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Changes in the Spoilage-Related Microbiota of Beef during Refrigerated Storage under Different Packaging Conditions

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The microbial spoilage of beef was monitored during storage at 5°C under three different conditions of modified-atmosphere packaging (MAP): (i) air (MAP1), (ii) 60% O_2 and 40% CO_2 (MAP2), and (iii) 20% O_2 and 40% CO_2 (MAP3). *Pseudomonas*, *Enterobacteriaceae*, *Brochothrix thermosphacta*, and lactic acid bacteria were monitored by viable counts and PCR-denaturing gradient gel electrophoresis (DGGE) analysis during 14 days of storage. Moreover, headspace gas composition, weight loss, and beef color change were also determined at each sampling time. Overall, MAP2 was shown to have the best protective effect, keeping the microbial loads and color change to acceptable levels in the first 7 days of refrigerated storage. The microbial colonies from the plate counts of each microbial group were identified by PCR-DGGE of the variable V6-V8 region of the 16S rRNA gene. Thirteen different genera and at least 17 different species were identified after sequencing of DGGE fragments that showed a wide diversity of spoilage-related bacteria taking turns during beef storage in the function of the packaging conditions. The countable species for each spoilage-related microbial group were different according to packaging conditions and times of storage. In fact, the DGGE profiles displayed significant changes during time and depending on the initial atmosphere used. The spoilage occurred between 7 and 14 days of storage, and the microbial species found in the spoiled meat varied according to the packaging conditions. *Rahnella aquatilis*, *Rahnella* spp., *Pseudomonas* spp., and *Carnobacterium divergens* were identified as acting during beef storage in air (MAP1). *Pseudomonas* spp. and *Lactobacillus sakei* were found in beef stored under MAP conditions with high oxygen content (MAP2), while *Rahnella* spp. and *L. sakei* were the main species found during storage using MAP3. The identification of the spoilage-related microbiota by molecular methods can help in the effective establishment of storage conditions for fresh meat.

Owing to its high water content and abundance of important nutrients available on the surface, meat is recognized as one of the most perishable foods. Spoilage can be defined as any change in a food product that makes it unacceptable to the consumer from a sensory point of view (24). Apart from physical damage, oxidation, and color change, the other spoilage symptoms are ascribable to the undesired growth of microorganisms to unacceptable levels. In the case of meat, microbial spoilage leads to the development of off-odors and often slime formation, which make the product undesirable for human consumption (26, 28, 29). The organoleptic changes may vary according to the microbial association contaminating the meat and to the conditions under which the meat is stored. The development of organoleptic spoilage is related to microbial consumption of meat nutrients such as sugars and free amino acids and the release of undesired volatile metabolites. Microbial loads from 10^7 CFU cm^{-2} are usually associated to occurrence of off-odors such as "cheesy" or "buttery" odors; these may evolve into "fruity" odors when the loads increase and become putrid smells as the result of free amino acid consumption at loads as high as 10^9 CFU cm^{-2} (11, 29). In fact, once the glucose present in the aqueous phase has been utilized, other substrates are sequentially consumed until odorous nitrogenous compounds such as ammonia and dimethylsulfide are released (51).

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Different spoilage-related species and strains can colonize the meat surface through different stages involving adsorption to the meat surface (8, 20) and attachment by glycocalix formation (10). The development of these phases depends on the intrinsic and extrinsic ecological factors of a particular meat ecosystem such as pH, meat surface morphology, O_2 availability, temperature, and the presence and development of other bacteria (12, 13, 24, 27, 28).

