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Aerobic deterioration stimulates outgrowth of spore-forming *Paenibacillus* in corn silage stored under oxygen-barrier or polyethylene films

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ABSTRACT

The occurrence of *Bacillus* and *Paenibacillus* spores in silage is of great concern to dairy producers because their spores can survive pasteurization and some strains are capable of subsequently germinating and growing under refrigerated conditions in pasteurized milk. The objectives of this study were to verify the role of aerobic deterioration of corn silage on the proliferation of *Paenibacillus* spores and to evaluate the efficacy of oxygen-barrier films used to cover silage during fermentation and storage to mitigate these undesirable bacterial outbreaks. The trial was carried out on whole-crop maize (*Zea mays* L.) inoculated with a mixture of *Lactobacillus buchneri*, *Lactobacillus plantarum*, and *Enterococcus faecium*. A standard polyethylene film and a polyethylene-polyamide film with an enhanced oxygen barrier were used to produce the silage bags for this experiment. The silos were stored indoors at ambient temperature (18 to 22°C) and opened after 110 d. The silage was sampled after 0, 2, 5, 7, 9, and 14 d of aerobic exposure to quantify the growth of endospore-forming bacteria during the exposure of silages to air. *Paenibacillus macerans* (gram-positive, facultatively anaerobic bacteria) was able to develop during the aerobic exposure of corn silage. This species was present in the herbage at harvesting, together with clostridial spores, and survived ensiling fermentation; it constituted more than 60% of the anaerobic spore formers at silage opening. During silage spoilage, the spore concentration of *P. macerans* increased to values greater than 7.0 $\log_{10}$ cfu/g of silage. The use of different plastic films to seal silages affected the growth of *P. macerans* and the number of spores during aerobic exposure of silages. These results indicate that the number of *Paenibacillus* spores could greatly increase in silage after exposure to air, and that oxygen-barrier films could help to reduce the potential for silage contamination of this important group of milk spoilage microorganisms by delaying the onset of aerobic deterioration.

Key words: corn silage, aerobic deterioration, spore-forming bacteria, oxygen-barrier film

INTRODUCTION

The presence of psychrotolerant spore-forming bacteria in high-temperature short-time pasteurized milk (minimum processing conditions of 72°C for at least 15 s) is the most limiting factor in extending shelf life beyond 14 d (Ranieri et al., 2009). The harmful effects on food safety and product quality caused by spore formers are the production of toxins, the production of spoilage enzymes, and interference with cheese making due to gas formation (cheese blowing; De Jonghe et al., 2010). *Paenibacillus* and *Bacillus* have been identified as the prominent gram-positive spore-former genera in dairy environments and pasteurized milk (Fromm and Boor, 2004; Scheldeman et al., 2004; Ivy et al., 2012). In the last few decades, studies have shown that many organisms formerly classified as the *Bacillus* genus actually represent several genera within the *Bacilli* class (Durak et al., 2006). At least 12 new genera of spore-forming bacteria have been defined, including *Paenibacillus* (Ash et al., 1993). Members of the *Paenibacillus* genus are facultative anaerobic organisms that produce spores in swollen sporangia and some of these organisms excrete diverse assortments of extracellular polysaccharide-hydrolyzing enzymes (Shida et al., 1997). The heat-resistant nature of the spores of aerobic and anaerobic spore formers highlights that it is particularly important to prevent an initial contamination of raw milk on dairy farms. The sources of contamination of spore formers at a farm level could be soil, silages, feces, feeds, and milking equipment (Colombari et al., 2001; Coorevits et al., 2008). Controlling and reducing the presence of spore formers is a challenging task because the introduction of these organisms can occur from many points in the food system (Durak et al., 2006). The spore contamination of milk in part occurs via soil and in part via the cow's diet. The latter route is the same as that of butyric acid bacteria spores: excretion in the cow's feces and transmission to milk during milking via dirt attached to the exterior of the teats (Vissers et al., 2007b). The occurrence of *Bacillus* and *Paenibacillus*

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spores in silage is of great concern to dairy producers (te Giffel et al., 2002), because their spores can survive pasteurization and some strains are capable of subsequent germination and growth under refrigeration conditions in pasteurized milk (Meer et al., 1991). Huck et al. (2008), studying dairy environmental samples collected on a commercial farm in New York State, found that a considerable subtype diversity of spore-forming bacteria was present in multiple locations on the farm (feed mix, bedding sawdust, manure, soil, water, and milk) and that each location represented a likely source of raw milk contamination. Furthermore, they found that about 13% of bacteria isolates from the dairy farm environment and raw milk samples consisted of *Paenibacillus* spp. The identification of the points of entry of *Paenibacillus* into milk could lead to the development of effective strategies to reduce or eliminate its presence in milk production systems (Huck et al., 2008). It is well known that all silages exposed to air deteriorate as a result of aerobic microbial activity during feedout (Woolford, 1990). This aerobic deterioration process is usually initiated by yeasts, which mainly utilize lactic acid as a substrate, and is followed by mold. Yeast activity is known to increase silage pH and temperature, thus creating an easier environment for less-acid-tolerant microorganisms, such as *Clostridium* and *Bacillus* species (Vissers et al., 2007a). Silage that has spoiled because of exposure to air is undesirable, due to the lower nutritive value (Tabacco et al., 2011) and to the risk of negative effects on animal performance, which are also connected to the proliferation of potentially pathogenic or otherwise undesirable microorganisms, such as spore-forming bacteria (Vissers et al., 2007a; Tabacco et al., 2009). Borreani and Tabacco (2010), in a survey on 54 dairy farms, found that anaerobic spores were at a lower level than 10^3 spores per gram of silage [from <1.18 to 2.36 $\log_{10}$ most probable number (**MPN**)/g] in the well-fermented central areas of corn silages, whereas their content reached and even passed 10^6 spores per gram of silage in the peripheral areas of the same silages, especially when aerobic deterioration took place. The number of endospores in silage determines the number of endospores that leave the animal, the subsequent magnitude of contamination of the milk, and finally the treatment technologies for their elimination (Pahlow et al., 2003).

