

DETECTION OF *LISTERIA* SPP. IN RAW MILK AND DAIRY PRODUCTS RETAILED IN ANKARA

Pınar Şanlıbaba^{1*}, Başar Uymaz Tezel², Gürcü Aybige Çakmak³

¹Ankara University Engineering Faculty, Department of Food Engineering, Gölbaşı, Ankara, Turkey

²Çanakkale Onsekiz Mart University Bayramiç Vocational School, Food Technology Program, Bayramiç, Çanakkale, Turkey

³Akdeniz University Engineering Faculty, Department of Food Engineering, Antalya, Turkey

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ABSTRACT

The objective of this study was to determine the prevalence of *Listeria* species in raw milk, and dairy products. A total of 110 samples were obtained from randomly selected retail stores and local bazaars located in Ankara. Using TS EN ISO 11290-1 method, 23 samples were found positive for *Listeria* spp. The overall prevalence of *Listeria* spp. was 20.91%, in which *L. innocua* was the most commonly recovered species (6.36%). The remaining isolates were identified as *L. ivanovii* (5.45%), *L. monocytogenes* (4.55%), and *L. welshimeri* (4.55%). The *L. monocytogenes* isolates were positive for the presence of *hlyA* gene. The highest prevalence of *Listeria* spp. was found in homemade cheese (9.09%), followed by raw milk (8.19%), and white cheese (3.64%). *L. monocytogenes* was isolated from raw milk and homemade cheese in this study.

In conclusion, the low hygienic quality dairy products may lead to listeriosis surveillance in Ankara.

Keywords: *Listeria*, milk, dairy products, identification

**ANKARA'DA SATIŞA SUNULAN ÇİĞ SÜT VE SÜT ÜRÜNLERİNDE
LISTERIA SPP. VARLIĞININ BELİRLENMESİ**

ÖZ

Bu çalışmada çiğ süt ve süt ürünlerinde *Listeria* türlerinin yaygınlığının saptanması amaçlanmıştır. Ankara'da bulunan ve rastgele seçilen market ve pazarlardan toplam 110 örnek alınmıştır. TS EN ISO 11290-1 yöntemi kullanılarak, toplam 23 örnek *Listeria* spp. varlığı bakımından pozitif bulunmuştur. Analize alınan örneklerde *Listeria* türlerinin bulunma sıklığı %20.91 olarak tespit edilmiş, *L. innocua* (%6.36) ise en fazla rastlanan tür olarak belirlenmiştir. Geri kalan izolatlar ise *L. ivanovii* (%5.45), *L. monocytogenes* (%4.55) ve *L. welshimeri* (%4.55) olarak tanımlanmıştır. *L. monocytogenes* suşları *hlyA* gen bölgesi varlığı bakımından pozitif olarak saptanmıştır. En yüksek *Listeria* spp. prevalansı ise, sırasıyla ev yapımı peynir (%9.09), çiğ süt (%8.19) ve beyaz peynirde (%3.64) saptanmıştır. Bu çalışmada *L. monocytogenes* suşları çiğ süt ve ev yapımı peynirlerden izole edilmiştir.

Sonuç olarak hijyenik kalitesi düşük süt ürünlerinin, listeriosis surveyansını Ankara'da artırabileceği gözlemlenmiştir.

Anahtar kelimeler: *Listeria*, süt, süt ürünleri, tanımlama

* Corresponding author/Yazışmalardan sorumlu yazar;

✉sanlibab@ankara.edu.tr,

☎ (+90) 312 203 3300/3617,

☎ (+90) 312 317 8711

INTRODUCTION

Listeria spp. is Gram positive and facultative anaerobic organisms. They are also non-spore forming, and rod-shaped bacteria (Momtaz and Yadollahi, 2013; Odetokun and Adetunji, 2016). All six *Listeria* spp. (*L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. welshimeri*, *L. innocua*, *L. grayi*) which were identified before 1985 can be isolated from foods. These strains referred to as “classic” *Listeria* spp. (Barre et al., 2016). *L. monocytogenes* and *L. ivanovii* are the two known pathogenic species within this genus. Although *L. monocytogenes* may lead to illness and death in humans and other mammals, *L. ivanovii* is primarily associated with ruminant animals (Hellberg et al., 2013). The genus *Listeria* consists of 17 species and 4 subtypes on the basis of 16S rRNA sequences so far (Anonymous, 2017).

