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Raw milk products such as soft cheeses, are considered high-risk products for *Listeria monocytogenes*, human pathogen of primary concern. Bale silages could be a source of *Listeria* contamination of milk at a farm level. The paper shows that moldy patches due to air penetration in silages increase the risk of *L. monocytogenes* that can contaminate the cow diets. All efforts to reduce the risk of air penetration in bale silages, such increasing layers of plastic and store bales in a safe way, could contribute to reducing the potential for *Listeria* contamination within dairy rations and the risk of milk contamination.

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, need pathogen growth and mycotoxin production. Silage may represent an ideal substrate for *Listeria monocytogenes*, pathogen of primary concern in a number of cheeses. The aim of this research was to investigate the occurrence of Listeria in baled silage fed to cows producing milk for a PDO cheese, and to characterize isolates by Repetitive sequence based PCR (rep-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 non molded areas, indicating that contamination may have occurred during production. The application of PCR allowed the unambiguous identification of *Listeria* isolated from baled silages, and rep-PCR allowed a rapid and effective typing of isolates. Results disclose the potential of the systematic typing of *Listeria* in primary production, needed for the understanding of its transmission pathways.

Keywords: 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 US 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, the plastic consumption and the 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 (four layers) to 105 μm (six 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 (Borreani and Tabacco, 2008a; O'Brien et al., 2007). Silage that has suffered aerobic spoilage has increased the probability to be 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 dairy farm environment (Vilar et al., 2007; Fox et al., 2009). *L. monocytogenes* is a food borne pathogen, agent that causes listeriosis: a serious invasive disease which 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, etc.) has also been widely reported (Kells and Gilmore, 2004; Lianou and Sofos, 2007). In most cases, contamination is due to post-processing 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 prior to 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 in order 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 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 in order to investigate pathogen dissemination and putative routes of contamination.

MATERIALS AND METHODS

A survey was carried out over two 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 (Lat. 45°29’19’’N, Long. 8°39’07’’E). The farms were all located in a 20 km area around the processing plant: 14 farms were as close to the plant as 2 km, other four were 8 km apart, and the remaining two, 15 and 20 km. Each farm was visited four times (one for each season), 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 damage. 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 four replicates using a micrometer (Digimatic Micrometer MDC-lite series 293, Mytutoyo Corp., Kamagawa, Japan). In order to obtain samples for microbiological, chemical, and fermentative analyses, four samples were taken using a steel core sampler (45 mm diameter) from a depth of 0 to 540 mm from the bale surface, in four 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 four samples were combined to provide one sample per bale (unaltered parts). When surface patches covered by mold were present, two to six 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 prior to 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, so as 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 three 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, USA) 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 x 6.25), for neutral and acid detergent fiber (NDF and ADF), according to the published protocol (Robertson and Van Soest 1981), and for ash by combustion at 550°C for 3 h. The hemicellulose content was calculated as the difference between NDF and ADF.

Considering the second wet silage sub-sample, thirty grams were transferred to a sterile homogenization bag, suspended 1:10 w/v in a peptone physiological salt solution (PPS: 1 g of neutralized bacteriological peptone and 9 g of sodium chloride per liter) and homogenized for 4 min in a laboratory Stomacher blender (Seward Ltd, London, UK) for the microbial counts. The mold and yeast counts were determined by preparing serial dilutions and using the pour plate technique with 40.0 g/L of yeast extract glucose chloramphenicol agar (YGC agar, DIFCO, West Molesey, Surrey, UK). Petri dishes were incubated at 25°C for 3 and 5 d for yeast and mold, respectively, and then the mold and yeast colony-forming units (cfu) were enumerated separately on plates that yielded 1–100 cfu per Petri dish. Mold and yeast cfu were enumerated separately, according to their

148 macromorphological features. The water activity (aw) of the silage was measured at 25°C on a fresh
149 sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopted the
150 chilled-mirror dew point technique.
151 Considering the third wet silage sub-sample, thirty grams were transferred to a homogenization
152 bag, suspended 1:10 w/v in deionized water and homogenized for 4 min in a laboratory Stomacher
153 blender (Seward Ltd, London, UK), for quantification of pH and ammonia-nitrogen (NH3-N),
154 through the use of specific electrodes. The nitrate content was determined in the water extract,
155 through semi-quantitative analysis, using Merckoquant test strips (Borreani and Tabacco, 2008b).
156 About 50 g of the same sub-sample were homogenized and extracted for 4 min in a Stomacher
157 blender in H_2SO_4 0.05 M at an acid-to-sample material (fresh weight) ratio of 5:1. An aliquot of 40
158 mL of silage acid extract was centrifuged at 3,622 × *g* for 4 min, and the supernatant was filtered
159 with a 0.20-μm syringe filter and used for quantification of lactic and monocarboxylic acids (acetic,
160 propionic, and butyric acids) with an HPLC (Agilent Technologies, Santa Clara, CA) (Canale et al.,
161 1984). Ethanol was determined by HPLC, coupled to a refractive index detector, on a Aminex
162 HPX-87H column (Bio-Rad Laboratories, Richmond, CA). The analyses were performed
163 isocratically under the following conditions: mobile phase 0.0025M H_2SO_4, flow rate 0.5 ml/min,
164 column temperature 37°C, and injection volume 100 μl. Duplicate analyses were performed for all
165 the determined parameters.
166
167 **Listeria Detection and Identification**
168 The ISO 170 method (EN ISO 11290-1:1996+A1:2004) was applied to all collected samples,
169 using 25 g aliquots. The enriched broth for each sample was then streaked onto Oxoid chromogenic
170 listeria agar (OCLA CM 1080; Oxoid, Milan, Italy) and selective PALCAM Agar (CM 0877 B;
171 Oxoid, Milan, Italy). Plates were incubated at 37°C for 24 to 48 h. The most probable number
172 (MPN) technique was also used, together with the ISO method, in order to retrieve *Listeria* present
173 in low concentration in silage. Briefly, 10 g samples were 1:10 diluted in buffered peptone water
174 (BPW; CM1049, Oxoid, Milan, Italy) and then two serial dilutions were prepared. Three ml of each
175 dilution was inoculated in 3 tubes with 9 ml of Fraser broth, for a final total of 9 tubes. These were
176 incubated at 31°C for 48 ± 2h and then each sample tube was streaked onto OCLA and PALCAM
177 Agar. Five colonies, if present, were selected from OCLA (*L. monocytogenes*) or PALCAM (other
178 *Listeria* species) for all the positive samples, and then used for further analyses. In particular,
179 colonies on OCLA with a typical *L. monocytogenes* appearance were confirmed through a species-
180 specific PCR (D'Agostino et al., 2004). The other colonies presenting typical *Listeria* spp.
181 morphology on PALCAM but no lecithinase halo on OCLA, were identified through 16SrDNA

