Important Vectors for \textit{Listeria monocytogenes} Transmission at Farm Dairies Manufacturing Fresh Sheep and Goat Cheese from Raw Milk

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ABSTRACT

The aim of this study was to determine the transmission routes of \textit{Listeria} spp. in dairy farms manufacturing fresh cheese made from ovine and caprine raw milk and to evaluate the impact of \textit{Listeria monocytogenes} mastitis on raw milk contamination. Overall, 5,799 samples, including 835 environmental samples, 230 milk and milk product samples, and 4,734 aseptic half-udder foremilk samples were collected from 53 dairy farms in the dairy intensive area of Lower Austria. Farms were selected for the study because raw milk was processed to cheese that was sold directly to consumers. A total of 153 samples were positive for \textit{Listeria} spp., yielding an overall prevalence of 2.6%; \textit{L. monocytogenes} was found in 0.9% of the samples. Bulk tank milk, cheese, and half-udder samples were negative for \textit{Listeria} spp. Because none of the sheep and goats tested positive from udder samples, \textit{L. monocytogenes} mastitis was excluded as a significant source of raw milk contamination. \textit{L. monocytogenes} was detected at 30.2% of all inspected farms. Swab samples from working boots and fecal samples had a significantly higher overall prevalence (\(P < 0.001\)) of \textit{L. monocytogenes} (15.7 and 13.0%, respectively) than did swab samples from the milk processing environment (7.9%). A significant correlation was found between the prevalence of \textit{L. monocytogenes} in the animal and in the milk processing environment and the silage feeding practices. Isolation of \textit{L. monocytogenes} was three to seven times more likely from farms where silage was fed to animals throughout the year than from farms where silage was not fed to the animals.

\textit{Listeria monocytogenes} is a gram-positive facultative intracellular pathogen responsible for severe foodborne infections in humans, resulting in 20 to 50% mortality in susceptible populations such as newborn children, the elderly, and immunocompromised persons (24). Outbreaks of \textit{L. monocytogenes} infection associated with the consumption of milk and milk products have been reported in recent years (7, 9). Generally, the prevalence of \textit{L. monocytogenes} in the dairy cattle environment is well documented. However, information on the contamination patterns of \textit{Listeria} spp. on small ruminant farms is scarce (13, 27).

Within Europe, Austria ranks second in direct selling of milk and milk products to end users (3). A small but growing group of people consume unpasteurized sheep’s or goat’s milk for practical reasons (e.g., dairy farm families), medical reasons (allergies to cow’s milk), or the perceived health benefits of natural and unprocessed foods (18). However, unlike cow’s milk, which is subject to stringent hygiene and quality regulations, microbiological standards for the production and distribution of goat’s and sheep’s milk are less stringent in Europe (16).

In recent years, farm cheese manufacture has become a thriving business. A previous Austrian study revealed that because of the simplicity of the manufacturing procedure the vast majority of dairy farms produce fresh cheese made from raw milk (23). However, management of the sanitary quality of sheep’s and goat’s milk is difficult because of the low production per animal, the type of milking system used, and the difficulties involved in machine milking for these animals (22).

The ecology and transmission of \textit{L. monocytogenes} in ruminants is poorly understood (17). Only a few researchers have found a direct association between cases of listerial mastitis in sheep and contamination of raw cheese and the cheese production environment (21, 22). Sheep suffering from listerial mastitis shed \textit{L. monocytogenes} at high rates over a long period without showing any symptoms of disease, posing a potential threat to public health (23).

The aims of this study were (i) to determine the transmission routes of \textit{Listeria} spp. in dairy farms that manufacture fresh cheese made from raw sheep’s and goat’s...
milk and (ii) to evaluate the impact of *L. monocytogenes* mastitis on raw milk contamination.

**MATERIALS AND METHODS**

**Farm selection and inspection.** Lower Austria is a dairy intensive area, with 20.1% of the national dairy output and 31.6% of the gross agricultural output. In 2009, Austria had 22,400 dairy sheep and 28,900 dairy goats, and Lower Austria contained 46.7 and 29.5% of the national herds, respectively. (3). Fifty-three farms were selected from a database of registered dairies maintained at the veterinary directorate county council. Farms were selected because their raw milk was processed to cheese and sold directly to consumers. In general, farms were visited once. The farms were notified of the forthcoming visit, and the proprietors were told to maintain routine cleaning procedures.

