Journal of Food Protection, Vol. 83, No. 11, 2020, Pages 1929–1933
https://doi.org/10.4315/JFP-20-023

Research Note

Occurrence of *Listeria monocytogenes* and *Escherichia coli* in Raw Sheep's Milk from Farm Bulk Tanks in Central Italy

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MS 20-023: Received 16 January 2020/Accepted 8 June 2020/Published Online 9 June 2020

ABSTRACT

For milk hygiene and safety, the milking phase is a critical moment because it is a probable pathway for the introduction of unwanted microorganisms in the dairy chain. In particular, *Listeria monocytogenes* and *Escherichia coli* are known as possible microbial contaminants of raw sheep's milk, although extensive knowledge regarding their contamination dynamics on sheep farms is still lacking. This study aimed to examine the occurrence and concentration of these microorganisms in milk samples collected from farm bulk tanks in the region of Lazio (Central Italy) and to investigate the related risk factors. Over a period of 1 year, we collected 372 milk samples from 87 sheep farms and administered a questionnaire to acquire information regarding relevant farm management variables. *L. monocytogenes* was not found in any of the samples, which indicates a low occurrence of this pathogen in sheep's bulk tank milk. In contrast, *E. coli* was found in almost two-thirds of milk samples (61%) but at levels below 10^2 CFU/mL in most of them (approximately 75%). Statistical analysis indicated that, during the warmest seasons, *E. coli* presence is more probable and counts are significantly higher. Unexpectedly, milk collected by hand milking had a lower level of contamination. Although further studies are necessary to clarify some aspects, the reported data add to the knowledge about the occurrence of *L. monocytogenes* and *E. coli* in raw sheep's milk and will be useful for future risk assessments.

HIGHLIGHTS

- *Listeria monocytogenes* was not isolated from 372 samples of bulk tank milk.
- *Escherichia coli* was a frequent contaminant of raw sheep's milk (61%).
- *Escherichia coli* occurrence and concentration were higher during the warmest seasons.

Key words: *Escherichia coli*; *Listeria monocytogenes*; Sheep's milk

It is well known that consumption of raw milk and dairy products made with unpasteurized milk is a possible cause of foodborne disease *(8)*. A wide range of different pathogens have been isolated in these foods and, although contamination can potentially occur at any step of the food chain, milking is considered the most crucial phase because of its impact on the hygienic quality and safety of the final product *(18)*. Unwanted microorganisms present in the milk collected at the farm level may come from the environment or may be excreted from the udders of infected animals *(23)*.

In the first case, the transfer of the microorganisms into the bulk tank derives from the direct contact of milk, during the milking step, with contaminated materials or surfaces (e.g., feces, soil, equipment, and fleece). The frequency of the contamination events as well as the magnitude of the microbial load depend on several factors, such as the use of good milking practices, the type of equipment and its maintenance, the cleanliness of the animals, and meteorological conditions. In the second route, the microbial contaminants originate in the animal itself. Some of the unwanted microorganisms found in raw milk are capable of colonizing the mammary gland and, consequently, are shed directly by the animal during milk harvesting.

Listeria monocytogenes is one of the most feared microbial contaminants of raw milk, especially when it is used to produce raw milk products. This gram-positive foodborne bacteria is the causative agent of human invasive listeriosis, a disease characterized by severe symptoms such as sepsis, abortion, and meningitis that can lead to death in some cases (fatality rate of 12.7 to 20.5%) *(9)*.

In addition, *Escherichia coli,* a gram-negative bacteria belonging to the *Enterobacteriaceae* family, is often isolated from raw milk. *E. coli* is considered an indicator of fecal contamination because it is harbored in the gastrointestinal tract of animals. As with other microorganisms, *E. coli* presence in bulk tank milk is mainly due to failure to use good milking practices, which leads to the product's contamination. In general, *E. coli* is considered

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nonpathogenic, but some pathotypes, such as Shiga toxin–producing *E. coli,* can be extremely dangerous for humans. The mechanism that leads to bulk milk contamination is assumed to be the same for both pathogenic and nonpathogenic *E. coli (25)*.