sequencing (600bp), according to the manufacturer's instructions (Micro seq 500 16S rDNA Bacterial Sequencing Kit). The generated sequences were compared with those present in the GeneBank database, using BLASTn sequence similarity searching. Before PCR were performed, colonies were broth cultured (using Brian Hearth Infusion Broth – BHI; Oxoid, Milan, Italy) at 37°C overnight and then 2 ml of broth culture was used for DNA extraction, by means of MBIO microbial kit (CABRU, SAS). The extracted DNA was used for both the identification and the characterization PCR assays. The amplifications generated by 16SrDNA PCR were purified and then sent to an external laboratory (IGA Technology Services, Udine, Italy) for sequencing.

Listeria Characterization

Listeria characterization was performed using ERIC and REP primers, according to the published protocol (Jersek et al., 1999) with minor modifications (Nucera et al., 2013). Fingerprints were analyzed with Bionumerics software (v 2.0, Applied Maths, Kortrijk, Belgium); similarity between fingerprints was determined by means of the Dice coefficient using optimization and position tolerance values, as previously described (Nucera et al., 2013). Results were then combined and a dendrogram was generated by the Unweighted pair group method with arithmetic mean (UPGMA). Shared profiles were defined as those found in one or more strains, whereas unique profiles were those found in a single strain.

Statistical Analysis

All the analyses were performed using SAS v. 9.1 for windows (SAS Institute Inc., Cary, NC, USA) and differences were considered statistically significant when $P<0.05$. A first set of analyses was performed comparing the altered and the unaltered areas considering: DM (g/kg), crude protein (g/kg DM), pH, a_w, nitrate concentration (mg/kg DM), organic acids (lactic, acetic, propionic, and butyric, g/kg DM), ethanol (g/kg DM), ash (g/kg DM), mold and yeast counts (log cfu/g), NDF and ADF (g/kg DM), and hemicelluloses (g/kg DM). The analyses were carried out using the t-test for equal/non equal variances, where the homogeneity of variance assumption was met/not accomplished. The frequency of Listeria in the two categories of samples was assessed by means of the χ^2 test. In addition, Kruskall-Wallis, and Mann-Whitney tests were used to investigate the effects of management factors such as wilting time, baler chamber used, plastic layers applied (n), storage location, bale orientation, height of bale storage, and days of conservation, on holes in the plastic cover, and bale surface covered by molds (expressed in %). Furthermore, silage that was positive and negative for the presence of *L. monocytogenes* and *Listeria* spp., within altered and

unaltered parts, were compared considering the features that could influence microbiological quality: DM concentration (g/kg), crude protein (g/kg DM), pH, aw, nitrate concentration (mg/kg DM), organic acids (lactic, acetic, propionic, and butyric, g/kg DM), ethanol (g/kg DM), ash (g/kg DM), mold and yeast counts (log cfu/g), NDF and ADF (g/kg DM), and hemicelluloses (g/kg DM). The analyses were carried out using the t-test for equal/non equal variances, where the homogeneity of variance assumption was met/not accomplished.

RESULTS

Chemical and Fermentative Composition of Bale Silage

A total of 80 bales were examined and sampled in the survey. Bales were made from three different crops: permanent meadow, alfalfa and Italian ryegrass. The mean values and the range of variations of DM content, nutritional characteristics in terms of NDF, ADF, hemicelluloses, crude protein, nitrate and ash contents, and fermentative profiles in terms of pH, lactic and volatile fatty acids, ethanol, and NH3-N contents are reported in Table 1. Both the chemical composition and fermentative profiles encompassed a wide range of situations that are representative of bale silage quality in northern Italy. The DM content showed a wide range of variability, with values typical of direct ensiling to high DM haylage for permanent meadow silages, whereas alfalfa and Italian ryegrass showed less variability. The fermentation showed pH ranging from 4.12 to 6.22, a_w from 0.78 to 1.00, lactic acid from <0.01 to 74.7 g/kg DM, acetic acid from <0.01 to 20.2 g/kg DM, and butyric acid from <0.05 to 17.8 g/kg DM.

Bale Management Factors

Data on the bale silage production and management, the effects of managerial factors on plastic damage and bale surface covered by mold are reported in Table 2. The majority of forages were wilted for at least 24 h and baled with a fixed chamber baler (89% and 83%, respectively). Farmers declared that the bales were wrapped within 4 h after baling on all the farms. All bales were wrapped using commercial polyethylene stretch film from different manufacturers and of different colors (16 were wrapped in white, 30 in light green, 34 in greenish-brown). About 2/3 of the bales were wrapped in 5 to 7 layers of plastic film, as established from a measurement of the plastic thickness on the curved side of the bale, whereas 20 bales were wrapped with less than 5 layers of plastic and 19 with more than 7 layers. Just over half the bales (58%) were wrapped and stored in the field with the remainder being transported to the storage area on the farm before wrapping. About half of the bales were stored in a single tier on the ground and on their flat end (59% and

53%, respectively). No farms used net nor other protection on their bales during storage. The plastic cover was visibly damaged on 39 bales, with 22 bales having more than two visible holes in their plastic cover. Visible mold growth was present on 57 out of 80 bales, with 21 bales having more than 5% of the surface covered by molds. Damage to the plastic cover and the fungal coverage on the bale surface were affected by the amount of plastic applied and by the time of conservation, with bales being wrapped in less than 5 layers and conserved for more than 200 d having the highest number of holes and the largest surface covered by molds. The proportion of bales positive to *Listeria* spp. was higher than 0.30 when wilting time was less than 48 h, the bales were baled with a variable chamber, the bales were wrapped with less than 5 layers of plastic, and the bales were conserved on their curved side.