The objectives of this study were (1) to quantify the spore populations of *Paenibacillus* spp. in corn silage, (2) to verify the role of aerobic deterioration of corn silage on the outgrowth of *Paenibacillus* spores, and (3) to study whether using an oxygen-barrier (**OB**) film to cover silage could contribute to reducing the growth of *Paenibacillus* spp. after exposure of the silage to air, thereby limiting the risk of contamination of the milk production system.

MATERIALS AND METHODS

Crop and Ensiling

The trial was carried out at the experimental farm of the University of Turin in the western Po Plain in northern Italy (44°50′N, 7°40′E, altitude 232 m above sea level) during 2008 with corn (*Zea mays* L.) harvested as a whole-corn crop, at a 50% milkline stage. The forage was chopped with a precision forage harvester to a 10-mm theoretical length of cut, inoculated with a mixture of *Lactobacillus buchneri* (strain ATCC PTA2494), *Lactobacillus plantarum* (strains ATCC 53187 and 55942), and *Enterococcus faecium* (strain ATCC 55593) inoculum (11C33; Pioneer Hi-Bred International, Des Moines, IA) to supply 1×10^5, 8×10^3, and 2×10^3 cfu/g of fresh forage for *Lb. buchneri*, *Lb. plantarum*, and *E. faecium*, respectively. A standard 120-μm-thick black-on-white polyethylene (**PE**) film and a 120-μm-thick Silostop (Bruno Rimini Ltd., London, UK) black-on-white co-extruded PE-polyamide film with an enhanced OB were used to produce the silage bags for this experiment. The bags were heat sealed at the closed end and were equipped with a one-way valve for CO_2 release. A portion of a polyvinyl chloride (PVC) tube (internal dimensions: 300-mm diameter and 300-mm height, 21-L volume) was used to contain the plastic bag to avoid mechanical damage to the plastic film and to obtain a similar silage density to that observed in commercial bunkers. Each bag was then filled with about 12 kg of fresh forage, which was compacted manually, secured with plastic ties, and left in the PVC tube so that just the top and the bottom of the bag had access to air. Four replications were prepared for each treatment. The density of the silage was 576 kg of fresh material/m^3 and 192 kg of DM/m^3. The oxygen permeabilities of the plastic materials, determined on the basis of the American Society for Testing and Materials (ASTM) standard method D 3985-81 (ASTM, 1981), were 1,480 and 70 cm^3/m^2 per 24 h at 100 kPa at 23°C, 0% relative humidity, for the PE and OB films, respectively. The silos (4 per treatment) were stored indoors at ambient temperature (18 to 22°C) and opened after 110 d. Silages were subjected to an aerobic stability test. Aerobic stability was determined by monitoring the temperature increases due to the microbial activity of the samples exposed to air. About 3 kg of each silo were allowed to aerobically deteriorate at room temperature (22 ± 1.6°C) in 17-L polystyrene boxes (290-mm diameter and 260-mm height) for 14 d.

A single layer of aluminum cooking foil was placed over each box to prevent drying and dust contamination, but also to allow air penetration. The room and the silage temperatures were measured each hour by a data logger. Aerobic stability was defined as the number of hours the silage remained stable before rising more than 2°C above room temperature (Ranjit and Kung, 2000). The difference between silage temperature and ambient temperature (**dT**) was determined. During the aerobic stability test, hours with temperature greater than 30°C were also calculated to better describe the optimum temperature for spore germination in the absence of inhibitory conditions (Spoelstra, 1983). The silage was sampled at opening of the silo and after 2, 5, 7, 9, and 14 d of aerobic exposure from the same polystyrene boxes for each replication to quantify the outgrowth of spore-forming bacteria during exposure of silages to air.