**Sampling.** Samples were taken from animals and from the milk processing environment. All samples were divided into five sample categories (SC): SC-I was samples from the animal and barn environment (feces, feedstuffs, and milk filters); SC-II was samples from working boots; SC-III was samples from the milk processing environment (floor, work surfaces, cheesemaking equipment, refrigerators, storage, and selling areas); SC-IV was samples from milk products (bulk tank milk and cheese); and SC-V was samples from each mammary gland (aseptic half-udder foremilk samples).

Fresh feces from 10 animals were collected from the barn floor with sterile plastic gloves. Silage was taken from the center of a cut surface. If no silage was fed, hay or freshly cut grass was sampled. For feces, feedstuffs, and cheese samples, the sample size was 25 g. To enable highly sensitive detection, the sample volumes of tank milk were increased from 1 ml to 10 ml and 100 ml.

Environmental samples (SC-II and SC-III) were taken by swabbing 900 to 1,000 cm² of surface with sterile cotton swabs (5 by 6 cm) and sterile buffered peptone water. The swab samples and milk filters were collected in plastic bags and covered with 100 ml of half-Fraser (HF) medium (Merck, Darmstadt, Germany).

For testing the microbiological status of the udder, milk samples from each half were collected aseptically (SC-V). After thorough cleaning of teat ends with alcohol and discharging the first milk, the samples were milked into sterile glass tubes.

Each farmer was given a training demonstration in hygiene methods required to collect and send the bulk tank milk and milk filter samples to be analyzed by the laboratory. Farmers were asked to send the samples at regular intervals until the animals were dried off. Milk samples were collected in the morning, 1 or 2 h after milking. The milk in the bulk tank included the milk from that morning and from the previous evening. All milk samples were transported to the laboratory at 4°C within 24 h of collection and were tested for the presence of *L. monocytogenes*.

**Isolation of *L. monocytogenes*.** Half-udder foremilk samples (SC-V) were centrifuged at 2,500 × g for 10 min, and the sediment (0.01 ml) was directly plated onto Columbia blood agar (5% sheep blood; bioMérieux, Marcy l’Étoile, France) and Palcam agar (Oxoid Ltd., Basingstoke, UK). Samples from SC-I through SC-IV were enriched according to ISO 11290-1 and ISO 11290-2 methods, respectively (1, 2). The 25-g samples (feces, feedstuffs, and cheese) and the 1-, 10-, and 100-ml samples of milk were added to 225 and 9, 90, and 900 ml of HF broth and incubated for 24 h at 30°C. After incubation, 0.1 ml of the HF broth was transferred to tubes containing 10 ml of Fraser broth (Merck) and incubated at 37°C for 48 h. Both enrichment cultures (HF and Fraser) were streaked onto duplicate plates of Palcam and chromogenic *Listeria* agar (Oxoid). The plates were incubated for 48 h at 37°C and observed for the presence of typical *Listeria* colonies.

**Molecular biological analysis.** The isolated *Listeria* strains were identified with a PCR assay as described by Rubert et al. (6). For molecular typing, the frozen stock culture was resuscitated by plating onto tryptone soy agar (Oxoid) and incubating the plates overnight at 37°C. A single *L. monocytogenes* colony was selected with a sterile loop and grown in tryptone soy broth containing 0.6% yeast extract (Oxoid). Preparation of agarose plugs, restriction of DNA using endonucleases *Ascl* and *Apal*, and subsequent separation of restriction fragments by pulsed-field gel electrophoresis (PFGE) was performed as described previously (11). All *L. monocytogenes* strains were typed based on *Ascl* and *Apal* fragments.

**Statistical analysis.** The χ² test was used to test the significance of uneven distributions in prevalence in various sample categories and parameters. The significance level for all statistical tests was *P* = 0.05.

**RESULTS**

**Prevalence of *Listeria* spp. and *L. monocytogenes* on all farms.** Overall, 5,799 samples, including 835 environmental samples (SC-I, SC-II, and SC-III), 230 milk and milk products (SC-IV), and 4,734 aseptic half-udder foremilk samples (SC-V) were collected from 53 small ruminant farms. All tank milk and cheese samples (SC-IV) were negative for *Listeria* spp., and no *Listeria* isolates were obtained from the aseptic half-udder foremilk samples (SC-V). A total of 153 samples (47 milk filters, 23 fecal samples, 13 feedstuff samples, and 70 swab samples from working boots and cheesemaking facilities) were positive for *Listeria* spp., yielding an overall prevalence of 2.6%; *L. monocytogenes* was found at a rate of 0.9% (50 of 5,799 samples).

