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Prevalence of *Listeria* spp and *L. monocytogenes* in Home made Pottery Cheese

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Presence of *Listeria* spp and *L. monocytogenes* in traditional pottery cheeses produced at eight different provinces in Turkey was investigated. A total of 279 samples were surveyed. Apart from *L. monocytogenes*, *L. ivanovii* and *L. gravyi*, *L. innocua* were determined from the cheese samples, while *L. welshimeri* and *L. seeligeri* were not detected in any of the samples. 21.5% of the samples were contaminated with *Listeria spp*.. However %15.77 of samples was positive for *L. monocytogenes*. The incidence of *L. grayi*, *L. innocua* and *L. ivanovii* isolated from samples were 2.15%, 1.43% and 2.15%, respectively. A total of 60 *Listeria spp*. positive samples were analyzed and it was determined that. *L. monocytogenes* was the most prevalent species with 44 (73.3 %) isolates recovered, while 16 (26.6 %) belonged to other *Listeria spp*.

Key words: Cheese, *Listeria monocytogenes, Listeria* spp, Pottery cheese

Ev Yapımı Küp Peynirinde *Listeria spp* ve *L. monocytogenes* Yaygınlığı

Türkiye'nin sekiz değişik illerinde toplanan geleneksel Küp peynirinde *Listeria spp* ve *L. monocytogenes* varlığı ve isnsidansı araştırılmıştır. Toplanan 279 peynir örneğinin *Listeria spp* % 21.5, *L. monocytogenes* %15.77'de, *L. grayi* %2.15*, L. innocua %1.43 ve L. ivanovii % 2.15* oranında kontamine olduğu belirlenmiş, *L. welshimeri* ve *L. seeligeri* ise tespit edilmemiştir. *Listeria spp.* pozitif toplam 60 örnekte en yaygın tür olarak 44 (% 73.3)'ünde *L. monocytogenes* ve 16 (% 26.6)'sında ise diğer *Listeria spp.* belirlenmiştir.

Anahtar kelimeler: *Listeria monocytogenes, Listeria* spp, Küp peyniri

Introduction

Listeria is a rod shaped, non-spore-forming, Gram positive, facultative anaerobic bacterium (Davis and Mauer, 2011; Wang et al., 2015), enviroment (raw foods, soil, stream water, silage, sewage and plants) it has wide range of hosts (Swaminathan and Gerner-Smidt, 2007) . The genus *Listeria* consists of ten species, namely; *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, *L. grayi*, *Listeria marthii*, *L. rocourtiae*, *L. fleischmannii* and *L. weihenstephanensis*, (Shantha and Gopal, 2014). Among them, two are pathogenic*. L. monocytogenes* is pathogenic to humans and animals; *L. ivanovii* primarily infects animals and very rarely causes disease in humans. *L. monocytogenes* is considered as one of the most important food-borne bacteria that cause listeriosis in animals and humans (Li et al., 2015). Although the incidences are low, *L. monocytogenes* is one of the most deadly human food-borne pathogens. *L. monocytogenes* is more likely to cause death than any other bacteria that can cause food poisoning. In the case of outbreaks, a mortality rate of more than 30% has been reported, and that rate could increase in high-risk individuals such as pregnant women, newborns, and the elderly (Elinav et al., 2014; Poulsen and Czuprynski, 2013). Several categories of dairy products have been associated with listeriosis outbreaks including unpasteurized dairy products. Recently, outbreaks of listeriosis associated with the consumption of camembert cheese (of 17 cases, 3 were fatal) and Mexican-style cheese (8 cases, 7 were pregnant Hispanic females) contaminated with *L. monocytogenes* have been reported (Jackson et al., 2011; Johnsen et al., 2010). The behaviour of *L. monocytogenes* in different kinds of cheese during ripening has been widely studied by challenge testing. *L. monocytogenes* mostly grows in soft cheeses and washed smear cheeses (Millet et al., 2006; Rodulf and Scherer, 2001; Schvartzman et al., 2014; Swaminathan and Gerner-Smidt, 2007). Some of the dairy products are well known in the world such as cheese and yogurt despite high diversity in their types. On the other hand, there are other dairy products (conventionally) which are not internationally known and recognized but within a

definite society or region. These conventional products are a primary source of dairy consumption for some people, particularly for those living in rural areas.