The correlations between the surface covered by molds, bale management factors and chemical and microbial composition of the silage samples from unaltered parts are reported in Table 3. The surface covered by molds showed negative correlation with the nominal number of plastic layers applied on the curved side, whereas it was positively correlated with the length of conservation, the number of holes in the plastic cover, and the butyric acid concentration. Interestingly, the butyric acid concentration was also positively correlated with the number of holes in the plastic cover and the conservation time. The DM content of silages was positively correlated with wilting time and pH and negatively correlated with NH_3-N, lactic and acetic acids. Other silage variables were variously intercorrelated.

Altered parts had lower DM and ethanol contents and higher values of pH, water activity, yeast and mold counts, and NDF and ADF concentrations, than the unaltered parts (Table 4). *Listeria* spp. presence was higher in the bale parts that were visibly altered and where fungal growth was evident.

When the unaltered parts of silages were considered in respect of the presence or absence of *L. monocytogenes* and *Listeria* spp. (Table 5); pH tended to be higher (P = 0.10) and yeast count greater (P = 0.030) in samples that were positive for the presence of *Listeria*. When the altered parts were considered, propionic acid was greater (P = 0.044) in samples that were positive for the presence of *Listeria*. It was interesting to note that samples that were negative for the presence of *Listeria* had a numerically greater nitrate content than those that were positive for both altered and unaltered parts.

Listeria Detection and Identification

Listeria spp. was detected in 22 bales (27.5%) considering jointly MPN and ISO methods. The microrganism was isolated both from altered and unaltered parts in 4 bales. It was isolated from the altered parts in 13 bales and in the unaltered parts in 5 bales. Only 1 sample (1.3%) yielded *L.*

monocytogenes and *Listeria* spp. together , whereas 5 samples (6.3%) yielded *L. monocytogenes* but no other *Listeria* species, and 16 samples (20%) yielded only non pathogenic *Listeria* spp. In 3 samples, colonies were retrieved by the MPN method but no growth was observed when the ISO procedure was applied. In these samples the MPN ranged from 3 to 80 MPN/g. In all other samples (n = 19), tested positive with ISO method, the MPN value was higher than 300 MPN/g. All other samples (n = 58) tested negative with ISO method; the MPN was below 0.3/g. The PCR confirmed all *L. monocytogenes* positive samples (OCLA) and sequencing (on PALCAM suspected colonies) allowed 4 and 12 samples harboring *L. seeligeri* and *L. innocua* to be identified, respectively. The one sample that showed colony 271 morphology characteristics for both *L. monocytogenes* and *Listeria* spp. was contaminated by *L. monocytogenes* and by *L. innocua*.

Statistical analyses demonstrated a significant difference in *Listeria* detection between the altered and unaltered areas (Table 4). The comparison between areas also allowed highly significant differences to be identified in the DM concentration, pH, a_w, yeast and mold counts, NH3-N, ethanol, NDF and ADF.

Listeria Characterization

A total of 80 colonies were processed with both PCRs: 48 were *L. innocua*, 24 *L. monocytogenes*, and 8 *L. seeligeri*. All strains were typeable and produced fingerprints characterized by 10 to 25 and 8 to 18 bands, for the ERIC and REP primers, respectively (data not shown).

The dendrogram of the combination of the two PCR results showed a high genetic homogeneity among the isolates: 78.8% (for *L. monocytogenes*) and 72.9% (for *L. innocua* and *L. seeligeri*). All *L. monocytogenes* strains were grouped into 2 clusters gathering highly similar isolates; one, gathered 42% of the isolates (similarity 86.2%) and the other 58% (similarity 87.1%). As far as *L. innocua* and *L. seeligeri* were concerned, 85% of *L. innocua* strains were grouped at a similarity of 76.6%, while 88.0% of *L. seeligeri* strains shared a 77.1% similarity level (Fig. 1). The analysis of the dendrogram allowed to the identification of 12 unique types (7 *L. innocua*, 4 *L. seeligeri*, and 1 *L. monocytogenes*) and 17 profiles (grouping 68 strains) shared with one or more strains (10 *L. monocytogenes*, 5 *L. innocua*, and 2 *L. seeligeri*). Of the shared profiles, 5 (38% of the strains) grouped together strains isolated from molded surfaces with those isolated from the center of the bales, and 8 (44% of the strains) grouped samples collected from different farms. Considering the total number of types and the number of tested samples, it appears that the former was greatly increased. Multiple colonies selected from the same sample did not always produce the same PCR profile. One single colony was selected for the PCR characterization from 7 samples, while several

colonies were selected (from 2 up to 6) from other 10 samples. These resulted in only one PCR profile. Considering the remaining 10 samples, even if from 2 to 5 colonies were selected from each, more PCR profiles were detected (from 2 to 5). The highest PCR profiles-per-sample was attributed to *L. innocua* (1 samples showed 5 profiles), whereas, considering the results of *L. seeligeri* and *L. monocytogenes*, 2 PCR profiles were detected in 4 samples each.