Species discrimination among the clones revealed that 65.4% of all *Listeria* isolates were *Listeria innocua* and 27.4% were *L. monocytogenes*; 10.5% of the *Listeria*-positive samples were contaminated by more than one species; 15 samples contained two *Listeria* species, and 1 swab sample (working boots) contained three *Listeria* species.

*L. monocytogenes* was detected on 30.2% of all inspected farms. Significant differences in the prevalence of *Listeria* spp. and *L. monocytogenes* by sample type were found (Fig. 1). Swabs from working boots had the highest prevalence; 51.0% of the samples were positive for *Listeria* spp. (Fig. 1). However, the prevalence of *Listeria* spp. in feces (42.6% of samples) and swab samples from the floor of the cheesemaking room (39.3%) were not significantly different (*P* > 0.05). *Listeria* spp. were found less frequently in silage (25.7% of samples), other feedstuffs (16.6%), the working bench of the cheesemaking room (14.1%), and milk filters (9.1%).

Working boots and fecal samples had a significantly higher overall prevalence (*P* < 0.001) of *L. monocytogenes* (15.7 and 13.0% of samples, respectively) than did swab samples from the milk processing environment (7.9%). Overall, the frequency of *Listeria* spp. and *L. monocytogenes*...
Incidence of *Listeria* spp. and *L. monocytogenes* in relation to silage feeding. Seven of 53 small ruminant farms fed no silage, 13 farms fed silage throughout the year, and 33 farms fed silage only during the winter feeding period. Based on the feeding practices and the time of farm inspection, four groups of farms were defined: F-1, silage was fed throughout the year; F-2, silage was fed only during winter and samples were taken when silage was being fed; F-3, silage was fed only during winter and samples were taken when silage was no longer fed; F-4, silage was not fed. The prevalences of *Listeria* spp. and *L. monocytogenes* in the animals and the milk processing environment relative to silage feeding practices are shown in Figure 2. F-1 farms had a significantly higher overall prevalence (*P* < 0.05) of *Listeria* spp. (35%) and *L. monocytogenes* (7.4%) than did farms without silage feeding (F-4). Isolation of *Listeria* spp. and *L. monocytogenes* was three to seven times more likely from farms where silage was fed throughout the year (F-1) than from farms without silage feeding (F-4). Shortly after silage feeding was stopped (F-3), the incidence of *L. monocytogenes* decreased from 9.4% (F-2) to 3.7% (F-3) (*P* = 0.07). *L. monocytogenes* was not isolated from farms on which no silage was fed (F-4; Fig. 2).

Seasonal incidence of *Listeria* spp. and *L. monocytogenes*. A total of 467 milk filters and 131 tank milk samples were sent in from 91% (48 of 53) and 17% (9 of 53) of all farms until the dairy animals were dried off. Although tank milk samples were constantly negative for *Listeria*, 39 milk filter samples were positive for *Listeria* spp., yielding an overall prevalence of 8.4%. *L. monocytogenes*, *L. innocua*, and other nonpathogenic *Listeria* species were found at a frequency of 30.1, 61.5, and 5.1%, respectively; 2.6% of the *Listeria*-positive milk filter samples were contaminated by more than one *Listeria* species. Compared with September and October, frequencies found in March and April and in May and June differed significantly (*P* < 0.01 and *P* < 0.1, respectively) (Fig. 3). The prevalence of *L. monocytogenes* declined continuously from 6.8% in March and April to 2.7% in May and June, 2.5% in July and August, and 0% in September and October.

**FIGURE 1.** Frequency (%) of all *Listeria* species (entire bar), *L. monocytogenes* (hatched part of the bar), and other *Listeria* species (open part of the bar) in the animal and milk processing environments (sample categories I through III). The number at the top of the bar is the absolute frequency of *Listeria* spp.

**FIGURE 2.** Relationship between silage feeding practice and incidence (%) of *Listeria* spp. (entire bar) and *L. monocytogenes* (hatched part of the bar) in the animal and milk processing environments. The number at the top of the bar is the absolute frequency of *Listeria* spp. * F-3, silage was fed only during winter and samples were taken when silage was no longer fed; F-2, silage was fed only during winter and samples were taken when silage was being fed.

