacticaseibacillus casei* and 2 as *Lacticaseibacillus paracasei*. Out of the 2 *Lecuconostos* spp. were classified as 1 *Leuconostoc lactis* and as 1 *Leuconostoc mesenteroides*. Out of the other 15 strains were classified as 6 *Lactiplantibacillus plantarum*, as 5 *Levilactobacillus brevis*, as 3 *Pediococcus acidilactici*, and as 1 *Weissella cibaria*.

In addition to the counts of the microbial populations, the distribution of their morphological types (cocci, bacilli and coccobacilli colonies) and the distribution of their species may vary depending on environmental and genetic parameters [10]. The *Enterococcus* spp. (47.2%) were the predominant group which is in line with the study results of Aktaş and Erdoğan [19] *Enterococccus* strains show tolerance to salt concentration and acidic conditions. The presence of *Enterococcus* strains in cheese resulted from insufficient hygiene and sanitation practices during the handling of raw milk or processing equipment. Additionally, *Enterococcus* strains are present in milk because of contamination from the external surface of dairy animals, unhygienic dairy equipment, dairy storage tanks, or water sources contaminated with feces. These strains affect quality properties of traditional cheeses such as mainly flavor and texture [7]. In line with the present results, Demirci *et al*. [20] stated that *Enterococcus* spp. is the dominant species in traditional cheese. Similarly, in another study, it was reported that *Enterococcus* isolates from Ezine cheese were mostly *E. faecium* (64.3%) [11]. *Enterococcus* strains are part of the natural microflora in cheese produced from raw milk and induce the ripening process and improve sensory quality. In addition to this, the presence of enterococci in dairy products results from the process of milking and storing [21].

FIGURE 2, gives distribution of lactic acid bacteria present in cheese samples. In the present study the greatest prevalence among genera was of *Enterococcus* spp. Particularly, *Enterococcus feacium* is the common strain identified for all cheese samples. Additionally, Şor cheese and Tecen cheese contain higher amount of *E. feacium* according to the other cheeses. *Lactiplantibacillus plantarum* was the second most prevalent lactic acid bacteria

TABLE II
Identification of lactic acid bacteria in cheese samples

Cheese samples	Isolate	Similarity based on 16S rDNA	Similarity (%)
Kesik cheese	OK–M17-1	*Enterococcus faecium*	100
	OK–M17-5	*Levilactobacillus brevis*	99
	OK–M17-9	*Leuconostoc lactis*	99
	OK–M17-13	*Lactiplantibacillus plantarum*	100
	OK–M17-15	*Leuconostoc mesenteroides*	97
	OK–MRS-2	*Lactiplantibacillus plantarum*	99
	OK–MRS-4	*Weissella cibaria*	100
	OK–MRS-14	*Lactiplantibacillus plantarum*	100
Şor cheese	AŞ–M17-2	*Pediococcus acidilactici*	100
	AŞ–M17-5	*Pediococcus acidilactici*	99
	AŞ–M17-11	*Enterococcus faecium*	98
	AŞ–M17-15	*Enterococcus faecium*	99
	AŞ–MRS-7	*Pediococcus acidilactici*	99
Tecen cheese	TC–M17-3	*Enterococcus faecium*	99
	TC–M17-5	*Enterococcus faecium*	99
	TC–M17-7	*Enterococcus faecium*	99
	TC–M17-8	*Enterococcus faecium*	99
	TC–M17-11	*Lactiplantibacillus plantarum*	99
	TC–MRS-2	*Enterococcus faecium*	99
	TC–MRS-3	*Enterococcus faecium*	99
	TC–MRS-7	*Enterococcus faecium*	99
	TC–MRS-9	*Enterococcus durans*	100
	TC–MRS-15	*Enterococccus faecium*	100
Kargı Tulum cheese	KT–M17-2	*Levilactobacillus brevis*	100
	KT–M17-8	*Levilactobacillus brevis*	100
	KT–M17-10	*Levilactobacillus brevis*	99
	KT–M17-12	*Enteroccocus faecium*	99
	KT–MRS-3	*Lacticaseibacillus casei*	99.8
	KT–MRS-6	*Lactiplantibacillus plantarum*	100
	KT–MRS-13	*Levilactobacillus brevis*	100
	KT–MRS-14	*Lactiplantibacillus plantarum*	99
Telli cheese	TP–MRS-5	*Lacticaseibacillus paracasei*	100
	TP–MRS-6	*Lacticaseibacillus casei*	99.57
	TP–MRS-11	*Lacticaseibacillus paracasei*	100
	TP–MRS-12	*Lacticaseibacillus casei*	100
	TP–MRS-15	*Enterococcus faecium*	100
	TP–M17-3	*Enterococcus durans*	99
	TP–M17-7	*Enterococcus durans*	98
	TP–M17-8	*Enterococcus faecium*	98