DISCUSSION

The aim of the present study was to elucidate the role of baled silage in *Listeria* transmission/maintenance in farm environment, by evaluating the prevalence of *Listeria* and the frequency and distribution of PCR subtypes in baled silage. The results have highlighted that bales may be contaminated by pathogenic and non pathogenic *Listeria* species, with a predominance of *L. innocua* over *L. monocytogenes*, as also previously reported (Fenlon et al., 1989; Husu et al., 2000; Mohammed et al., 2009). This could be considered positive, even if the World Health Organization (1988) indicated that the presence of non pathogenic species indicates that environmental conditions are favorable for the survival and growth of the pathogenic species as well. Therefore, particular care should be given in silage preparation and storage, as emphasized by the presented results showing that silages which has suffered aerobic spoilage showed to harbor *Listeria* species.

The presence of *Listeria* in bale silage may be due to its presence in the soil and on vegetation, and it has been suggested that these bacteria are a normal part of the microflora of the plant in the field (Husu et al., 1990; Pahlow et al., 2003), and its survival/growth during ensiling occurs, especially in poor management conditions that favor air penetration (Fenlon et al., 1989; Pauly and Tham, 2003). *Listeria monocytogenes* is resistant to drying, can survive up to 2 years in dry soil and feces, and is capable of growing over a wide range of temperatures, from 4°C to 44°C (Membre et al. 1997; Allan et al. 2004). Once the herbage is contaminated by *Listeria*, its count may increase rapidly after ensiling when the microrganism utilizes the residual oxygen and the acidic condition characteristics of well made bales are not yet established (Fenlon and Wilson, 1998). The presence of *Listeria* on the bales may indicate that the process of acidification had not occurred properly, perhaps in relation to the poor quality of the ensiled forages, which reflects a slow acidification that allows *Listeria* survival as it could commonly happen in wrapped bale silages. When the anaerobiosis constraint is removed, *Listeria* may start to multiply and reach hazardous doses (Fenlon and Wilson, 1998).

Results of the present paper support this hypothesis: there was a significant difference in pH between bales which allowed the isolation of *Listeria*, compared to those in which the

352 microrganism was not isolated. Similarly, visible fungal-contaminated silage had a higher pH, mold
353 and yeast counts than silage parts that were free of visible fungal contamination, in agreement with
354 other reported results (O'Brien et al., 2007). Moreover, the presence of *Listeria* was highly
355 significantly associated to visually contaminated areas. Overall, the results presented in Table 4
356 have shown that the presence of *Listeria* spp. and *L. monocytogenes* was higher in bales
357 characterized by an altered profile due to air penetration in silage. Other authors (Vilar et al., 2007)
358 confirmed the relationship between poor silage quality (indicated by high pH values) and the
359 presence of *Listeria* spp. in silage (29.5% vs. 6.2% for pH above or below 4.5, respectively).
360 Similarly, several studies have highlighted that clinical listeriosis in ruminants is often associated
361 with feeding poor-quality silage (Adams and Moss 1995; Driehuis and Oude-Elferink, 2000;
362 Boerlin et al., 2002). Therefore, spoilage prevention of feeds is an important point to prevent herd
363 economic losses due to a spectrum of clinical conditions, including septicemia, meningitis,
364 meningoencephalitis, abortion and, in some instances, death (Ramaswamy et al., 2007). Data from
365 the present research showed that the main managerial factor that could contribute to reduce the
366 incidence of mold growth over the bale surface is the number of stretch-plastic layers applied,
367 which reduced the risk of damage to cover and increased the anaerobic status of the bale. Data are
368 in agreement with previously reported results (Keller et al., 1998; Borreani and Tabacco, 2008a),
369 which showed that increasing the number of film layers from four to six or even eight often
370 improved air-tightness of the bale coverage and significantly reduced mold growth over the bale
371 surface.
372 The presented results also show that *L. innocua* and *L. monocytogenes* can be present in areas
373 with low pH and yeast and mold counts. Similar results were found by other researchers (Ryser et
374 al., 1997) who showed that high-quality corn silage (pH < 4.0) also contains *Listeria* spp., including
375 *L. monocytogenes* strains belonging to Ribotypes of clinical importance in cases of food-borne
376 listeriosis. In the present study, in all the bales where *Listeria* was detected in the unaltered parts
377 alone, whereas the ISO qualitative method allowed the detection, the MPN results indicated very
378 low concentration of cells (3 to 80 MPN/g). This finding probably indicated the presence of the
379 microorganism in the ensiled herbage where it was not able to actively multiply given the ensiling
380 conditions, but it still remained present in the bale in its quiescent phase. On the other hand, the
381 same result could be explained by the soil being contaminated by low density of quiescent cells
382 which may persist over time (Welshimer and Donker-Voet, 1971). These cells are then revitalized
383 by the ISO method, being then able to grow in laboratory media, as it happened in the majority of
384 the herein analyzed samples. However, some cells may be also sub-lethally injured therefore the
385 standard selective enrichment steps do not promote their recovery as efficiently as for the uninjured

