The presence of *Listeria* species in dairy cattle farms in Bandırma province, Turkey

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Geliş Tarihi / Received: 21.10.2014, **Kabul Tarihi** / Accepted: 01.12.2014

Summary: In this study, the presence of *Listeria* species in dairy cattle farms was investigated. In this context, a total of 340 samples including milk, fresh feces, silage, soil, water and swabs from milking parlour were obtained from 21 dairy cattle farms between November 2013 and June 2014. For the isolation of *Listeria* species from samples, AOAC/IDF method in milk samples and USDA/FSIS method in other samples were used. As a result of the study, *Listeria* spp. was isolated from 11 dairy cattle farms (52.38%), while in other 10 dairy cattle farms (47.62%) *Listeria* spp. was not isolated. Isolation of *Listeria* spp. occurred from at least one of samples, including the milk samples from 5 dairy cattle farms, the fresh feces samples from 7 dairy cattle farms, the silage samples from 3 dairy cattle farms, the soil samples from 10 dairy cattle farms, the water samples from 8 dairy cattle farms and the milking parlour samples from 2 dairy cattle farms. *Listeria* spp. was isolated from 15% (51/340) of all the analyzed samples. Isolation of *Listeria* spp. was carried out from 8 of 105 milk samples (7.61%), 12 of 105 fresh feces samples (11.42%), 3 of 21 silage samples (14.28%), 15 of 42 soil samples (35.71%), 11 of 42 water samples (26.19%) and 2 of 25 milking parlour samples (8%). Isolates were identified by cultural and biochemical characters, and 8 of 51 isolates *L. monocytogenes*, 1 of 51 isolates *L.ivanovii*, 17 of 51 isolates *L. innocua*, 7 of 51 isolates *L. seeligeri*, 7 of 51 isolates *L. welshimeri*, 11 of 51 isolates *L. grayi* were identified. Isolation rates of *L. monocytogenes* were found as 0.95%, 0.95%, 4.76%, 7.14% and 4% respectively in milk, fresh feces, soil, water and milking parlour samples, however *L. monocytogenes* were not isolated from silage samples. Consequently, the presence of *Listeria* species in milk and environmental samples of dairy cattle farms pose a risk for the animal health as well as for human health.

Key words: *Listeria* spp., Dairy cattle farm, Prevalence

Bandırma ve çevresinde bulunan süt sığırı işletmelerinde *Listeria* türlerinin varlığı

Özet: Bu çalışmada, Bandırma ve çevresinde bulunan süt sığırı işletmelerinde *Listeria* türlerinin varlığı araştırıldı. Bu kapsamda, Kasım 2013-Haziran 2014 döneminde, 21 adet işletmeden süt, taze dışkı, silaj, toprak, su ve süt sağım odası sıvap örnekleri olmak üzere toplam 340 adet örnek alındı. *Listeria* izolasyonu amacıyla süt örneklerinde AOAC/IDF metodu, diğer örneklerde USDA/FSIS metodu kullanıldı. Çalışma sonucunda, 10 (%47.62) işletmede *Listeria* spp. izolasyonu gerçekleşmezken, 11 işletmede (%52.38) *Listeria* spp. izolasyonu yapıldı. Beş işletmenin süt, 7 işletmenin taze dışkı, 3 işletmenin silaj, 10 işletmenin toprak, 8 işletmenin su ve 2 işletmenin süt sağım odası örneklerinin en az birinden *Listeria* spp. izolasyonu gerçekleşti. İncelenen örneklerin %15'inden (51/340) *Listeria* spp. izolasyonu yapıldı. Yüz beş adet süt örneğinin 8'inden (%7.61), 105 adet taze dışkı örneğinin 12'sinden (%11.42), 21 adet silaj örneğinin 3'ünden (%14.28), 42 adet toprak örneğinin 15'inden (%35.71), 42 adet su örneğinin 11'inden (%26.19) ve 25 adet süt sağım odası örneğinin 2'sinden (%8) *Listeria* spp. izolasyonu gerçekleştirildi. İzolatlar kültürel ve biyokimyasal karakterlerine göre identifiye edildi ve 51 adet izolatın 8'i *L. monocytogenes*, 1'i *L.ivanovii*, 17'si *L. innocua*, 7'si *L. seeligeri*, 7'si *L. welshimeri*, 11'i *L. grayi* olarak tanımlandı. *L. monocytogenes* izolasyon oranı süt, taze dışkı, toprak, su ve süt sağım odası örneklerinde sırasıyla %0.95, %0.95, %4.76, %7.14 ve %4 olarak tespit edilirken silaj örneklerinden izole edilmedi. Sonuç olarak, süt sığırı işletmelerinin süt ve çevre örneklerinde *Listeria* türlerinin varlığı gerek hayvan sağlığı gerekse halk sağlığı açısından risk oluşturmaktadır.

