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Microbial Dynamics during Aerobic Exposure of Corn Silage Stored under Oxygen Barrier or Polyethylene Films▽

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The aims of this study were to compare the effects of sealing forage corn with a new oxygen barrier film with those obtained by using a conventional polyethylene film. This comparison was made during both ensilage and subsequent exposure of silage to air and included chemical, microbiological, and molecular (DNA and RNA) assessments. The forage was inoculated with a mixture of *Lactobacillus buchneri*, *Lactobacillus plantarum*, and *Enterococcus faecium* and ensiled in polyethylene (PE) and oxygen barrier (OB) plastic bags. The oxygen permeability of the PE and OB films was 1,480 and 70 cm^3 m^{-2} per 24 h at 23°C, respectively. The silages were sampled after 110 days of ensilage and after 2, 5, 7, 9, and 14 days of air exposure and analyzed for fermentation characteristics, conventional microbial enumeration, and bacterial and fungal community fingerprinting via PCR-denaturing gradient gel electrophoresis (DGGE) and reverse transcription (RT)-PCR-DGGE. The yeast counts in the PE and OB silages were 3.12 and 1.17 log_{10} CFU g^{-1}, respectively, with corresponding aerobic stabilities of 65 and 152 h. *Acetobacter pasteurianus* was present at both the DNA and RNA levels in the PE silage samples after 2 days of air exposure, whereas it was found only after 7 days in the OB silages. RT-PCR-DGGE revealed the activity of *Aspergillus fumigatus* in the PE samples from the day 7 of air exposure, whereas it appeared only after 14 days in the OB silages. It has been shown that the use of an oxygen barrier film can ensure a longer shelf life of silage after aerobic exposure.

Forage ensiling is based on the natural fermentation of water-soluble plant carbohydrates by lactic acid bacteria (LAB) under anaerobic conditions (24). The most important single factor that can influence the preservation efficiency of forage ensiling is the degree of anaerobiosis reached in the completed silo (36). Anaerobic conditions are not always achieved in silos on individual farms, especially in the outer layer of a silo, because of the difficulty of sealing it efficiently (6). The aerobic deterioration of silages is a significant problem for farm profitability and feed quality throughout the world. All silages exposed to air deteriorate as a result of aerobic microbial activity during feed-out (8, 31, 35). These losses can reach 70% of the stored dry matter in the top layer and near the sidewalls of the bunkers and are related to the depletion of the digestible carbohydrate and organic acid fractions (5). Spoilage of silage due to exposure to air is undesirable, due to the resulting decrease in nutritive value and to the risk of negative effects on animal performance (18), which are also connected to the proliferation of potentially pathogenic or otherwise undesirable microorganisms (20) and mycotoxin synthesis (28).

Polyethylene (PE) films have been used for many years to seal bunker silos and drive-over piles because of their suitable mechanical characteristics and low costs. The high O_2 permeability of PE films can contribute to the low quality of silage in the top layer of horizontal silos (6). A new silage sealing plastic film, which uses a new plastic formulation with an 18-fold-lower oxygen permeability than the PE film usually used on farms, has recently been developed (7). Polymers different from PE, such as polyamides (PA) and ethylene-vinyl alcohol (EVOH), help create an excellent barrier against oxygen, combined with good mechanical characteristics (puncture resistance), and are suitable for blown coextrusion with PE to produce 45- to 200-μm-thick plastic films.

Monitoring microbiota during the ensiling process has become more reliable and accurate, thanks to recently developed culture-independent methods (25, 29). In recent years, DNA-based community fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP), have been applied to investigate the microbial community composition of silage (9, 22). Advanced molecular biological techniques have been used to further our understanding of the structure of complex microbial community dynamics (25). However, to the best of our knowledge, no study has used a community fingerprinting approach to investigate the population dynamics of corn silage during aerobic deterioration.

In this study, a culture-independent technique, PCR-DGGE, was used to study microbial dynamics, whereas reverse transcription (RT)-PCR-DGGE allowed us to investigate the metabolically active populations. These techniques were performed together with conventional microbiological enumeration in order to investigate the effects of a new oxygen barrier film on the microbial community of ensiled and aerobically deteriorated corn silages.

MATERIALS AND METHODS

Crop and ensiling. The trial was carried out at the experimental farm of the University of Turin on the western Po plain in northern Italy (44°50′N, 7°40′E;

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altitude, 232 m above sea level) in 2008 on corn (*Zea mays* L.) harvested as a whole-corn crop, at a 50% milk line stage, and at 333 g dry matter (DM) kg^{-1} of fresh forage. The forage was chopped with a precision forage harvester to a 10-mm theoretical length and 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 yield 1×10^5, 8×10^3, and 2×10^3 CFU per gram of fresh forage, respectively. Standard black-on-white polyethylene film, 120 μm thick (PE), and 120-μm-thick Silostop (Bruno Rimini Ltd., London, United Kingdom), black-on-white coextruded polyethylene-polyamide film with an enhanced oxygen barrier (OB), were used to produce the silage bags for this experiment. Bags were heat sealed at the closed end and were equipped with a one-way valve for CO_2 release. Each bag was inserted into a portion of a PVC (polyvinyl chloride) tube (internal dimensions, 300-mm diameter and 300-mm height; 21-liter volume) so that just the top and the bottom of the bag had access to air. All bags were then filled with about 12 kg of fresh forage, which was compacted manually, and secured with plastic ties. Four replicates were prepared for each treatment. The density of the silage was 576 kg fresh matter (FM) m^{-3} and 192 kg DM m^{-3}. The oxygen permeability of PE and OB, determined by ASTM standard method D 3985-81 (4), was 1,480 and 70 $cm^3\ m^{-2}$ per 24 h at 100 KPa at 23°C and 0% relative humidity, respectively. The silos were stored at ambient temperature (18 to 22°C) indoors and opened after 110 days. The final weights were recorded at silo opening, and the silage was mixed thoroughly and subsequently sampled. The DM concentration (three replicates) and fermentation end products (two replicates) were determined for each sample. Microbiological counts (two replicates) and culture-independent techniques (two replicates) were also carried out. The 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 three kilograms from each silo was allowed to aerobically deteriorate at room temperature (22 ± 1.6°C) in 17-liter polystyrene boxes (290-mm diameter and 260-mm height) for 14 days. A single layer of aluminum cooking foil was placed over each box to prevent drying and dust contamination but also allowed air penetration. The temperature of the room and of the silage was 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 (27). The silage was sampled after 0, 2, 5, 7, 9 and 14 days of aerobic exposure to quantify the microbial and chemical changes of the silage during exposure to air.

