Dairy farm management practices and the risk of contamination of tank milk from *Clostridium* spp. and *Paenibacillus* spp. spores in silage, total mixed ration, dairy cow feces, and raw milk

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ABSTRACT

The occurrence of *Paenibacillus* and *Clostridium* spores in silage is of great concern for dairy producers because their spores can contaminate milk and damage processed milk and semi-hard cheeses. Spoiled silage is considered to be the main contamination source of the total mixed ration (TMR), feces of dairy cows, and consequently bulk tank milk via the contamination of cow teats by dirt during milking. The presence of an anaerobic and facultative anaerobic sporeformer population in different matrices (soil, corn silage, other feeds, TMR, feces, and milk) and its transmission pathway has been studied on 49 dairy farms by coupling plate count data with 16S-DNA identification. The different matrices have shown a high variability in the anaerobic and facultative anaerobic spore count, with the highest values being found in the aerobically deteriorated areas of corn silages. *Clostridium tyrobutyricum*, *Paenibacillus macerans*, and *Paenibacillus thermophilus* were detected in all the matrices. The TMR spore count was influenced by the amount of spoiled corn silage in the TMR and by the care taken when cleaning the spoiled silage before feed-out. Most of the farms that prevent the presence of visible moldy silage in the silo and carefully clean to remove molded spots were able to maintain their TMR spore counts below 4.0 log spores/g. When a level of 4.5 log spores/g of TMR was exceeded, the feces presented a greater contamination than 3.0 log spores/g. Moreover, the higher the number of spores in the feces was, the higher the number of spores in the milk. Most of the farms that presented a feces contamination greater than 5.0 log spores/g had a higher milk spore contamination than 1,000 spores/L. Careful animal cleaning and good milking practices have been found to be essential to maintain low levels of contamination in bulk tank milk, but it has emerged that only by coupling

these practices with a correct silage management and cleaning during TMR preparation can the contamination of milk by spores be kept at a low level. It has been found that aerobically deteriorated silage has a great capacity to contaminate TMR and consequently to increase the risk of milk spore contamination, even when routine milking practices are adopted correctly.

Key words: silage aerobic deterioration, management practice, *Paenibacillus* spp., *Clostridium* spp

INTRODUCTION

The occurrence of *Paenibacillus* and *Clostridium* spores in silage is of great concern for dairy producers because their spores can survive milk and cheese processing; thus, after germination, some strains are capable of spoiling processed milk and dairy products (te Giffel et al., 2002; Masiello et al., 2017). The ubiquitous nature of these sporeformers and the shift caused by the pasteurization processes may result in sporeformers being the dominant viable microflora, even when the total microbial count in raw bulk milk is at least 100- to 1,000-fold higher than the spore count (Wells-Bennik et al., 2016). Butyric acid fermentation in cheese (late-blowing), caused by the outgrowth of the clostridial spores present in raw milk (Klijn et al., 1995; Le Bourhis et al., 2005), which commonly originate from silage (Vissers et al., 2007c; Julien et al., 2008), can create a considerable loss of product, especially in the production of semi-hard cheeses (Stadhouders et al., 1983; Colombari et al., 2001). Several studies have reported that the initial contamination of milk with spore-forming bacteria occurs on dairy farms (Vissers et al., 2007c; Julien et al., 2008; Masiello et al., 2017) and is mainly due to the presence of spores in soil, silage, and other feeds. Spores are concentrated in the feces of dairy cows and transferred to the raw milk via the contamination of cow teats with dirt during milking (Vissers et al., 2007b). It has been shown that the high risk of sporeformer contamination of bulk tank milk could be attributable to aerobically deteriorated silages,

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which can contribute to increasing the spore contamination of TMR and consequently of cow feces (Jonsson, 1991; Vissers et al., 2007c; Driehuis et al., 2016). When the aerobic deterioration of silages takes place, oxygen penetration indirectly stimulates the growth of anaerobic microorganisms (Jonsson, 1989, 1991). Aerobic microorganisms, mainly yeasts, consume oxygen close to the surface and cause an increase in the silage temperature. Moreover, the aerobic/anaerobic zone moves toward the surface of silage exposed to air and anaerobiosis is restored in the deeper parts (Jonsson, 1989). *Clostridium tyrobutyricum* and other anaerobic or facultative anaerobic sporeformers (mainly clostridia and *Paenibacillus* spp.) can grow and multiply in this ecosystem in micro-niches with less inhibitory activity (Jonsson, 1989; Borreani et al., 2013). The increase in the anaerobic spore content of silage, due to air penetration, has been observed for whole-plant corn silage (Vissers et al., 2007a; Borreani and Tabacco, 2008), wilted alfalfa (Colombari et al., 2001), whole-plant grain sorghum (Tabacco et al., 2009), and grass silage (Jonsson, 1991; Vissers et al., 2007a). Furthermore, the multiplication of *Paenibacillus* spp. has also been reported in aerobically deteriorated corn (Borreani et al., 2013) and grass silages (Driehuis et al., 2016). This leads to a different distribution of spores at the silage feed-out face when aerobic deterioration takes place (Tabacco and Borreani, 2002; Vissers et al., 2007a). The improper incorporation of any deteriorated part from the top layers of the silo in the feed mixer could increase contamination of the ration with filamentous fungi and anaerobic spores (Borreani and Tabacco, 2014; Borreani et al., 2018). Borreani and Tabacco (2014) found that the anaerobic spore count in corn silage increased linearly with an increase in the mold count, thus showing that the inclusion of parts of the silage that are visually spoiled (higher mold count than 5.0 log cfu/g) increases the risk of worsening the spore contamination of the TMR fed to lactating dairy cows.

When dairy cows are fed with contaminated feeds or TMR, the sporeformers are concentrated in the gastrointestinal tract, due to digestive processes, and they then pass to the feces, whereby the risk of milk contamination becomes greater, especially when low hygiene practices are adopted during milking (Stadhouders and Jørgensen, 1990; Vissers et al., 2007b).

Among the clostridia that are considered of concern for milk and cheese processing, *C. tyrobutyricum* is the most frequently detected in late-blown cheeses, and it is considered the principal causative agent of butyric acid fermentation in cheese (Klijn et al., 1995). However, spores of other *Clostridium* species, such as *C. butyricum*, *Clostridium beijerinckii*, and *Clostridium sporogenes*, which occur in raw milk, have been associ-

ated with butyric acid fermentation and are believed to be responsible for causing late-blowing defects (Le Bourhis et al., 2005; Driehuis et al., 2016). Spores of other clostridia, and *Clostridium bifermentans*, *Clostridium perfringens*, and *Clostridium tertium* in particular, have also been isolated from natural and processed cheeses and raw milk, and their role as enhancers of the late-blowing defect has been suggested (Le Bourhis et al., 2007). In the last few decades, studies have shown that many organisms that were formerly classified in the *Bacillus* genus actually represent several genera of the *Bacilli* class (Durak et al., 2006). Members of the *Paenibacillus* genus can survive pasteurization in spore form and are able to grow under refrigeration, thus resulting in product spoilage and limiting the shelf life of HTST pasteurized fluid milk (Ivy et al., 2012). Among the *Paenibacillus* spp., the most frequently reported are *Paenibacillus odorifer*, *Paenibacillus graminis*, *Paenibacillus amylolyticus*, *Paenibacillus* c.f. *peoriae*, and *Paenibacillus polymyxa* in milk (Ivy et al., 2012; Driehuis et al., 2016), and *P. polymyxa*, *Paenibacillus pabuli*, and *Paenibacillus macerans* in silages (Borreani et al., 2013; Driehuis, 2013; Driehuis et al., 2016).