Many groups of organisms contain members that potentially contribute to meat spoilage under appropriate conditions. This makes the microbial ecology of spoiling raw meat very complex and the spoilage thus very difficult to prevent. Under aerobic conditions, a few species of the genus *Pseudomonas* are generally recognized to dominate the meat system and to actively contribute to spoilage owing to their capability for glucose and amino acid degradation, even at refrigeration temperatures (13, 34, 35, 48). *Brochothrix thermosphacta* is a microorganism for which meat is considered an ecological niche, even though it can also occur in spoiled fish. The capability of *B. thermosphacta* to grow on meat during both aerobiosis and anaerobiosis makes it a significant meat colonizer and an important member of the spoilage-related flora due to off-odor production (11, 45). Many members of the *Enterobacteriaceae*, belonging to the genera *Serratia*, *Enterobacter*, *Pantoea*, *Proteus*, and *Hafnia*, often contribute to meat spoilage (5, 24, 30, 35, 43). Moreover, lactic acid bacteria (LAB) such as *Lactobacillus*, *Carnobacterium*, and *Leuconostoc* can play an important role in the spoilage of refrigerated raw meat (35) and are also recognized as important competitors of the other spoilage-related microbial groups under appropriate conditions (5, 22).

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Packaging can be an effective method for meat shelf life extension that avoids the use of chemical preservatives (7, 42). These methods of preservation include the use of modified-atmosphere packaging (MAP) using gas mixtures containing variable O_2 and CO_2 concentrations in order to inhibit the different spoilage-related bacteria and are often associated with the use of low temperatures during storage (19). Substantial fractions of CO_2 are used to retard the growth of organisms produced by aerobic spoilage, and a certain concentration of O_2 is employed for red meat MAP to preserve meat color (23, 32). This kind of packaging is normally associated with the use of packs made of materials that provide a barrier to the exchange of gases between the pack and the external atmosphere (23). Improved storage of meat can be achieved by using CO_2, allowing the growth of LAB such as *Lactobacillus* spp. and *Leuconostoc* spp. and thus outcompeting *Enterobacteriaceae*, *Pseudomonas* spp., and *Brochothrix thermosphacta* (51). Moreover, the use of high CO_2 concentrations, together with low pH and chill storage, can more readily inhibit the growth of food pathogens than vacuum packaging (21).

In addition, novel technologies of active packaging are currently under exploitation; these techniques can employ natural antimicrobials such as essential oils or bacteriocins to inhibit microbial growth in meat products (18, 38, 49, 50).

Most of the work performed on the changes of microbial associations in meat during storage have focused on comparisons between viable counts of spoilage-related microbial groups. However, microbial analysis alone as a spoilage index may not be exhaustive enough to understand the actual shifts of the microbial ecology of raw meat in response to different storage conditions. Certain taxa may be differently influenced by the specific storage conditions, and the different microbial species may unpredictably develop during storage, thus influencing the time and type of spoilage development. There is still a need to assess, within each spoilage-related microbial group, which species are actually involved in the spoilage of meat. Moreover, molecular methods such as PCR-denaturing gradient gel electrophoresis (DGGE) are seldom optimized to monitor changes in spoilage-related microbial flora in food, while they are widely exploited for the characterization of fermented foods (17). The aim of this work was to assess the microbial diversity of the spoilage-related microbial groups that develop during refrigerated storage of beef at the species level under different MAP conditions by considering quality indices of significant importance for the acceptability of fresh meat by the consumer.

MATERIALS AND METHODS

Meat samples. Freshly cut beefsteaks, all arising from the same meat piece (*Longissimus dorsi*), were purchased in a local retail store. They were transported to the laboratory at 4°C within 30 min and modified-atmosphere packaged by using a packaging machine (TSM, 105 Minipack Torre; Cava dei Tirreni [SA], Italy). Beefsteaks were packed in polystyrene trays whose interior was covered with a multilayer barrier film (volume, 750 ml) (CoopBox, Bologna, Italy), and a barrier polyethylene film (PO_2 = 1.3 cm^3/m^2/24 h/atm at 23°C, 0% rH) was used as a sealing top. An absorbent sheet was placed on the bottom of the tray to avoid excessive accumulation of exudates. The ratio between the volume of gas and weight of food product (G/P ratio) was 3:1 (vol/wt). The beef was packed using (i) air (MAP1), (ii) 60% O_2–40% CO_2 (MAP2), and (iii) 20% O_2–40% CO_2–40% N_2 (MAP3). All samples were stored in a refrigerator at a constant temperature (5°C) for 2 weeks. Viable microbial count, color, and headspace gas analyses were performed at time zero and after 2, 4, 7, and 14 days of storage under each atmosphere condition.