Sample preparation and analyses. Each of the pre-ensiled samples of each herbage and the samples of silage taken from each bag of silage were split into three subsamples. One subsample was oven-dried at 65°C to constant weight to determine the DM content and air equilibrated, weighed, and ground in a Cyclotec mill (Tecator, Herndon, VA) to pass through a 1-mm screen. The dried samples were analyzed for total nitrogen (TN) by combustion (30), according to the Dumas method, using a Micro-N nitrogen analyzer (Elementar, Hanau, Germany), and for ash by complete combustion in a muffle furnace at 550°C for 3 h. A portion of the second subsample was extracted using a stomacher blender (Seward Ltd., London United Kingdom) for 4 min in distilled water at a ratio of water to sample material (fresh weight) of 9:1, and another portion was extracted in H_2SO_4 (0.05 mol $liter^{-1}$) at a ratio of acid to sample material (fresh weight) of 5:1. The nitrate (NO_3) contents were determined in the water extract, through semiquantitative analysis, using Merckoquant test strips (7). The ammonia nitrogen (NH_3-N) content, determined using a specific electrode, was quantified in the water extract. The lactic and monocarboxylic acids (acetic, propionic, and butyric acids) were determined by high-performance liquid chromatography (HPLC) in the acid extract (10). Ethanol, for which the HPLC was coupled to a refractive index detector, was also measured using an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA). The analyses were performed isocratically under the following conditions: mobile phase, 0.0025 mol $liter^{-1}$ H_2SO_4; flow rate, 0.5 ml min^{-1}; column temperature, 37°C; injection volume, 100 μl. Duplicate analyses were performed for all the determined parameters. The duplicates were averaged, and the four means (one for each silo) were considered four observations in the statistical analysis. The water activity (a_w) of the silage was measured at 25°C using an AquaLab series 3TE instrument (Decagon Devices Inc., Pullman, WA) on a fresh sample at silo opening. The weight losses due to fermentation were calculated as the difference between the weight of the plant material placed in each silo at ensiling and the weight of the silage at the end of conservation.

A third subsample was used for the microbiological analyses. For the microbial counts, 30 g of sample were transferred into sterile homogenization bags, suspended at 1:10 (wt/vol) in peptone salt solution (PPS; 1 g of bacteriological peptone and 9 g of sodium chloride per liter), and homogenized for 4 min in a stomacher blender (Seward Ltd., London, United Kingdom). Serial dilutions were prepared, and the following counts were carried out: (i) aerobic spores after pasteurization at 80°C for 10 min followed by double-layer pour plating with 24.0 g $liter^{-1}$ nutrient agar (NUA; Oxoid, Milan, Italy) and incubation at 30°C for 3 days; (ii) mold and yeast on 40.0 g $liter^{-1}$ of yeast extract glucose chloramphenicol agar (YGC agar; Difco, West Molesey, Surrey, United Kingdom) after incubation at 25°C for 3 and 5 days for yeast and mold, respectively. The mean count of the duplicate subsamples was recorded for the microbial counts on plates that yielded 10 to 100 CFU per petri dish.

Microbial community fingerprinting by PCR-DGGE and RT-PCR-DGGE. (i) Sampling and nucleic acid extraction. Two milliliters of the supernatant of the above-described 1:10 diluted sample suspension were collected for each sampling point and centrifuged at 13,400 × *g* for 5 min to pellet the cells. After the supernatant had been discarded, the pellet was subjected to DNA and RNA extraction using DNeasy and RNeasy plant minikits (Qiagen, Milan, Italy), respectively, according to the manufacturer's instructions. The presence of residual DNA in the RNA samples was checked by PCR (12).

(ii) PCR and RT-PCR. The dominant bacterial microbiota was investigated, at both the DNA and RNA level, by PCR-DGGE and RT-PCR-DGGE. The primers 338fGC and 518r were used to detect and amplify the bacterial variable V_3 region of 16S rRNA gene (1). In order to investigate the dominant fungal microbiota, the D1-D2 loop of the 26S rRNA gene was amplified by PCR using the primers NL1GC and LS2 (11).

(iii) DGGE analysis. The Dcode universal mutation detection system (Bio-Rad) was used to perform DGGE analysis. The amplicons obtained from the PCR and RT-PCR were applied to an 8% (wt/vol) polyacrylamide gel (acrylamide-bisacrylamide, 37.5:1) with a 30%-to-60% denaturant gradient (13). Some selected DGGE bands were excised from the gels and incubated overnight at 4°C in 50 μl of sterile water. The eluted DNA was reamplified and analyzed in DGGE (1). The amplicons that gave a single band comigrating with the control were then amplified with a 338f primer and NL1 primer, respectively, for bacterial and fungal microbiota without a GC clamp and purified with Perfectprep gel clean-up (Eppendorf, Milan, Italy) for sequencing.