Turkey has a wide range of traditional dairy products based on raw or pasteurised milk that have been known and highly appreciated by consumers for centuries. Natural cheeses, regardless of their varying characteristics, can support the growth of microorganisms including food-borne pathogens. Given the continuous increase of natural cheese consumption in regions associated with pottery cheese, there is concern for the microbiological safety of domestically produced natural cheeses (from cows' and sheep milk, or a mixture of the two). Pottery cheese, (It's in English is called as Küp cheese in Turkish),, which is matured underground in pots (earthenware materials), is widely produced in the provinces of Central Anatolia and its vicinity.

Pottery cheese is a semi-hard cheese with a crumbly texture, white-cream color and a "quite favorable taste" (personal communication with local producer). This cheese is usually produced from in small farms and private village houses by the farmer women, using raw whole sheep and/or cows' milk, with no added lactic starters. The traditional production method of Pottery cheese begins with fresh raw milk that is coagulated by adding rennin and salt (2 to 3% w/w). The indigenous microflora contained in raw milk increases the acidity of the curd. Afterwards, the curd is cut into small pieces by hand ("pea size", approximately 1 cm^3) and put into small cotton bags. A weight is then put over the bags to drain the whey (at 20-25°C for 24 h). The drained curd is kneaded with salt and put into larger cotton bags. Any remaining whey is drained by putting a weight over the cotton bags. Afterwards, the curd is compressed into the pot so that no entrapped air remains. Pottery cheese prepared as such, is then ripened in said pots for at least five months (at 4-6°C) in the caves and subsoil. This specific type of cheese has been in high demand at the local markets, and is greatly appreciated by consumers. Because of their unique tastes, artisan cheeses produced through spontaneous fermentation of unpasteurized milk, receive a great deal of attention from consumers, not only in Turkey but also in other countries around the world. Although Pottery cheese is a popular cheese type, unfortunately there is no standard production technique for it. Pottery cheese may be consumed while ripening, but it is generally consumed after it has ripened (in its earthenware jug or pot). Since the Pottery cheese is produced from unpasteurized raw milk and during its production there is no heating process, pathogenic bacteria may also grow and survive with this decline, in these cheeses. Even though there is a lot of evidence about the contamination of milk and dairy products by *L. monocytogenes* worldwide, there is no data about the contamination of Pottery cheese. There are a few reports on the microbiological quality of Pottery cheese (Pekel and Korukluoğlu, 2006, 2009). Although *L. monocytogenes* have not been analyzed in these researches. Therefore, the aim of this present study was the detection of *Listeria spp* and *L. monocytogenes* in order to evaluate the possible health and hygiene risks to the consumer, within traditional homemade Pottery cheeses produced from raw cow, sheep or inadequately pasteurized milk.

Materials and Methods

Cheese samples

A total of 279 homemade Pottery cheese samples, manufactured from raw milk or inadequately pasteurized milk were taken from village bazaars, located in the provinces of Ankara, Kayseri, Kırşehir, Nevşehir, Niğde, Sivas and Yozgat (in Central Anatolia, Turkey) during the period of March 2013 through to February 2014. All samples were placed directly in sterile bags and transported (in refrigeration set at 4°C) to the laboratory within 1-2 h of the time of collection. The microbiological analysis (of samples) was carried out immediately.