The objectives of this study were (1) to verify on farm the role of the aerobic deterioration of corn silage on the proliferation of *Clostridium* and *Paenibacillus* spores; (2) to identify, through 16S-DNA sequencing, the dominant species of anaerobic and facultative anaerobic sporeformers along the milk chain from the farm soil to the bulk tank milk; and (3) to evaluate whether careful management practices, aimed at reducing the amount of aerobic deteriorated corn silage that reaches TMR, could mitigate the carry-over of anaerobic and facultative anaerobic spores in milk.

MATERIALS AND METHODS

Farm Selection and Survey Design

A survey was carried out over a 2-yr period (2013–2014) in the western Po plain (Italy) on 49 dairy farms (Italian Friesian breed) that produced milk destined for Grana Padano Protected Designation of Origin (PDO) cheese. The farms were selected in agreement with the technicians from the “Consorzio di Tutela Grana Padano” to be representative of the Grana Padano production area (around 150,000 t of cheese produced in 2017 in 127 dairy plants in the Lombardia, Veneto, Piemonte, and Emilia-Romagna regions of Italy). The farms were selected on the basis of the farmers' willingness to participate in the survey, and a previous classification of the anaerobic spore levels in the bulk tank milk from a group of 156 farms that supply milk to 2 cheese factories in Lombardy (Italy), and in which

Table 1. Summary of the surveyed management practices and samples collected during farm visits to 49 dairy farms in Lombardy (Italy)

Factor	Description	No. of samples
Soil	Information: crop succession in the last 5 yr; slurry and manure application rates, method, and timing of the application. Samples: 5 to 10 randomly selected 0- to 30-cm cores from a representative cultivated area.	49 (soil)
Corn silage	Information: amount of silage consumed daily and feeding rates, filling and packing methods, silo cover, materials used to hold the cover on the top of the silo surface, silo face area, visible spoiled area (area visibly spoiled/face area), temperatures at the silo face according to Borreani and Tabacco (2010). Samples: samples collected from the core (C) of the silo (pool of 5 cores), from the top close to the sealant film (A1, pool of 5 grabs), from the zone immediately above the top (BA1, pool of 5 grabs).	49 (C); 47 ¹ (A1); 49 (BA1)
TMR	Information: composition of the rations of dairy cows, number of distributions per day. Samples: 5 to 6 randomly selected grabs, pooled to form 1 sample per farm.	49 (TMR)
Herd and cow hygiene	Information: number of milking cows, average milk production per cow, average milk production per year, cow hygiene score in the housing area, percent dirty of cow udders [teat cleanliness scoring system: 5 = clean (no visible dirt); 4 = almost clean (approximately <10% of the area dirty); 3 = slightly dirty (10 to 20% of the area dirty); 2 = dirty (20 to 50% of the area dirty); and 1 = extremely dirty (>50% of the area dirty)] on 10% milking cows randomly selected in the housing area. Samples: feces from 10% milking cows randomly selected from the herd and pooled.	49 (feces)
Milking routine	Information: gloves worn during milking, average milking time, use, type, and product used for pre-milking teat disinfection, if and how udders were dried, use of forestripping, and use, type, and product of postmilking dipping. Samples: pooled milk sample from the farm tank, pooled sample of drinking and cleaning water drawn from the tap.	49 (milk); 49 (water)

¹Two farms did not present any visible molded area.

MPN spores are tested monthly. All the farmers were fully informed about the design of the experiment, the nature of the data being collected, and their future use. A detailed questionnaire (with questions on feed production and management practices) was presented to the farmers on each farm and the information collected on each farm is summarized in Table 1.

Sample Preparation and Analyses

The number of sub-samples and resulting pooled samples collected on the farms are reported in Table 1.

The corn silages used in the lactating cow rations at the time of the visits were sampled on each farm. Samples were collected from 3 areas in each silo: the core, at least 1 m from the surface ($n = 49$); and 2 peripheral areas, close to the sealant film (**A1**), the outermost 0.15 m of visibly spoiled silage, which is usually discarded by farmers before feeding ($n = 47$, since 2 farms did not present any visible molded area); and below A1 (**BA1**, 0.15 to 0.30 m immediately below the A1 layer, which is always fed to dairy cows; $n = 49$). The samples were collected using a sterile corer (45 mm diameter; 250 mm depth) in the core area (mixing 5 randomly collected samples) and by means of 5 hand grabs (around 100 g) in the A1 and BA1 sections. The temperature was measured at each sampling point of the silo to a depth of 200 mm (Borreani and Tabacco, 2010) using a probe thermometer. The working face

area that was visibly molded/spoiled was also determined and measured to calculate the percentage of the working face that was aerobically deteriorated. Each silo was then inspected to determine whether the aerobically deteriorated silage that was discarded before silage was included in the TMR, and the farms were classified into 3 categories (farms on which deteriorated silage was not, partially, or well discarded). Farm silage were assigned to each class following these assumptions: well-discarded silage = no visible spoiled silage was included in the TMR; partially = some traces of spoiled silage were included in the TMR; not discarded = most of the spoiled silage present in the outer layer still remained and was included in TMR. The resulting TMR fed to the lactating cows was collected immediately after unloading (5 to 6 sub-samples of about 200 g, pooled in one farm sample; $n = 49$). Other feeds were also sampled if their incidence in the TMR was higher than 20% on a DM basis ($n = 46$, of which 19 were commercial mixes, 14 were corn grain meals, 1 was a soybean meal, and 12 were grass or alfalfa hays).

Five to 10 random soil sub-samples (0–300 mm) were collected on each farm and pooled into one composite soil sample, which was representative of where corn silage was grown ($n = 49$). Feces from a random 10% of lactating cows were collected and pooled into one farm sample ($n = 49$). A pooled drinking and cleaning water sample was collected from the farm taps ($n = 49$). One milk sample (1 L) from the farm bulk tank was also

collected after 15 min of continuous mechanical mixing on each farm ($n = 49$).

Each corn silage was analyzed for its chemical, microbial, and fermentative characteristics, whereas the other feeds, TMR, feces, soil, bulk tank milk, and water were only analyzed for anaerobic and facultative anaerobic sporeformers.

Each silage sample was mixed thoroughly and divided into 3 sub-samples. The first sub-sample was analyzed to determine the DM concentration, which was achieved by oven drying the sample at 65°C until constant weight was reached.

Thirty grams of the second wet silage sub-sample was transferred to a sterile homogenization bag, suspended 1:10 wt/vol in a peptone physiological salt solution (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 were enumerated separately on plates that yielded 1 to 100 cfu per Petri dish. The mold and yeast colony-forming units were enumerated separately, according to their macro-morphological features. The anaerobic and facultative anaerobic spores were counted after pasteurization of the serial dilutions at 80°C for 10 min to inactivate vegetative cells and to trigger the germination of spores, and this was followed by the streak plate technique, which was conducted with 50.0 g/L of reinforced clostridial medium agar (RCM; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 0.005% of neutral red (Sigma-Aldrich, Merck KGaA), and 200 mg/kg of D-cycloserine (Sigma-Aldrich, Merck KGaA) incubated anaerobically for 7 d at 35°C (Jonsson, 1990). Colonies showing evidence of gold-yellow fluorescence under UV light were considered for identification.

Thirty grams of the third wet silage sub-sample was transferred to a homogenization bag, suspended 1:10 wt/vol in deionized water, and homogenized for 4 min in a laboratory Stomacher blender for quantification of the pH value and nitrate content. The pH value was determined through the use of specific electrodes. The nitrate content was determined in the water extract, through semiquantitative analysis, using Merckoquant test strips (Merck KGaA; Borreani and Tabacco, 2008).

About 50 g of the same sub-sample was homogenized and extracted for 4 min in a Stomacher blender in H_2SO_4 0.05 M at an acid-to-sample material (fresh weight) ratio of 5:1. The solution was filtered with a

0.20- μm syringe filter and used for quantification of the lactic and monocarboxylic acids (acetic, propionic, and butyric acids) with an HPLC (Agilent Technologies, Santa Clara, CA; Canale et al., 1984). Ethanol and 1,2-propanediol were determined by means of HPLC, coupled to a refractive index detector, on an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA). The analyses were performed isocratically under the following conditions: mobile phase 0.0025 M H_2SO_4 , flow rate 0.5 mL/min, column temperature 37°C, and injection volume 100 μL . Duplicate analyses were performed for all the determined parameters.