(iv) Sequence analysis. The PCR-DGGE and RT-PCR-DGGE bands were sent for sequencing to Eurofins MWG Operon (Ebersberg, Germany), and the gene sequences obtained were aligned with those in GenBank using the BLAST program (2) to establish the closest known relatives of the amplicons run in DGGE.

Statistical analysis. All microbial counts and hours of aerobic stability were $\log_{10}$ transformed to obtain log-normal-distributed data. The fermentative characteristics, microbial counts, pH, nitrate contents, dry weight losses, and hours of aerobic stability were subjected to a one-way analysis of variance (Statistical Package for Social Science, version 16; SPSS Inc., Chicago, IL) to evaluate the statistical significance of the differences between the two treatments. Between-treatment comparisons were made using an unpaired Student's *t* test, and differences were considered significant at $P < 0.05$.

DGGE profiles were normalized and subjected to cluster analysis using BioNumerics software (Applied Maths, Kortrijk, Belgium). The Pearson product moment correlation coefficient was used to calculate the similarities in DGGE patterns, and dendrograms were obtained via the unweighted pair group method with arithmetic averages.

RESULTS

Fermentative quality and microbial counts of the silages. The results of the chemical and microbial determination of the corn forage prior to ensiling are shown in Table 1. The values are typical of those of corn harvested at a 50% milk line. The fermentation quality and microbial composition of the silages, after 110 days of conservation, are shown in Table 2. All the silages were well fermented. The main fermentation acids found were lactic and acetic acids, whereas butyric acid was below the detection limit (less than 0.1 g kg^{-1} DM) in all the silages. The silages sealed with the PE film led to silages with higher pH ($P < 0.002$), and lower concentrations of lactic acid ($P < 0.033$) in comparison to the OB silages. The nitrate levels in the corn crop were lower in the silages than in the corresponding herbage. The yeast counts were lower below the OB film, whereas the mold count was below 2 $\log_{10}$ CFU g^{-1} silage in both treatments. The a_w of the silages at opening had a

TABLE 1. Chemical and microbial composition of the corn forage prior to ensiling

Parameter[a]	Value
DM (g kg^{-1})	333
pH	5.84
TN (g kg^{-1} DM)	12.6
Starch (g kg^{-1} DM)	262
NDF (g kg^{-1} DM)	443
ADF (g kg^{-1} DM)	248
Ash (g kg^{-1} DM)	39.0
Nitrate (mg kg^{-1} herbage)	1,181
a_w	0.98
Yeasts ($\log_{10}$ CFU g^{-1} herbage)	6.90
Molds ($\log_{10}$ CFU g^{-1} herbage)	6.13
Aerobic spores ($\log_{10}$ CFU g^{-1} herbage)	3.57

[a] a_w, water activity; ADF, acid detergent fiber; DM, dry matter; NDF, neutral detergent fiber; TN, total nitrogen.

TABLE 2. Fermentation quality and microbial composition at unloading of silages sealed with oxygen barrier (OB) and standard polyethylene (PE) films after 110 days of conservation

Parameter[a]	Value for:		SE	*P* value
	PE	OB		
pH	3.78	3.73	0.011	0.002
DM (g kg^{-1})	297	310	8.22	0.500
Lactic acid (g kg^{-1} DM)	45.3	53.2	2.08	0.033
Acetic acid (g kg^{-1} DM)	27.6	22.7	1.23	0.019
Butyric acid (g kg^{-1} DM)	<0.10	<0.10		
Propionic acid (g kg^{-1} DM)	0.45	0.76	0.164	0.402
1,2-Propanediol (g kg^{-1} DM)	10.5	10.4	0.706	0.981
Ethanol (g kg^{-1} DM)	12.3	11.2	0.651	0.443
Lactic-to-acetic acid ratio	1.64	2.34	0.165	0.004
Nitrate (mg kg^{-1} silage)	837	1026	156	0.603
NH_3-N (g kg^{-1} TN)	46.8	45.8	0.112	0.746
Ash (g kg^{-1} DM)	40.6	40.2	0.031	0.656
a_w	0.99	0.99	0.001	0.947
Yeasts ($\log_{10}$ CFU g^{-1} silage)	3.12	1.17	0.443	<0.001
Molds ($\log_{10}$ CFU g^{-1} silage)	1.74	1.41	0.118	0.189
Aerobic spores ($\log_{10}$ CFU g^{-1} silage)	2.65	2.97	0.095	0.095
Weight loss (g kg^{-1} DM)	37.5	30.6	0.178	0.035
Aerobic stability (h)	65	152	19.9	0.001

[a] a_w, water activity; C, control treatment; DM, dry matter; LAB, lactic acid bacteria; NH_3-N, ammonia nitrogen; TN, total nitrogen.

mean value of 0.99, and there was no difference between the two treatments. The weight losses were lower in the OB silages than in the PE silages. The aerobic stabilities of the silages exposed to air were 65 and 152 h in the PE and in OB silages, respectively.