Isolation of *Listeria spp*

Listeria spp were isolated in accordance to the standard method recommended by United States' Food and Drug Administration (FDA) (Hitchins and Jinneman, 2011).
For the isolation of *Listeria spp*., 25 g of each sample was taken aseptically and homogenized in 225 mL*Listeria* Enrichment broth (Oxoid, CM862 + SR141) using a stochmacher (IUL Instruments,

Barcelona, Spain) for 1 min and incubated at 30 C for 48 h. Following that, 100 mL of inocula from the enrichment culture was streaked in duplicate, onto three selected agars, Oxford agar (Oxoid CM856), PALCAM agar (Oxoid CM877) and Brilliance *Listeria* agar (Oxoid CM1080). All selected plates were incubated at 35 C for 48 h. At least five colonies resembling *Listeria* (black colonies with black sunken centres) from each of three plates were transferred onto Tryptic Soy agar (Oxoid CM131) with Yeast extract (Oxoid L21), and incubated at 37 °C for 24 h. All the colonies were examined microscopically by Gram's staining, catalase activity, oxidase reaction, the presence of hemolysis in blood agar, and motility in SIM medium at 25 °C, and were further identified using the API *Listeria* identification kit (API-*Listeria*, bioMerieux), following the manufacturer's instructions (McLauchlin et al., 1997)

Identification of *Listeria* using the API test procedure

The API *Listeria* system (Microbact *Listeria* 12 L identification system, MB1128 A; Oxoid, Hampshire, UK) consists of the following 12 tests: Hydrolysis of esculin, hemolysis of red blood cells, and acid production from D-arabitol, D-xylose, L-rhamnose, α-methyl-D-glucose, α-methyl-D-mannose, Dribose, glucose-1-phosphate, D-tagatose, trehalose, and mannitol. Bacterial inocula were prepared by emulsifying a single isolated colony from a culture (of 18-24h) in the buffered *Listeria* enrichment broth base (BLEB; Oxoid), then mixed thoroughly to prepare a homogenous suspension.

A reaction strip was removed from packaging and placed in a holding tray and the bacterial suspension (100 µL) was distributed into each well of the strip. The hemolysin reagent (MB 1249 A; Oxoid) for hemolysis testing was warmed to room temperature and a drop of the reagent was added to well number 12. After inoculation, the strip box was closed and incubated at 35±2°C for 24 h. Reaction results were determined according to color change indicators, as per the manufacturer's instructions (Table 1). Identification of different species of *Listeria* was further confirmed using the diagnostic key for *Listeria* in Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986)

Table 1. Identification scheme for species of *Listeria* based on the Microbact *Listeria* identification system (API-*Listeria* system)

Species of *Listeria*						
Test	*L. monocytogenes*	*L. innocua*	*L. seeligeri*	*L. ivanovii*	*L. grayi*	*L. welshimeri*
Esculin hydrolysis	+	+	+	+	+	+
Hemolysis	+	-	-	+	-	-
Acidification of:						
D-Arabitol	+	+	+	+	+	+
α-Methyl-D-glucose	+	+	+	+	-	+
α-Methyl-D-mannose	+	+	-	-	+	+
Trehalose	+	+	+	+	+	+
D-Xylose	-	-	+	+	-	+
L-Rhamnose	+	V1	-	-	-	V1
D-Tagatose	-	-	-	-	-	+
D-Ribose	-	-	-	+	+	-
Mannitol	-	-	-	-	+	-
Glucose-1-phosphate	-	-	-	+	-	-

Results and Discussion

Identification of different species of *Listeria*

Identification of *Listeria* isolates was achieved by using an API-*Listeria* identification kit (Oxoid) and was confirmed by using the diagnostic key of Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986). Members of the genus *Listeria* are Gram-positive, motile at 25°C, non-spore forming, rod-shaped bacteria Bacteriology (Seeliger and Jones, 1986). Results of the API-*Listeria* kit are shown in Table 1.