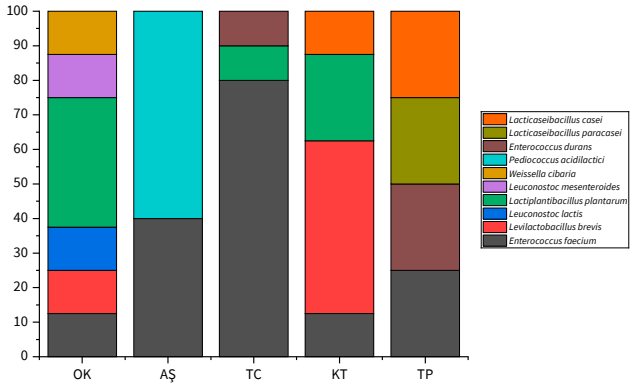

FIGURE 2. Distribution of lactic acid bacteria present in cheese samples

in cheese samples of the present study. The highest diversity of lactic acid bacteria species was verified in the Ordu Kesik cheese samples with 6 different species including *Enterococcus faecium, Levilactobacillus brevis, Leuconostoc lactis, Lactiplantibacillus plantarum, Leuconostoc mesenteroides* and *Weissella cibaria*. Furthermore, *Weissella cibari* and *Leuconostoc lactis* were identified in only Ordu Kesik cheese. *Pediococcus pentosaceus* (FFH20) was only isolated from the Artvin Şor cheese samples. Telli cheese is rich in *Lacticaseibacillus* spp. compared with other cheese samples. Similar to the results in TABLE I, and FIG. 2 shows that in terms of similarity in the distribution of LAB, Şor cheese, Tecen cheese and Kargı Tulum cheese form a similar group while Telli cheese and Kesik cheese form another group. In this study, *Enteroccus durans* was identified in Tecen cheese and Telli cheese.

Medeiros *et al.* [10] highlighted that high lactic lactic bacteria counts indicate microbial richness. On the contrary, in this study, kesik cheese with low LAB counts had much more microbial richness involving a large variety of different lactic acid bacteria species. As known from literature reports, microorganisms have antagonistic activity and dominant microflora may inhibit other species of strains (Rençber *et al.* [7]). This explains the high variety of LAB and low variety of LAB in Ordu kesik cheese and Artvin Şor cheese, respectively.

Similarly, previous researchers reported that *Lactiplantibacillus* spp. was second dominant microflora in Tulum cheeses [7, 22]. They have great potential to utilize as starter culture since *Lactiplantibacillus* species are resistant to low pH and high amounts of salt in cheese [11]. *Lactobacillus* was another dominant species in cheese manufactured from raw milk due to their growth under hard selective conditions and proteolytic activities [23].

Chourasia *et al.* [24] stated that *Enterococcus durans* was the main LAB in chhurpi cheese. The excessive use of antibiotics in agricultural and clinical practices has boosted the prevalence of antibiotic–resistant bacteria in food products. Antibiotic–resistant enterococci are among the most crucial microorganisms associated with infections [24].

The distribution of LAB genus of traditional cheeses was not homogeneous in all locations or regions. The deficiency of standardization of the traditional cheeses manufactured in all regions causes heterogeneity in distributions of LAB genera. This heterogeneity provided specific sensory characteristics of this traditional food product [10].

Diversity of LAB strains resulted from specific to traditional cheese, commonly processed with raw milk. Raw milk or non–pasteurized milk is abundant in all lactic acid bacteria including non–starter lactic acid bacteria. The main source of the cheese microflora is considered as the environment, water, the mammary gland, processors of dairy products or other materials during the milk manufacturing process [10]. Traditional cheeses involve indigenous lactic acid bacteria populations which cause unique texture and aroma of cheese. Pasteurization damages the natural microflora of milk and dairy products. Hence, traditional starter cultures are added into milk after the pasteurization process [16]. Nonstarter lactic acid bacteria are naturally present in raw milk microflora. Particularly they improve aroma and flavor in long–term ripened cheese [21].

CONCLUSIONS

The present results indicated a great and important diversity of LAB in the traditional cheeses of the Black Sea Region. The sequencing of the 16S rRNA gene was a highly effective technique for the identification of lactic acid bacteria in cheeses. The most prevalent genera in cheese samples were *Enterococcus* spp. and *Lactiplantibacillus* spp. In general, the distribution of the LAB species in cheese samples was different from each other. In the next study, the influence of identified lactic acid bacteria on the technological properties of cheeses and the potential for use as a starter culture may be focused on.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Ethics approval

Not required. Any of the author conducted no human or animal studies in this article.

Consent for publication

Not required.

Compliance with ethical standards

There are no studies with human or animal subjects in this article

Author Contributions

S.K.: Conceptualization, supervision, methodology, statistical analysis, writing–original draft, review & editing, K.Ö.: Methodology, data curation and analysis, writing–original draft, review & editing. G.Ö.: Review, writing–original draft, data curation and analysis E.U.T: Methodology, supervision, writing–original draft, review & editing

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