Sample Preparation and Analyses

Samples from the preensiled material, the silage at silo opening, and the aerobically exposed silage were each split into 3 subsamples. One subsample was oven dried at 65°C to constant weight to determine the DM content. The second subsample (about 300 g) was extracted as a wet sample, using a Stomacher blender (Seward Ltd., Worthing, UK) for 4 min in distilled water at a water-to-sample material (fresh weight) ratio of 9:1 or in 0.05 *M* H_2SO_4 at an acid-to-sample material (fresh weight) ratio of 5:1. The nitrate (NO_3) content was determined in the water extract, through semiquantitative analysis, using Merckoquant test strips (Merck KGaA, Darmstadt, Germany; Borreani and Tabacco, 2008). The lactic, acetic, and butyric acid contents were determined by HPLC in the acid extract (Canale et al., 1984). The water activity of the herbage and silages was measured at 25°C using an AquaLab series 3TE water activity meter (Decagon Devices Inc., Pullman, WA) on a fresh sample. The third subsample was used for the microbiological analyses. For the microbial counts, a 30-g sample was transferred into sterile homogenization bags, suspended in 270 g of peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride per liter), and homogenized for 4 min in a laboratory Stomacher blender. When the spore count was below the detection limit, a 50-g sample was resuspended in 25 g of peptone salt solution and homogenized for 4 min. Serial dilutions were prepared and the mold and yeast numbers were determined using the pour plate technique with 40.0 g/L of yeast extract glucose chloramphenicol agar (YGC agar; Difco Laboratories Ltd., West Molesey, Surrey, UK) after incubation at 25°C for 3 and 5 d for yeasts and molds, respectively. Mold and yeast colony-forming units were enumerated separately, according to their macromorphological features. The anaerobic spores were counted after pasteurization of the serial dilutions at 80°C for 10 min followed by the streak plate technique with reinforced clostridium medium (**RCM**) agar (Oxoid CM149; Oxoid Ltd., Basingstoke, UK), 0.005% neutral red (Sigma N-7005; Sigma-Aldrich Co., St. Louis, MO), and 200 ppm of D-cycloserine (Sigma C-6880; Jonsson, 1990) and incubation at 37°C for 7 d in anaerobic jars (Gas-Pak jar; BBL Microbiology Systems, Cockeysville, MD). The mean count of the duplicated subsamples was recorded for the microbial counts on plates that yielded 1 to 100 cfu per Petri dish. The gas-forming spore-former concentration was determined by the MPN procedure with 3 tubes per dilution. Tubes from each dilution step, containing 9 mL of sterilized RCM supplemented with sodium lactate (10 mL/L) and agar (1.5 g/L), were each inoculated with 1 mL of diluted sample. The tubes were sealed with agar and incubated for 7 d at 37°C. A tube scored positive if it exhibited abundant gas formation after incubation.

Bacterial Isolation

The bacterial colonies present on the RCM were visually examined for each silage sample. A colony representative of each distinct morphology was chosen for isolation and later for identification. Generally, 5 to 10 colonies per sample were selected and streaked for purity on RCM agar and anaerobically incubated for 7 d at 37°C. The purified isolates were transferred to brain heart infusion (BHI) broth (Oxoid Ltd.) and the overnight cultures were stored at −80°C in 20% glycerol.

Bacterial Identification

Bacterial identification was performed by sequencing of the V1 to V3 region of the 16S rRNA gene, after PCR-denaturing gradient gel electrophoresis (**DGGE**), which was used to group isolates on the basis of their migration. Bacterial isolates were submitted to DNA extraction, according to the FTA Protocol BD02 (Whatman, Kent, UK). Briefly, 10 μL of each overnight culture was applied to FTA cards impregnated with a patented chemical formula that lyses cell membranes and denatures proteins on contact. A sample disc from the dried spots was placed in a PCR amplification tube with FTA Purification Reagent (Whatman), which washes all cell debris and inhibitors, leaving the DNA immobilized in disc cellulose fibers. A PCR-DGGE approach was adopted for identification purposes. The PCR was performed directly in the tubes containing

the sample discs and using the 338fGC and 518r primers (Ampe et al., 1999) to amplify the V3 region of the 16S rRNA gene, according to the protocol described by Cocolin et al. (2004). The DCode universal mutation detection system [Bio-Rad Laboratories S.r.l., Segrate (Milan), Italy] was used for DGGE analysis. The PCR products were applied to an 8% (wt/vol) polyacrylamide gel (acrylamide/bis-acrylamide, 37.5:1) with a denaturant gradient of 30 to 60%, in a 1× TAE buffer [2 *M* Tris base, 1 *M* glacial acetic acid, and 50 *M* EDTA (pH 8)]. The gels were subjected to a constant voltage of 120 V for 3.5 h at 60°C and DNA bands were stained in 1× TAE containing 1× SYBR Green I (Sigma), and analyzed under UV light using UVIpro Platinum 1.1 Gel software (Eppendorf, Hamburg, Germany). The DGGE profiles were grouped and representative strains of each group were amplified with the P1V1 and P4V3 primers, as described by Klijn et al. (1991), targeting 700 bp of the V1 to V3 region of the 16S rRNA gene. The PCR products were sequenced by a commercial facility (MWG Biotech AG, Ebersberg, Germany) and the resulting sequences were aligned with those in GenBank using the Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 1997) to determine the closest known relatives, on the basis of partial 16S rRNA gene homology.