Silage quality during the air exposure test. The changes in temperature, pH, lactic acid, yeast and mold counts, and numbers of aerobic spores in the silages for 14 days of aerobic exposure are reported in Fig. 1. The temperature at silo opening was about 22°C for both treatments. After 65 h of aerobic exposure, the temperature of the PE silages started to rise, whereas the temperature in the OB silages did not increase over the first 6 days of exposure to air. The PE silages showed temperatures above 35°C after 4.7 days (113 h) and reached the highest temperature of 42.2°C after 9.5 days (229 h). Under the OB film, temperatures above 35°C were reached only after 11.4 days (274 h) of air exposure. Simultaneously with the variation in silage temperatures, a pH increase was observed in the two treatments. The pH was always lower in the OB than in the PE silages. The lactic acid concentration started to decrease after 2 days in the PE silage and after 7 days in the OB silage. The yeast count increased from the second day of air exposure in both treatments and reached 6 $\log_{10}$ CFU g^{-1} silage after 5 days of air exposure. The mold counts remained almost constant till day 5 of air exposure and started to increase at day 7, with higher values in the PE silage than in the OB silage. They reached similar values after 14 days of air exposure. The aerobic spore count increased with air exposure time in the PE silages, reaching a maximum value of 9.3 $\log_{10}$ CFU g^{-1} after 14 days of air exposure. The aerobic spore count in the OB silages remained almost constant till day 9 of air exposure and reached a value of 7.8 $\log_{10}$ CFU g^{-1} after 14 days of air exposure.

Bacterial community fingerprinting of the silages and aerobically exposed silages. The bacterial microbiota dynamics was well described through the PCR-DGGE and RT-PCR-DGGE profiles. The DNA and RNA gels are shown in Fig. 2, and the band identification results are reported in Table 3. The inoculated starter, *L. buchneri*, was present for 14 days, at the DNA level, in the OB silage samples (Fig. 2c, band a), whereas it was found for 5 days in the PE silages (Fig. 2a, band a). *L. plantarum* was detected in the samples ensiled in OB only between days 5 and 14 (Fig. 2c, band g). Faint bands were found at the RNA level for these two species. *L. buchneri* was not detected beyond 7 days (Fig. 2d, band a), and *L. plantarum* was detected in the OB samples from days 5 to 9 (Fig. 2d, band g). *Acetobacter pasteurianus* was clearly present at both DNA and RNA levels in the PE silage samples, except for the days immediately subsequent to air exposure (Fig. 2a and b, band b). *A. pasteurianus* was found in OB silages only after 7, 9, or 14 days of aerobic exposure (Fig. 2c and d, band b). Faint bands corresponding to *Bacillus subtilis* were detected at the DNA level in the PE silage samples from day 5 to day 14 (Fig. 2a, band c), whereas *B. subtilis* was recovered in the DNA extracted from the OB samples and at the RNA level only at day 14 (Fig. 2b, c, and d, band c). *Lactobacillus amylovorus* was found at the RNA level in PE silage upon opening (Fig. 2b, band d), together with a band corresponding to an uncultured bacterium (Fig. 2b, band e). Finally, a more persistent band, again identified by sequencing as uncultured bacterium, was observed in the same samples, from day 5 to day 14.

Fungal community fingerprinting of the silages and aerobically exposed silages. The fungal population was detected at the DNA level, in both the PE and OB silages, with a band present from opening to day 7 (Fig. 3a and c, band i, and Table 4). After sequencing, the band was determined to be *Kazachstania exigua*. *Aspergillus fumigatus* was found after 14 days of air exposure (Fig. 3a and c, band h). DNA bands corresponding to *Pichia kudriavzevii* were revealed in PE silages at day 7 and day 9 (Fig. 3a, band l). RT-PCR-DGGE revealed activity of *A. fumigatus*, in particular in the PE silages, where it was detected from day 7 to day 14 of air exposure (Fig. 3b, band h). Unlike the DNA analysis, RNA identified a new band, which was sequenced as *Aureobasidium pullulans*. This species was present for 14 days of aerobic exposure in OB silage (Fig. 3d,