Gas analysis. O_2 and CO_2 concentrations (percent, volume/volume) in the package headspace were monitored using a portable PBI Dansensor A/S (Check Mate 9900 O_2/CO_2; Ringsted, Denmark) analyzer (accuracy, ±0.1%) by sampling 3 ml of gas from the package headspace with a syringe needle (0.8 mm by 40 mm; Thermo Europe N.V., Leuven, Belgium).

Weight loss. The weight loss of the samples was measured by a gravimetric method. Beefsteaks were weighed before packaging and at each storage time after removal from the packages. The difference of weight (Δg) was divided by the initial weight of the product (g) and expressed as $\Delta g/g\%$.

Colorimetric measurement. Surface beefsteak color (CIE L^*, a^*, and b^* values) was measured using a colorimeter tristimulus (Chroma Meter, model CR-300; Minolta, Osaka, Japan) with a circular measurement area (D = 8 mm). The colorimeter was calibrated using a white standard plate (L^* = 100). Ten readings were carried out for each beefsteak. Only the lean color was measured, and precaution was taken to avoid any intramuscular fat. Total color changes (ΔE) were calculated as follows:

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2}$$

Microbiological analysis and bulk cell formation. Samples (25 g) arising from each tray were aseptically weighed and homogenized in one-quarter-strength Ringer's solution (Oxoid) for 2 min in a stomacher (LAB Blender 400; PBI, Italy) at room temperature. Decimal dilutions were prepared, and aliquots of 0.1 ml of the appropriate dilutions were spread in triplicate onto the following media: plate count agar (PCA; Oxoid) incubated at 30°C for 72 h for the enumeration of mesophilic aerobic bacteria and incubated at 5°C for 10 days for the enumeration of psychrotrophic aerobic bacteria; violet red bile glucose agar (VRBGA; Oxoid) for the *Enterobacteriaceae* incubated at 30°C for 24 to 48 h; MRS agar (Oxoid) for LAB incubated at 30°C for 48 h; Pseudomonas agar with cetrimide-fucidin-cephaloridine selective supplement (Oxoid) for *Pseudomonas* incubated at 30°C for 48 h; and STAA medium (Oxoid) for *Brochothrix thermosphacta* incubated at 25°C for 48 h. Results were calculated as the means of three determinations. MRS and VRBGA plates were incubated under anaerobic conditions by using an Anaerogen kit (Oxoid).

After the microbial counts, the plates were used for bulk formation as previously described (14). Briefly, all the colonies present on the surface of each countable plate were suspended in a suitable volume of one-quarter-strength Ringer's solution, harvested with a sterile pipette, and stored by freezing at −20°C. When necessary, 100 μl of the bulk was used for DNA extraction.

DNA extraction from beef and bulk cells. For DNA extraction from beef and bulk cells, the protocol described by the manufacturer of the Wizard DNA purification kit (Promega, Madison, Wis.) was applied. Beef samples (10 g) were homogenized in a stomacher bag with 20 ml of one-quarter-strength Ringer's solution for 1 min; the large deposit was allowed to set for 1 min, and the supernatant was used for the DNA extraction. One milliliter of the beef homogenate suspension or 100 μl of the bulk cell sample was centrifuged at 17,000 × g for 5 min at 4°C, and the resulting pellet was resuspended in 100 μl of Tris-EDTA buffer (100 mmol $liter^{-1}$ Tris, 10 mmol $liter^{-1}$ EDTA); next, a solution containing 160 μl of 0.5 mol $liter^{-1}$ EDTA/Nuclei Lysis Solution (Wizard DNA purification kit; Promega) in a 1/4.16 ratio, 5 μl of RNase (10 mg ml^{-1}; Sigma), and 20 μl of pronase E (20 mg ml^{-1}; Sigma) was added, and the mixture was incubated for 60 min at 35°C. After incubation, 1 volume of ammonium acetate (5 mol $liter^{-1}$) was added to the sample, which was then centrifuged at 17,000 × g for 5 min at 4°C. The supernatant was precipitated with 0.7 volumes of isopropanol and centrifuged at 29,000 × g for 5 min. Finally, the pellet was dried and resuspended in 50 μl of DNA rehydration solution by incubation at 55°C for 45 min.