Anaerobic and facultative anaerobic spores of the farm soils, other feeds, TMR, feces, water, and tank milk were measured and identified, as reported for the silage samples. The detection limit of the anaerobic and facultative anaerobic spores in the silage, TMR, soil, and feces was 50 spores/g and it was 62 spores/L in the milk. To improve the detection limit of milk collected on farms with low levels of spores, 40 mL of milk was centrifuged ($3,600 \times g$ for 15 min at 20°C), the supernatant was removed, and the resultant pellet was re-suspended in 1 mL of a peptone physiological salt solution, in Eppendorf vials (Eppendorf AG, Hamburg, Germany), and re-centrifuged ($17,980 \times g$ for 3 min at 20°C). The pellet was then re-suspended in 0.5 mL of a peptone physiological salt solution, which was plated onto 2 RCM streak plates [1 colony in 0.2 mL (2 plates), and this resulted in a detection limit of 62 spores/L].

The milk fat, protein, lactose, urea, and casein contents were measured on a subsample by means of Fourier transform infrared spectroscopy (MilkoScan FT6000, Foss Electric A/S, Hillerød, Denmark). The SCC and total bacteria counts were automatically determined in the same subsamples by the mean of Fossomatic FC (Foss Electric A/S) and BactoScan FC (Foss Electric A/S), respectively.

Bacterial Isolation and Identification

Up to 6 yellow colonies were selected from each sample by visually identifying and picking out the distinct morphologies. Up to 10 colonies were selected in the milk with more than 1,000 spores/L. Each colony was purified by streaking on RCM agar and subsequently incubated for 7 d at 37°C under anaerobiosis. Phenotypic characterization was performed by conducting routine laboratory tests, and the isolates were analyzed by observing the colony morphology, Gram staining, the ability to grow anaerobically on RCM, and the presence of spores. Only gram-positive, obligate anaerobic, or facultative anaerobic endospore forming isolates were then studied in detail. Pure cultures were grown in BHI broth (Merck KGaA) for 7 d at 37°C under anaerobiosis

before freezing in 20% glycerol at -80°C . The cultures were grown in BHI for 7 d in anaerobic chambers (Anaerocult Kit, Biomerieux, Milan, Italy) and the DNA was then extracted following the extraction kit procedures (Ultra Clean Microbial Isolation Kit, Mo Bio Laboratories Inc., Cabru, Milan, Italy). Genomic DNA was amplified using 16S universal primers (527 bp), according to the manufacturer's instructions (Micro seq 500 16S rDNA Bacterial Sequencing Kit). The used primers, that is, primer sequence 5'-TGGAGAGTTT-GATCCTGGCTCAG-3' and reverse primer sequence 5'-TACCGCGGCTGCTGGCAC-3', have already been published by Hall et al. (2003). The PCR reactions were performed using a TGradient thermal cycler (VWR, Milan, Italy). Each 25 μL of PCR reaction contained 2.5 μL of DNA template (50 ng), 200 mM deoxynucleotide triphosphate, 5 μL of 10X buffer (Taq DNA Polymerase, Qiagen, Chatsworth, CA), 0.7 mM primer, and 1.0 U of Taq DNA Polymerase (Qiagen). The PCR program was 95°C , 3 min; 34 cycles: 94°C , 15 s; 55°C , 45 s; 72°C , 55 s; and a final extension at 72°C for 7 min. A 10- μL aliquot of the PCR products from each reaction was electrophoresed in 1.5% agarose gel and then stained with SYBR SAFE (Invitrogen, Eugene, OR). Gel images were acquired using a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA). The amplifications generated by 16S rDNA PCR were purified and then sent to an external laboratory (IGA Technology Services, Udine, Italy) for sequencing. The obtained chromatograms were visually inspected for the absence of background noises, and only sequences with clear and unambiguous peaks were used for the subsequent analysis steps. Any areas of the sequence characterized by unresolved peaks were removed from the analysis. The generated sequences were compared with those present in the GenBank database, using BLASTn sequence similarity searching [<https://blast.ncbi.nlm.nih.gov/Blast.cgi>; ribosomal RNA sequences (bacteria and archaea)]. The highest identity was selected as the identified species or genus when sequence similarity was $\geq 98\%$ (with no gaps). A match was considered appropriate when the query sequence only matched the selected level of one bacterial species. If more species matched at the same similarity level, identification was not considered to have been achieved at the species level, but only at the genus level.

Statistical Analysis

The microbial counts were $\log_{10}$ transformed and were presented on a wet weight basis. The values below the detection limit (that is, 50 spores/g in the soil, silage, TMR, and feces, and 62 spores/L in the milk) were

assigned a value corresponding to half of the detection limit to calculate the average value.

The data were analyzed for their statistical significance, via ANOVA, with their significance reported at a 0.05 probability level, using the General Linear Model of the Statistical Package for Social Science (v 25.0, SPSS Inc., Chicago, IL) with the following statistical model: $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where Y_{ij} = observation, μ = overall mean, α_i = the corn silage spore content (low, medium, high) or management practices (well, partially, or not discarded molded silage), and ε_{ij} = error. When the calculated values of F were significant, the Tukey post-hoc test ($P < 0.05$) was used to interpret any significant differences among the mean values. Pearson correlation coefficients of the milk spores, percentage of molded silo face, TMR spores, feces spores, and animal cleaning score, and their level of significance were determined for each management practice (well, partially, or not discarded molded silage).

RESULTS

Farm Characteristics

The number of lactating cows per farm ranged from 16 to 301 (mean value of 105 cows) and the main cow breed was Holstein. All the farms milked twice daily, as reported in the production disciplinary regulations for Grana Padano PDO cheese. Cows were housed in freestalls (77%) or tiestalls (23%). The average milk production across farms was 8,400 kg per cow per year and ranged from 4,150 to 10,970 kg. The utilized agricultural area of the surveyed farms ranged from 8 ha to 208 ha (mean value of 48 ha). The milk intensity reported as the fat- and protein-corrected milk produced per hectare of utilized agricultural area was on average 24 t/ha, with values ranging from 4 to 61 t of fat- and protein-corrected milk/ha. All the farms included corn silage in their milking cow TMR. The average inclusion was 33% on a DM basis, with values ranging from 9 to 49% of the DMI. Some farms ($n = 14$) had other silages (e.g., sorghum silages, Italian ryegrass silage), but they used them exclusively for replacement heifer feeding.

Anaerobic and Facultative Anaerobic Sporeformers Isolated in Different Matrices

The anaerobic and facultative anaerobic sporeformer counts (log spores/g) in the soil, corn silage, other feeds, TMR, and feces of the 49 surveyed dairy farms are reported in Figure 1. The spore content was below the detection limit in all the drinking and cleaning water samples. The soil spore content had a median value of around 2.9 log spores/g, with 50% of the samples

ranging from 2.7 to 4.1 log spores/g. The core area of the corn silages had a median spore count of 2.6 log spores/g, with 75% of the samples below 3.4 log spores/g. The peripheral samples, that is, BA1 and A1, had median values of 4.8 and 6.0 log spores/g, respectively, with only 25% of the samples being below 3.8 and 5.3 log spores/g. Other feeds included in the ration of lactating cows presented average spore counts of 1.98 ± 0.40 and 2.06 ± 0.48 log spores/g for dry forages (hays and straw) and concentrates (corn grain, soybean meal, and commercial mixes), respectively. The TMR had a median value of 4.3 log spores/g, with 50% of the samples ranging from 3.9 to 5.0 log spores/g. Feces showed a similar box plot to that of TMR, but with slightly lower values, and with a median value of 3.8 log spores/g.