Listeriosis is one of the most important bacterial infections worldwide. This infection arises mainly from the consumptions of contaminated foods. According to reports of Centers for Disease Control and Prevention (CDC), an estimated 1.600 people get sick from *Listeria* each year, and about 260 die (Anonymous, 2018a). Furthermore, listeriosis affected about 2.200 people in 2015, causing 270 deaths. The highest number of this rates ever reported in the EU. The proportion of cases in the over 64 age group steadily increased from 56% in 2008 to 64% in 2015 (Anonymous, 2018b). In Turkey, listeriosis was first detected in 1945, but rare cases were reported in human, and the epidemic was not found (Anonymous, 2018c). *L. monocytogenes* is one of the most important pathogens responsible for food-borne illness which may result in meningitis, septicemia, spontaneous abortion, perinatal infections and gastroenteritis. *L. monocytogenes* is characterized into 13 different serotypes. 1/2a, 1/2b, 1/2c, and 4b are pathogenic serotypes of which 1/2a, 1/2b, and 4b are responsible for 98% human listeriosis. While 1/2a, 1/2b, 4b are isolated from the clinical cases, 1/2a is mostly isolated from foods (Soni et al., 2013; Barre et al., 2016; Reda et al., 2016). The major risk population groups at risk for invasive listeriosis are the immunocompromised such as pregnant women, unborn or newly delivered infants, organ

transplant recipients, cancer and AIDS patients, and the elderly, with fatality rates of 20-30% (Yehia et al., 2016; Phraephaisarn et al., 2017). Different environments such as soil, surface water, sewage, animal feed, farm environments, food processing equipments and environments, urban and suburban settlements are found be contaminated with *Listeria* spp. (Korsak and Szuplewska, 2016). Various food types such as raw and processed foods also can become contaminated with *Listeria* species. These foods are raw milk and dairy products, various meats and meat products such as beef, fermented sausages, fish products, ready-to-eat foods, and vegetables (Saludes et al., 2015).

Milk and dairy products have high nutritional value. Hence, these food products are very suitable for development of microorganisms, including pathogenic bacteria. *Listeria* species are commonly found in the dairy environment, on the farm and in the processing plants (Sarfaz et al., 2017). One of the most common paths for transmission of *L. monocytogenes* is raw milk in dairy industry. Pasteurization of milk which occurs at the temperature of 62.8 °C for 30 minutes and 71.7 °C for 15 seconds is enough to destroy *Listeria* spp. present in the population of 10² CFU/mL. Soft, white and fresh cheeses are also suitable for growth of *L. monocytogenes*. Moreover, in semi-hard cheeses are excellent for reproduction of *L. monocytogenes* (Kasalica et al., 2011). *L. monocytogenes* can be transmitted by the consumption of homemade cheeses which are produced from unpasteurized milk (Arslan and Özdemir, 2008). According to CDC reports annually, about 800 laboratory-confirmed cases of listeriosis linked to several types of cheeses are reported each year in the United States, and typically 3 or 4 outbreaks are identified. In 2017, 8 confirmed listeriosis were reported in the United States as a result of consume of raw milk cheeses, and 2 die occurred (Anonymous, 2018a). In accordance with Turkish Food Codex, there should not be any *L. monocytogenes* in each 25 g/mL of dairy products (Anonymous, 2011).