PCR amplification. Primers U968 and L1401 were used (56) to amplify the variable V6-V8 region of the 16S rRNA gene, giving PCR products of about 450 bp. A GC clamp was added to the forward primers according to a method described previously by Muyzer et al. (41). Amplifications were performed in a programmable heating incubator (Techne; Progene, Italy). Each mixture (final volume, 50 μl) contained 20 ng of template DNA, each primer at a concentration of 0.2 μM, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM $MgCl_2$, 2.5 μl of 10× PCR buffer (Invitrogen, Milano, Italy), and 2.5 U of *Taq* polymerase (Invitrogen). Template DNA was denatured for 5 min at 94°C. A "touchdown" PCR was performed as previously described (14). The initial annealing temperature was 66°C, and this temperature was decreased 1°C every cycle for 10 cycles; finally, 20 cycles were performed at 56°C. The extension step for each cycle was carried out at 72°C for 3 min, while the final extension step was

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TABLE 1. Headspace gas composition of the packed beef during storage at 5°C

Days of storage	Headspace composition under conditions of:					
	MAP1		MAP2		MAP3	
	% O_2	% CO_2	% O_2	% CO_2	% O_2	% CO_2
0	21.0 ± 0.1	0.0 ± 0.1	57.7 ± 0.3	36.3 ± 0.3	22.0 ± 0.1	37.3 ± 0.3
2	18.6 ± 0.2	2.4 ± 0.2	55.0 ± 0.3	37 ± 1	22.2 ± 0.1	38.0 ± 0.3
4	18.3 ± 0.2	3.2 ± 0.2	56.4 ± 0.4	39.0 ± 0.2	22.7 ± 0.2	38.9 ± 0.4
7	9.0 ± 3	9.0 ± 2	56.9 ± 0.4	38.6 ± 0.3	23.0 ± 0.5	38.2 ± 0.9
14	0.0	25.0 ± 1	55.1 ± 0.5	40.2 ± 0.7	11.3 ± 0.7	54.9 ± 0.8

carried out at 72°C for 10 min. Aliquots (2 μl) of PCR products were routinely checked on 1.5% agarose gels.

DGGE analysis. PCR products were analyzed by DGGE using a Bio-Rad Dcode apparatus. Samples were applied to 7% (wt/vol) polyacrylamide gels in 1× Tris-acetate-EDTA buffer. Parallel electrophoresis experiments were performed at 60°C by using gels containing a 25 to 55% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40% [wt/vol] formamide). The gels were run for 10 min at 50 V, followed by 4 h at 200 V. They were then stained with ethidium bromide for 3 min, rinsed for 15 min in distilled water, and observed.

Sequencing of DGGE fragments. DGGE bands to be sequenced were purified in water according to a method described previously by Ampe et al. (1). One microliter of the eluted DNA of each DGGE band was reamplified by using the primers and the conditions described above. PCR products that gave a single band that comigrated with the original band were then purified by using a QIaex PCR purification kit (QIAGEN, Milano, Italy) according to the manufacturer's instructions and sequenced.

Sequencing was performed by using a Deoxy Terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems) with the primer L1401. To determine the closest known relatives of the partial 16S rRNA gene sequences obtained, searches were performed in public data libraries (GenBank) with the BLAST search program.