Overall, the dominant species of anaerobic and facultative anaerobic sporeformers identified in the different matrices belonged mainly to the *Clostridium* and *Paenibacillus* genera. All the identified species and their number are reported in Table 2, where they are clustered according to the different studied matrices (soil, corn silage, other feeds, TMR, feces, and bulk tank milk). Overall, 26 species of *Clostridium* and 12 species of *Paenibacillus* were found among the studied matrices. Furthermore, *Bacillus* spp., *Brevibacillus* spp., and *Lysinibacillus* spp. were also identified as

facultative anaerobic bacteria. *Clostridium tyrobutyricum*, *P. macerans*, and *Paenibacillus thermophilus* were observed in all of the matrices. Five species of clostridia (*C. tyrobutyricum*, *C. sporogenes*, *Clostridium aerotolerans*, *Clostridium celerecrescens*, and *Clostridium xylanolyticum*) and 2 species of *Paenibacillus* (*P. macerans* and *P. thermophilus*) were found in at least 4 matrices. Twelve species of clostridia and 4 of *Paenibacillus* were found in the corn silage.

Spore Population in Farm Soils

Figure 2 reports the distribution of the dominant species found in the soil, corn silage, other feeds, TMR, and feces. The anaerobic and facultative anaerobic spore-forming population of the soils was made up of 49% of clostridia species and 30% of *Paenibacillus* species. Of the dominant species, *C. tyrobutyricum*, *C. aerotolerans*, *C. celerecrescens*, *P. macerans*, and *P. thermophilus* presented higher frequencies than 5%.

Spore Population in Corn Silages

The corn silage samples were split, in relation to their anaerobic and facultative anaerobic spore-former contents, into 3 classes: low spore contamination (below 3.0 log spores/g), medium spore contamination (between

Figure 1. Box plot of the anaerobic and facultative anaerobic spore-forming bacteria of the soil, corn silage, other feeds, TMR, and feces of the 49 commercial dairy farms. Boxes = interquartile range; bold line = median; whiskers = minimum and maximum values; dots = outliers. A1 = silo peripheral areas close to the sealant film; BA1 = below A1.

Table 2. Number of isolates of the predominant anaerobic and facultative anaerobic spores from farm soil, corn silage, other feeds, TMR, feces, and tank milk¹

Bacterial species	Soil	Corn silage	Other feeds	TMR	Feces	Milk	Total isolates
<i>Clostridium</i> spp.							
<i>Clostridium acetobutylicum</i>		2					2
<i>Clostridium acidisoli</i>						1	1
<i>Clostridium aerotolerans</i>	13	10		10	14		47
<i>Clostridium alginifacis</i>					7		7
<i>Clostridium aminovalericum</i>		16				2	18
<i>Clostridium amygdalinum</i>		9			1		10
<i>Clostridium beijerinckii</i>				20		1	21
<i>Clostridium bif fermentans</i>			2			4	6
<i>Clostridium butyricum</i>				5	9	3	17
<i>Clostridium celerecrescens</i>	16	37	1	12	8		74
<i>Clostridium felsineum</i>		3					3
<i>Clostridium frigidicarnis</i>					8		8
<i>Clostridium guangxiense</i>		1		2			3
<i>Clostridium isatidis</i>	6						6
<i>Clostridium jejuense</i>		16		2			18
<i>Clostridium neuense</i>		4		1			5
<i>Clostridium perfringens</i>	15		1				16
<i>Clostridium saccharolyticum</i>				2			2
<i>Clostridium sardiniense</i>			1				1
<i>Clostridium sartagoforme</i>				1	1		3
<i>Clostridium schirmacherense</i>			1				1
<i>Clostridium sporogenes</i>	8	22	5	3	7		46
<i>Clostridium subterminale</i>			1		7		8
<i>Clostridium sulfidigenes</i>					5		5
<i>Clostridium tyrobutyricum</i>	23	27	1	24	9	20	104
<i>Clostridium xylanolyticum</i>	8	132	1	37	21		199
Other <i>Clostridium</i> spp. ²	9	23	1	22	17	2	74
<i>Paenibacillus</i> spp.							
<i>Paenibacillus amylolyticus</i>	14						14
<i>Paenibacillus barcinonensis</i>				2			2
<i>Paenibacillus barengoltzii</i>		3				1	4
<i>Paenibacillus cookii</i>				4		2	6
<i>Paenibacillus fonticola</i>					7		7
<i>Paenibacillus ginsegagri</i>				2			2
<i>Paenibacillus lactis</i>					13		13
<i>Paenibacillus lautus</i>				2			2
<i>Paenibacillus macerans</i>	18	173	19	16	13	42	281
<i>Paenibacillus pabuli</i>					6		6
<i>Paenibacillus polymyxa</i>			1	3		2	6
<i>Paenibacillus thermophilus</i>	15	23	8	32	25	3	106
Other <i>Paenibacillus</i> spp. ²	17	36	2	20	32	26	133
Others							
<i>Bacillus</i> spp.	28	26	2	27			83
<i>Brevibacillus</i> spp.	15			8			23
<i>Lysinibacillus</i> spp.		11		2			13
Other unclassified			4	5	79	12	100
Total <i>Clostridium</i> and <i>Paenibacillus</i> species	162	537	45	224	210	111	1,289
Total isolates	205	574	51	266	289	123	1,508

¹The species were identified by means of 16S universal primers when sequence similarity was $\geq 98\%$ in the GenBank database, using BLASTn sequence similarity searching (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

²Other *Clostridium* and *Paenibacillus* species that were not possible to classify at the species level.

3.0 and 5.0 log spores/g), and high spore contamination (above 5.0 log spores/g). The mean fermentative and microbial characteristics of the 3 silage classes were reported in Table 3. The low contaminated silages are mainly constituted by core silages (89.5%), and to a lesser extent by BA1 silages (10%); no silage collected from the A1 zone was present in this class. Most of the silages in the highly contaminated silage class were

collected in the peripheral areas of the silo (34.5 and 63.8% from BA1 and A1, respectively), whereas only one sample was from the silage core. The fermentative characteristics of the low contaminated silages were typical of well-fermented lactic acid silages, with a low incidence of butyric acid (only 2 samples had a higher concentration than 1 g/kg of DM), whereas the medium and high spore contamination silage classes presented

higher numbers of samples containing butyric acid, with 3 samples (one from A1 and 2 from BA1) having a higher butyric acid content than 10 g/kg of DM. Most of the medium and highly contaminated silages presented the typical fermentative profiles of aerobic deteriorated silages, with mean temperatures above 30°C, higher values of pH, yeast, and mold counts than the low contaminated silages, and low concentrations of fermentative products inhibiting the spoilage microorganisms (lactic and acetic acids and ethanol). Nitrates were present in 32% of the low contaminated silages, whereas a lower frequency (9.6% of the samples) was observed in the medium and highly contaminated samples.

The dominant clostridia found in the corn silages were *C. tyrobutyricum*, *C. aerotolerans*, *Clostridium aminovalericum*, *C. celerecrescens*, *Clostridium jejuense*, *C. sporogenes*, and *C. xylanolyticum*, whereas the *Paenibacillus* species were *P. macerans*, *P. thermophilus*, and *P. cookii*. Six species (*C. tyrobutyricum*, *C. sporogenes*, *C. celerecrescens*, *C. xylanolyticum*, *P. macerans*, and *P. thermophilus*) were present in all the zones of the silages (data not shown). The low contaminated silages were characterized by 5 dominant clostridia species, with *C. xylanolyticum* representing 38% of the total population. *Clostridium tyrobutyricum*, *C. sporogenes*, *C. aerotolerans*, and *C. celerecrescens* were observed, but at lower frequencies. *Paenibacillus macerans* was the main species of this genus and represented 25% of the total

anaerobic and facultative anaerobic sporeformers of the low contaminated silages. The medium contaminated silages presented greater frequencies of *C. celerecrescens* and *P. macerans* than the lower contaminated silages. *Clostridium tyrobutyricum* and *C. sporogenes* increased in frequency in the highly contaminated silages, whereas the frequency of *C. xylanolyticum* decreased from 40% in the medium contaminated silages to 3%.