The three biochemical characteristics that were positive for all *Listeria* isolates were; 1) hydrolysis of esculin, 2) acid production from D-arabitol, and 3) acid production from trehalose. The presence of hemolysis and acid production from D-xylose, L-rhamnose, D-ribose, glucose 1-phosphate, D-tagatose, and mannitol were used for individual species identification (Table 1). Identical findings were also reported by using the API commercial system for differentiation amongst species of *Listeria* (McLauchlin et al., 1997). Differential characteristics in different species of *Listeria* isolated in this study were identified on the basis of biochemical and carbohydrate fermentation results. Carbohydrate fermentation tests were also used to confirm results for acidification of carbohydrates in the API *Listeria* system (Table 1). All species of *Listeria* identified herein showed positive results for the catalase, Voges Proskauer (VP), and methyl red (MR) tests, and negative results for all other tests (Table 2).

Table 2. Biochemical description of isolated species of *Listeria*

Species of *Listeria*				
Test[(1)]	*L. monocytogenes*	*L. grayi*	*L. innocua*	*L. ivanovii*
Catalase test	+	+	+	+
MR test	+	-	-	+
Nitrate reduction	-	-	-	-
Urea hydrolysis	-	-	-	-
Oxidase test	+	+	+	+
VP test	+	+	+	+

[1)]MR, methyl red; VP, Voges Proskauer test

Table 3. Prevalence of *Listeria spp* in Pottery cheese obtained from 8 different provinces

	Pottery Cheeses							
The number of samples and provinces	n	*Listeria spp*	*L monocytogenes*	*L. grayi*	*L. innocua*	*L. ivonavii*	*L. seeligeri*	*L. welshimeri*
Ankara	40	+	+	+	-	+	-	-
Kayseri	52	+	+	-	+	+	-	-
Kırşehir	32	+	+	+	-	-	-	-
Konya	15	+	+	-	+	-	-	-
Nevşehir	38	+	+	+	-	+	-	-
Niğde	25	+	+	-	-	-	-	-
Sivas	35	+	+	+	-	+	-	-
Yozgat	42	+	+	+	+	+	-	-
Total	279	8	8	5	3	5	0	0

Incidence of *Listeria spp*. in Pottery cheese samples

Prevalence of *L. monocytogenes* and other *Listeria spp*. isolated from obtained Pottery cheeses in the 8 provinces of Turkey. A total of 279 Pottery cheese samples collected over a 8 month period from June of 2013 through to January of 2014, were analyzed for *Listeria spp*.

Results are summarized in Table 3. After isolation of *Listeria* species from cheese samples, the *Listeria* species from the cheeses obtained from locations related to the contaminated cheeses were tested. In the present study *Listeria spp*. was isolated from all cheese sources (100 %). As well as *Listeria, L. monocytogenes* was obtained from all the cheese sources investigated, *L. ivanovii* and *L. gravyi* from 5 (62.5 %), *L. innocua* from 3 (37.5 %), while *L. welshimeri* and *L. seeligeri* were not detected (Table 3).

A total of 279 samples of homemade Pottery cheese collected from various public bazaars were examined for the presence of *Listeria spp*. in the present work. *Listeria spp*. were isolated from 60 (21.50 %) samples. A total of 60 *Listeria spp*. were isolated and speciated. *L. monocytogenes* was the most prevalent species with 44 (73.3 %) isolates recovered, while 16 (26.6 %) belonged to other *Listeria spp*. The prevalence of *L. monocytogenes* and other *Listeria spp*. from different sampling sites is reported in Table 4. A total of 279 cheese samples were analyzed for the presence of *L. monocytogenes. L. monocytogenes* was detected in 44 (15.77%) out of 279 samples. Overall, 16 (5.73%) of the Pottery cheese samples examined were positive for the other *Listeria spp*.; 6 (2.15%) were positive for *L. grayi*; 4 (1.43%) for *L. innocua*; 6 (2.15%) for *L. ivanovii*. In our study conditions, *L. welshimeri* and *L. seeligeri* were not detected in any of the samples (Table 4).