Experimental design and data analysis. To study the effect of atmosphere and storage time on water loss and color change, a factorial design with two factors (time and atmosphere) was used. There were three levels of atmospheres and five levels of storage time (0, 2, 4, 7, and 14 days). Three replicates were performed for each experiment for a total of 45 samples, and the standard error (SE) was calculated. Analysis of variance was performed on the data to evaluate the effect of atmosphere (*A*), time (*B*), and the interaction effect ($A \times B$) on the quality attributes. Duncan tests were performed to find the source of the significant differences within samples. Significance of differences was defined at a P value of $\leq$0.05.

Nucleotide sequence accession numbers. The sequences reported here can be found in the GenBank database under accession numbers DQ405232 to DQ405261 (see Table 3).

RESULTS

Headspace gas composition. When the beefsteaks were packed by using air as the initial gas (MAP1), the oxygen in the headspace decreased from 21% to 0% and carbon dioxide increased from 0% to 25% in 14 days (Table 1). By contrast, if MAP2 (60% O_2–40% CO_2) conditions were used, both O_2 and CO_2 concentrations remained constant during the entire period of storage. The same was observed for MAP3 during the first week of storage, while between 7 and 14 days of storage, a dramatic drop of oxygen and increase of carbon dioxide could be observed.

Color variation. The color variation of the beef samples stored under MAP1, MAP2, and MAP3 conditions is well described by ΔE, which provides a measure of the total color change (Fig. 1). ΔE increased in all the cases, and the greatest variability was observed for the sample stored under MAP3 conditions. A fast increment of ΔE indicates that the beef color turned from red to brown. The results of analysis of variance suggested that there was a significant effect of the time (*A*) and of the atmosphere (*B*) composition as well as of the interaction $A \times B$ on the color change, ΔE ($F_{4,462} = 110$ [$P \ll 0.0001$]; $F_{2,462} = 25$ [$P \ll 0.0001$]; $F_{8,462} = 25$ [$P \ll 0.0001$] for *A*, *B*, and the $A \times B$ interaction, respectively). In the case of the samples packed under MAP1 conditions, ΔE increased linearly during the first 7 days, and it then approached a constant value during the second week of storage. Duncan results showed that the first significant differences were observed between 2 days and 4 days of storage. During the first week, storage under MAP2 and MAP3 conditions resulted in beef color variation similar to that occurring under MAP1 conditions; however, the sample color differentiated dramatically at the end of the second week. For all the samples packed in a protective atmosphere, a noticeable color change could be observed after 14 days of storage. However, the color change under MAP2 conditions remained acceptable until the seventh day of storage.

Weight loss. All the samples lost water during storage (Fig. 2). Both time and atmosphere had a significant effect on the weight loss, but the interaction effect was not significant ($F_{3,20} = 8.7$ [$P = 0.01$], $F_{2,20} = 7.5$ [$P = 0.003$], and $F_{6,20} = 0.8$ [$P = 0.5$] for time, atmosphere, and interaction effect, respectively). The difference between the samples and the control (MAP1) became significant after 7 days of storage, and the lowest weight loss was observed under MAP1 conditions (Fig. 2).

Microbiological analysis. After 14 days of storage, all the meat samples presented objective signs of spoilage and were sensorially not acceptable. The results of the viable counts of the targeted microbial groups from beef samples under the different storage conditions are reported in Table 2. At time

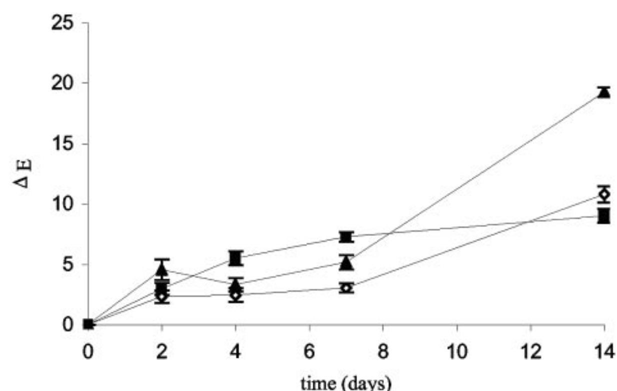

FIG. 1. Total color change ($\Delta E \pm$ SE) of beefsteaks during storage at 5°C under MAP1 (■), MAP2 (◇), and MAP3 (▲) conditions.