Spore Populations in Other Feeds, TMR, and Feces

The spore content of the other feeds ranged from 1.40 to 3.06 log spores/g. The dominant population was represented by the *Paenibacillus* genus (67%) and the *Clostridium* genus (33%). The most frequently detected dominant species were *P. macerans* (42%), *P. thermophilus* (18%), and *C. sporogenes* (11%).

The TMR spore counts ranged from 2.11 to 5.89 log spores/g. Moreover, 5 farms presented a TMR of below 3.0 log spores/g, 34 farms a TMR of 3.0 to 5.0 log spores/g, and 10 farms a greater TMR than 5.0 log spores/g.

The distribution of the dominant species for the TMR contamination level was divided into 3 classes [as suggested in previous works by Stadhouders and Jørgensen (1990) and Stadhouders and Spoelstra (1990)]: a low spore contamination below 3.0 log spores/g, a medium spore contamination between 3.0 and 5.0 log spores/g, and a high spore contamination above 5.0

Figure 2. Distribution of the dominant anaerobic and facultative anaerobic spore-forming bacteria isolates in the soil, corn silages (divided into 3 contamination classes), other feeds, TMR (divided into 3 contamination classes), and feces of the 49 commercial dairy farms in northern Italy.

Table 3. Mean values (ranges in parentheses) of chemical and microbiological characteristics when samples ($n = 145$) were partitioned into 3 classes on the basis of their spore content (low, medium, or high)¹

Item	Corn silage spore content				SE	P-value
	Low (<3.0 log spores/g; n = 38)	Medium (3.0 to 5.0 log spores/g; n = 49)	High (>5.0 log spores/g; n = 58)			
Anaerobe spore (log spores/g)	2.16 ^c	4.11 ^b	6.10 ^a	0.143	<0.001	
Core samples (%)	89.5	28.6	1.7	—	—	
BBA1 samples (%)	10.5	51.0	34.5	—	—	
A1 samples (%)	0	20.4	63.8	—	—	
DDM content (%)	35.7 ^a (27.9–43.3)	32.6 ^a (17.8–46.2)	26.1 ^b (13.8–44.6)	0.646	<0.001	
pH	3.78 ^c (3.50–4.15)	4.40 ^b (3.45–7.56)	6.01 ^a (3.81–8.19)	0.122	<0.001	
Yeast (log cfu/g)	1.53 ^c (<1.00–4.76)	3.75 ^b (<1.00–7.78)	5.89 ^a (3.15–8.02)	0.206	<0.001	
Mold (log cfu/g)	1.30 ^c (<1.00–4.41)	2.84 ^b (<1.00–8.07)	6.14 ^a (<1.00–8.44)	0.216	<0.001	
Nitrates (mg/kg of DM)	308 ^a (<100–2,200)	103 ^{ab} (<100–2,988)	52 ^b (<100–980)	36.2	0.014	
Lactic acid (g/kg of DM)	48.2 ^a (10.8–94.4)	32.2 ^b (<0.1–84.1)	9.5 ^c (<0.1–68.7)	2.01	<0.001	
Acetic acid (g/kg of DM)	24.7 ^a (9.8–49.3)	23.0 ^a (<0.1–102.8)	8.8 ^b (<0.1–43.9)	1.30	<0.001	
Propionic acid (g/kg of DM)	3.1 ^{ab} (<0.1–12.2)	5.4 ^a (<0.1–42.4)	1.9 ^b (<0.1–11.7)	0.416	0.002	
Butyric acid (g/kg of DM)	0.1 (<0.1–1.7)	1.0 (<0.1–24.7)	0.8 (<0.1–16.5)	0.238	0.385	
Ethanol (g/kg of DM)	6.8 ^a (<0.1–21.5)	2.5 ^b (<0.1–14.4)	0.3 ^c (<0.1–5.3)	0.363	<0.001	
1,2 Propanediol (g/kg of DM)	7.6 ^a (<0.1–25.9)	4.3 ^b (<0.1–27.8)	1.1 ^c (<0.1–11.6)	0.477	<0.001	
Sample temperature (°C)	24.4 ^c (17.8–48.8)	31.7 ^b (17.59–50.0)	37.6 ^a (22.4–59.8)	0.806	<0.001	
No. of samples with butyric acid between 1 to 10 g/kg of DM	2/38	3/49	7/58	—	—	
No. of samples with butyric acid above 10 g/kg of DM	0/38	2/49	1/58	—	—	

^{a,c}Within a row, mean values with the same superscript letter are not significantly different ($P < 0.05$).

¹Samples collected in 49 dairy farms of the Po plain of Italy; 2 farms did not present visible spoiled silage. Proportion of samples from core, below area 1 (BA1), and area 1 (A1) are reported for each contamination class.

log spores/g (Figure 2). Clostridia represented 63, 54, and 52% and *Paenibacillus* spp. 19, 17, and 19% of the total anaerobic and facultative anaerobic sporeformers for the 3 contamination classes, respectively. The dominant species of the low contaminated TMR were *C. tyrobutyricum*, *C. beijerinckii*, and *P. macerans*. A greater variability of the dominant species was observed in the medium and high contamination classes. *Clostridium xylanolyticum*, *C. tyrobutyricum*, and *P. thermophilus* had frequencies that were greater than or equal to 10% in the medium contamination class, and *C. aerotolerans*, *C. beijerinckii*, *C. celerecrescens*, *C. butyricum*, *C. sporogenes*, and *P. macerans* were present at lower frequencies. *Clostridium xylanolyticum*, *C. celerecrescens*, and *P. thermophilus* had greater frequencies than 10% in the high contamination class, and *C. aerotolerans*, *C. beijerinckii*, *C. butyricum*, and *C. tyrobutyricum* were present at lower frequencies.

The spore contamination of the feces ranged from 2.15 to 5.32 log spores/g. The anaerobic and facultative anaerobic spore-forming population of the feces was represented by 39% of the clostridia species and 33% of the *Paenibacillus* species. *Clostridium xylanolyticum*, *C. aerotolerans*, *P. macerans*, and *P. thermophilus* presented higher frequencies than 5% in the dominant species (Figure 2). *Clostridium aerotolerans*, *C. celerecrescens*, *C. beijerinckii*, *C. butyricum*, and *C. tyrobutyricum* were present at lower frequencies.

Spore Population in Bulk Tank Milk

The milk samples ranged from <1.79 to 3.82 log spores/L, with a median value of 1.79 log spores/L. The milk samples were split, in relation to the spore contamination level, into 3 classes: low spore contamination (below 200 spores/L); medium spore contamination (between 200 and 1,000 spores/L); and high spore contamination (above 1,000 spores/L). These classes represented 2 critical contamination levels that could influence the hard cheese making process: without any additive <200 spores/L (Bottazzi and Battistotti, 1978; Bergère and Sivelä, 1990) or with additives (nitrates or lysozyme) >1,000 spores/L (Walstra et al., 2005). The farms were distributed into classes as follows: 35 farms <200 spores/L (of which 20 farms were below 62 spores/L), 8 farms between 200 and 1,000 spores/L, and 6 farms >1,000 spores/L.

