Recovery and PCR-based characterization of *Listeria* strains from total mixed ration and maize silages with different silo management practices

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Introduction The penetration of air into a silage stimulates aerobic bacteria, yeasts and moulds, and causes aerobic deterioration. This aerobic activity results in dry matter (DM) and nutrient losses, the accumulation of pathogens and mycotoxins, and reduced DM intake (Borreani et al. 2018). Among pathogens, *Listeria monocytogenes* is a potentially dangerous foodborne pathogen, which represents a primary concern for the production of Gorgonzola, an Italian Protected Designation of Origin (PDO) blue-veined cheese produced only in the Piedmont and Lombardy regions. The aim of this research was to investigate the occurrence of *Listeria spp*. strains in maize silage fed to cows that produce milk destined for Gorgonzola production, and to characterize the isolated strains by PCR-based method.

Material and Methods A survey was carried out over 4 years in the western Po plain (Novara, Italy) on 18 dairy farms (Italian Friesian cows) that supply milk to a Gorgonzola producing plant. Each farm was visited seven times, and one maize silage (bunker or pile open for feed-out) was examined in detail on each farm (for a total of 120 maize silage silos). Total mixed ration (TMR) samples were collected at the same time as the visit (n = 117). A questionnaire was completed, on each studied farm, with information on the silage-making process, silo covering, silo management and dairy ration composition. Each silo face was examined in detail and silage samples were collected in central (CORE, n = 120) and peripheral (PERIPHERAL, n = 151) zones of the silo, as reported by Borreani and Tabacco (2010). The ISO 11290-1:1996/Amd 1:2004 (2004) method was applied to all collected samples for the isolation of *Listeria spp*. and PCR was adopted to identify *L. monocytogenes*. The other *Listeria* species were identified after 16S rRNA gene sequencing. All silage samples were analysed for DM content, pH, fermentative profile and microbial counts.

Results and Discussion Almost all maize silos had at least one visible spoiled area. The 16S rRNA sequencing resulted in all *Listeria spp*. being classified as *L. innocua*. On 10 farms out of 18, at least one strain of *L. monocytogenes* was isolated from maize silage or TMR, whereas at least one strain of *L. innocua* was isolated from all the surveyed farms. *L. innocua* was detected in 82 and *L. monocytogenes* in 8 out of 271 maize silage samples, respectively, and 91% of the positive samples were collected in peripheral zones or in a part of the silo where spoiling silage was visible (Figure 1). The silage sample collected in the peripheral areas of the silo that were positive to *Listeria spp*. had lower DM, and higher pH, yeast and mould counts than non-contaminated peripheral samples (Table 1). Six maize silage samples from the visible spoiled area were positive to both *L. monocytogenes* and *L. innocua*. More than half of the TMR samples (52%) were contaminated by *L. innocua*, whereas *L. monocytogenes* was detected in 11 out of 117 TMR samples. The contaminated TMR samples had a higher mould count than non-contaminated samples (Table 1). A total of 286 and 46 strains of *L. innocua* and *L. monocytogenes* were PCR-typed, respectively. Non-pathogenic *L. innocua* isolates clustered all at 35% similarity level, thus showing a great diversity. However, 94% of the isolates (n=268) clustered at a 70.9% similarity level, thus indicating a degree of relatedness which may be explained by considering an adaptation to the environmental conditions of the farm. This large cluster gathered strains belonging to TMR and maize silage collected over four different years. In 7 farms out of 18, there were isolates from TMR clustering with those isolated from maize silage at the same sampling date, with values of similarity ranging from 81% up to 94.4%, thus probably indicating a contamination pathway. *Listeria monocytogenes* strains were all clustered at 39.7%, with a less extent of variability than the other species, however the populations were considered quite heterogeneous, considering that all isolates belonged to the same species. However, 87% of the isolates (n=40) grouped at 69.7%, thus also indicating a degree of selection based on the farm environment. On 4

farms, the similarity of TMR and maize silage isolates ranged from 75.9% to 87.6%, probably indicating a route of transmission (Figure 2). The higher proportion of TMR positive to *L. monocytogenes* than silage from the peripheral area of the silo could indicate that some other sources of contamination were present on the farms, and these could have been found in not well preserved baled silage, as reported by Nucera et al. (2016).

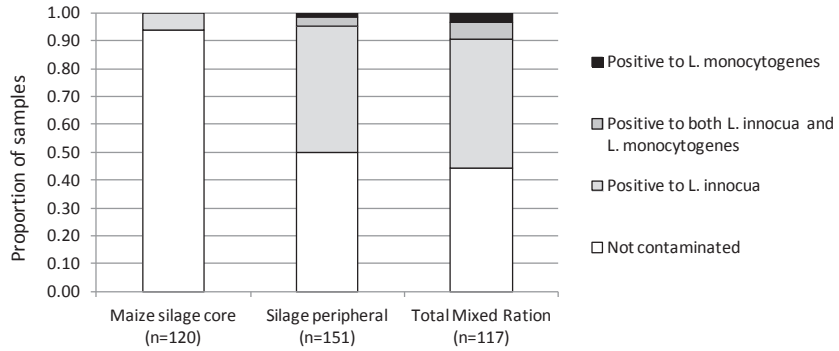

Figure 1. Proportion of samples not contaminated, or positive to *L. innocua*, to *L. monocytogenes* or both, in different zones of maize silage and in total mixed ration, on 18 dairy farms in Novara, Italy.

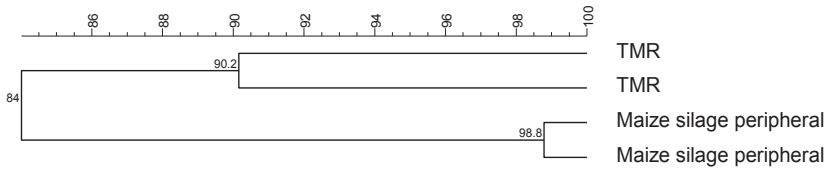

Figure 2. Example of cluster analysis of *L. monocytogene* strains isolated from samples collected in the peripheral area of the silages and TMR on one farm at the same sampling date.

Table 1. Characteristics of maize silage samples collected in the peripheral areas and TMR that were contaminated or not by *Listeria spp*. in the 4-year survey in Novara, Italy.

Variables	Maize silage peripheral (n=151)		*P value*	TMR (n=117)		*P value*
	Not contaminated	Contaminated		Not contaminated	Contaminated	
DM content (g kg^{-1})	31.9	28.0	***	52.9	51.2	NS
pH	4.44	5.84	***	4.90	5.03	NS
Yeast (log cfu g^{-1})	4.48	5.96	***	6.04	5.98	NS
Moulds (log cfu g^{-1})	3.79	6.22	***	3.87	4.47	*

Conclusion Results of this survey show that spoiled maize silage from the peripheral area of a silo could be one of the most relevant sources of the direct contamination of *L. innocua* and *L. monocytogenes* for TMR fed to dairy cows, even though some other farm sources could also be present.

References

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