Statistical Analysis

The microbial counts were $\log_{10}$ transformed to obtain log-normal distributed data. Data analysis was conducted for the DM concentration, pH, nitrate concentration, aerobic spore count, and anaerobic spore obtained during the aerobic exposure test was conducted in a repeated measure analysis using the general linear model (GLM) procedure of SPSS Statistics (version 17.0; SPSS Inc., Chicago, IL). The model included the effects of film type, time, and the interaction between the film type and time. Treatment effects were declared significant at $P < 0.05$. To account for differences at specific time points during aerobic exposure, post-hoc pairwise comparisons were performed using a Bonferroni correction based on the Šidák method (Šidák, 1967). Pearson correlation coefficients of fermentative characteristics, microbiological composition, temperature indices of the silage (dT and hours with temperature greater 30°C), and their level of significance were determined.

RESULTS

Crop at Ensiling

The results of the DM content, water activity, mold and yeast count, and aerobic and anaerobic spore-forming bacteria of the corn forage before ensiling are shown in Table 1. Yeast and mold counts were greater than 6 $\log_{10}$ cfu/g of silage. There were more aerobic spores than anaerobic spores, and the values were within the range of those commonly observed at harvesting on corn from commercial farms (Vissers et al., 2007a; Borreani and Tabacco, 2008).

Silage and Aerobic Deterioration

After 110 d of conservation, all silages were well fermented and the main fermentation acids found were lactic and acetic acids (Table 2). The butyric acid was always below the detection limit (data not shown). Silages sealed with the PE film had greater pH ($P = 0.002$), lower lactic acid ($P = 0.033$), and greater yeast count ($P < 0.001$) than OB silages. At opening, all silages had numerically lower aerobic and anaerobic spore-forming bacteria counts than the herbage at ensiling, without any differences between the 2 treatments.

During aerobic exposure, the pH increased to values greater than 4 after 5 and 9 d and the lactic acid concentration decreased to below 15 g/kg of DM after 7 and 14 d for PE and OB films, respectively (Table 2). Anaerobic spores remained almost stable in the first

Table 1. The DM concentration, nitrate concentration, and aerobic and anaerobic spore-forming bacteria of the corn forage before ensiling

Item	Amount
DM (%)	33.3 ± 0.54
pH	5.84 ± 0.028
Nitrate (mg/kg of herbage)	395 ± 44
Water activity (a_w)	0.98 ± 0.0010
Yeast ($\log_{10}$ cfu/g of herbage)	6.90 ± 0.054
Mold ($\log_{10}$ cfu/g of herbage)	6.13 ± 0.059
Aerobic spores ($\log_{10}$ cfu/g of herbage)	3.57 ± 0.13
Anaerobic spores ($\log_{10}$ cfu/g of herbage)	1.40 ± 0.14
Spore of gas-forming anaerobic bacteria ($\log_{10}$ MPN/g herbage)[1]	1.74 ± 0.16
Paenibacillus (% of anaerobe population)	50.0 ± 13

[1]MPN = most probable number.

Table 2. The DM concentration, nitrate concentration, and aerobic and anaerobic spore-forming bacteria in corn silages at silo opening and after 2, 5, 7, 9, and 14 d of air exposure

Item[1]	Treatment[2]	DM (%)	pH	Lactic acid (% of DM)	Acetic acid (% of DM)	Nitrate (mg/kg of silage)	Aerobic spores (log_{10} cfu/g of silage)	Anaerobic spores (log_{10} cfu/g of silage)	Spores of gas forming anaerobic bacteria (log_{10} MPN/g of silage)[3]	*Paenibacillus* spp. (% of anaerobe population)	Yeast (log_{10} cfu/g of silage)	Mold (log_{10} cfu/g of silage)	dT[4] (°C)	Hours temperature >30°C
Days of air exposure														
0	PE	29.7	3.78	4.53	2.76	258	2.65	0.31	<1.48	58.2	3.12	1.74	−0.8	0
	OB	31.2	3.73	5.32	2.27	323	2.97	0.44	<1.48	62.5	1.17	1.41	−0.7	0
2	PE	30.8	3.84	4.21	2.55	210	3.45	0.41	<1.48	35.7	4.74	1.44	0.0	0
	OB	31.7	3.76	5.14	1.96	274	2.95	0.63	<1.48	71.4	4.18	1.49	−0.3	0
5	PE	33.2	4.66	1.89	0.93	187	5.20	1.05	<1.48	48.7	6.84	1.74	15.2	47
	OB	32.5	3.77	5.25	2.28	210	2.94	0.33	<1.48	62.5	6.05	1.31	−0.7	0
7	PE	32.9	4.82	1.02	1.11	113	7.13	4.83	3.76	66.7	6.86	5.24	15.5	95
	OB	33.5	3.86	5.02	2.28	153	3.90	2.19	1.56	83.3	7.07	2.32	3.2	0
9	PE	34.3	5.76	0.29	0.27	96	8.90	7.73	3.60	70.0	6.97	8.10	19.3	143
	OB	33.7	4.23	3.88	1.63	129	3.12	1.50	<1.48	80.0	7.90	4.74	9.3	18
14	PE	32.2	6.56	0.45	0.18	0	9.30	7.81	<1.48	88.9	6.76	9.23	3.8	199
	OB	34.6	5.63	1.44	1.01	16	7.87	5.66	1.52	93.3	7.86	9.23	20.2	89
	SE	0.56	0.16	3.89	1.79	22	0.43	0.49	—	—	0.34	0.53	1.44	11
F		NS	0.008	0.003	NS	NS	0.004	0.017	—	—	NS	0.023	0.020	0.001
T		NS	<0.001	<0.001	0.035	<0.001	<0.001	<0.001	—	—	<0.001	<0.001	<0.001	<0.001
I × T		NS	0.003	NS	NS	NS	<0.001	<0.001	—	—	<0.001	<0.001	<0.001	<0.001

[1]F = film effect; T = air exposure time effect; I × T = interaction effect (*P*-values).