Among all the spore-forming species in the milk, clostridia represented 36, 58, and 0% of the 3 classes, respectively. The dominant anaerobic and facultative anaerobic sporeformers of the bulk tank milk are reported in Figure 3. The most frequently identified species in the low- and medium-contaminated milk was *C. tyrobutyricum*, whereas no clostridia were found as

Figure 3. Distribution of the dominant anaerobic and facultative anaerobic spore-forming bacteria in the tank milk (divided into 3 contamination classes) of the 49 commercial dairy farms in northern Italy.

the dominant species in the highly contaminated milk. *Clostridium butyricum*, *C. beijerinckii*, and *C. sporogenes* were present at lower frequencies. The *Paenibacillus* species represented 60, 38, and 100% in the milk of the 3 contamination classes, respectively. *Paenibacillus macerans* and *P. thermophilus* were the most frequently identified species.

Spore Contamination Pathway from Silage to Tank Milk

The scatter plot of the TMR spore count and the visibly molded silo face, as affected by the different management practices adopted to discard deteriorated silage, is reported in Figure 4. The higher the presence of moldy areas in the silage was, the higher the anaerobic and facultative anaerobic spore count in the TMR. Four out of 15 farms that managed aerobic deterioration in corn silage well were found to be able to keep the TMR spore count below 3.0 log spores/g, whereas the other farms that managed the aerobic deterioration of silage well (reduced aerobic spoiled silage and good cleaning/discarding of the spoiled part, if present) were always below 5.0 log spores/g. Farms that did not carry out an effective aerobic deterioration management or cleaning of the silo before feed-out always presented higher TMR spore counts than 3.0 log spores/g, with 18 of 22 farms having higher spore counts than 4.0 log spores/g, and the highest contamination level reaching 5.89 log spores/g.

The scatter plot of the anaerobic and facultative anaerobic spore count in the TMR and of the spores in the feces is reported in Figure 5. The higher the spore count in TMR is, the higher the spore count in the feces. Most of the farms that did not manage corn

Figure 4. Scatter plot of the anaerobic and facultative anaerobic sporeformers in the TMR and the percentage of visible aerobic deteriorated corn silage fed to the dairy cows on the 49 commercial dairy farms in northern Italy, in relation to the silage management practices before feed-out.

Figure 6. Scatter plot of the anaerobic and facultative anaerobic sporeformers in the feces and milk of the 49 commercial dairy farms in northern Italy, in relation to the silage management practices before feed-out.

silage in an appropriate way presented higher levels of spores in both the TMR and feces, but a lower TMR spore count did not always implicate a low spore count in the feces.

A scatter plot of the anaerobic and facultative anaerobic spore count in the feces and in the milk is reported in Figure 6. The higher the spore count in the feces is, the higher the spore count in the milk. When feces

contamination was greater than 5.0 log spores/g, most of the farms presented milk spore counts greater than 3.0 log spores/L (6 out of 7 farms). Farms with bulk tank milk spore counts greater than 3.0 log spores/L had generally not managed their silages well.

The practical management practices applied on farm that contribute to the safety of the entire milk chain, from the feed production in the field to the milking routine, are summarized in Table 4. Farms were divided into 3 groups according to their silage management practices (well, partially, or not discarded molded silage), and the proportion of farms applying each of the selected management practices in each group was calculated accordingly. The farms that discarded molded silages well before feeding had a lower spore count in the TMR, feces, and milk compared with those that partially or did not discard molded silages. Farms that did not discard silages before feed-out usually adopted fewer silage and milking management practices (accurate silo covering, number of films to cover the silo top, the use of film on the silo walls, pre-dipping treatments, treatments with additive) than those that discarded molded silages partially or totally.

The Pearson correlation coefficients of the spore contamination of the milk and visibly molded silo face, the spore contamination of the TMR and feces, and the hygienic score on farms that used well, partially, or not discarded corn spoiled silage before TMR preparation are reported in Table 5. The spore count in the milk of farms that did not discard molded silages before TMR preparation was positively correlated with the percent-

Figure 5. Scatter plot of the anaerobic and facultative anaerobic sporeformers in the TMR and feces of the 49 commercial dairy farms in northern Italy, in relation to the silage management practices before feed-out. The dotted line is the 1:1 line.

Table 4. Practices adopted for corn silage management, pre- and postmilking cleaning procedures, spore contamination of TMR, feces, and milk, and milk quality on farms that well discarded (well), partially discarded (partially), or did not discard (not) molded corn silage before TMR preparation (percentage of the farms applying the procedure within each group in parentheses)

Item	Not (n = 22)	Partially (n = 12)	Well (n = 15)	SE	P-value
Silage management factor					
No. of farms making corn silage in bunker with concrete walls	18 (81.8)	10 (83.3)	15 (100.0)	—	—
No. of farms using at least 2 plastic films to cover the silo top	10 (45.5)	9 (75.0)	12 (80.0)	—	—
No. of farms using film on the silo walls	6 (27.3)	5 (41.7)	6 (40.0)	—	—
No. of farms using soil/gravel to weigh down the silo cover	6 (27.3)	3 (25.0)	5 (33.3)	—	—
No. of farms having a feed-out rate of at least 0.8 m/wk	12 (54.5)	4 (33.3)	13 (86.7)	—	—
No. of farms with silage treated with additive ¹	3 (13.0)	3 (25.0)	3 (20.0)	—	—
No. of farms applying at least 3 silo management practices	12 (54.5)	7 (58.3)	13 (86.7)	—	—
No. of farms applying at least 4 silo management practices	3 (13.6)	4 (33.3)	8 (53.3)	—	—
No. of farms with silage in molded area below 5% of the front face	11 (50.0)	4 (33.3)	11 (73.3)	—	—
Average feed-out rate (m/wk)	0.82	0.86	1.32	0.114	0.067
Incidence of moldy silage on silage feed-out face (%)	6.07	6.23	3.27	0.705	0.126
Amount of corn silage in TMR (% on DM)	32.5	31.0	35.1	1.10	0.317
TMR spore (log spores/g)	4.35 ^a	4.55 ^a	3.71 ^b	0.123	<0.001
Feces spore (log spores/g)	4.35 ^a	3.88 ^{ab}	3.56 ^b	0.101	0.014
Udder hygiene management					
No. of farms with freestall and cubicles	13 (59.1)	9 (75.0)	14 (93.3)	—	—
No. of farms with milking parlor	12 (54.5)	10 (83.3)	14 (93.3)	—	—
No. of farms only wiping teat with a dry paper towel before milking machine attachment	13 (59.1)	4 (33.3)	4 (26.7)	—	—
No. of farms applying a germicidal solution to the teats before milking machine attachment	4 (18.2)	4 (33.3)	8 (53.3)	—	—
No. of farms applying 2 pre-dipping treatments	4 (18.2)	5 (41.7)	7 (46.7)	—	—
No. of farms applying manual postmilking teat dipping with an iodophor teat dip	14 (66.3)	10 (83.3)	12 (80.0)	—	—
No. of farms with milk spore contamination under detection limit	4 (18.2)	5 (41.7)	11 (73.3)	—	—
Animal cleaning score ²	2.61 ^b	2.89 ^{ab}	3.78 ^a	0.144	0.009
Structural and milk quality parameter					
Milking cows per farm (no.)	76 ^b	96 ^{ab}	150 ^a	10.5	0.010
Annual milk yield (kg/cow)	7,946 ^b	8,502 ^b	9,975 ^a	223.7	0.001
Milk intensity (t of FPCM/ha) ³	21.0	21.4	31.0	1.82	0.054
Milk spore count (log spores/L)	2.30 ^a	1.90 ^{ab}	1.62 ^b	0.086	0.006
Milk protein (%)	3.37	3.44	3.44	0.020	0.159
Fat content (%)	3.79	3.76	3.83	0.043	0.789
Lactose (%)	4.97	5.01	4.98	0.008	0.111
Total bacterial count (log/mL)	4.18	4.06	4.07	0.037	0.260
SCC (cells/mL)	261,000	247,000	236,000	9.86	0.354

^{a,b}Within a row, mean values with the same superscript letter are not significantly different ($P < 0.05$).
¹Corn silage treated with inocula (*Lactobacillus buchneri* based inocula or mixed inocula), or other silage additives (propionic acid, propionate, sodium chloride, milk liquid whey).
²Teat cleanliness scoring system: 5 = clean (no visible dirt); 4 = almost clean (approximately <10% of the area dirty); 3 = slightly dirty (10 to 20% of the area dirty); 2 = dirty (20 to 50% of the area dirty); and 1 = extremely dirty (>50% of the area dirty).
³FPCM = fat- and protein-corrected milk.