386 cells, producing negative results even when qualitative methods are applied (Lee et al., 2011). The
387 discrepancy observed between MPN and ISO methods could then be attributed to the presence in
388 some samples (n = 3) of damaged cells which did not grow (or were outgrown by other flora) in
389 Half-Fraser broth incubated overnight (following the ISO method) but they were able to multiply
390 when grown on Fraser broth for 48 hours (as required by MPN). The higher selective pressure of
391 Fraser broth and the longer incubation time may have allowed the outgrowth of *Listeria* on the other
392 flora, therefore its detection on plates. The typing results also seem to confirm this hypothesis:
393 finding the same PCR profiles in both the bale centers and altered areas may reflect the presence of
394 strains on the herbage at the moment of the ensiling, but which were still viable after 150 d of
395 conservation. This finding could disclose the presence of a strain "reservoir" that is able to survive
396 in correctly fermented bales. The presence of a minimal percentage of oxygen inside the silage mass
397 may allow not only survival, but also growth of the microorganism, which could reach levels as
398 high as 10^6 cfu/g in more than 100 d (Fenlon and Wilson, 1998). In addition, when the pH increases
399 (i.e. aerobic deterioration), these strains start to multiply, therefore becoming a putative
400 contamination route for ruminants. In 90's (Fenlon et al., 1996) found that silage was the likely
401 source of the introduction of *L. monocytogenes* in the farm environment: they showed that soil
402 samples from pastures of silage-fed ruminants were more likely to host *L. monocytogenes* than
403 other pastures. The hypothesis of crops as a source of *Listeria* could be supported by the present
404 work which has shown that more than one PCR type was retrieved in 10 out of 27 tested samples,
405 suggesting a multiple source of contamination. Forage crops may indeed be contaminated by
406 manure produced on the farm, or by any sort of wildlife, mostly birds and small mammals
407 (Nightingale et al., 2004). The application of ribotyping (Wiedmann et al., 1996), also highlighted a
408 high number of *L. monocytogenes* strains on farms linked to a listeriosis outbreak; however, they
409 did not explain this finding.
410 Despite the high number of papers published on the successful application of REP-PCR on
411 *Listeria* isolated from foods (e.g. Jersek et al., 1999; Harvey et al., 2004; Zunabovic et al., 2012)
412 typing data on silage collected samples lack in literature, and the presented resulted were difficult to
413 compare. However the present data are in agreement with those reported by an earlier study using
414 Ribotyping (Ryser et al., 1997). The authors reported a lower number of profiles than samples,
415 indicating that many samples shared the same profile, even though retrieved from different sources.
416 The herein presented results have shown that 17 PCR profiles were shared by two or more isolates,
417 and that eight of them indicated the presence of identical strains in bale silages present on the
418 different farms, as far as 20 km, as shown in the dendrogram of Figure 1. This observation could
419 underline the presence of strains that are able to disseminate from farm to farm, probably through

farm machinery or the movement of people, feed, and animals. It should be considered in fact that the samples were all collected in farms that were located in a small geographic areas and that the two farthest farms were located 20 km apart. Moreover, some of the farmers belonging to the cheese producing plant are sharing the machinery needed to produce silage and, sometimes, the feed they produce. It should be considered also that the REP-PCR method herein applied does not allow to differentiate highly similar strains, given the 95% similarity cut-off chosen for the identification of identical strains. However, researches previously published (Chou and Wang, 2006; Zunabovic et al., 2012; Nucera et al., 2013) showed that the discriminatory power of PCR typing is comparable to that of PFGE (the current gold standard typing method for *L. monocytogenes*). For these reasons, its applicability as a screen typing tool in field research cannot be denied, considering that results on large scale sampling can be obtained in less time and with the investment of less resources, in comparison to what PFGE would require.

CONCLUSION

The results herein presented highlight that bales may represent a potential hazard mostly when they are not properly conserved (i.e. damage to the plastic film which ensures a safety preservation), as showed by the presence of *Listeria* more frequently in molded areas than in unaltered ones. Hence, emphasis on the application of good agricultural practices during preparation and of the good hygienic practices (**GHP**) during management and distribution of baled silage should be given. In fact, considering the current food law (Regulation (EC) 178/2002; Regulation (EC) 852/2004) the farm to fork approach requires the food business operators to be responsible for the quality and the safety of products all along the food chain. Therefore, the proper silage preparation and storage should be part of the GHP manual that the farms are now required to have in order to enter in the food chain in EU. The control of pathogen spread (as evidenced by REP-PCR data) in primary production is necessary mostly in products (such as the one we selected in this paper) in which the presence of *L. monocytogenes* represents a primary concern, in order to prevent dissemination of the pathogen firstly in primary production, and then in food-producing factories.

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614

615 **Table 1.** Chemical composition and fermentation variables of bales (n = 80) sampled on 20 farms in
616 North-West Italy.

Parameter[1]	Permanent meadow (n = 41)		Alfalfa (n = 20)		Italian ryegrass (n = 19)	
	Mean	Range	Mean	Range	Mean	Range
DM, g/kg	572	(159-849)	521	(331-715)	577	(360-738)
Crude protein, g/kg DM	137	(77-192)	178	(118-226)	96	(63-148)
Nitrate, mg/kg silage	491	(0-3470)	516	(0-3357)	314	(0-2513)
NDF, g/kg DM	448	(294-668)	412	(311-530)	556	(453-702)
ADF, g/kg DM	328	(239-441)	348	(264-478)	390	(298-501)
Hemicelluloses, g/kg DM	126	(34-280)	64	(20-151)	166	(127-220)
Ash, g/kg DM	124	(82-209)	114	(71-179)	103	(75-177)
a_w	0.92	(0.78-1.00)	0.95	(0.88-0.98)	0.93	(0.80-0.97)
pH	5.51	(4.12-6.22)	5.41	(4.66-6.10)	5.59	(4.56-6.13)
Lactic acid, g/kg DM	11.4	(<0.01-74.7)	13.9	(<0.01-46.9)	6.6	(<0.01-27.4)
Acetic acid, g/kg DM	3.3	(<0.01-16.1)	8.5	(1.4-20.2)	3.4	(<0.01-8.9)
Butyric acid, g/kg DM	0.3	(<0.05-8.8)	0.6	(<0.05-7.7)	1.3	(<0.05-17.8)
Propionic acid, g/kg DM	1.0	(<0.05-6.7)	1.7	(<0.05-11.9)	1.2	(<0.05-7.4)
Ethanol, g/kg DM	8.0	(<0.01-55.3)	6.4	(0.3-19.6)	18.5	(1.1-30.7)
NH_3-N, g/kg TN	63	(9-165)	79	(32-173)	70	(26-147)

617 [1] a_w = water activity; DM = dry matter; NDF = neutral detergent fiber; ADF = acid detergent fiber; NH_3-N = ammonia
618 nitrogen; TN = total nitrogen.
619

Table 2. Crop type and bale management factors and relationship with plastic cover damages, extent of spoiled area on the bale surface and proportion of bales positive to *Listeria* spp.