Anahtar kelimeler: Listeria spp., Süt sığırı işletmesi, Prevalans

Introduction

The bacteria of the genus *Listeria* are widespread in nature and found in many different environments including soil, water, vegetation, sewage, animal feeds, farm environments and food-processing environments [23,40]. To date, ten species have been recognized within the genus: *L. monocytogenes*, *L.ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi, L. marthii*, *L. rocourtiae*, *L. fleischmannii*

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and L. weihenstephanensis [6,26]. Two species, *L. monocytogenes* and *L.ivanovii*, are pathogenic for animals and humans [19]. However, sporadic human infections due to *L. seeligeri* and *L. innocua* have also been reported [32,34]. There is also some evidence for very rare infections caused by *L. innocua* in domestic animals [48].

Listeria monocytogenes is an important foodborne pathogen in terms of public health risk [16]. Human listeriosis occurs as sporadic disease or outbreaks, and predominantly occurs as a result of consumption of contaminated ready-to-eat and raw food products [35,41]. In general, *L. monocytogenes* contamination of processed ready-to-eat food products occurs by cross-contamination of the finished product from the food processing plant environment. Infected animals and contaminated agricultural environments rarely cause to directly human infections. However, animal-derived food products that are not processed before consumption (e.g., raw milk) and raw foods of plant origin that have been contaminated by manure from infected or shedding animals can play an important role for human infections [29,31].

Listeriosis has been reported a wide range of species of domestic and wild animals including birds. The most susceptible domestic species are sheep, goats and cattle. Infection in farm animals usually appears to be linked to consumption of contaminated silage [23,29]. In previous studies, *Listeria* species were isolated from the silage samples at the varying rates up to 60% [10,14,36,44]. Listeriosis manifests itself clinically in ruminants as encephalitis, abortion and septicaemia. *Listeria monocytogenes* may also cause eye infections and mastitis. Mastitis is usually subclinical, but bacterial shedding into milk is possible. *Listeria monocytogenes* can be shed in the fecal material of clinically affected animals, however, asymptomatic animals from farms with an outbreaks of listeriosis and healthy animals from farms without a record of listeriosis cases can shed the bacterium also [45,46]. Studies have shown that up to 50% of asymptomatic cattle shed *L. monocytogenes* in their feces [29]. Fecal shedding of *L. monocytogenes* may pose a risk for contamination of milk, animal feed, and agricultural environment [20]. Kalorey et al. [24] reported that 6.74% of 2060 milk samples from dairy cows were positive for *Listeria* spp. Also, many studies shown that *Listeria* spp. was found in dairy cattle farm environment samples such as soil, water troughs, commodity feeds, feed bunks, bedding, cow path, surface runoff, yard dust/debris, milk sock/filters, etc. [18,21,28,43] and, it has been reported that ruminant, particularly bovine, farms are a reservoirs for human *L. monocytogenes* infections [29].

The aim of the present study was to investigate the presence of *Listeria* species in dairy cattle farms in Bandirma province, Turkey. Thus it will be determined that whether there is a risk in terms of animal health and hence public health in the region of the study conducted. In addition, this study will provide to determination of possible source of *Listeria* spp. infection for animals.

Material and Methods

***Sample Collection*:** The research was carried out from November 2013 to June 2014, and a total of 340 samples were obtained from the 21 dairy cattle farms randomly selected. The herd size ranged from 20 to 300 cows and cows were clinically healthy appearance. There was no record for listeriosis cases in all farms. Approximately 15 samples were collected from each farm, including 5 milk, 5 fresh feces, 1 silage, 2 soil, and 2 drinking water samples. In addition, total 25 swab sample were taken from milking parlour of 5 dairy cattle farms. Milk (100 mL) and fresh feces (approximately 100 g) samples were taken from the randomly selected cows. Silage samples were taken from the surface, interior of silos and in manger. Soil samples were also collected from different parts of farms including the main entrance and the farmyard. Each of silage and soil sample was taken approximately 500 g. Water samples (1 liter) were taken from each of the source water used to fill water troughs or drinking cups and the drinking water from water troughs or drinking cups. Swab samples were taken from different regions of the milking parlour including floors, walls, and milking units (the inner surface of milking liner and milk hoses). All samples were collected aseptically and quickly transported to the laboratory under chilled condition and stored 4 °C until processed. Samples were processed within 4 h of collection.