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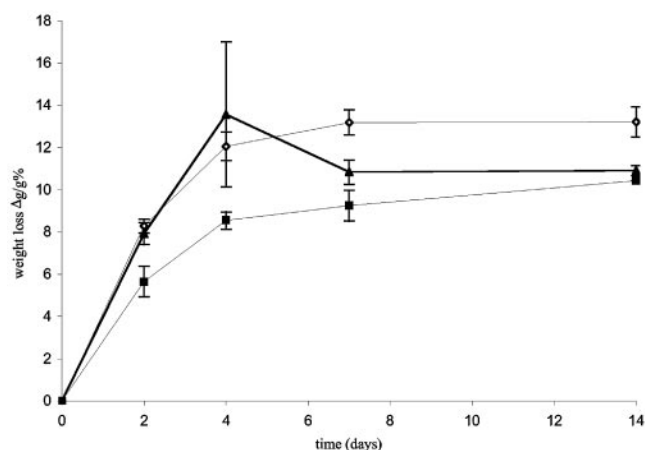

FIG. 2. Weight loss (Δg/g%) changes (average ± SE) of beef during storage at 5°C under MAP1 (■), MAP2 (◇), and MAP3 (▲) conditions.

zero, the highest counts were shown by mesophilic and psychrotrophic aerobic bacteria, whose loads ranged between 10^2 and 10^3 CFU/g. *Enterobacteriaceae* and LAB displayed loads below 10^2 CFU/g, while *Pseudomonas* species and *B. thermosphacta* were below the detection limit at this stage. After 4 days of refrigerated storage under MAP1 conditions (Table 2), the loads of all the targeted microbial groups increased to above 10^3 CFU/g. By contrast, the beef stored under MAP2 and MAP3 conditions showed a completely different trend: mesophilic and psychrotrophic aerobic bacteria along with LAB increased their initial number to about 10^4 CFU/g in 2 days, while the *Enterobacteriaceae*, *Pseudomonas* species, and *B. thermosphacta* increased only to 10^2 CFU/g (Table 2). The difference between these groups of spoilage-related bacteria remained constant under MAP2 and MAP3 conditions up to at least 7 days. After this time, *Pseudomonas* species and *B. thermosphacta* reached values of 10^4 CFU/g under both MAP2 and MAP3 conditions. By contrast, *Enterobacteriaceae* displayed values of 10^4 CFU/g under MAP3 conditions, while the counts were 1 log lower under MAP2 conditions. At the same time of sampling, LAB and mesophilic and psychrotrophic aerobic bacteria grew up to about 10^6 CFU/g. Conversely, after 7 days of storage in air packaging (MAP1), the viable counts of all the groups were higher: *Pseudomonas* and mesophilic and psychrotrophic aerobic bacteria grew to over 10^7 CFU/g, while the other groups reached values between 10^5 and 10^6 CFU/g. These loads increased even more after 14 days of storage, with the highest values above 10^8 CFU/g for all the bacterial groups. On the other hand, the beef stored under MAP2 and MAP3 conditions showed lower counts after 14 days, when all the groups reached and overcame 10^7 CFU/g, except for *Enterobacteriaceae* and *B. thermosphacta*, which kept their loads at about 10^5 CFU/g in beef stored under MAP2 conditions.

Identification of microbial species by PCR-DGGE. After each viable count for every targeted group, the microbial colonies from the countable plates were collected in bulk cells as described in Materials and Methods and processed by PCR-DGGE after DNA extraction and amplification of the V6-V8 region of the 16S rRNA gene. The PCR-DGGE profiles obtained for each microbial group are shown in Fig. 3, while the results of band identification by DNA sequencing are reported in Table 3.