The aim of the present work was to provide information about *Listeria* spp. strains isolated from raw milk and dairy products produced in Ankara (Turkey), focusing on their prevalence, phenotypic and genotypic characteristics.

MATERIALS AND METHODS

Sampling

In the period February 2016 to July 2016, a total of 110 samples of which 25 were raw milk, 25 pasteurized milk, 30 white cheeses, and 30 homemade cheeses, which were randomly purchased from various local bazaars and supermarkets in Ankara, Turkey. The food samples were transported to the laboratory under cold conditions on the sampling day and analyzed immediately.

Bacterial Strains and Culturing

Listerial strains isolated in this study and the reference strain (*L. monocytogenes* ATCC 7644) were propagated on Tryptic Soy Broth supplemented with 0.6% of yeast extract (TSB-YE) (Sigma, Germany). They were grown at 35 °C for 24 h. The initial isolates of strains were stored at -20 °C with 30% (v/v) glycerol (Merck, Germany).

The reference strain of *L. monocytogenes* ATCC 7644 was obtained from the culture collection of Food Microbiology Culture Collections, Department of Food Engineering, Engineering Faculty, Ankara University, Ankara, Turkey.

Isolation and identification of *Listeria* spp.

Isolation and identification of *Listeria* spp. were carried out according to the International Organization for Standardization (IS EN ISO 11290-1) procedure. Two-step method for enrichment of *Listeria* spp. was performed in accordance with the standard. 25 grams of cheeses were added to 225 mL of ½ Fraser broth (Merck, Germany) as the first selective enrichment medium. It was homogenized in a stomacher-400 (London, UK) at high speed for two minutes and incubated for 24±2 h at 30 ±1 °C. Similarly, 25 mL of milk was sampled and pH adjusted to neutral and thoroughly mixed with 1:10 ratio to ½ Fraser broth and incubated at

30±1 °C for 24 h. After first enrichment step, 0.1 mL of ½ Fraser broth culture was transferred to 10 mL of Fraser broth as a secondary enrichment medium and incubated at 37 °C for 48±2 h. At the same time, after primary enrichment incubation, a loopfull of culture was streaked onto ALOA (Agar *Listeria* Ottaiani Agosti) agar (Merck, Germany) and PALCAM (Polmixin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol) agar (Merck, Germany) and incubated for 24-48 h at 37 °C. In a similar vein, incubation a loopfull of secondary enrichment culture was streaked onto ALOA and PALCAM agar plates and incubated, at 37 °C for 24-48 h. It was observed grey-green colonies with black background on PALCAM agar plates, which is typical for *Listeria* spp. Typical green-blue colored colonies with and without a distinctive opaque colonies were determined on ALOA agar. Three to five presumptive colonies from ALOA and PALCAM agar were re-streaked on Tryptic Soy Agar supplemented with 0.6% of yeast extract (TSA-YE) (Sigma, Germany) at 37 °C for 24-48 h. Typical colonies from TSA-YE (1 mm to 2 mm in diameter, convex, colourless and opaque) were subjected to standard biochemical tests including gram staining, determination of catalase activity, oxidase activity, and stabbed into *Listeria* Motility Medium (Sigma, Germany) at 25 °C and 35 °C for observing the characteristics umbrella motility. The isolated and characterized strains were identified using API *Listeria* test system according to the manufacturer recommendations (BioMeriux, France). The reference strain *L. monocytogenes* ATCC 7644 was used in all biochemical tests.