***Isolation and identification of Listeria spp. from samples*:** For the isolation of *Listeria* species from samples, Association of analytical Chemists/ The International Dairy Federation (AOAC/IDF) method [5] was used in milk samples and US Department of Agriculture (USDA/FSIS) method [7] was used in other samples. Briefly, approximately 25 mL of each milk sample was directly inoculated into 225 mL of Listeria Enrichment broth (Oxoid, CM862, SR141) and incubated at 30 °C for 48 h. Then, 100 mL inocula from the enrichment culture was surface streaked in duplicate on Oxford agar (Oxoid, CM856, SR140) and also *Brillance*™ Listeria agar (Oxoid, CM1080, SR227, SR228). All selective plates were incubated at 35 °C for 48 h. Each of the feces, silage and soil samples (approximately 25 g) were directly inoculated into 225 mL of University of Vermont Listeria Enrichment broth (UVM I, Oxoid, CM863, SR142). Water samples (approximately 1 liter) were filtered through 0.22 µm membrane filters (Millipore, GSWG047S1) using membrane filtration system (Sartorius AG) and the filter was placed in 100 mL of UVM I broth. Swab samples from milking parlour were directly inoculated into 100 mL of UVM I broth. All enrichments were homogenized and then incubated at 30 °C for 24 h. One millilitre of primary enrichments were transfered to 9 mL UVM II broth (Oxoid, CM863, SR143) and Fraser broth (Oxoid, CM895, SR156), and incubated at 35 °C for 24 h. Secondly enrichments were streaked onto Modified Oxford agar (Oxoid, CM856, SR206) and also *Brillance*™ Listeria agar (Oxoid, CM1080, SR227, SR228), and incubated at 35 °C for 48 h. All selective plates were examined for typical *Listeria* colonies and colonies surrounded by a brownish green and/or black halo were taken as possible *Listeria* spp. One typical suspected *Listeria* spp. colonies from each plate were subcultured to Tryptic Soy agar (Oxoid, CM131) suplemented with 0.6% Yeast extract (Oxoid, L21) for purity and incubated at 37 °C for 24 h. Presumptive *Listeria* isolates were confirmed and identified at the species level on the basis of Gram staining, catalase and oxidase reaction, H_2S production, indole test, urease activity, motility in SIM medium (Oxoid, CM435) at 25°C, β-haemolysis, nitrate reduction, methyl-red-Voges Proskauer test, CAMP test and fermentation of sugars (rhamnose, xylose, mannitol and α-methyl-D-mannopyranoside) [4,6].

***Measurement of the pH values of silage samples*:** Twenty five grams of silage samples were mixed with 100 mL of distilled water with blender and filtered through two layers of cheesecloth. The pH value of the filtrate was immediately measured with a laboratory pH meter (pH 211, Hanna Instruments) [12].

Findings

Listeria species were isolated from 11 (52.38%) out of the 21 dairy cattle farms and were not isolated in the other 10 (47.62%) dairy cattle farms. Overall, *Listeria* spp. were found in 51 (15%) out of the 340 samples. Of these 51 isolates, eight isolates were identified as *L. monocytogenes*, one was *L.ivanovii*, seventeen were *L. innocua*, seven were *L. seeligeri*, seven were *L. welshimeri* and eleven were *L. grayi.* Results of isolation and identification of *Listeria* spp. from samples were shown in Table 1.