**FIGURE 3.** Portion of milk filters positive for *Listeria* spp (entire bar) and for *L. monocytogenes* (hatched part of the bar) by the season of sampling. The number at the top of the bar is the absolute frequency of *Listeria* spp.
and O through W) for the AscI patterns and numbers (1 through 25, 27, and 28) for the ApaI patterns. A total of 28 AscI, 27 ApaI, and 32 AscI-ApaI patterns were identified among the 50 L. monocytogenes isolates analyzed (Table 1). PFGE type A/1 predominated and included 14 isolates (4 working boot isolates, 6 isolates from the milk processing environment, and 4 isolates from the animal environment) followed by types B/1 and F/4 (2 isolates each from the milk processing environment), type B/6 (1 milk filter isolate and 1 fecal isolate), type A2/3 (1 isolate from a working boot and 1 isolate from the cheesemaking room), and type K/17 (two milk filter isolates). Twenty-six PFGE types (e.g., I/5, E/15, and C/7) were represented by only one isolate. Two clonal types (A/1 and A2/3) were found on different farms (Table 1). PFGE type A2/3 was detected on a working boot isolate from a cheesemaking room on farm 37. L. monocytogenes strains with PFGE profile A/1 were isolated from the animal environment and the milk processing environment on four different farms (farms 8, 10, 25, and 49) (Table 1). Three of the four farms (farms 8, 10, and 49) were located adjacent to each other, and the same veterinarian managed the animal health on these three farms. On one farm (farm 25), we traced the contamination route of L monocytogenes A/1. The foodborne pathogen was detected in the animal environment (silage and feces) and was then transferred to the milk processing environment (cleaning brush and working bench of the cheesemaking room) via working boots.

**DISCUSSION**

The concepts of selling and buying local produce and the demand for natural and unprocessed foods are growing consumer trends that have resulted in an increased interest in cheeses made from raw milk. However, dairy farms are an important reservoir of foodborne pathogens such as L monocytogenes (26). Pasteurization is regarded as an effective method for eliminating foodborne pathogens from milk, but because many dairy farms (100% of the farms inspected in the present study) usually process raw milk for cheese without any pasteurization, traditional raw milk cheese is associated with a significant food safety risk (23).

Two possible routes for L. monocytogenes contamination of raw milk and cheese have been suggested: (i) contamination from feces or the environment due to poor hygiene (19) and (ii) direct contamination from animals with listerial infections or mastitis (22, 28). Mammary listeriosis is typically subclinical, with apparently healthy animals excreting the pathogen in the milk for long periods of time (10, 25). One ewe with L. monocytogenes mastitis can contaminate the entire production chain, from the farm and milk processing environment to the final product (22). None of the sheep and goats in our study tested positive for Listeria based on analysis of the half-udder foremilk samples, and subclinical mastitis was excluded from being a significant source of raw milk contamination in this study.

Apart from mammary infection of single dairy animals, the prevalence of L. monocytogenes in raw milk and milk products is influenced by numerous factors such as farm size, number of animals on the farm, hygiene, farm management practices, sampling methods, types of samples evaluated, detection methods, geographical location, and season (19). In our study, Listeria spp. and L. monocytogenes were isolated from 42.6 and 13.0% of fecal samples, respectively (Fig. 1). Our data are similar to those obtained

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**TABLE 1. Pulsotypes of 50 L. monocytogenes strains isolated from the animal and milk processing environments on small ruminant farms that sell cheese directly to consumers**

<table>
<thead>
<tr>
<th>Farm no.</th>
<th>Strains</th>
<th>Pulsotypes</th>
<th>No. of strains/no. of pulsotypes</th>
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<tr>
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<td>703, 707, 7M3</td>
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<td>3/2</td>
</tr>
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<td>9M2</td>
<td>E/27</td>
<td>1/1</td>
</tr>
<tr>
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<td>1009, 1027, 1007, 1008, 1011, 1021, 10212, 10215, 10M1</td>
<td>A/1, A4/12, C/7, B2/8, E1/5, I/5, B1/13, D1/28, R/24</td>
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</tr>
<tr>
<td>11</td>
<td>1103</td>
<td>S/23</td>
<td>1/1</td>
</tr>
<tr>
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<td>1908</td>
<td>T/22</td>
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</tr>
<tr>
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<td>13/3</td>
</tr>
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<td>50</td>
<td>5002</td>
<td>W/28</td>
<td>1/1</td>
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by others, who also have reported massive contamination of the farm animal environment with *Listeria* spp. (4, 12, 17). Fecal contamination is the most common route of bulk tank milk contamination. Our data are consistent with those of previous studies, which generally were indicative of a high prevalence of *Listeria* spp. (up to 39.3%) in drains and floors of dairy and food processing facilities (13, 15).