Molecular identification

Bacterial genomic DNA was extracted from the bacterial cells grown at 35 °C overnight in TSB-YE using genomic DNA extraction kit (Thermo Fisher Scientific), following the manufacturer's instructions. The DNA was stored at -20 °C. The primer pairs designated as 907r (CCGTCAATTCCTTTGAGTTT) and 27f (AGAGTTTGATCCTGGCTCAG) proposed by Beasley and Saris (2004) were used to amplify a 900 bp region in the 16S rRNA gene for the detection of *Listeria* genus. In addition, primer

pairs designated as F:GCAGTTGCAAGCGCTTGGAGTGAA and R:GCAACGTATCCTCCAGAGTGATCG were used to detect *L. monocytogenes* isolates harbouring *hlyA* gene that amplify a 456 bp fragment (Paziak-Domanska et al., 1999). Polymerase chain reaction (PCR) amplification was performed in 50 µL of a reaction mixture containing 5 µL of PCR buffer, 1 µL of 2 mM of deoxynucleoside triphosphate mix, 1 µL of each primer, 34.75 µL of sterile distilled water, 0.25 µL of Tag DNA polymerase, 4 µL of 25 mM MgCl₂ and 3 µL of the DNA template solution (Blaiotta et al., 2002). PCR amplification was carried out in a programmed ThermoCycler (Techne TC-512, Staffordshire, UK) with following by 35 cycles each, of 2 minutes denaturation at 95 °C, 45 sec annealing at 55 °C, 2 minutes extension for 72 °C, and final extension at 72 °C for 7 minutes. PCR products were electrophoresed in 1% agarose gel, stained with ethidium bromide at 80 V for 45 minutes and visualized under UV illuminator (SYNGENE, Biosystems UK). A 10000 bp DNA molecular ladder was included to determine the size of the amplified products.

RESULTS AND DISCUSSION

A total of 110 samples were examined for the presence of *Listeria* spp. using two step selective enrichment recommended by TS EN ISO 11290-1 method, which is based on biochemical identification of suspected colonies on ALOA and PALCAM agar plates. Of 110 samples analyzed, 23 were found as *Listeria* spp. positive. All isolates were found to be Gram positive, catalase positive, oxidase negative, and the characteristics umbrella motility was observed in a motility medium. In addition, API®*Listeria* test kit was used to species-level identification of 23 isolates (data not shown). The PCR results were used as final confirmation to identify of presumptive colonies isolated in this study (Figure 1). Incidence of *Listeria* spp. and *L. monocytogenes* was summarized in Table 1. The counts of *Listeria* spp. were distributed as follows: 6.36% to *L. innocua*, 5.45% to *L. ivanovii*, 4.55% to *L. monocytogenes*, and 4.55% to *L. welshimeri*. All of the five *L. monocytogenes* isolates were determined to have the *hlyA* gene (Figure 2). The remaining

Listeria isolates tested negative for the *hlyA* gene according to PCR. *L. monocytogenes* was isolated from raw milk and homemade cheese in this study. The highest prevalence of *Listeria* spp. was detected in homemade cheese (9.09%), followed by raw milk (8.19%), and white cheese (3.64%). As it can be seen from Table 1, *L. innocua* was the most prevalent species isolated from the samples, which is followed by *L. ivanovii*. This finding was in agreement with earlier reports (Gebretsadik et al., 2011; Rahimi et al., 2012; Jamali et al., 2013). The incidence of *L. innocua* was 6.36%. *L. innocua* is an indicator of the presence of *L. monocytogenes*. Furthermore, this strain has been used as a surrogated for the study of *L. monocytogenes* in a variety of food systems (Milillo et al., 2012). *L. ivanovii* cause animal and human infections with *L. monocytogenes* (Seyoum et al., 2015). Incidence of *L. ivanovii* (5.45%) obtained in this study was also worrying. Therefore, this prevalence rate is a risk factor in human body causing infections. Moreover, the identification of non-pathogenic *Listeria* spp. in current study was important. These non-pathogenic species have been found to cause disease in both immunocompetent and immunocompromised individuals (Usman et al., 2016).