Table 1. Results of Listeria spp. isolation from samples in dairy cattle farms

Sample type and number (n)	Number of positive fams n (n/21%)	Number of Positive samples n (%)	Number of *Listeria* species isolated from samples (%)					
			L.monocytogenes	*L.ivanovii*	*L.innocua*	*L.seeligeri*	*L.welshimeri*	*L.grayi*
Milk (n:105)	5 (23.8)	8 (7.61)	1 (0.95)	-	3 (2.85)	-	2 (1.9)	2 (1.9)
Fresh feces (n:105)	7 (33.33)	12 (11.42)	1 (0.95)	-	5 (4.76)	2 (1.9)	1 (0.95)	3 (2.85)
Silage (n:21)	3 (14.28)	3 (14.28)	-	-	2 (9.52)	1 (4.76)	-	-
Soil (n:42)	10(47.61)	15 (35.71)	2 (4.76)	1 (2.38)	4 (9.52)	2 (4.76)	2 (4.76)	4 (9.52)
Water (n:42)	8 (19.04)	11 (26.19)	3 (7.14)	-	1 (2.38)	-	2 (4.76)	1 (2.38)
Milking parlour (n:25)	2 (40)*	2 (8)	1 (4)	-	-	1 (4)	-	-
TOTAL (n:340)	11 (52.38)	51 (15)	8 (2.35)	1 (0.29)	17 (5)	7 (2.05)	7 (2.05)	11 (3.23)

*Milking parlour samples were taken from only 5 dairy cattle farms.

In this study, the pH values of the silage samples varied between 3.9 and 7.7, likewise, the pH values of the silage samples that isolated Listeria spp. varied between 6.2 and 7.7.

Discussion and Conclusion

The region where the study was conducted, Bandirma province, is located in the Southern Marmara Region of Turkey, and in this region, domestic animals breeding, especially dairy cattle, are performed quite intensively. Domestic animals are likely to be exposed to the organisms which are widely distributed in soil and environment. *Listeria* is a common contaminant in the dairy environment, both on the farm and in the processing plant, and dairy cattle farms play a bigger role in the spread of *Listeria* between animals or humans [20,29,38]. For this reason, investigation of *Listeria* contamination of the dairy cattle farms from the standpoint of animal and public health are very important. This study was planned considering to the literature informations and also to the absence of a previously comprehensive study in this region, and investigated the presence of *Listeria* spp. in dairy cattle farms samples including milk, faeces, silage and environment samples. In the study, the presence of *Listeria* spp. was determined in a least one of the samples taken from 11 (52.38%) of the 21 dairy cattle farms examined, and was not determined in the other 10 dairy cattle farms. In a study conducted by Fox et al. [18], *L. monocytogenes* was isolated from five (55%) of the nine farms which are collected water trough, soil, and fecal samples for analysis. Husu [22] and Esteban et al. [15] reported that *Listeria* spp. was isolated from at least one of the dairy cattles from 45.8% of the 249 herds and 46.3% of 82 herds, respectively. Also, in the studies performed by Takai et al. [43] and Sasaki et al. [39], *Listeria* species were isolated from 20% and 12% of the farms examined, respectively. These results show that of the probability of the presence of *Listeria* spp. in dairy farms is high. However, in the many studies, the rate of positive farms for *Listeria* spp. was not usually reported.

In the present study, *Listeria* spp. was isolated from 8 (7.61%) of 105 milk samples and these milk samples were belonged to the 5 dairy cattle farms. These isolates were identified as: 1 *L. monocytogenes*, 3 *L. innocua*, 2 *L. welshimeri* and 2 *L. grayi* (Table 1). In addition, in these 5 dairy cattle farms, *Listeria* spp. was found in faecal, soil, water and milking parlour samples from the 2 farms, and it was also found in from faecal, silage, soil and water samples from the 3 farms. Recently studies in Turkey reported that *Listeria* spp. were found between 0% and 2% of isolation rate in cow's milk from dairy farms [3,8,14,44]. Result of this study is high in this range. This high isolation rate may be a result of a widely presence of *Listeria* spp. in these 5 dairy cattle farms. As mentioned above, *Listeria* spp. have also been isolated from the other samples of these farms. *L. monocytogenes* may directly contaminate milk as a consequence of listerial mastitis, and also asymptomatic or healthy cows can also shed *L. monocytogenes* in their milk for many months [29,45,46]. Furthermore, there is evidence supporting a role of silage in the contamination of raw milk with *L. monocytogenes* [37]. Animals fed with *Listeria*-contaminated silage can shed the organism in their milk [13], and *Listeria* species can be isolated from the milk samples of animals fed with silage [44,46]. Also, it has been known that environmental contamination and barn hygiene are important risk factors for the milk contamination [24,37]. Fox et al. [18] detected a correlation between the level of hygiene standards on the farm and the occurrence of *L. monocytogenes* in the environment (water, soil, silage, cow faeces, milk, etc.). However, some authors in other countries reported higher prevalence of *Listeria* spp. in raw milk from bulk tank in the dairy farms [11,25,30,49]. Infected cows [21], cattle feces, silage and farm environment could be sources of the bulk tank milk contamination with *L. monocytogenes* in the dairy farms [28,49]. Another possible origin of bulk tank milk contamination with *L. monocytogenes* can be the milking equipment. Latorre et al. [25] presented evidence that a source of *L. monocytogenes* contamination was milking equipment, 67.6% of in-line milk filter samples and 19.7% of bulk tank milk samples were positive for *L. monocytogenes*. In the same study, *Listeria* spp. and *L. monocytogenes* were also isolated 35% and %15 of milking parlour and milking equipment samples, respectively. In a different study, 32.2% of milk filter samples and 5.6% of in-line milk samples were positive for *L. monocyto-*