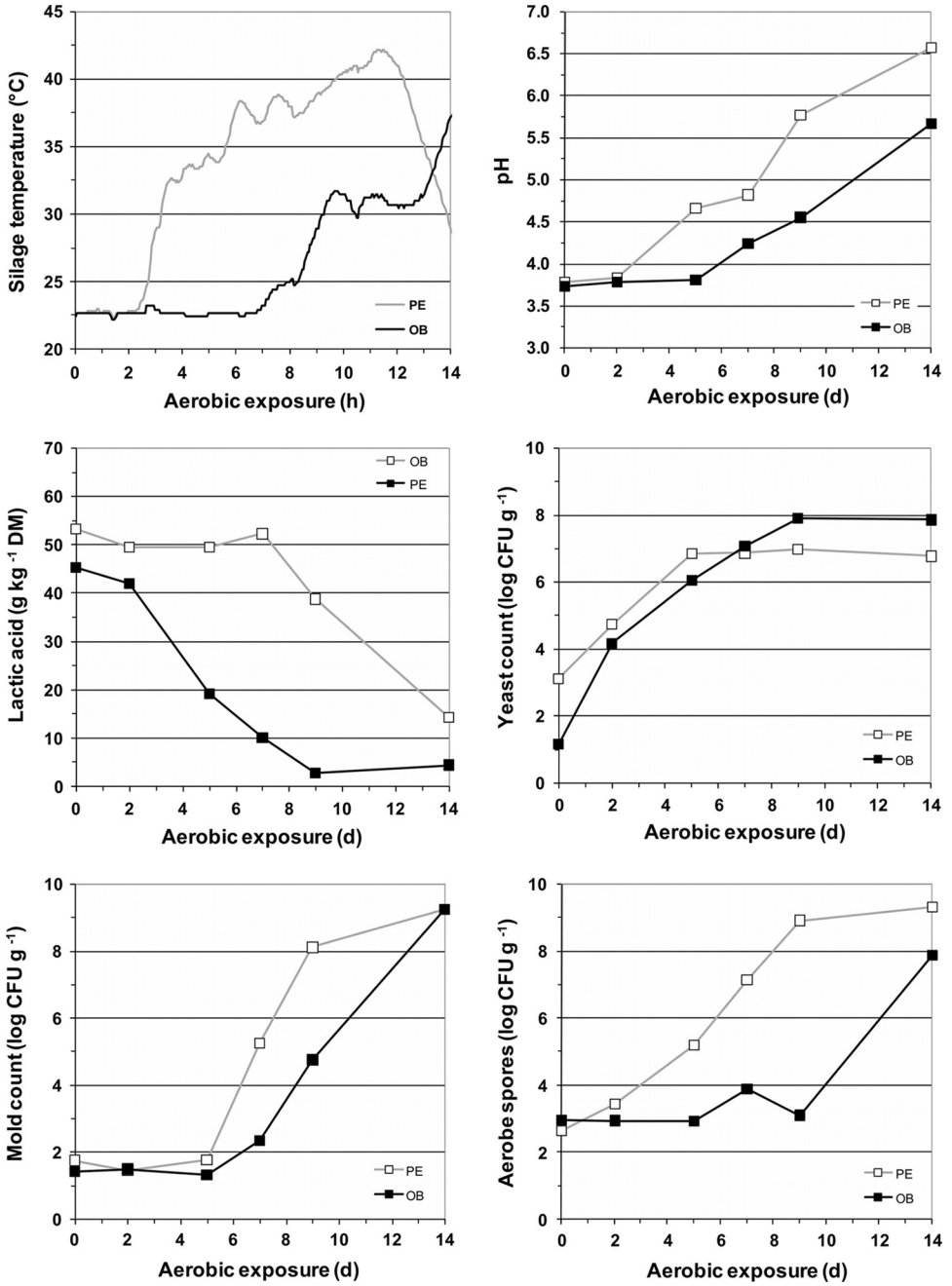

FIG. 1. Dynamics of silage temperature, pH, lactic acid, yeast count, mold count, and aerobe spore count during air exposure of silages. PE, polyethylene film; OB, oxygen barrier film.

band m), whereas it was found for only 5 days of air exposure in the PE silages (Fig. 3b, band m). Furthermore, a band that could not be matched to any fungal species was present in OB silages from day 2 to 9 (Fig. 3d, band n). Finally, at the DNA level, bands run in the middle of the lanes were observed and excised, but when they were reamplified, unclear profiles were obtained, and thus, they were interpreted as heteroduplexes. No important differences in fingerprints were observed between DNA and RNA among the replicates for each treatment.

The cluster analysis highlighted the influence of the PE and OB films on the bacterial DGGE profiles (Fig. 4). A clustering related to DNA and RNA analysis within each treatment can be observed. The DGGE profiles of the mycobiota clustered in two main groups, according to the nucleic acid analyzed (Fig. 5). Clusters correlated to both the sealing treatment and temporal dynamics were noted at the RNA level.

DISCUSSION

An anaerobic environment is the most important individual factor that can influence silage conservation (36). Most of the