We did not found any *Listeria* spp. from pasteurized milk, in agreement with Sarker and Ahmed (2015). In contrast to our findings, 16.7% of unclean pasteurized milk was obtained by Silva et al. (2003). Natratilova et al. (2004) stated that *Listeria* spp. was found to be 5% in Czech Republic. In addition, 40% of pasteurized milk was found to be contaminated with *Listeria* spp. in Ethiopia (Seyoum et al., 2015). Contamination after pasteurization or faults of technology during pasteurization are responsible for the presence of *Listeria* spp., specially *L. monocytogenes*. These reports show that thermal process like pasteurization does not give any way guarantee the absolute safety of milk and dairy products.

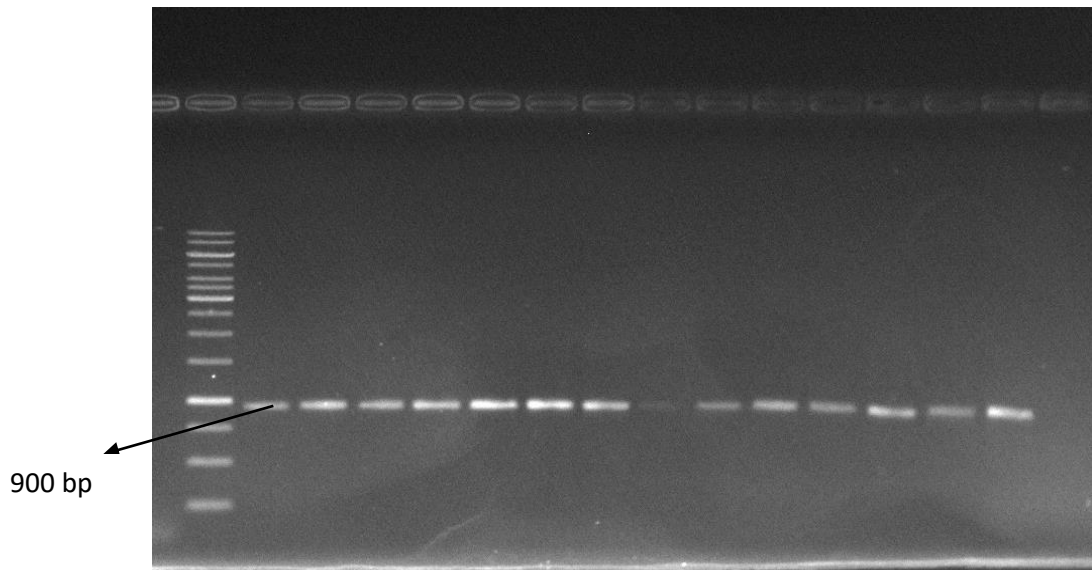


Figure 1. PCR screening of 16S rRNA gene from *Listeria* species
Lanes 1:10000 bp (O'Gene Ruler DNA marker); 2: Positive control (*L. monocytogenes* ATCC 7644); 3-15: L6, L10, L20, L28, L29, L32, L37, L38, L46, L47, L48, L49, L50; 16: Negative control

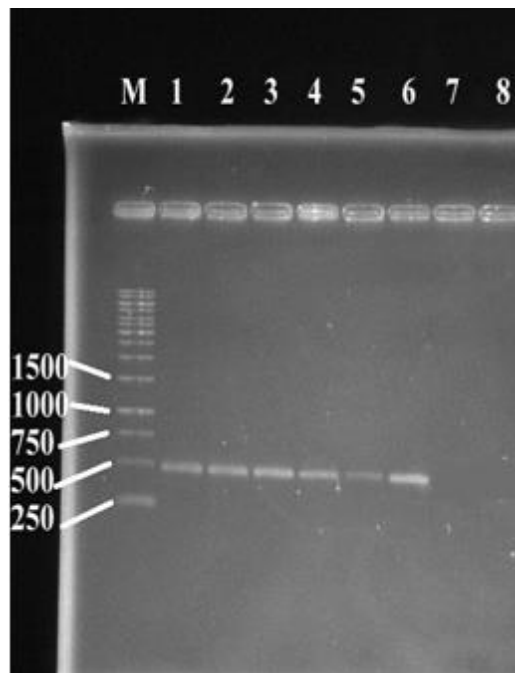


Figure 2. PCR screening of *hlyA* fragments from *L. monocytogenes*
Lanes M:10000 bp (O'Gene Ruler DNA marker); 1: Positive control (*L. monocytogenes* ATCC 7644); 2-6: L32, L37, L38, L46, L47; 7-8: Negative control