Because of the impact of *E. coli* on the safety and hygienic quality of milk, several studies have investigated its presence in farm bulk milk; although a number of studies are available for bovine species, only a few have focused exclusively on sheep. Condoleo et al. *(4)* also highlighted a scarcity of data regarding the occurrence of *L. monocytogenes* when they studied the risk of human disease associated with the consumption of raw sheep's milk cheeses. Likewise, to the best of our knowledge, only Bogdanovičová et al. *(3)* and de Garnica et al. *(7)* have published data regarding the presence of *E. coli* in sheep's bulk milk collected at the farm level.

Therefore, the present study aimed to acquire further information regarding the occurrence of *L. monocytogenes* and *E. coli* in bulk milk collected from 87 sheep farms to fill such knowledge gaps and provide useful data for future risk assessments. Another purpose was to evaluate the relevant variables associated with a greater risk of contamination.

MATERIALS AND METHODS

Study population. Our study was conducted over a period of approximately 1 year (October 2018 to September 2019) in Lazio, the Italian region with the third-largest sheep population; 590,207 head (9.44% of the country's population) are reared on about 7,700 farms (data retrieved from the National Livestock Registration System, reference date 30 June 2019). In Italy, sheep are generally raised for both milk and meat; for commercial reasons, lamb births, and consequently milk production, are concentrated in three periods: February to April, May to July, and November to January. According to European legislation, sheep farmers must periodically check their milk through laboratory tests to ensure compliance with milk hygiene rules. Our study population contained 87 sheep farms randomly selected from those that routinely rely on our laboratory. A questionnaire was administered to the farmers to collect specific information about farm management, such as farm size, breeds raised, number of milked animals, milking system, breeding system, and usage of silage. This study was approved by the Ethics Committee of Istituto Zooprofilattico Sperimentale del Lazio e Toscana "M. Aleandri."

Microbiological analyses. Samples of bulk milk were aseptically collected from the tank or bin of these farms by trained technicians during the high milk production periods (see previous paragraph). They were kept at 4°C until consignment to the laboratory (within 24 to 48 h) and were tested for the presence of *Listeria* spp. (including *L. monocytogenes*) and *E. coli*. Sample preparation, initial suspension, and dilutions were performed following ISO EN ISO 6887-5 *(12)*.

Detection and enumeration of *L. monocytogenes* and other *Listeria* spp. were carried out according to UNI EN ISO 11290-1:2017 and UNI EN ISO 11290-2:2017, respectively *(14, 15)*. In the first case, 25 mL of each sample was cultured in the preenrichment broth (half Fraser broth, Liofilchem, Roseto, Italy) and incubated at 30°C for 24 h. Then, the preenriched samples were transferred into a secondary enrichment broth (Fraser broth, Liofilchem) and incubated at 37°C for 24 h. Finally, the samples were transferred directly onto two different selective media, agar Listeria according to Ottaviani and Agostini (ALOA) and *Listeria* selective agar (Oxford formulation), and were incubated at 37°C for 48 h. For enumeration, 1 mL of each sample was diluted into 9 mL of half Fraser broth, and then 1 mL of the resultant broth was streaked on ALOA plates, which were incubated at 37°C for 24 h.

For both methods, presumptive *L. monocytogenes* colonies, after being cultured in tryptone soya yeast extract agar (Microbiol, Uta, Italy), were confirmed using appropriate morphological and biochemical tests (API *Listeria* and Vitek 2 Compact System, bioMérieux, Marcy-l'Étoile, France). In each case, at least one colony suspected to belong to *Listeria* spp. was taken from both types of selective media and tested using the abovementioned morphological and biochemical tests to verify the presence of atypical colonies of *L. monocytogenes* and the presence of other *Listeria* spp. Positive and negative controls were used to test the protocol. The detection and quantification limit of the methods were, respectively, 0.04 and 10 CFU/mL.