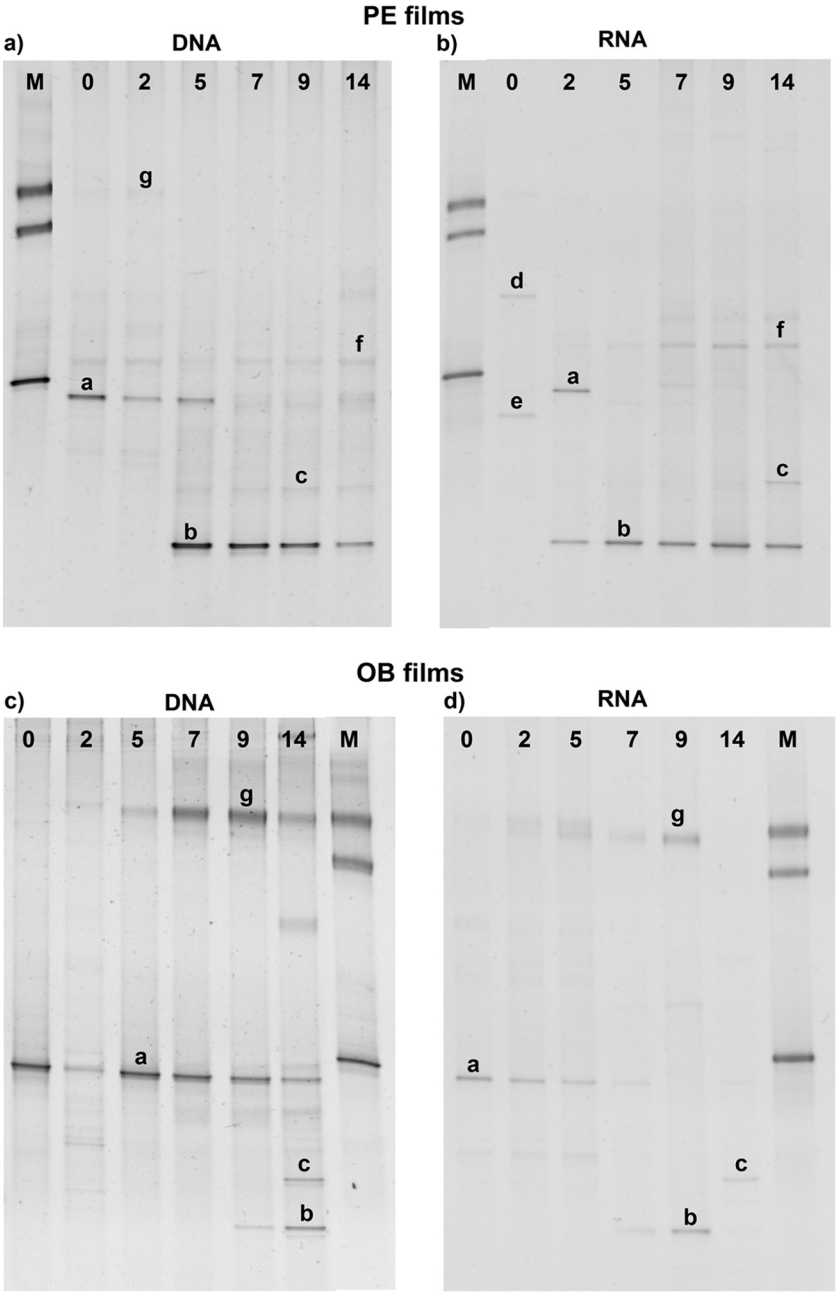

FIG. 2. DGGE profiles of bacterial DNA (a and c) and RNA (b and d) extracted from silage samples exposed to air for 0, 2, 5, 7, 9, and 14 days and ensiled in polyethylene plastic bags (PE) (a and b) or oxygen barrier bags (OB) (c and d). Letters indicate bands that were subjected to sequencing as described in Materials and Methods, and the results are reported in Table 3. Lanes M, markers.

silages on individual farms 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 a poor management of the exposed silo surface (3). These observations highlight risks in terms of: spoilage with losses in nutritional value (33), multiplication of potentially pathogenic microorganisms, and production of mycotoxins (16). Since aerobic microbial populations increase during aerobic deterioration in an exponential manner, the silages from the spoiled top corner and from the molded spots have the potential for contaminating feed-out silage to a great extent, even when it is included in very small amounts. To address the issue of aerobic stability, inoculants containing *L. buchneri* have been used over the last decade with the primary purpose of increasing the amount of acetic acid and, as a consequence, of decreasing yeast counts in silages (17). Plastic oxygen barrier films are also now available to cover silages and to improve the anaerobic environment during conservation (6).

In our study, the fermentative profiles of silages stored under both OB and PE films were typical of fermentation driven by *L. buchneri*, with a relatively high content of acetic acid, low

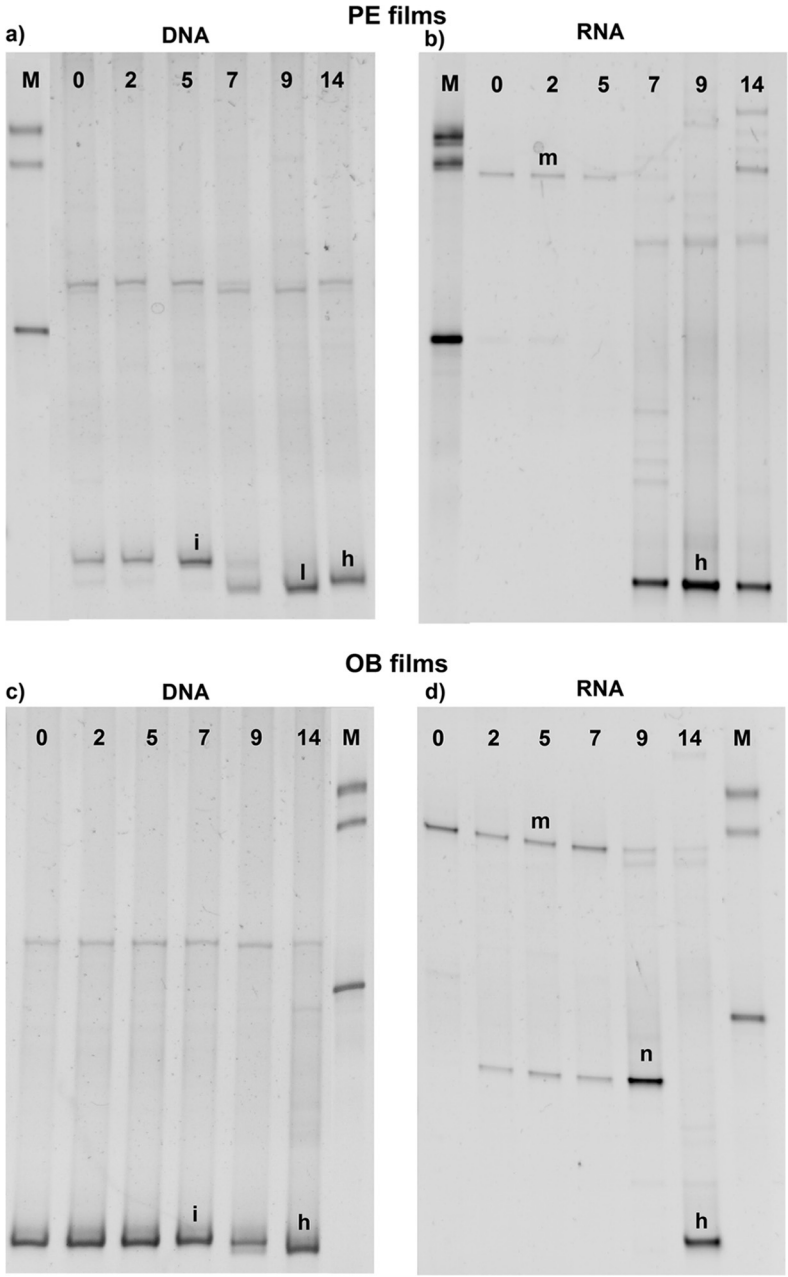

FIG. 3. DGGE profiles of fungal DNA (a and c) and RNA (b and d) extracted from silage samples exposed to air for 0, 2, 5, 7, 9, and 14 days and ensiled in polyethylene plastic bags (PE) (a and b) or oxygen barrier bags (OB) (c and d). Letters indicate bands that were subjected to sequencing as described in Materials and Methods, and the results are reported in Table 4. Lanes M, markers.