*L. monocytogenes* also is commonly present in silage (14). The pathogen can multiply only in silage that has been inadequately fermented (pH above 5.0 to 5.5) with pockets of aerobic deterioration, and big bales of silage present a greater risk for listerial contamination because of lower density and a greater risk for mechanical damage to the plastic covering (5).

Reported prevalences of *L. monocytogenes* in silage vary widely from 3 to 39%, apparently because of differences in sampling procedures and analytical methods (26). Nevertheless, numerous study results emphasize the strong link between fecal shedding and silage feeding practices (12, 17, 20). In our study, *L. monocytogenes* was three to seven times more likely to be isolated from farms where silage was fed throughout the year than from farms without silage feeding (Fig. 2). The use of contaminated silage most likely explains the observed seasonality, because *L. monocytogenes* prevalences dropped in autumn when silage was not included in the feed (Fig. 3). Contaminated silage is a source of listerial infection for sheep and goats and contributes to massive contamination of the farm and milk processing environment (12).

*L. monocytogenes* is widely distributed in the natural environment and has been isolated from soil surfaces, rotten vegetables, and pasture herbage. The natural habitat of these bacteria is thought to be decomposing plant material, in which they live as saprophytes. Many authors agree that a likely scenario for *L. monocytogenes* transmission on farms includes initial contamination of crops and soil by wildlife, birds, or rodents (8). Fecal material from wild birds living close to agricultural environments may contain high levels of *L. monocytogenes* that may contaminate bedding, soil, water, and feeds, and sheep and cattle may be directly exposed through grazing and may become latent carriers, shedding *L. monocytogenes* via feces. Transmission on the dairy farm includes the domestic ruminants and their manure used to fertilize fields, which plays a key role in the maintenance of *Listeria* in the rural environment through a continuous fecal-oral enrichment cycle.

In our study, working boots were the items most often contaminated with *Listeria* spp. (51.0%) and *L. monocytogenes* (15.7%), respectively. Overall, the frequency of *Listeria* spp. and *L. monocytogenes* on working boots was always about twice the frequency in silage (Fig. 1). Transmission of *Listeria* spp. from the farm into the milk processing environment is strongly associated with the movement of farmers, who work both with the animals and in the dairy area. Contaminated milk processing environments always pose a significant hygiene problem and can lead to surface contamination of the final product (12, 23). In this study, all tank milk and cheese samples (SC-IV) were negative for *L. monocytogenes*. However, sampling of a high enough number of cheeses to detect contamination at a high confidence level is rarely practicable. Our data suggest that exclusive end-product sampling programs are not the answer to protecting consumers from listeriosis. Consequently, the cheese processing environment at the dairy farm must be monitored more precisely.

Two indistinguishable pulsortypes of *L. monocytogenes* isolates (A/1 and A2/3) were recovered from the entire production chain at different farms, thus substantiating an epidemiological link (Table 1). Pulsotype A/1 was isolated from the animal and milk processing environments of four farms, and at three of these four farms the animals were managed by the same veterinarian. According to Hoe and Ruegg (13), visitors such as veterinarians, stock handlers, and nutritionists pose a significant biosecurity risk to dairy farms by neglecting basic aspects of hygiene such as washing their boots and changing their overalls. Our data indicate that at least one veterinarian may have been a significant vector for the transmission of *L. monocytogenes* among farms.

Additional educational approaches for both dairy farmers and farm visitors such as veterinarians and stock handlers are necessary. Risk management strategies should be pursued at various levels along the food production chain, and hazard analysis and critical control point programs should be implemented and specific hygienic recommendations should be made to high risk groups. As a prerequisite for safe dairy practices, the milk processing area should be protected from the animal environment by developing strict hygiene barriers including disinfection trays and mandatory changes of working boots and clothes. Boots and clothes in colors specific to the animal or milk processing environment would be best.

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