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Detection, identification, and typing of *Listeria* species from baled silages fed to dairy cows

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ABSTRACT

Anaerobiosis, critical for successful ensilage, constitutes a challenge in baled silages. The loss of complete anaerobiosis causes aerobic deterioration and silages undergo dry matter and nutrient losses, pathogen growth, and mycotoxin production. Silage may represent an ideal substrate for *Listeria monocytogenes*, a pathogen of primary concern in several cheeses. The aim of this research was to investigate the occurrence of *Listeria* in baled silage fed to cows producing milk for a protected designation of origin cheese, and to characterize isolates by repetitive sequence-based PCR. *Listeria* spp. were detected in 21 silages and *L. monocytogenes* in 6 out of 80 of the analyzed silages; 67% of positives were found in molded zones. Results of the PCR typing showed genotypic homogeneity: 72.9 and 78.8% similarity between strains of *Listeria* spp. (n = 56) and *L. monocytogenes* (n = 24), respectively. Identical profiles were recovered in molded and nonmolded areas, indicating that contamination may have occurred during production. The application of PCR allowed the unambiguous identification of *Listeria* isolated from baled silages, and repetitive sequence-based PCR allowed a rapid and effective typing of isolates. Results disclose the potential of the systematic typing of *Listeria* in primary production, which is needed for the understanding of its transmission pathways.
Key words: baled silage, *Listeria* contamination, mold count, aerobic deterioration, plastic film damage

INTRODUCTION

In intensive dairy farms, forage crops are harvested as silage throughout the world to reduce feeding costs. Among the various silage conservation methods, wrapped bales are commonly used in Europe to preserve the quality of forage from meadows (Wilkinson and Toivonen, 2003; McEniry et al., 2007) and are gaining popularity in the United States in the last decade (Han et al., 2006; Arriola et al., 2015). Baled silage is often made from herbage that is wilted more extensively and presented more limited fermentation than conventional bunker silo silage, as it reduces the number of bales per hectare, plastic consumption, and costs, and can be more convenient when fed to animals (Han et al., 2006; McEniry et al., 2007; Tabacco et al., 2013). Unfortunately, the increased DM content also tends to increase fungal growth in wrapped forages (O'Brien et al., 2008; Tabacco et al., 2013), thus increasing hygienic issues as well as the risk of mycotoxicosis (O'Brien et al., 2007) and *Listeria* contamination (Fenlon et al., 1989). In bale silages, more than 40% of the silage DM stored is within 120 mm of the film cover and the reduced total thickness of the combined layers of stretch film on the bale side, usually 70 μm (4 layers) to 105 μm (6 layers), could be expected to make individually wrapped bales more susceptible to oxygen ingress (Forristal and O'Kiely, 2005). Even small holes that can occur on farm due to both mechanical and wildlife factors can result in quantitative DM losses because of mold growth, especially in conserved forages with higher DM contents (McNamara et al., 2001; Müller et al., 2007). Air penetration into the silage stimulates aerobic bacteria, yeasts, and molds and causes aerobic deterioration (O'Brien et al., 2007; Borreani and Tabacco, 2008a). Silage that has suffered aerobic spoilage has an increased probability of being contaminated by *Listeria* spp. (Fenlon et al., 1989; Borreani et al., 2012).

Listeria monocytogenes is a gram-positive, rod-shaped microorganism that is ubiquitous in the dairy farm environment (Vilar et al., 2007; Fox et al., 2009). *Listeria monocytogenes* is a foodborne pathogen, an agent that causes listeriosis: a serious invasive disease that affects both humans and a wide range of animals (Adams and Moss, 1995). Its occurrence in raw milk and food processing environments (dairy, fish, pork, and so on) has also been widely reported (Kells and Gilmore, 2004; Lianou and Sofos, 2007). In most cases, contamination

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is due to postprocessing environmental cross-contamination of foods, as heat treatments kill the organism. Because of the absence of a pasteurization step, raw milk products, especially soft cheeses, are considered high-risk products (Lunden et al., 2004). Dairy cows may be directly exposed to *L. monocytogenes* through the ingestion of improperly fermented silage (pH >5.0) contaminated before ensiling (Fenlon, 1988), and *L. monocytogenes* may then reach bulk tanks as a result of fecal contamination during milking, as also reported for spore-forming bacteria (Vissers et al., 2007). Other than *Listeria* detection, currently strain typing has largely been applied to explore subtype frequency and distribution: some authors applied repetitive element sequence-based PCR (**REP**) for characterizing isolates collected from dairy primary production as well as from the food processing environment (Harvey et al., 2004; Van Kessel et al., 2005; Chou and Wang, 2006), indicating the putative transmission/contamination paths.