TABLE 3. Sequence information for fragments detected on DGGE gels obtained by analyzing the bacterial population through direct DNA and RNA analysis of silage samples

Band	Closest sequence relative	Identity (%)	GenBank accession no.
a	*Lactobacillus buchneri*	99	HM162413
b	*Acetobacter pasteurianus*	98	AP011156
c	*Bacillus subtilis*	100	HQ009797
d	*Lactobacillus amylovorus*	100	EF439704
e	Uncultured bacterium	98	GU343612
f	Uncultured bacterium	100	GQ233026
g	*Lactobacillus plantarum*	100	HQ117897

lactic-to-acetic acid ratio (<3), and the presence of more than 10 g kg^{-1} DM of 1,2-propanediol. These values are in agreement with those reported by Kleinschmit and Kung (17), who summarized the effects of *L. buchneri* on silage quality in 43 experiments. Furthermore, the low permeability to oxygen of the OB film helped create a more anaerobic environment, and this was reflected in a silage with a higher lactic acid content, a lower pH and acetic acid content, and lower weight losses. The better anaerobic environment under the OB film also contributed to reducing yeast counts to below 2.0 $\log_{10}$ CFU g^{-1} of silage. The reduction in yeast counts was reflected in an

TABLE 4. Sequence information for fragments detected on DGGE gels obtained by analyzing the fungal population through direct DNA and RNA analysis of silage samples

Band	Closest sequence relative	Identity (%)	GenBank accession no.
h	*Aspergillus fumigatus*	99	HM807348
i	*Kazachstania exigua*	100	FJ468461
l	*Pichia kudriavzevii*	99	GQ894726
m	*Aureobasidium pullulans*	98	GQ281758
n	Synthetic construct, ankyrin repeat protein E2_17 gene, partial CDS[a]	100	AY195852

[a] CDS, coding sequence.

increase in the aerobic stability of the OB silages, when exposed to air. It is well known that lactate-assimilating yeasts (*Saccharomyces*, *Candida*, and *Pichia* spp.) are generally the main initiators of the aerobic spoilage of silages (24), and under aerobic conditions, they utilize lactic acid, thus causing an increase in silage temperature and pH. In our study, the dominant yeast species after exposure to air, as observed from the DGGE profiles of fungal DNA and RNA, was *Kazachstania exigua*, in both the PE and OB silages. Yeasts of the genus of *Kazachstania* were previously observed in aerobically deteriorating corn silages (21). Furthermore, *Pichia kudriavzevii* was observed in PE silages after 7 days of air exposure. Yeasts of the genus *Pichia* are usually reported to be the initial cause of aerobic deterioration of different silage crops (24). *P. kudriavzevii* has recently been found in Italian ryegrass silage treated with *L. buchneri* (19). From the DGGE profiles of bacterial RNA at silage opening and during 14 days of air exposure, apart from the presence of LAB, *A. pasteurianus* was also seen to be present from the second day of air exposure in the PE silages, while it appeared at day 7 in the OB silages. This could partially explain the more rapid degradation that occurred in the PE silage after exposure to air. Spoelstra et al. (32) found that *Acetobacter* spp. could be involved in the aerobic spoilage of corn silage, by oxidizing ethanol to acetate or by oxidizing lactate and acetate to carbon dioxide and water. Furthermore, the selective inhibition of yeasts, due to the addition of acetic or propionic acid, could also increase the proliferation of acetic acid bacteria in silage (15). Here, the use of *L. buchneri* as a silage inoculant provoked a heterolactic fermentation with an increase in the acetic acid concentration. This could have indirectly stimulated the activity of *A. pasteurianus*. The presence of *A. pasteurianus* in silages was recently reported by Nishino et al. (23), who identified two strains of *A. pasteurianus* in whole-crop corn silage which contained significant amounts of acetic acid and which had been stored for 18 months.

When the yeasts and *Acetobacter* had consumed most of the lactic acid (Fig. 1) and acetic acid (data not shown), the pH level increased and the growth of other aerobic bacteria and filamentous fungi became possible, which caused further spoilage (36). This secondary aerobic spoilage microbiota, which principally consist of mold and bacilli, not only decreases the nutritive value of the silage but also presents a risk to animal health and the safety of milk (34). In this study, the aerobic