[2]PE = polyethylene film; OB = oxygen-barrier film.

[3]MPN = most probable number.

[4]Difference between silage temperature and ambient temperature.

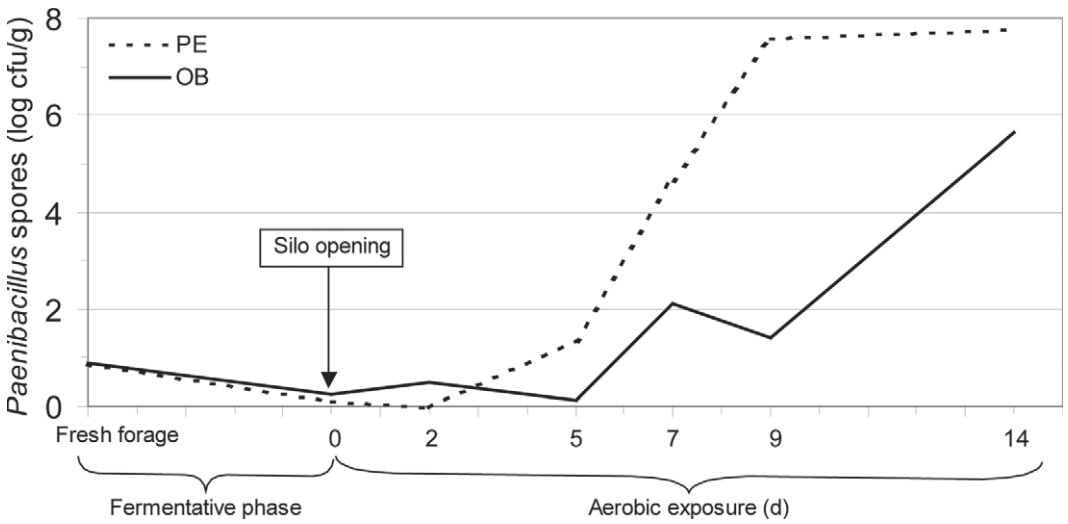

Figure 1. Dynamics of *Paenibacillus* spp. spores during air exposure of corn silages below polyethylene (PE) or oxygen-barrier (OB) films.

5 d of aerobic exposure, and then started to increase in PE film silages, reaching 7.73 $\log_{10}$ cfu/g of silage after 9 d of aerobic exposure. The OB film delayed spore outgrowth until d 9, and values of 5.66 $\log_{10}$ cfu/g silage were observed after 14 d of aerobic exposure. The anaerobic gas-forming spores were below 2.0 $\log_{10}$ MPN/g of silage at silage opening and started to increase in the PE film silages after 7 d of aerobic exposure, reaching values of 3.76 $\log_{10}$ MPN/g of silage at d 7. At d 14, anaerobic gas-forming spores again decreased to values below 2 $\log_{10}$ MPN/g of silage. The OB silages did not show any increase in anaerobic gas-forming spores, which always remained below 2.00 $\log_{10}$ MPN/g of silage. *Paenibacillus* spore counts during silage conservation and after exposure of silages to air are reported in Figure 1. *Paenibacillus* spores started to increase at 5 d of air exposure in the PE film treatment and reached values greater than 7 $\log_{10}$ cfu/g after 9 d of conservation, whereas the OB treatment delayed the increase in spores to values greater than 3 $\log_{10}$ cfu/g until d 9. The yeast count increased from the second day of air exposure in both treatments and reached 6 $\log_{10}$ cfu/g of silage after 5 d of air exposure, whereas the mold counts remained almost constant until 5 d of air exposure and then started to increase at d 7, with greater values in the PE film silage than in the OB silage. When the yeast count became greater than 6.5 $\log_{10}$ cfu/g of silage, the silage temperature started to rise and dT reached values greater than 15°C after 5 d of air exposure for PE film and 14 d for OB film. The temperature of PE film silages remained greater than 30°C for about 200 h, from d 3 to 14, whereas OB silage temperatures rose above 30°C on d 9 and remained over 30°C until d 14 of aerobic exposure. Pearson correlation coefficients of fermentative characteristics, microbiological composition, and temperature indices of the silages after opening and during air exposure are reported in Table 3. *Paenibacillus* spores showed a positive correlation with pH, yeast count, mold count, dT, hours with temperature greater than 30°C, and aerobic and anaerobic spores, whereas it was negatively correlated with the nitrate, lactic acid, and acetic acid concentrations. Furthermore, the mold count was highly positively correlated with the pH, hours with temperature greater than 30°C, and aerobic and anaerobic spores.