Table 1. Incidence of *L. monocytogenes* and other *Listeria* spp. in raw milk and dairy products

Nature of Samples	Number of Samples	<i>L. monocytogenes</i>		<i>L. innocua</i>		<i>L. ivanovi</i>		<i>L. welshimeri</i>		Total	
		n ^a	%	n	%	n	%	n	%	n	%
Raw Milk	25	3	12.00	3	12.00	1	4.00	2	8.00	9	8.19
Pasteurized Milk	25	- ^b	-	-	-	-	-	-	-	-	-
White Cheese	30	-	-	1	3.33	1	3.33	2	6.67	4	3.64
Homemade Cheese	30	2	6.67	3	10	4	13.33	1	3.33	10	9.09
Total	110	5	4.55 ^c	7	6.36 ^c	6	5.45 ^c	5	4.55 ^c	23	20.91 ^c

^a number of positive samples

^b not detected

^c calculated for total of 110 samples

The contamination rate of *Listeria* spp. was observed in 36 % of raw milk samples. Among these contaminated samples, 12% harbored *L. monocytogenes*. The isolates *L. innocua* (12%), *L. welshimeri* (8%), and *L. ivanovi* (4%), were observed in raw milk. The number of the isolates of *L. monocytogenes* obtained in our study was not similar to the other investigators from Turkey. Vardar-Ünlü et al. (1998), Sağun et al. (2001), Aygun and Pehlivanlar (2006), Taşçı et al. (2010), Abay et al. (2012), Kevenk (2014), and Durmaz et al. (2015), reported that raw milk contained *L. monocytogenes* were found to be 4% in Sivas, 1.2 % in Van, 0% in Antakya, 2.4% in Burdur, 0% in Kayseri, 5% in Samsun, and 2.1% in Southeastern Anatolia, respectively. Our findings were higher than these results. Our results were also comparable to other surveys on raw milk conducted in other countries. Kongo et al. (2006) indicated that 1.9% of raw milk samples analyzed in Portugal were positive for *L. monocytogenes* and also no other *Listeria* spp. were isolated. Furthermore, isolation rates of *L. monocytogenes* from raw milk was 1.9% in Portugal (Kongo et al., 2006), 10.96% in Syria (Al-Mariri et al., 2013), 8.33% in Morocco (Marnissi et al., 2013), 5.8% in India (Soni et al., 2013), 2.04% in Ethiopia (Seyoum et al., 2015), 6.55% in Nigeria (Usman et al., 2016), and 1.46% in Cyprus (Botsaris et al., 2016).

Contamination of raw milk may occur in a dairy farm. In addition, the source of *L. monocytogenes*

contaminations could be: i) poor quality of silage, ii) inadequate cleanliness of animals, iii) poor hygienic conditions which could occur during milking, storage and transport, iv) infected cows in dairy farms (Marnissi et al., 2013; Telli et al., 2016).