Management factor [1]		No. of sampled bales	Holes in the plastic cover (n)	Bale surface Covered by mould (%)	Presence of *Listeria* spp.
Crop	Permanent meadow	41	1.1	2.2	0.24
	Alfalfa	20	1.8	5.1	0.25
	Italian ryegrass	19	3.9	8.8	0.37
Wilting time, h	< 24	9	1.1	4.0	0.33
	24 - 48	35	2.7	4.9	0.34
	> 48	36	1.4	4.1	0.19
P			*NS*	*NS*	
Baler chamber	Fixed	66	1.8	4.7	0.26
	Variable	14	2.6	3.6	0.36
P			*NS*	*NS*	
Plastic layers applied, n	< 5	20	4.1	11.2	0.40
	5 - 7	41	1.8	3.5	0.26
	> 7	19	0.3	1.0	0.20
P			*0.001*	*0.001*	
Storage location	On farm	34	1.5	4.9	0.26
	In the field	46	2.3	4.1	0.28
P			*NS*	*NS*	
Bale orientation	Flat end	42	2.2	4.6	0.21
	Curved side	38	1.7	4.3	0.34
P			*NS*	*NS*	
Height of bale storage	Ground tier	47	1.6	4.1	0.26
	More than 1 tier	33	2.4	5.0	0.30
P			*NS*	*NS*	
Days of conservation	< 100	18	1.1	1.2	0.28
	100-200	41	1.1	2.7	0.27
	> 200	21	4.3	10.7	0.29
P			*0.001*	*0.001*	

Table 3. Pearson correlation coefficients of bale silage chemical and microbiological composition and management factors (n = 80, samples from unaltered parts).

Item	Surface covered by mold	DM	pH	NH_3-N	Yeast	Mold	Lactic acid	Acetic acid	Butyric acid	Wilting time	Plastic layers	Conservation length
DM, g/kg	NS [1]											
pH	NS	0.653**										
NH_3-N, g/kg TN	NS	-0.617**	-0.461**									
Yeast, log cfu/g	NS	NS	NS	NS								
Mold, log cfu/g	NS	NS	0.222**	NS	NS							
Lactic acid, g/kg DM	NS	-0.791**	-0.763**	0.622**	NS	NS						
Acetic acid, g/kg DM	NS	-0.548**	-0.488**	0.545**	NS	NS	0.631**					
Butyric acid, g/kg DM	0.400**	NS	NS	NS	NS	NS	NS	NS				
Wilting time, h	NS	0.373**	0.373**	-0.250**	NS	NS	-0.239**	NS	NS			
Plastic layers, n	-0.339**	NS	NS	NS	NS	0.270**	NS	NS	NS	NS		
Conservation length, d	0.557**	NS	NS	NS	NS	NS	NS	0.229**	0.501**	NS	-0.329**	
Holes in plastic cover, n	0.663**	NS	NS	NS	NS	NS	NS	NS	0.645**	NS	-0.286**	0.582**

[1] DM = dry matter; NH_3-N = ammonia nitrogen; TN = total nitrogen; * $P < 0.05$; ** $P < 0.01$; NS = $P > 0.05$.

Table 4. Microbiological, chemical and fermentative characteristics of unaltered and altered parts of silage bales (only bales with altered parts were considered in the comparison; n = 57).

Item [1]	Unaltered parts		Altered parts		*P*
	mean	SEM	mean	SEM	
DM, g/kg	562	16.3	510	15.5	0.028
pH	5.72	0.08	6.57	0.16	<0.001
a_w	0.93	0.005	0.95	0.004	<0.001
Nitrate, mg/kg silage	530	121.9	459	131.7	NS
NH_3-N, g/kg TN	67.6	4.70	95.5	10.2	0.015
Yeast, log cfu/g	3.57	0.22	6.09	0.28	<0.001
Mold, log cfu/g	2.45	0.14	4.93	0.26	<0.001
Lactic acid, g/kg DM	11.2	1.92	7.6	1.71	NS
Acetic acid, g/kg DM	4.8	0.5	5.1	0.77	NS
Propionic acid, g/kg DM	1.2	0.84	1.1	0.25	NS
Butyric acid, g/kg DM	0.7	0.3	0.5	0.24	NS
Ethanol, g/kg DM	10.6	1.67	4.5	1.29	0.005
Ash, g/kg DM	117	3.7	109	4.5	NS
Crude protein, g/kg DM	137	4.6	140	5.2	NS
NDF, g/kg DM	469	10.9	502	13.5	0.057
ADF, g/kg DM	348	6.7	384	9.0	0.002
Hemicelluloses, g/kg DM	121	6.7	119	7.5	NS
Listeria spp. presence	0.11	-	0.28	-	0.021