Anaerobic Spore Identification

The anaerobic spore-forming bacteria population mainly consisted of *Paenibacillus* (50%) and *Clostridium* spp. (25%; Figure 2). The *Paenibacillus* population on forage before ensiling mainly consisted of *P. macerans* (95% of the selected colonies) and *Clostridium* species were mainly ascribed to *C. proteoclasticum*, *C. intestinale*, and *C. beijerinckii*. The anaerobic spore-forming bacteria after 110 d of silage conservation mainly consisted of *P. macerans* (58.2 and 62.5% for the PE film and OB film, respectively) and *Clostridium* spp. (18.2 and 4.2% for the PE film and OB film, respectively; Figure 2). *Paenibacillus* spores constituted from 36 to 93.3% of the anaerobic spore-former population during air exposure, and was the dominant species in the anaerobic spore-former population after 7 d of air exposure.

DISCUSSION

The presence of *Paenibacillus* in farm and processing environments suggests several different potential sources of milk contamination from these organisms

Table 3. Pearson correlation coefficients of the chemical and microbiological composition of corn silage and temperature (temp.) indices of the silage after air exposure

Item	*Paenibacillus* spores	pH	Nitrate	Lactic acid	Acetic acid	Yeast	Mold	Aerobic spores	dT[1]	Hours temp. >30°C[2]
pH	0.931**									
Nitrate	−0.691**	−0.672**								
Lactic acid	−0.716**	−0.751**	0.545**							
Acetic acid	−0.712**	−0.792**	0.538**	0.879**						
Yeast	0.520**	0.530**	−0.626**	−0.516**	−0.497**					
Mold	0.878**	0.867**	−0.644**	−0.658**	−0.597**	0.573**				
Aerobic spores	0.866**	0.836**	−0.565**	−0.798**	−0.680**	0.519**	0.875**			
dT	0.628**	0.617**	−0.507**	−0.623**	−0.557**	0.654**	0.610**	0.619**		
Hours temp. >30°C	0.940**	0.949**	−0.633**	−0.765**	−0.739**	0.462**	0.843**	0.880**	0.562**	
Anaerobic spores	0.999**	0.903**	−0.689**	−0.722**	−0.713**	0.515**	0.872**	0.868**	0.613**	0.943**

[1]Difference between silage temperature and ambient temperature.

[2]Number of hours with temperature greater than 30°C.