In white cheese, *L. welshimeri* (6.67%), *L. innocua* (1.33%), and *L. ivanovi* (1.33%) were detected. The contamination rate of *Listeria* spp. was observed in 16 % of white cheese samples. *L. monocytogenes* was not isolated from white cheese. In other reports conducted with white cheese sold in Turkey, isolation rates of *L. monocytogenes* was 6% in Afyonkarahisar (Akkaya and Alişarlı, 2006), 9.5% in Kayseri (Kum et al., 2011), 3% in Erzincan (Azak et al., 2012), 2% in Balıkesir (Gökmen et al., 2016), 15.77% in Tekirdağ (Kaptan, 2016), and 3.53% in Konya (Telli et al., 2016). These results mentioned above were higher than our findings. In contrast to our results, prevalence of *Listeria* spp. in white cheese sold in Turkey was 33.1% in Bolu (Arslan and Özdemir, 2008) and 21.5% in Tekirdağ (Kaptan, 2016). A lower incidence of *Listeria* spp. was found by Gökmen et al. (2016) in Balıkesir and Telli et al. (2016) in Konya, who determined that 14% and 8.85% of white cheese samples were contaminated, respectively. The prevalence of 16% obtained for *Listeria* spp. in this study was comparable to other surveys conducted in other countries on white cheese. Kongo et al. (2006) did

not detect *Listeria* spp. in cheese samples in Portugal. The report from Seyoum et al. (2015) indicated that 60% cheese samples were contaminated with *Listeria* spp. The study performed by Elshinaway et al. (2017), *Listeria* spp. was detected in 12.5% in the white cheese. Large numbers of viable *Listeria* cells present in milk are killed by heat treatment applied through cheese-making (Coroneo et al., 2016). However, cross contamination is a major problem during the cheese production. Contamination sources may be: i) the contaminated raw milk, ii) environment conditions and unclean equipments, and iii) insufficient heat treatment of milk to kill the organisms (Kasalica et al., 2011; Telli et al., 2016). In addition, there are several factors of reproducing of *Listeria* spp. and specially *L. monocytogenes* in cheese: i) the type and composition of cheese, ii) the resistance of *Listeria* to the decreased pH during cheese production, iii) moisture percentage, iv) salt percentage, v) ripeness of cheese, vi) storage conditions, and vii) starter cultures (Elshinaway et al., 2017).

The contamination rate of *Listeria* spp. was observed in 33.33% of homemade cheese samples. The most common species isolated in homemade cheese was *L. ivanovii* (13.33%); the remaining *Listeria* isolates were *L. innocua* (10%), *L. monocytogenes* (6.67%), and *L. welshimeri* (3.33%). In other report conducted with homemade cheeses in Turkey presented by Kaptan (2016) showed that 21.50% of homemade cheese samples were contaminated with *Listeria* spp. and *L. monocytogenes* was the most prevalent species with 73.3% isolates recovered. In countries other than Turkey, the prevalence of *Listeria* spp. in traditional cheese samples was reported as 16.7% in Brazil (Silva et al., 2003), 12.2% in Brazil (Abrahao et al., 2008), 9.8% in Spain (Arrese and Arroyo-Izaga, 2012), 15% in Iran (Rahimi et al., 2012), 50% in Iran (Moosavy et al., 2014), and 10% in Egypt (Elshinaway et al., 2017). In this study, a higher frequency of *L. ivanovii* in homemade cheese than white cheese was observed. The highest presence of *L. ivanovii* in home-made cheese samples could be linked with listeriosis risk. Traditional cheeses are currently produced from pasteurized milk in modern dairy

industry. Small-sized factories produced dairy products may uncontrol and therefore, they produce non-hygienic cheeses (Kaptan, 2016).

CONCLUSIONS

The results of current study provide information about the contamination status of raw milk and dairy products sold in Ankara with *Listeria* spp. This study demonstrated that raw milk and dairy products was not safe for the presence of *Listeria* spp. We found that *L. innocua* was the most prevalent species, which is followed by *L. ivanovii*. The highest prevalence of *Listeria* spp. was found in homemade cheeses, followed by raw milk, and white cheese. According to microbiological criteria of cheese in Turkish Food Codex, *L. monocytogenes* must not found in cheese samples analyzed. However, in this present study, homemade cheeses were found with *L. monocytogenes*. *L. monocytogenes* was also isolated from raw milk. We suggest that hygienic conditions should still be enforced in order to minimize the count of *Listeria* spp. and *L. monocytogenes* in dairy industry.

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