genes [30]. However, in a study by Fox et al. [18], *L. monocytogenes* was not isolated from milk filter samples. In the present study, swab samples were taken from different regions of the milking parlour in 5 dairy cattle farms, and one of the swab samples from 2 farms were found positive for *Listeria* spp. Of them, one from floor samples of milking parlour were found positive for *L. monocytogenes* and one from milk hoses samples were found positive for *L. seeligeri* (Table 1). All these results supports that the milking parlour and milking equipments could be a potential source for *Listeria* spp. including *L. monocytogenes*.

Listeria spp. may be isolated from fecal, soil and silage samples in dairy cattle farms. In current study, *Listeria* species were detected in the fresh feces samples from 7 farms, in the soil samples from 10 farms and in the silage samples from 3 farms. While *L. monocytogenes* was isolated from feces and soil samples, not isolated from silage samples. However, *L. innocua* was isolated at the highest rate from each three sample group (Table 1). Many studies showed that *L. monocytogenes* and *L. innocua* are the most common species in natural environment [23], dairy cows feces and silage [1,14,46,47] and raw milk [24]. But, some studies also reported that of the prevalence of other species in the different environments is high [9,23]. The prevalence of *Listeria* spp. in feces of dairy cattles is variable and higher [1,2,15,18,28]. In these studies, the prevalence of *Listeria* spp. in feces of dairy cattles reported between 6% and 61%. Finding of this study (11.42%) is included in this range. It has been reported that the different results of the prevalence of *Listeria* spp. in feces of healthy dairy cattles may be a result of the varieties of sampling procedure, analytical methods, geography and seasonal variations [1,8,28]. In this study, *L. monocytogenes* were detected from 4.76% of soil samples (Table 1). Previous studies were also carried out the isolation of *L. monocytogenes* from the soil samples of cattle farms. In a study performed by Fox et al. [18], the occurrence of *L. monocytogenes* in of soil samples from dairy farms was reported as 3%. Also, in a case-control study conducted by Nightingale et al. [29], *L. monocytogenes* was found in %14,6 of soil samples from cattle control farms, but was found in 35.3% of soil samples from cattle case farms. It is known that *L. monocytogenes* as well as *Listeria* spp. to be present in soil in the natural environment. *L. monocytogenes* can survive in the soil for months and even grow in favourable conditions, but soil is not a general or true reservoir for *L. monocytogenes* [17,23]. The presence of *L. monocytogenes* in soil might be probably related to contamination by plant decay or faecal materials of the infected farm animals and wild animals, including wild birds [17,38]. The presence of Listeria spp. in silage samples has been demonstrated in several studies [2,14,28,44,46], and the isolation rates of Listeria spp. were reported between 0% and 33.2%. Finding of this study (14.28%) is included in this range (Table 1). Similar to this study, Şahin et al. [42] detected Listeria spp. (*L. welshimeri* and *L. grayi*) in silage samples, but has not detected *L. monocytogenes*. Similarly, in a study by Pantoja et al. [30], *L. monocytogenes* was not isolated from silage samples in dairy farms, but Listeria spp. was isolated from 16.7% rate. However, in the studies by Vilar et al. [46] and Taşçı et al. [44], *L. monocytogenes* was isolated from 6.0% and 6.66% of the silage samples, respectively. The pH of the silage is an important factor for the presence of Listeria spp. and the the poor quality silage with a pH value higher than 5.5 supports the growth of Listeria spp. [36]. In the present study, the pH values of the silage samples from which Listeria spp. (L. innocua and L. seeligeri) were isolated ranged from 6.2 to 7.7, and *L. monocytogenes* were not isolated from these silage samples. Similarly, Ryser et al. [36] could not identified *L. monocytogenes* in Listeria spp.-positive (L. innocua and *L. welshimeri*) grass silage samples were all of poor-quality, ranging in pH from 5.78 to 5.89. In a different study, Vilar et al. [46] detected Listeria spp. in 29.5% of silage samples with a pH≥4.5 and in 6.2% of samples with a pH<4.5. The pH values of the silage samples which have been isolated Listeria spp. in other studies were 3.8 to 5.2 in Rea et al. [33], 4.05 to 5.77 in Durmaz et al. [14], 5.1 to 8.3 in Taşçı et al. [44]. It is well known that, there is an association between silage consumption and listeriosis in ruminants, and Listeria spp., including *L. monocytogenes*, most commonly found in poorly fermented silage, and well-preserved silage generally has a pH<4.5 [23,36,46].