**$P < 0.01$.

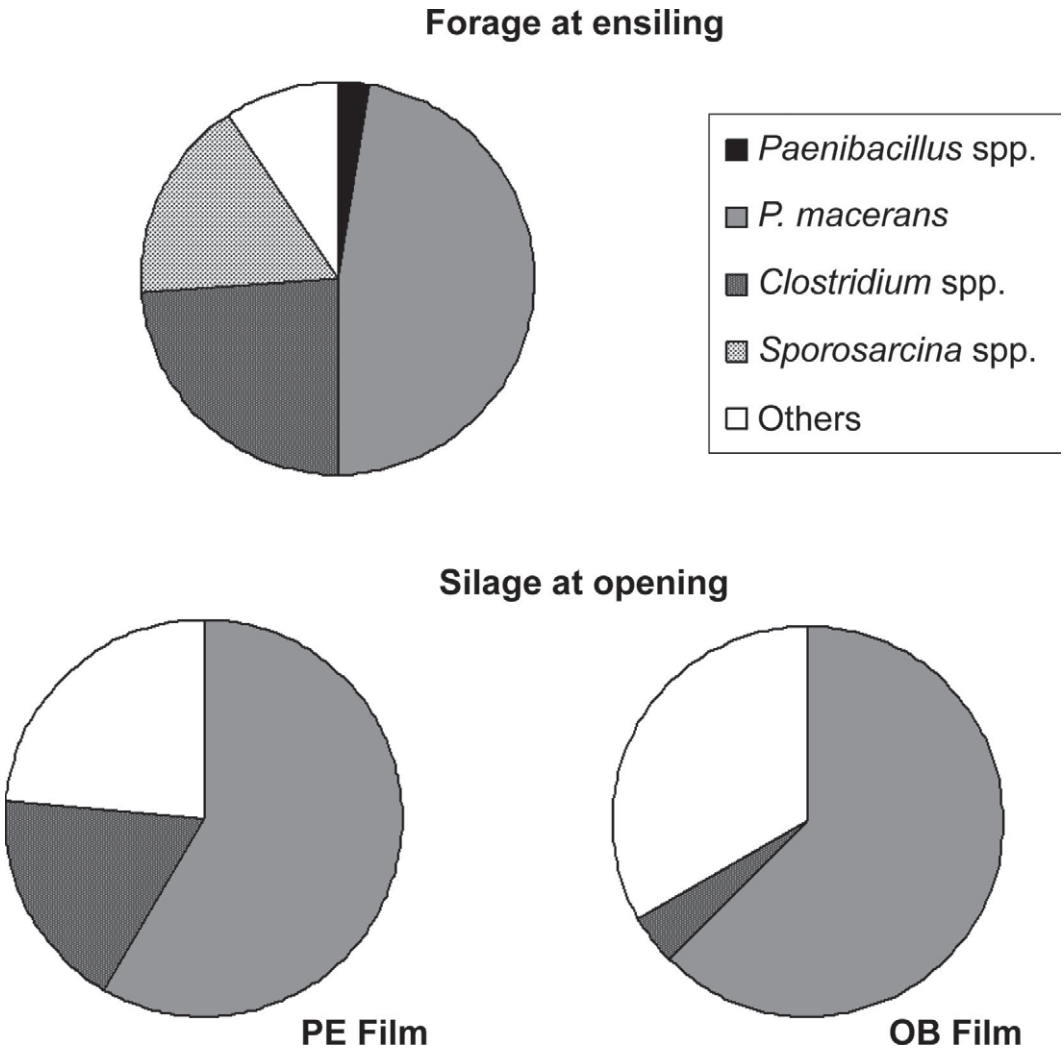

Figure 2. Distribution of anaerobic spore-forming bacteria isolates in forage at harvesting before ensiling (n = 55) and in silage after 110 d of conservation below polyethylene (PE) or oxygen-barrier (OB) films (n = 37 and 20, respectively).

(Ivy et al., 2012). To prevent spoilage of pasteurized dairy products, spore-forming bacteria should be controlled through a chain management approach (Vissers et al., 2007c).

The primary objective of this research was to identify, through sequencing of the V1 to V3 region of 16S rRNA, the spore populations of *Paenibacillus* spp. in corn silage and to study whether aerobic deterioration could influence the amount of *Paenibacillus* spores in the spoiled parts, because corn silage is the main source of forage for lactating dairy cows in Europe and North America. In this work, the presence of *Paenibacillus* spores was found on corn before ensiling and at silo opening at levels below 1 $\log_{10}$ cfu/g of silage, whereas they increased during aerobic exposure to values greater than 6 $\log_{10}$ cfu/g. The large increase in *Paenibacillus* spores during aerobic deterioration showed that if this deteriorated silage were to be accidentally supplied to animals, it could contaminate the feed ration. Silages are known to be an important contamination source of other aerobic and anaerobic spore formers, such as *Clostridium* spp. or *Bacillus cereus*, in raw milk, through dung contamination during milking (Stadhouders and Spoelstra, 1990; Magnusson et al., 2007), especially when aerobic deterioration takes place during the feed-out period (Vissers et al., 2007a; Borreani and Tabacco, 2008). Only a few previous studies have reported the presence of *Paenibacillus* in grass silage (te Giffel et al., 2002) and in corn silage (Rossi and Dellaglio, 2007), but until now it has not been clear whether the presence of *Paenibacillus* is due to environmental contamination from the soil or to growth during the anaerobic silage fermentation or aerobic feed-out phases.

Results from this work have shown that the anaerobic spore formers decreased during conservation, from harvesting to silo opening by 12.3 and 9.1 fold for silages stored under PE and OB films, respectively, and aerobic spore formers decreased by 8.3 and 4.0 fold under PE and OB films, respectively. These data are consistent with results from previous work by Borreani and Tabacco (2008) and Tabacco et al. (2009), who observed a 5- to 11-fold reduction in anaerobic spore-former count on corn silage from harvesting to silo opening in those parts of the silo that were surely under anaerobiosis (i.e., the core of bunker silos and laboratory macro-silos). This reduction could be ascribed to recent advances in corn silage management and technology, which allow the time of air exposure during silo filling to be reduced and the level of anaerobiosis during conservation until silo opening to be enhanced. These factors greatly reduce the risk of spore-former contamination and growth during the fermentative phase (Vissers et al., 2007a; Borreani and Tabacco, 2008), whereas the control of the activity of these microorganisms after exposure of silage to air continues to be a great challenge, especially at the farm level (Muck, 2013). The growth of anaerobic gas-forming spores during the feed-out phase of corn silages, when aerobic deterioration takes place, has already been shown by the MPN method for anaerobic gas-forming spore formers (Vissers et al., 2007a; Borreani and Tabacco, 2008).

To reduce the incidence of aerobic deterioration, inoculants containing *Lb. buchneri* have been used over the last decade to decrease yeast counts in silages through an increase in the amount of acetic acid produced during fermentation (Kleinschmit and Kung, 2006). Furthermore, Borreani et al. (2007) have confirmed the relevance of silage management factors, such as the daily feed-out rate, the use of a *Lb. buchneri*-based inoculant and the use of an OB film to cover the silage mass to improve fermentation quality in the peripheral area of bunker silos. In the present study, the fermentative profile of silages, stored under OB and PE films, was typical of fermentation driven by *Lb. buchneri*, with a greater acetic acid content than 2% of DM and lower lactic acid-to-acetic acid ratio (<3). Furthermore, the low permeability to oxygen of the OB film contributed to increased anaerobic conditions of the silo. Consequently, OB silages had a greater lactic acid content, and lower pH and acetic acid content than PE film silages. The better anaerobic environment under the OB film also reduced yeast counts to below 2.0 $\log_{10}$ cfu/g of silage, and consequently increased the aerobic stability of silages after exposure to air. All these findings are in agreement with results of Borreani et al. (2007), who reported, for corn bunker silage treated with *Lb. buchneri*, a greater lactic acid content and lower pH, lower yeast count, and greater aerobic stability in silages stored under the OB film than silages stored under PE film.