Table 5. Pearson correlation coefficients of milk spore contamination, silo face visibly molded, TMR and feces spore contamination, and animal cleaning score on the 49 dairy farms¹

Item	Milk spores	% of molded silo face	TMR spore	Feces spores
All farms (n = 49)				
% of molded silo face	0.423**			
TMR spores	0.419**	0.504**		
Feces spores	0.766**	0.420**	0.553**	
Animal cleaning score	-0.503**	-0.075	-0.339*	-0.470**
Spoiled silage well discarded (n = 15)				
% of molded silo face	-0.003			
TMR spores	0.290	0.210		
Feces spores	-0.293	0.188	0.074	
Animal cleaning score	-0.313	0.202	0.040	0.221
Spoiled silage partially discarded (n = 12)				
% of molded silo face	-0.089			
TMR spores	0.571	0.477		
Feces spores	0.711**	0.385	0.717**	
Animal cleaning score	-0.649*	-0.171	-0.620*	-0.480
Spoiled silage not discarded (n = 22)				
% of molded silo face	0.473*			
TMR spores	0.128	0.531*		
Feces spores	0.746**	0.427	0.453*	
Animal cleaning score	-0.299	0.148	-0.084	-0.313

¹Data are reported aggregated or split out by farms that well, partially, or do not discard corn spoiled silage before TMR preparation.

* $P < 0.05$; ** $P < 0.01$.

age of molded silo face and the feces spore count. A relation between the percentage of molded silo face, and spores in the TMR and feces was also determined. No significant effect was found for the analyzed parameters on farms that discarded the molded silages completely before TMR preparation.

DISCUSSION

Recent evidence from several milk production chains for PDO cheeses, milk powder, and long conservation pasteurized milk throughout the world has pointed out the need to reduce the sporeformer contamination of bulk tank milk to a minimum because of the difficulties in reducing their contamination level later on in the dairy plant, in part due to the fact that milk heat treatments exacerbate the activation of spore germination (Doyle et al., 2015; Masiello et al., 2017).

This study has analyzed the contamination pathways of anaerobic and facultative anaerobic sporeformers, from silage to bulk tank milk, on 49 dairy farms in northern Italy that produce milk destined for Grana Padano PDO cheese, and has coupled the plate count method with 16S-DNA sequencing of the dominant isolated anaerobic and facultative anaerobic sporeformer species.

In the present experiment, the low contaminated silages presented a very similar anaerobic and facultative anaerobic spore population to those of the considered soil samples. Most of the species observed in the soil (7 vs. 11) were found in both the well-conserved silage

(with low pH, high lactic acid content, and an absence of butyric acid) and in the deteriorated silage. However, most of the samples from the peripheral areas of the corn silage, which were found to be aerobically spoiled, had a higher number of anaerobic and facultative anaerobic spores that was several magnitudes higher (up to 7.76 log spores/g) than the core samples. The dominant *Clostridium* and *Paenibacillus* species in the spoiled samples were *C. aerotolerans*, *C. celerecrescens*, *C. jejuense*, *C. sporogenes*, *C. tyrobutyricum*, *C. xylanolyticum*, *P. macerans*, and *P. thermophilus*. This suggests that these species have the capacity to outgrow in silage during aerobic deterioration, as previously suggested for *C. tyrobutyricum* in grass silage (Jonsson, 1991). Other authors (Vissers et al., 2007a; Borreani and Tabacco, 2008) suggested, on the basis of tests conducted with the most probable number (MPN) method, that the aerobic deterioration of corn silage led to an increase in butyric acid bacteria, which in turn produced gas under anaerobiosis on lactate acetate agar. Borreani et al. (2013), in trials on the deterioration of corn silage, then found the outgrowth of *Paenibacillus macerans*, which was identified by 16S-DNA sequencing as the dominant anaerobic and facultative anaerobic sporeformer isolated on an RCM medium. Driehuis et al. (2016) found *C. tyrobutyricum*, *C. beijerinckii*, and *Paenibacillus* spp. (identified by means of a PCR assay on *C. tyrobutyricum*, *C. beijerinckii*, and *Paenibacillus* spp.) as the dominant species, in both low and highly contaminated corn silage (less or more than 4 log MPN/g). This result was achieved using

MPN tubes in which gas formation was observed in a Van Beynum and Pette medium. Zucali et al. (2015), in a survey on 23 dairy farms in northern Italy, reported the presence of *C. tyrobutyricum*, *C. beijerinckii*, *C. butyricum*, and *C. sporogenes* in corn. This result was achieved using a multiplex PCR of colonies purified from MPN gas positive tubes (lactate-acetate agar plus reconstituted skim milk). In the present study, *C. beijerinckii* was not found among the dominant anaerobic and facultative anaerobic sporeformers in any of the corn silage samples, even though it was present in some of the TMR fed to dairy cows. The contrasting results between the present study and the studies of Zucali et al. (2015) and Driehuis et al. (2016) about the presence of *C. beijerinckii* among the dominant anaerobic sporeformers in corn silage could be due to the MPN medium, containing lactate acetate, that was used in those studies, which could have favored a selective multiplication of saccharolytic clostridia.

Most of the dominant sporeformer species in the low contaminated TMR (below 3.0 log spores/g) were found to belong to *C. tyrobutyricum*, *C. beijerinckii*, and *P. macerans*. The higher contaminated TMR (>3.0 log spores/g) instead showed less incidence of *C. tyrobutyricum* and *P. macerans* and an equal presence of *C. beijerinckii*. Furthermore, *C. aerotolerans*, *C. butyricum*, *C. celerecrescens*, *C. xylanolyticum*, and *P. thermophilus* were among the dominant isolated species. The frequency of most of these clostridia species (except *C. aerotolerans*) increased in the highly contaminated TMR (>5.0 log spores/g), and it seems that *P. thermophilus* partially substituted *P. macerans*.

No *Clostridium* species were found in milk with a higher spore count than 1,000 spores/L. All the identified anaerobic and facultative anaerobic species belonged to *Paenibacillus* spp., with *P. macerans* and *P. thermophilus* representing 62% of the sporeformer population. This result could be connected to the isolation of colonies from the plates that selected only the dominant population; therefore, the clostridia presence at a lower level cannot be excluded. The reason why *Paenibacillus* species dominate bulk tank milk of farms with spore count higher than 3 log spores/L still remains unexplainable with the data obtained in this study. Bermúdez et al. (2016) found, on 5 commercial dairy plants in Uruguay, that *C. tyrobutyricum* was the most frequently isolated species in milk (ranging from 50 to 58%), *C. sporogenes* was the second (17 to 21%), whereas *C. beijerinckii* and *C. butyricum* were present at lower levels, and they also observed several other species of clostridia, that is, *C. bifermentans*, *C. acetobutylicum*, *C. xylanolyticum*, and *C. aminovalericum*. Masiello et al. (2017), in a longitudinal assessment of dairy farms in New York State, found that *Paenibacil-*

lus spp. was widely present in bulk tank milk. *Paenibacillus* spp. (which is a facultative anaerobe) is one of the main sporeformer genera that limits the shelf life of pasteurized fluid milk under refrigeration (Masiello et al., 2017).