Enumeration of *E. coli* was carried out according to ISO 16649-2:2001 *(13)*. A 1-mL aliquot of each sample and serial 10-fold dilutions were prepared using peptone tryptone water (Condalab, Madrid, Spain) and were transferred into petri dishes, to which tryptone bile X-glucuronide medium (Biolife, Milan, Italy) was immediately added. The samples were incubated at 44°C for 24 h, and then the presumptive colonies were enumerated. The quantification limit was 1 CFU/mL.

Statistical analysis. An Excel spreadsheet (version 2016, Microsoft Corporation, Redmond, WA) was used to record the collected data and carry out the descriptive statistics, along with WinEpi *(28)*. Qualitative data was described using frequencies and percentages; mean values and percentiles were used for quantitative data. We used nonparametric statistical tests (χ^2 and Kruskal-Wallis) to investigate the relationship between occurrence or bacterial concentration and the information acquired through the questionnaires, including the sampling period: spring (February to April), summer (May to July), and winter (November to January). Such analyses were performed using SPSS software (version 21, IBM, Armonk, NY) *(11)*.

RESULTS

Data collected through the questionnaire showed that the number of sheep in the investigated farms ranged from 50 to 4,300 (mean = 621, median = 400) and that, on average, 70.5% of them were milked daily. The majority of the farms were equipped with a pipeline milking system (82.5%) to convey the milk directly to the tank, whereas the others used a bucket trolley milker (16.3%). Only one farmer (1.2%) milked the animals by hand. Almost all farmers ($n = 85$, 97.7%) adopted a semiextensive breeding system, as used in most parts of Italy; in this system, animals graze in the pasture for most of the year. Silage was not used to feed the animals.

Overall, we analyzed 372 milk samples. No *L. monocytogenes* or other potentially pathogenic species, such as *L. ivanovii,* were detected (maximum possible prevalence 0.8%, CL 95%), but one milk sample was positive for *L. innocua*. In contrast, *E. coli* was detected in 227 samples (61.0%, CL 95% [56.1 to 66.0%]) from 80 farms. The distribution of the positive samples according to the concentration is illustrated in Figure 1. Bacterial load ranged between 0 and 4.1 Log CFU/mL, whereas mean, 5th,