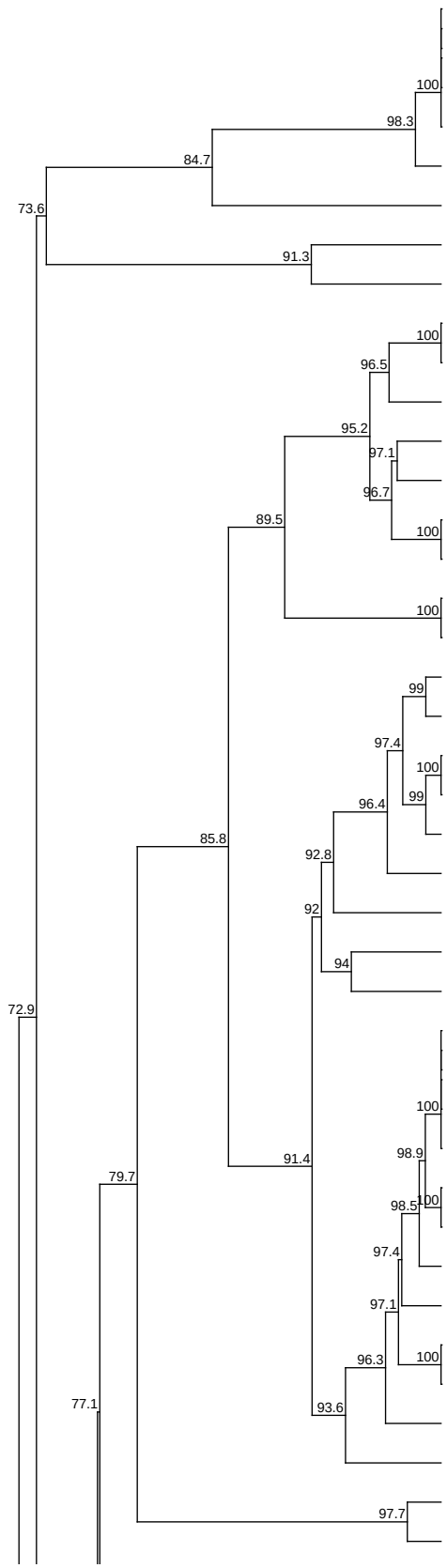
[1] ADF = acid detergent fiber; a_w = water activity; DM = dry matter; NDF = neutral detergent fiber; NH_3-N = ammonia nitrogen; NS = not significantly different; TN = total nitrogen.

Table 5. Comparison of microbiological, chemical and fermentative characteristics of samples that were positive (+) or negative (–) for the presence of *L. monocytogenes* and *Listeria* spp. within the unaltered and altered parts of the bales.

Item [1]	Unaltered parts (n = 80)			Altered parts (n = 57)		
	Listeria + mean (SEM)	*Listeria* − mean (SEM)	*P*	*Listeria* + mean (SEM)	*Listeria* − mean (SEM)	*P*
DM, g/kg	580 (50)	560 (20)	NS	490 (40)	520 (20)	NS
pH	6.09 (0.26)	5.67 (0.08)	0.10	6.62 (0.3)	6.56 (0.19)	NS
a_w	0.93 (0.015)	0.93 (0.005)	NS	0.96 (0.008)	0.95 (0.004)	NS
Nitrate, mg/kg silage	133 (88)	580 (136)	NS	263 (186)	535 (167)	NS
NH_3-N, g/kg TN	62.2 (12.0)	68.3 (5.11)	NS	98 (25.8)	94.5 (10.3)	NS
Yeast, log cfu/g	4.56 (0.4)	3.44 (0.23)	0.030	6.52 (0.55)	5.92 (0.32)	NS
Mold, log cfu/g	2.94 (0.45)	2.39 (0.15)	NS	5.22 (0.55)	4.82 (0.28)	NS
Lactic acid, g/kg DM	9.94 (5.1)	11.3 (2.07)	NS	6.90 (3.0)	7.86 (2.1)	NS
Acetic acid, g/kg DM	4.67 (1.8)	4.76 (0.52)	NS	5.66 (1.0)	4.93 (1.0)	NS
Propionic acid, g/kg DM	1.03 (0.54)	1.23 (0.27)	NS	2.16 (0.68)	0.62 (0.19)	0.044
Butyric acid, g/kg DM	0.14 (0.14)	0.79 (0.33)	NS	< 0.1 (-)	0.63 (0.33)	0.07
Ethanol, g/kg DM	14.07 (6.03)	10.25 (1.75)	NS	3.24 (1.99)	4.69 (1.49)	NS
Ash, g/kg DM	11.07 (0.44)	11.74 (0.41)	NS	10.13 (0.44)	11.28 (0.61)	NS
Crude protein, g/kg DM	136 (16.3)	137 (4.8)	NS	139 (12.6)	140 (5.6)	NS
NDF, g/kg DM	442 (30.3)	473 (11.6)	NS	512 (29.6)	489 (15.2)	NS
ADF, g/kg DM	330 (16.2)	350 (7.2)	NS	382 (18.7)	384 (10.4)	NS
Hemicelluloses, g/kg DM	11.2 (20.2)	12.2 (7.1)	NS	13.0 (12.9)	11.4 (9.1)	NS

[1] ADF = acid detergent fiber; a_w = water activity; DM = dry matter; NDF = neutral detergent fiber; NH_3-N = ammonia nitrogen; NS = not significantly different; TN = total nitrogen.