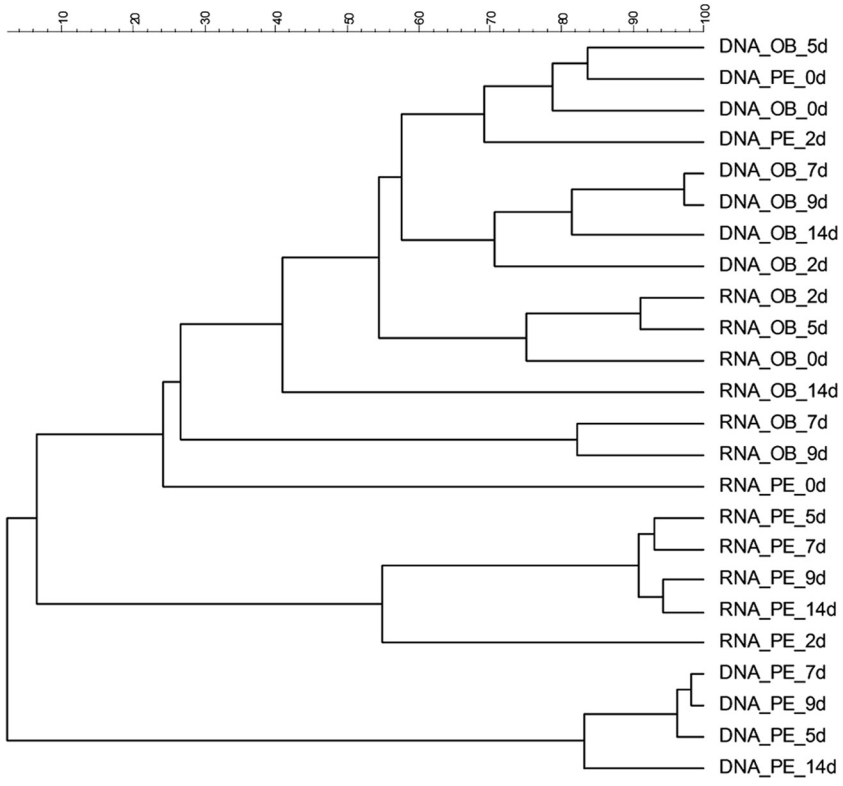

FIG. 4. Dendrograms obtained from cluster analysis of DGGE profiles of the bacterial microflora detected on PE- and OB-treated silage samples, at both the DNA and RNA levels, during aerobic exposure.

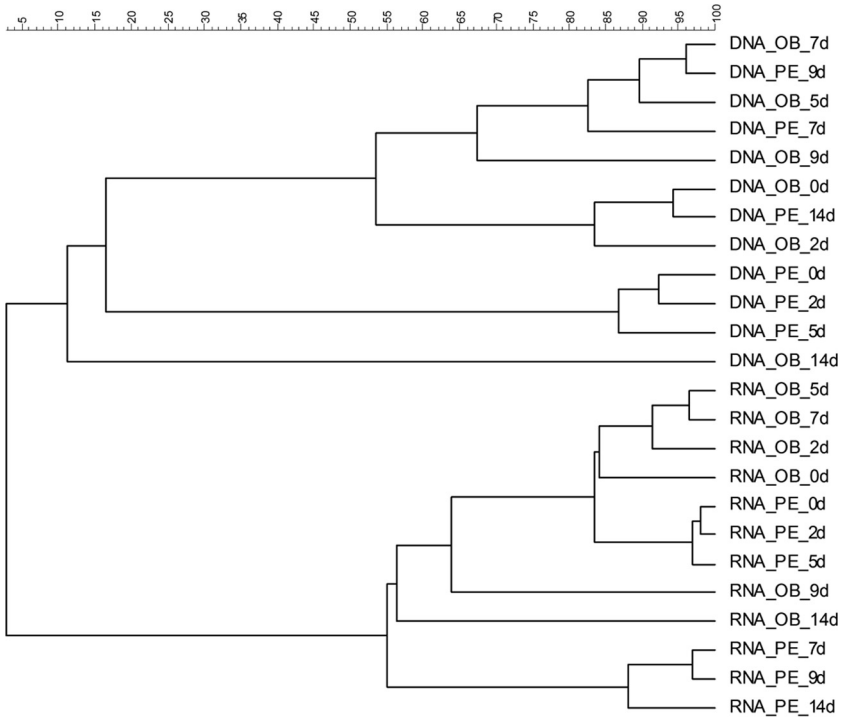

FIG. 5. Dendrograms obtained from cluster analysis of DGGE profiles of the fungal microflora detected on PE- and OB-treated silage samples, at both the DNA and RNA levels, during aerobic exposure.

spore counts increased above 8 $\log_{10}$ CFU g^{-1} from day 3 of air exposure and beyond in the PE silage, whereas they tended to increase in the OB silage only after 9 days of air exposure. *Bacillus* sp. counts of up to 9 $\log_{10}$ CFU g^{-1} silage have been detected in deteriorating silage and from the face layer of opened bunker silages (24). The DGGE profiles showed that *B. subtilis* was present in both the PE and OB silages. The presence of *A. fumigatus* was observed after 7 days of aerobic exposure in the PE silages and after 14 days in the OB silages, when mold counts exceeded 6 $\log_{10}$ CFU g^{-1} silage. *A. fumigatus* is a well-known human and animal pathogen that causes aspergillosis, and it can produce gliotoxin, a toxic compound that has potent immunosuppressive, genotoxic, cytotoxic, and apoptotic effects (14, 26).

Overall, the cluster analysis highlighted the influence of PE and OB films on the metabolic activity of microbiota throughout aerobic exposure. At the RNA level, clusters corresponding to the sealing treatment were detected for both the bacterial and fungal populations.

In this study, it has been shown that the use of oxygen barrier plastic films for ensiling can ensure a longer shelf life of silage, protecting it from spoilage. Moreover, an important feature of OB use is the delay in growth of pathogenic molds, which are able to produce potent mycotoxins that are harmful to animals and humans.

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