Hence, the aims of the study were to assess the occurrence of *Listeria* spp. and *L. monocytogenes* in baled silage fed to dairy cows destined for cheese production, and identify management and silage production practices associated with the presence of the microorganism. Moreover, typing by rep-PCR has been carried out to investigate pathogen dissemination and putative routes of contamination.

MATERIALS AND METHODS

A survey was carried out over 2 agricultural years (2007–2008 and 2009–2010) in the western Po plain (Italy) on 20 dairy farms (Italian Friesian breed) that produced milk (about 28,000 kg/d) for a cheese-producing plant (45°29′19″N, 8°39′07″E). The farms were all located in a 20-km area around the processing plant: 14 farms were as close as 2 km from the plant, another 4 were 8 km away, and the remaining 2 were 15 and 20 km away. Each farm was visited 4 times (one for each season), and a detailed questionnaire (with questions on feed production and management) was presented to the farmers in each visit, and one already sealed bale, ready for feeding, was examined and sampled on each farm (for a total of 80 bales).

Bale Sample Collection

One bale per visit was randomly selected from those ready to be fed to animals. The diameter and height of each bale were measured and the polyethylene cover was carefully examined, looking for visible holes or damages. After the wrapping film had been removed, the visible surface area of each mold patch was measured, according to the published method (Borreani and Tabacco, 2010). The percentage of the total surface area affected by mold growth was then calculated for each bale. The thickness of the wrapping film covering the curved side was measured with 4 replicates using a micrometer (Digimatic Micrometer MDC-lite series 293, Mytutoyo Corp., Kamagawa, Japan). To obtain samples for microbiological, chemical, and fermentative analyses, 4 samples were taken using a steel core sampler (45 mm diameter) from a depth of 0 to 540 mm from the bale surface, in 4 positions in which no molds or spoilage were visible. The sampling points of this set of samples were spaced around the circumference of the bale at positions of about 0, 90, 180, and 270° on the bale side, at mid-point between the ends. The 4 samples were combined to provide one sample per bale (unaltered parts). When surface patches covered by mold were present, 2 to 6 samples were also taken from these parts from a depth of 0 to 120 mm from the surface and combined to provide one sample per bale (altered parts). Two different steel corers were used to sample the bale parts that were with or without visible fungal contamination. The corers were disinfected after each sampling operation using 95% industrial methylated spirit. The samples were immediately stored at 4 to 6°C before analysis, which was conducted later the same day. Sampling was performed, according to literature (Müller et al., 2011), by a limited number of people (the authors ET and GB). The same sampling protocol was followed at the same sampling time in each farm to avoid differences in sampling procedure that could have influenced the analytical result. On each farm visit, farmers were requested to complete a pre-determined questionnaire giving details on the history of the sampled silage bale, with information on the forage crop, wilting management, ensiling and wrapping procedures, the type and color of film wrap used, the number of film-wrap layers applied, bale hauling, and bale storage management. Days of conservation were calculated from the day the bale was wrapped to when it was sampled in the survey.

Sample Preparation and Analyses

Each silage sample was thoroughly mixed under aseptic conditions and divided into 3 sub-samples. The first sub-sample was analyzed for DM concentration, by oven drying it at 60°C until constant weight. The dried samples were air equilibrated, weighed, ground in a Cyclotec mill (Tecator, Herndon, VA) to pass a 1 mm screen, and analyzed for total nitrogen (**TN**) by combustion (nitrogen analyzer, Primacs SN, Skalar, Breda, the Netherlands), for crude protein (TN × 6.25), for NDF and ADF, according to the published protocol (Robertson and Van Soest 1981), and for ash by com-