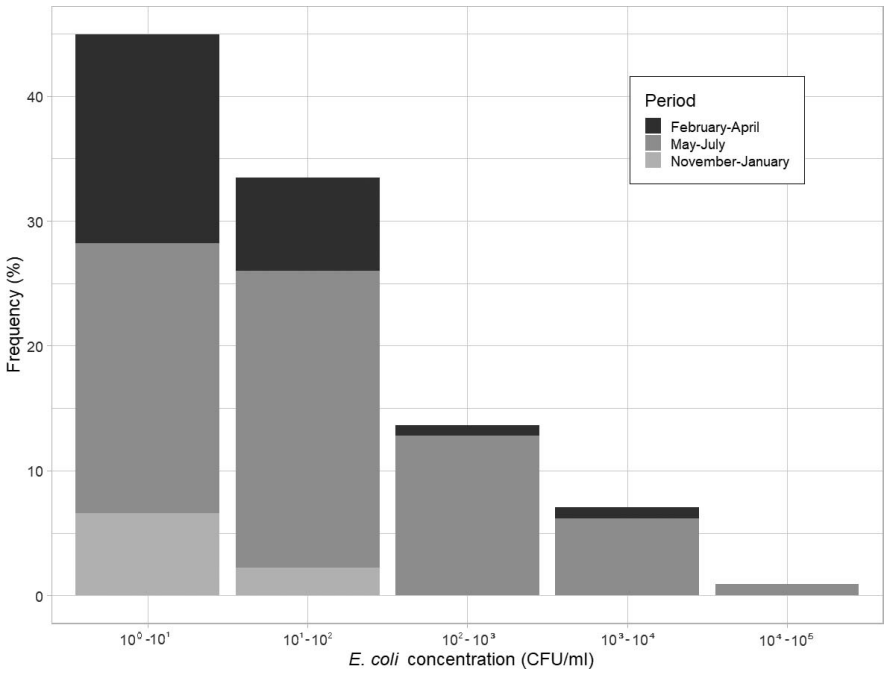

FIGURE 1. Escherichia coli *concentration ranges and seasonal frequencies of positive samples from milk tanks on sheep farms* (n = *227).*

50th, and 95th percentiles were 1.31, 0, 1.17, and 3.41, respectively.

Sampling period and milking system were the only variables found to be statistically associated with the presence of *E. coli* ($P < 0.05$). The isolation rate was higher during the warmest periods (spring, 54.6%; summer, 78.3%) compared with winter (26.6%). The Kruskal-Wallis statistical test also showed that *E. coli* concentration significantly increased in the hottest periods ($P < 0.05$); average values for spring, summer, and fall were 1.8, 2.6, and 0.3 Log CFU/mL, respectively. As for milking technique, no *E. coli* was isolated from the bulk tank milk that originated from the one farm where animals were milked by hand; however, *E. coli* was detected in 70.0 and 59.2% of the samples collected from the farms that used a bucket trolley milker and a pipeline system, respectively.

DISCUSSION

The present study reduces the knowledge gaps concerning the presence and concentration of *L. monocytogenes* and *E. coli* in raw sheep's milk at the bulk tank level. Our findings suggest that the prevalence of *L. monocytogenes* in sheep's bulk milk should be considered sporadic or, at least, as a low probability event, as reported by other authors. Although such bacteria are regularly isolated from the farm environment and domestic animals, Amagliani et al. *(2)* and D'Amico and Donnelly *(6)* did not find *L. monocytogenes* in milk, and other studies reported a low prevalence *(3, 16, 27)*. Such low values might be the consequence of low levels of milk contamination. In fact, several studies, albeit conducted on cow's milk, indicated a low level of contamination on farms, unless there was an animal suffering from mastitis due to *L. monocytogenes* infection present *(17)*. Thus, we can fairly assume that *L. monocytogenes* might be present in a certain number of milk samples but that, because the concentration is extremely low, sampling and detection methods are unable to detect it. The negative effects of competitive microbial flora on the growth, or even the survival, of *L. monocytogenes* in raw milk might also explain why the bacteria were not isolated.

Ready-to-eat foods produced with raw milk, such as cheeses, are generally considered to pose a greater risk than analogous products manufactured with pasteurized milk, especially for human listeriosis, as demonstrated by numerous outbreaks due to the consumption of raw milk products *(5, 10, 18, 20, 22)*. However, in a previous assessment that specifically addressed the risk of listeriosis associated with the consumption of raw sheep's milk cheese in Italy, a low probability of illness was estimated when no animal shedders were present on a farm and when the product did not allow the growth of *L. monocytogenes (4)*. Because that assessment used a higher bulk tank prevalence than the maximum possible value reported in this study, our results suggest that the risk associated to these products could be even lower if the abovementioned assumptions were respected.

E. coli is considered an important direct and indirect indicator of fecal contamination of raw milk; it can also cause mastitis (clinical or subclinical) and be shed through the udder. However, the frequency of potentially unnoticed subclinical mastitis in sheep seems to be rather low *(1, 24)*. Therefore, it is reasonable to suppose that the counts reported in this study are mainly due to environmental contamination because farmers do not usually use the milk produced by animals suffering from clinical mastitis for human consumption, due to its poor quality.

Studies that report data about *E. coli* presence in sheep's milk at the farm level are rare because investigations usually focus on other microorganisms that are considered reliable indicators of milking hygiene, such as coliforms or those belonging to the *Enterobacteriaceae* family. However, unlike *E. coli,* such bacteria do not indicate a specific contamination of the product with fecal matter *(26)*. In fact,

to date, our research is one of the largest investigations, and few studies are available for comparison.