In the present study, *L. monocytogenes* was isolated from the water samples at the highest rate (7.14%) in compared with other samples. However, other Listeria species (*L. innocua, L. welshimeri* and *L. grayi*) were also isolated in the ratio of 9.52% (Table 1). In Turkey, Atıl et al. [8] reported that *L. monocytogenes* as well as *L. innocua*, *L. welshimeri* and *L. seeligeri* were isolated from water samples in cattle farms. In the studies conducted in other countries, the isolation rates of *L. monocytogenes* were reported between 6.1% and 66% in water samples from dairy cattle farms [18,28,29]. Also, Pantoja et al. [30] and Latorre et al. [25] reported that *Listeria* species were isolated from 45.5% and 61.7% of water samples, respectively. These high isolation rates in water samples suggest that water may be a significant source for *Listeria* spp. including *L. monocytogenes*. However, in the study by Latorre et al. [25], *L. monocytogenes* was isolated from the drinking water samples, but was not isolated from the source water samples throughout the study, and researchers, taking into considering the results of PFGE, reported that source of the drinking water contamination is feces. In this study, water samples were taken from two different points in each farm; source water (used to fill water troughs or drinking cups) and drinking water (from the water troughs or the drinking cups). As a result; in the 8 dairy cattle farms which are water samples positive for *Listeria* spp., *Listeria* spp. were isolated from the drinking water samples of 6 farms and, the source water samples of 1 farm and, the drinking water as well as the source water samples of 1 farm. Also, *L. monocytogenes* was isolated from drinking water (one farm) as well as source water (two farms) samples. Waters of these two farms which are source water samples positive for *L monocytogenes* are provided from artesian wells, and both farms are situated fairly close to lake and there are agricultural lands of around. Obviously, in the scope of the present study, it is not possible to make suggestions about how to being contaminated of the source waters of two farm with *L. monocytogenes.* However, it is known that *L. monocytogenes* is present in the water environments such as lake, ditches, effluent from a sewage treatment plant, canals leading from this sewage treatment plant to the sea, the sea and river [23]. In addition, springs and groundwater wells may be harbour to the bacterium [27].

As a conclusion, the results of this study showed that *Listeria* spp. including *L. monocytogenes* are widely among dairy cattle farms. This status also indicates that there is a potential risk in terms of animal health and public health. Therefore, necessary measures must be taken for the main sources of *Listeria* such as silage, soil, water, manure, effluents and milking parlour. Biosecurity and hygiene practices are important for reducing the risk of introduction and perpetuation of *Listeria* spp. on farms. Briefly, for the control of listeriosis, it can be taken measures such as the control of silage fermentation and feed quality, the improvement of water quality, the manure treatment to inactivate organisms, and the hygienic practises at the every stage on dairy farms [38]. Continuous monitoring and surveillance programs are needed to evaluate trends of the occurrence of *Listeria* spp. in dairy cattle farms.

Acknowledgements

The author would like to thank to the cooperation of all farmer who allowed sampling, and also thank to Ismail Kanburoğlu for his help in sample collection.

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