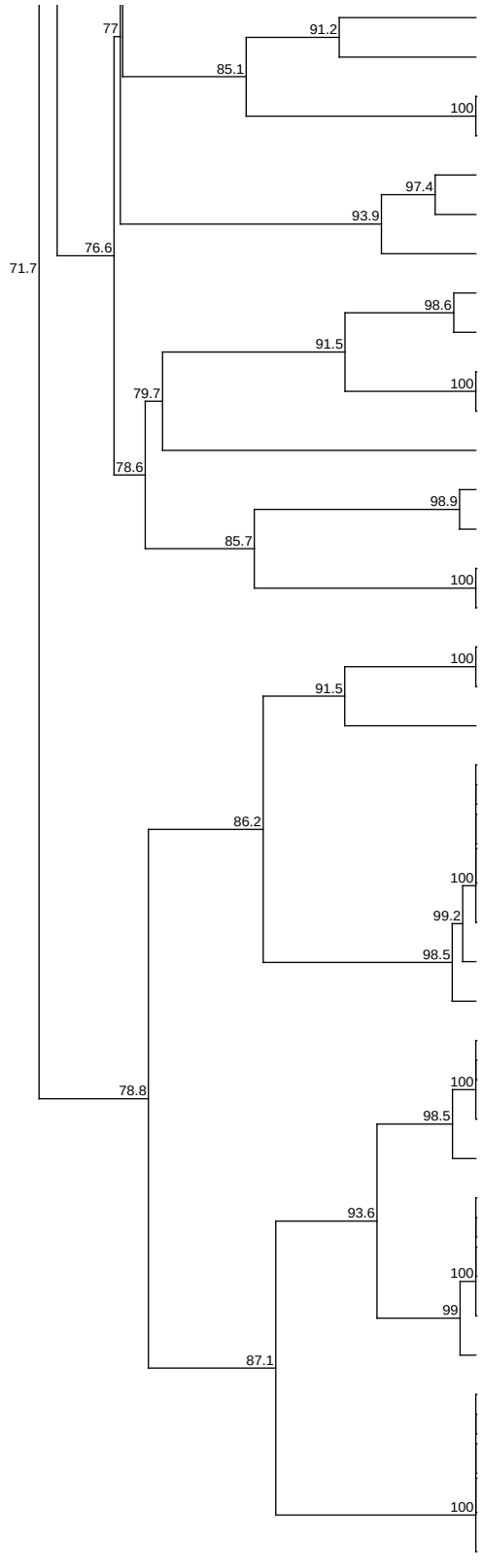
Nucera Figure 1



	Species	Collection site	Bale status	sampling year
	L. innocua	Farm 1	Altered	2007
	L. innocua	Farm 1	Altered	2007
	L. innocua	Farm 1	Altered	2007
	L. innocua	Farm 1	Altered	2007
	L. innocua	Farm 1	Altered	2007
	L. innocua	Farm 2	Altered	2007
	L.seeligeri	Farm 3	Unaltered	2009
	L. innocua	Farm 4	Altered	2010
	L. innocua	Farm 5	Altered	2008
	L. innocua	Farm 5	Altered	2008
	L. innocua	Farm 3	Altered	2008
	L. innocua	Farm 3	Unaltered	2008
	L. innocua	Farm 3	Altered	2008
	L. innocua	Farm 3	Altered	2008
	L. innocua	Farm 3	Altered	2008
	L. innocua	Farm 5	Altered	2008
	L. innocua	Farm 6	Unaltered	2008
	L. innocua	Farm 6	Altered	2008
	L. innocua	Farm 6	Altered	2008
	L. innocua	Farm 6	Altered	2008
	L. innocua	Farm 6	Altered	2008
	L. innocua	Farm 6	Altered	2008
	L. innocua	Farm 5	Altered	2008
	L. innocua	Farm 6	Unaltered	2008
	L. innocua	Farm 6	Unaltered	2008
	L. innocua	Farm 7	Altered	2008
	L. innocua	Farm 7	Altered	2008
	L. innocua	Farm 8	Unaltered	2008
	L. innocua	Farm 8	Unaltered	2008
	L. innocua	Farm 7	Altered	2008
	L. innocua	Farm 7	Altered	2008
	L. innocua	Farm 8	Unaltered	2008
	L. innocua	Farm 7	Altered	2008
	L. innocua	Farm 8	Unaltered	2008
	L. innocua	Farm 8	Unaltered	2008
	L. innocua	Farm 6	Unaltered	2008
	L. innocua	Farm 6	Unaltered	2008
	L. innocua	Farm 5	Altered	2010
	L. innocua	Farm 9	Altered	2010

Continued

Nucera Figure 1 (Continued)



	Species	Collection site	Bale status	sampling year
	L.seeligeri	Farm 1	Altered	2007
	L.seeligeri	Farm 1	Altered	2007
	L.seeligeri	Farm 1	Unaltered	2007
	L.seeligeri	Farm 1	Unaltered	2007
	L.seeligeri	Farm 10	Altered	2009
	L.seeligeri	Farm 11	Altered	2009
	L.seeligeri	Farm 11	Altered	2009
	L. innocua	Farm 1	Altered	2007
	L. innocua	Farm 2	Altered	2007
	L. innocua	Farm 1	Altered	2007
	L. innocua	Farm 1	Altered	2007
	L. innocua	Farm 9	Altered	2010
	L. innocua	Farm 12	Unaltered	2010
	L. innocua	Farm 5	Altered	2010
	L. innocua	Farm 9	Altered	2010
	L. innocua	Farm 9	Altered	2010
	L. monocytogenes	Farm 3	Unaltered	2009
	L. monocytogenes	Farm 3	Unaltered	2009
	L. monocytogenes	Farm 4	Unaltered	2009
	L. monocytogenes	Farm 3	Unaltered	2009
	L. monocytogenes	Farm 3	Altered	2009
	L. monocytogenes	Farm 3	Altered	2009
	L. monocytogenes	Farm 3	Altered	2009
	L. monocytogenes	Farm 3	Altered	2009
	L. monocytogenes	Farm 3	Altered	2009
	L. monocytogenes	Farm 3	Altered	2009
	L. monocytogenes	Farm 4	Unaltered	2009
	L. monocytogenes	Farm 4	Unaltered	2009
	L. monocytogenes	Farm 4	Unaltered	2009
	L. monocytogenes	Farm 5	Altered	2009
	L. monocytogenes	Farm 5	Altered	2009
	L. monocytogenes	Farm 5	Altered	2009
	L. monocytogenes	Farm 2	Altered	2009
	L. monocytogenes	Farm 2	Altered	2009
	L. monocytogenes	Farm 2	Altered	2009
	L. monocytogenes	Farm 3	Unaltered	2008
	L. monocytogenes	Farm 3	Unaltered	2008
	L. monocytogenes	Farm 3	Unaltered	2008
	L. monocytogenes	Farm 3	Unaltered	2008
	L. monocytogenes	Farm 3	Unaltered	2008

Figure caption

Figure 1. Dendrogram generated by the combination of REP and ERIC primers. Each tested strain is reported together with the species it belongs to, the status of the area of the bale from where the strain was isolated, and the detection year. Boxes show PCR profiles with similarity >95%, therefore indistinguishable.