Clostridium spp. that were most frequently identified in the different matrices considered in the present research belonged to 2 clusters (I and XIVa), according to the classification proposed by Collins et al. (1994). Cluster I is a family of *Clostridiaceae*, which included the following identified species: *C. tyrobutyricum*, *C. beijerinckii*, *C. butyricum* (in the group of nonliquefying gelatin), and *C. sporogenes* and *C. acetobutylicum* (liquefying gelatin). The XIVa family cluster of *Ruminococcaceae* included the following species: *C. aerotolerans*, *C. aminovalericum*, *C. celerecrescens*, *C. xylanolyticum* (Collins et al., 1994), and *C. jejuense* (Jeong et al., 2004). *Clostridium aerotolerans*, *C. celerecrescens*, and *C. xylanolyticum* are able to hydrolyze and then hydrogenate hydroxycinnamic acids (ferulic and p-coumaric acids), which are esterified to arabinoxylan in plant cell walls and are thus involved in the anaerobic biotransformation of the fiber components linked to lignin (Chamkha et al., 2001). Furthermore, *C. xylanolyticum* is also able to utilize a wide range of carbohydrates and lactate as an energy source. In the present study, these *Clostridium* species were commonly identified in soil, deteriorated corn silages, and feces samples. It can be speculated that these species are involved in the advanced degradation of OM in deteriorated silages together with filamentous fungi. The pH is not too acidic (around 6.0) in these silages, the temperature is around 37°C, and there is a limited availability of oxidizable free soluble nutrients, such as organic acids and sugars. Therefore, the main sources of substrate for microbial growth are linked to the structural carbohydrates of fiber, which are less available due to cell wall lignification (McSweeney et al., 1999). Phenolic acids are ester-linked to arabinoxylan and lignin, or cross-linked to lignin and polysaccharide through ether and ester bonds, respectively. It is thought that the ester linkages between phenolic acids and polysaccharide limit the anaerobic degradation of fiber through the microorganisms in the gut of ruminants, and these groups of clostridia share a role in the degradation of fiber in the gastrointestinal tract of cows (Dowd et al., 2008).

The role of corn silage management in increasing the risk of bulk tank milk contamination from anaerobic and facultative anaerobic spores has been analyzed in detail on 49 commercial dairy farms. It has been stated, in recent farm surveys, that corn silage is the main source of the anaerobic and facultative anaerobic spore contamination of TMR, in particular when aerobically spoiled areas are included during feed-out (Tabacco

and Borreani, 2002; Vissers et al., 2007c; Driehuis et al., 2016). The present research has shown a high variability in the anaerobic and facultative anaerobic spore counts in the different studied matrices, with greater values being observed in samples from the peripheral areas of corn silage, especially when aerobic deterioration had taken place. Most of the forage conserved in the core mass of the silo (75% of the farms) contained lower numbers of spores than 3.0 log spores/g. This unavoidable contamination has mainly been attributed to soil contamination during corn growth and harvesting (Julien et al., 2008). The passage of spores from manured soil to silage has been proved in several studies on grass silage, and many of these studies related clostridia spore contamination to the slurry application timing, rates, and methods (e.g., Östling and Lindgren, 1991; Davies et al., 1996). Östling and Lindgren (1991) enumerated the clostridia in manure and in manured crops used for silage and found clostridia counts of around 4.2 log spores/g in manure, a number that decreased by 83% in grass silage crops at harvesting. Lango and Heinonen-Tanski (1995), in a work on cattle slurry on Finnish dairy farms, reported that the occurrence of *Clostridium tyrobutyricum* spores in an anaerobic and facultative anaerobic sporeformer population was unexpectedly low (around 5%).

In the present study, the TMR spore count was found to be influenced by the amount of deteriorated corn silage that was included in the TMR during feed-out and preparation. Figure 4 clearly suggests this hypothesis: the spore count of TMR increased as the incidence of spoiled corn silage (visible moldy silage) present in the silo increased, and it was also connected to the care taken in cleaning the spoiled silage before feed-out. Most of the farms (9 out of 13 farms) that coupled a good silage management, to prevent the presence of spoiled silage in their silos (below 5% of the silage front face), with a careful discarding of all the molded spots, were able to keep their TMR spore counts below 4.0 log spores/g.

The influence of TMR on the anaerobic and facultative anaerobic spore content in the feces of dairy cows indicates that the feces presented a greater contamination than 3.0 log spores/g when the TMR spore count was greater than 4.5 log spores/g. Nineteen farms out of the 21 that had feces contaminations with more than 4.0 log spores/g did not clean the silo face properly before feed-out, and the majority of them (18 out of 19) presented a higher TMR spore count than 4.0 log spores/g. A higher concentration in feces than in TMR is generally explained by the digestion of feed components during passage through the intestinal tract (Driehuis, 2013). Driehuis et al. (2016) reported a ratio

between spores in feces and in TMR of around 3. In the present study, 4 farms showed a ratio higher than 4 between the spores in the feces and the spores in the TMR. This large increase in spores in the feces (feces/TMR spore ratio from 14 to 59) could be explained, not only by sampling problems that could have led to an overestimation of the feces spore count on these farms, but also by a multiplication of clostridia in the digestive tracts, as previously suggested by Contrepois et al. (1971). It has recently been reported that clostridia generally account for approximately 20% of the total microbial population in the intestinal tract of cattle (Dowd et al., 2008; Callaway et al., 2010) and can become problematic in the case of dietary stress, injury, changes in management, parasitism, or other unusual circumstances (McGuirk, 2015). Therefore, new roles of clostridia in the intestinal tract may be speculated and would suggest their beneficial function in improving the digestion of complex OM, such as cellulose (Dowd et al., 2008).

The passage of spores into milk occurs from the exterior of the cow's teats, through feces contamination, and they are transferred to milk during the milking process (Vissers et al., 2007c). Therefore, the critical points are feces spore contamination and the accuracy of the cleaning routines during milking (Vissers et al., 2007c; Zucali et al., 2015).

The data of the present study have confirmed the role of feces in spore milk contamination and the role of management practices to reduce the risk of carry-over of spores from spoiled corn silages to milk. In particular, a positive correlation was found between milk spore count in farms that did not discard molded silages before TMR preparation and feces spore count. There is clear evidence that the majority of farms that discarded spoiled silages well before feed-out also adopted management practices that were considered to be effective in reducing silage spoilage and the carry-over of anaerobic and facultative anaerobic spores from feeds to milk, with more than 70% of the farms having milk spore contamination values under the detection limit. On the other hand, more than 50% of the farms that partially discarded or did not discard spoiled silage failed to reach this goal (Table 4). The silage management practices (such as corn silage conserved in a bunker with concrete walls, at least 2 plastic films used to cover the silo top, plastic film on the silo walls, and a feed-out rate of at least 0.8 m/wk) helped to reduce the incidence of aerobic deterioration (Borreani et al., 2018), and consequently, the reduction of the discarded spoiled silage led to lower spore contamination of the TMR, feces, and milk. It is also evident that farms that carried out cleaning management procedures before

silage feeding also adopted pre- and postmilking procedures to reduce the risk of microbial contamination of milk during milking.

The present data have confirmed that the anaerobic and facultative anaerobic spore count of milk is influenced to a great extent by the inclusion of aerobically deteriorated corn silage in TMR. This leads to the conclusion that it is not the presence of silage itself in the TMR that increases the risk of milk contamination, but the undervalued capability of aerobic deteriorated silage to contaminate the TMR. When spoiled silage is included in the TMR, it contributes to a great extent to increasing the spore content of TMR and consequently to increasing the risk of a higher feces spore count. This makes any further control of spore contamination along the milk production chain very difficult, even when the milking and cleaning practices are done properly. Processing milk to reduce spore contamination in dairy plants is expensive, time consuming, and labor intensive, and even though it is impossible to eliminate the risk of spore contamination of milk on farms, the results of this work show that it is possible to reduce this risk through the implementation of good farm management practices and specific processing steps from silage to milking.

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