The presence of *L. monocytogenes* in different types of traditional cheeses produced in Turkey, was reported in many previous studies. In a study performed by Arslan and Ozdemir (2008), 142 homemade white cheese samples were analysed and 33.1% and 9.2% were found to be positive for *Listeria spp*. and *L. monocytogenes* respectively.

In other studies, *L. monocytogenes* was isolated 2.5 % of 40 çeçil cheese (Gülmez and Güven, 2001), 3.93 % of 254 herbed cheese (Sağun et al., 2001), 4.8 % of 250 tulum cheese (Colak et al., 2007) and 8% of 50 herbed cheese samples (Erkan et al., 2007), The result of this study and these results show similarity. In a recent study performed by Kum et al.(2011), it was reported that *L. monocytogenes* was detected in 22.7% of homemade pre-cut wedge cheese, 20% of uncured cheese and 14.2% of fibre type cheese. Also, in a study conducted by Güner and Telli (2011) *L. monocytogenes* was determined in

30% of herbed cheese, 46.6% of carra cheese, 23.3% of küflü cheese and 13.3% of tulum cheese. In general, the results of this study agree with those from other surveys, with the exception of herb cheese, its survey found higher incidence of *Listeria spp*.

Table 4. The occurrence percent of *L. monocytogenes* and other *Listeria spp* in Pottery cheese

The number of samples and provinces	Pottery Cheeses									
		Listeria spp		*L monocytogenes*		*L. grayi*		*L. innocua*		*L. ivonavii*	
	n	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Ankara	40	10	25.0	8	20.0	1	2.5	-	-	1	2.5
Kayseri	52	14	26.9	11	21.2	-	-	1	1.9	2	3.8
Kırşehir	32	6	18.8	5	15.6	1	3.1	-	-	-	
Konya	15	4	26.7	3	20.0	-	-	1	6.7	-	
Nevşehir	38	9	23.7	7	18.4	1	1.6	-	-	1	2.6
Niğde	25	3	12.0	3	12.0	-	-	-	-	-	
Sivas	35	5	14.3	3	8.6	1	2.9	-	-	1	2.9
Yozgat	42	9	21.4	4	9.5	2	4.8	2	4.8	1	2.4
Total	279	60	21.5	44	15.77	6	15	4	1.43	6	2.15

These results disagree with ours. On the other hand, some studies reported that *L. monocytogenes* was not detected in traditional cheeses, tulum cheese (Cetinkaya et al., 1999), skim milk cheese and village type brined cheese (Kum et al., 2011) The findings of the study and the results of studies mentioned above, show that traditional cheeses have a risk for *L. monocytogenes* contamination in Turkey.