We found that a remarkable proportion of milk samples (almost two-thirds) were contaminated by *E. coli*. Our value is definitely higher than the one reported by de Garnica et al. *(7),* who found *E. coli* in 17.4% of bulk milk samples collected from 205 herds in Spain. This important difference is difficult to explain but could be due, at least partially, to the higher detection limit of the method used by the Spanish research group (10 CFU/mL) compared with the detection limit of the method described in this study (1 CFU/mL). Our findings regarding *E. coli* concentration seem to support such a hypothesis because it was quantified below 10 CFU/mL in almost half of the positive samples and below 10^2 CFU/mL in about 75% of them (Fig. 1).

In addition, we compared our findings with those reported by the only quantitative study concerning sheep until now *(3),* in which researchers examined 23 bulk milk samples from Czech farms and found that *E. coli* counts reached as high as 10^6 CFU/mL. Our range only partially overlaps theirs because we did not reach that level of contamination; however, in some samples we found values of approximately 10^4 CFU/mL, which could also be considered a relevant level of contamination. Indeed, consumption of unpasteurized products manufactured with such milk could represent a nonnegligible food safety issue to consumers if the harbored *E. coli* strains belong to a pathogenic group such as Shiga toxin–producing *E. coli*.

No mandatory hygiene criteria regarding the presence of *E. coli* contamination in raw milk has been established worldwide *(19)*. However, the Food Safety Authority of New Zealand, in a code of practice published to assist the local dairy processors, suggests that a concentration of *E. coli* above 100 CFU/mL in farm bulk milk should be considered unacceptable *(21)*. By this standard, a considerable proportion of bulk milk samples we tested would have been noncompliant.

The nonparametric statistical tests showed a significant increase in the isolation rate and concentration of *E. coli* during spring and summer months. This was in contrast with the findings of de Garnica et al. *(7),* who reported a significantly higher prevalence in winter and autumn, determined by a higher fecal contamination of milk as a result of rainy weather and confinement of the animals. The reasons for these different results cannot be fully clarified but could be due to dissimilarities in management practices between Italian and Spanish sheep farms as well as diverse ecological conditions in the two countries. However, we can hypothesize that a combination of high temperatures during the warm seasons and a nonrigorous respect of the cold chain could have led to *E. coli* growth and, consequently, to higher concentrations and more frequent opportunity of detection.

Surprisingly, we found that harvesting milk by hand was significantly associated with lower milk contamination compared with the other milking methods. Hand milking is recognized to carry a higher likelihood of contamination because the milker's hands may be a vehicle for germs and because the milk is not immediately placed in a closed container but is exposed to numerous potential sources of contamination, such as fecal material, fleece, and soil. However, in our study, this technique was used on only one farm and the milker may have been exceptionally rigorous about hygiene during the milking operations.

Data from the questionnaire revealed that our study population was mainly composed of medium to large sheep farms. Presumably, these farms, compared with smaller ones, tend to rely more often on a laboratory for their own quality checks, and for this reason, they were recruited more frequently. This represents a limit of the investigation because milk from small farms could pose additional hygienic and safety concerns due to the restricted use of modern equipment or less stringent application of good milking practices. For instance, Mezher et al. *(20)* reported that hand milking was used by 45% of small milk producers in Central Italy; therefore, additional studies should be conducted to acquire data from this specific category of sheep farms.

In conclusion, this study enriches the knowledge about the presence and concentration levels of two well-known microbial contaminants of sheep milk at the farm level. Few data about these contaminants, concerning sheep exclusively, have been published until now, and further investigations are needed. However, the data produced in this study can be used to refine the existing risk assessments and to develop new ones.

ACKNOWLEDGMENT

This study has been funded by the Italian Ministry of Health—Ricerca corrente LT1016.

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