The main results of the present study refer to the characterization of the anaerobic spore-former population. Among spore formers, *P. macerans* (a gram-positive, facultative anaerobic organism) has shown to be able to develop during aerobic exposure of corn silage and, in this experiment, became the dominant anaerobic spore former after 7 and 14 d of air exposure in PE and OB film silages, respectively. This species was present in the herbage at harvesting, together with clostridia spores, and survived ensiling fermentation. At silage opening, *P. macerans* constituted more than 60% of the anaerobic spore count. Spores of *P. macerans* were found in a farm silage survey by Rossi and Dellaglio (2007) on a sample of corn silage at opening after 50 d of conservation that had a pH of 3.77, and the colony was picked in the 1/10 dilution tube of the

MPN method used to isolate clostridia. In the present experiment, the spore concentration of *P. macerans* increased to >7.0 $\log_{10}$ cfu/g of silage during aerobic spoilage of the silage. For the first time, the results reported in this work show evidence of the outgrowth of *P. macerans* in corn silage during aerobic exposure. Some other authors have hypothesized that *Paenibacillus* spp. outgrowth could occur in silage during the later stages of aerobic spoilage (Driehuis, 2013).

Conversely, the outgrowth of clostridia, which were present at ensiling (25% of anaerobic spore formers) and at silo opening (4 and 18% for PE and OB film silages, respectively) did not dominate during aerobic exposure, and this was probably due to the presence of nitrate in the silage until 9 d of aerobic exposure. Data are consistent with results reported by Tabacco et al. (2009), who reported that the growth of clostridia during aerobic deterioration was only observed when the nitrate was exhausted to lower values than the detection limit (i.e., 100 mg/kg of herbage).

The different plastic films used to seal silages affected the growth of *P. macerans* and the number of spores during aerobic exposure of silages. In particular, silages below the OB film needed more than 9 d of aerobic exposure to reach a greater level of *P. macerans* spores than 3 $\log_{10}$ spores/g; however, this limit was surpassed after 5 to 7 d of aerobic exposure with the PE film. The results have highlighted the role of aerobic spoilage in the growth of *P. macerans* in silage, because the Pearson correlation coefficients between *Paenibacillus* and pH and mold count were 0.931 and 0.878, respectively. The yeast and mold activity, through the consumption of organic acids (lactic and acetic acids), increased the pH and temperature of silages, which stimulated the growth of aerobic and anaerobic spore formers. Among anaerobic spore formers, *Paenibacillus* resulted to be dominant when the pH was greater than 4.5 and temperature was above 30°C for at least 75 h, irrespective of which plastic film was used to cover silages. Most silages at the farm level are exposed to air during conservation, due to the permeability of plastic to air and difficulties in sealing the outer layer of silage properly, or during the feed-out phase, due to an inadequate amount of silage being removed and to poor management of the exposed silo surface. Consequently, severely deteriorated parts coexist at a farm level with well-preserved silage at the feed-out face (Borreani and Tabacco, 2012). In this experiment, when *Paenibacillus* spores had increased to greater values than 5 $\log_{10}$ spores/g (after 7 and 14 d for PE and OB film silages, respectively), the pH, silage temperature, and yeast and mold counts were similar to those observed in the peripheral areas of farm corn silages by Borreani and Tabacco (2010). The presence of these deteriorated parts have led to the recognition of silage as the main source for endospores relevant to the dairy sector (Stadhouders and Spoelstra, 1990; Pahlow et al., 2003), and it is evident that it is always preferable to keep their presence to a minimum from the very beginning through good ensiling practices (Pahlow et al., 2003). Furthermore, Mahanna and Chase (2003) reported that the role of silages on the contamination of TMR with spore formers could be important, especially when aerobically deteriorated silages are not perfectly discarded and then included in the TMR. We speculate that 100 g of silage with a greater *Paenibacillus* spore count than 6 $\log_{10}$ cfu/g (as was found in the present work) could contaminate 30 kg of TMR at a level of 3.5 $\log_{10}$ cfu/g, thus increasing the risk of transmission of this microorganism to the milk. For aerobic spore formers, Walstra et al. (2005) reported that to achieve a shelf life of 7 d for pasteurized milk, the raw milk before processing should always contain less than 3 $\log_{10}$ spores/L, but this level of contamination cannot be achieved when aerobic spore-former counts are above 3 $\log_{10}$ spores/g of feed (Vissers et al., 2007c).

CONCLUSIONS

Results from this experiment indicate that *Paenibacillus* spores could greatly increase in silage during aerobic deterioration. The study also confirms that technical solutions, such as the use of inoculants containing *Lb. buchneri* (Schmidt and Kung, 2010) and (or) the use of OB films to cover silage (Borreani et al., 2007), which could help improve silage quality and its aerobic stability, especially in the silo parts that are more at risk of spoilage, could also reduce the risk of having silage parts heavily contaminated with aerobic and anaerobic spore formers. Further work is needed to determine the incidence of *Paenibacillus* spores in different areas of commercial silos to understand the role on *Paenibacillus* spore outgrowth of different forages, nitrate content, and management factors in practical-scale silages.

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