In countries other than Turkey, results also indicated that the presence of *L. monocytogenes* in cheeses. The mean prevalence of *L. monocytogenes* in cheese was reported as 2.4% in Italy (Prencipe et al., 2010) and 8% in Greece (Filiousis et al., 2009). *L. monocytogenes* was detected in 12.1 % of 123 traditional cheese samples in Japan (Makino et al., 2005), 5.9% of 351 farmhouse cheese in Ireland (O'Brien et al., 2009) and 15% of 60 traditional cheese in Iran (Rahimi et al., 2010). In a study in Noorabad, Iran, *L.monocytogenes* was detected in 3.3 % and 6.7% of white cheese samples that were collected from two traditional dairy manufacturers (Mahmoodi, 2010). According to research conducted by many authors, cheeses produced from raw milk are more often contaminated with *L. monocytogenes,* compared to cheeses produced from pasteurized milk. In Sweden, *L.monocytogenes* was isolated in 6% of samples, of which 42% were produced from raw milk, and 2% from heat treated milk in samples (333) of soft and semi-soft cheeses collected from retail stores (Loncarevic et al., 1995). Rudolf and Scherer (2001), results concluded that repeated contamination of milk with *L. monocytogenes,* in 8,0% of cheeses, was produced from pasteurized milk,. Jacquet et al. (1993), in that period of 1988-1990, 340 analysis samples were collected from dairy plant equipment, dairy production facilities and different types of cheeses, the presence of *L. monocytogenes* was isolated in 44 tested samples of cheese, and from dairy plant equipment/facilities; cheese was contaminated during the ripening process. The incidence of *L. monocytogenes* in soft and semi-soft cheese varied from 0.0 % to 46.00 % in Chile (Cordano and Rocourt, 2001), Iran (Mahmoodi, 2010; Rahimi et al., 2010), Turkey (Arslan and Özdemir, 2008; Colak et al., 2007), Canada (Farber et al., 1987), Norway (Rørvik and Yndestad, 1991), and Portugal (Pintado et al., 2005). A survey of herb cheese showed an incidence rate of 5.1% for *Listeria spp*. out of 254 samples, 1 (0.4%) was found to be positive for both of *L. ivanovii and L. innocua* (Sağun et al., 2001). In another study conducted in Isfahan province of Iran, *Listeria spp*. was identified in 18.9% of 90 cheese samples in which L. innocua was detected in 8.2% of samples (Rahimi et al., 2010). The incidence of *Listeria spp*. in different types of cheeses has been reported. Silva et al. (1998) examined 103 cheese samples in three different types, including the homemade Minas Frescal cheeses. Of the analyzed samples, 11 (10.7 %) were contaminated with *L. innocua*, 6 (5.8%) with *L. grayi*.

Although these traditional cheeses are currently produced from pasteurized milk in modern dairy plants, it is noted that smaller operators and home chefs are uncontrolled and therefore producıng non-hygienic cheeses. According to the microbiological criteria of cheese in Turkish Food Codex, *L. monocytogenes* must not be found (zero tolerance) in cheese samples analyzed (Turkish Food Codex, 2011). Thus in the present study, the high numbers of contaminated samples with *L. monocytogenes* 15.75 % (44/279) and other *Listeria spp*. 5.73% (16/279) could be assessed as noteworthy. *L. monocytogenes* contamination of cheeses may come from two main sources; the milk (or other ingredients used to make cheeses) and the environment during production and post-production. Differences between the results above may be related with the use of raw or pasteurized milk in cheese production, cheese production techniques, and hygienic conditions during the production, storage and sales processes, and some characteristics of cheese such as pH and moisture content. *L. monocytogenes* can be found more frequently in high-moisture cheeses, soft and semi-soft cheeses which have water activity higher than hard cheeses (Arslan and Özdemir, 2008; Rodulf and Scherer, 2001).

Conclusion

In conclusion, this study demonstrates the presence of some pathogens including *L. monocytogenes,* in retail Pottery cheese. Even though pasteurization of raw milk is effective in the destruction of *Listeria*, there are reports of listeriosis caused by the consumption of pasteurized dairy products. However, this contamination is usually related to post-pasteurization contamination. Traditional dairy products in Turkey are generally non-pasteurized and produced in small scale dairy plants or by home chefs. There is also no reliable data

available on the level of hygienic practices in these small scale dairy plants, they are known to have poor food safety standards. Therefore, these traditional foods are a serious risk to the public's health. Also, the presence of these organisms indicated that there were poor hygienic conditions during the manufacturing, storage and sales process of these traditional cheeses. Manufacturing procedures adhering to the HACCP, use appropriate hygienic measures to avoid (processing and post processing) cross-contamination and of the raw milk quality. All of which are critical for controlling pathogens in this cheese. The levels of contamination found in Pottery cheeses showed that there were inadequate conditions and sanitization measures during production. Control of pathogenic microorganisms during artisanal production of Pottery cheese is a problem. With this type of artisanal cheese ; a safety management system, adequate temperatures during processing, storage, and transportion to the retail markets, production equipment being properly disinfected after contact with cheese, are measures that should be implemented to avoid contamination of cheese.

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