The analysis of bulk cells from Pseudomonas agar showed that two different bands occurred in the beef at time zero (Fig. 3A); these two entities were both shown to be close relatives of *Pseudomonas* species (Table 3). These two entities occurred during the whole period of storage in air, but *Rahnella* spp. and *Serratia proteamaculans* could also be identified from the bulk cells after 7 and 14 days of storage, respectively (Fig. 3A and Table 3). The beef stored under MAP2 conditions with a high O_2 concentration already showed a significant degree of diversity after 2 days of storage; apart from the *Pseudomonas* doublet shown, in the profile of beef at time zero, fragments belonging to *S. proteamaculans*, *Pseudomonas* spp., and *Rahnella* spp. were also identified. However, the latter species were not found from day 4, and after 14 days, only one band identified as *Pseudomonas* spp. was detected (Fig. 3A). The beef

TABLE 2. Viable counts of different spoilage-related microbial groups detected on beef during storage under MAP1, MAP2, and MAP3 conditions at 5°C for 14 days

MAP	Storage time (days)	pH ± SD	Log CFU g^{-1} ± SD					
			Psychrotrophic bacteria	Mesophilic bacteria	*Pseudomonas* spp.	*B. thermosphacta*	*Enterobacteriaceae*	LAB
1	0	5.35 ± 0.02	2.44 ± 0.03	2.77 ± 0.10	<1.00	<1.00	1.00	1.47
	2	5.34 ± 0.05	4.41 ± 0.05	4.30 ± 0.05	3.39 ± 0.03	4.00 ± 0.04	1.84 ± 0.08	3.25 ± 0.07
	4	5.34 ± 0.03	7.60 ± 0.01	6.38 ± 0.07	5.00 ± 0.10	5.00 ± 0.05	5.00 ± 0.20	6.14 ± 0.05
	7	5.45 ± 0.03	8.00 ± 0.10	7.17 ± 0.15	7.50 ± 0.20	5.96 ± 0.15	5.30 ± 0.20	5.46 ± 0.10
	14	5.53 ± 0.02	8.90 ± 0.03	9.39 ± 0.20	9.00 ± 0.30	8.00 ± 0.20	7.00 ± 0.15	8.30 ± 0.15
2	0	5.35 ± 0.02	2.44 ± 0.03	2.77 ± 0.10	<1.00	<1.00	1.00	1.47
	2	5.35 ± 0.03	3.89 ± 0.08	3.77 ± 0.20	2.00 ± 0.10	1.95 ± 0.03	1.47 ± 0.03	3.66 ± 0.02
	4	5.34 ± 0.02	5.83 ± 0.01	5.99 ± 0.03	4.07 ± 0.08	3.47 ± 0.05	3.47 ± 0.10	5.50 ± 0.03
	7	5.42 ± 0.02	6.25 ± 0.10	6.17 ± 0.18	3.60 ± 0.05	4.11 ± 0.10	2.47 ± 0.05	5.90 ± 0.05
	14	5.36 ± 0.05	7.54 ± 0.07	7.00 ± 0.08	7.11 ± 0.08	5.07 ± 0.03	5.00 ± 0.02	7.17 ± 0.10
3	0	5.35 ± 0.02	2.44 ± 0.03	2.77 ± 0.10	<1.00	<1.00	1.00	1.47
	2	5.36 ± 0.02	4.55 ± 0.05	4.23 ± 0.10	2.30 ± 0.20	2.30 ± 0.08	1.47 ± 0.03	3.27 ± 0.18
	4	5.40 ± 0.02	5.43 ± 0.02	5.61 ± 0.05	2.83 ± 0.10	3.62 ± 0.20	2.51 ± 0.10	5.34 ± 0.06
	7	5.46 ± 0.03	7.43 ± 0.15	6.32 ± 0.10	3.95 ± 0.05	4.00 ± 0.25	3.95 ± 0.05	6.47 ± 0.20
	14	5.23 ± 0.02	8.39 ± 0.20	7.90 ± 0.03	6.90 ± 0.03	6.90 ± 0.08	7.17 ± 0.03	7.60 ± 0.03

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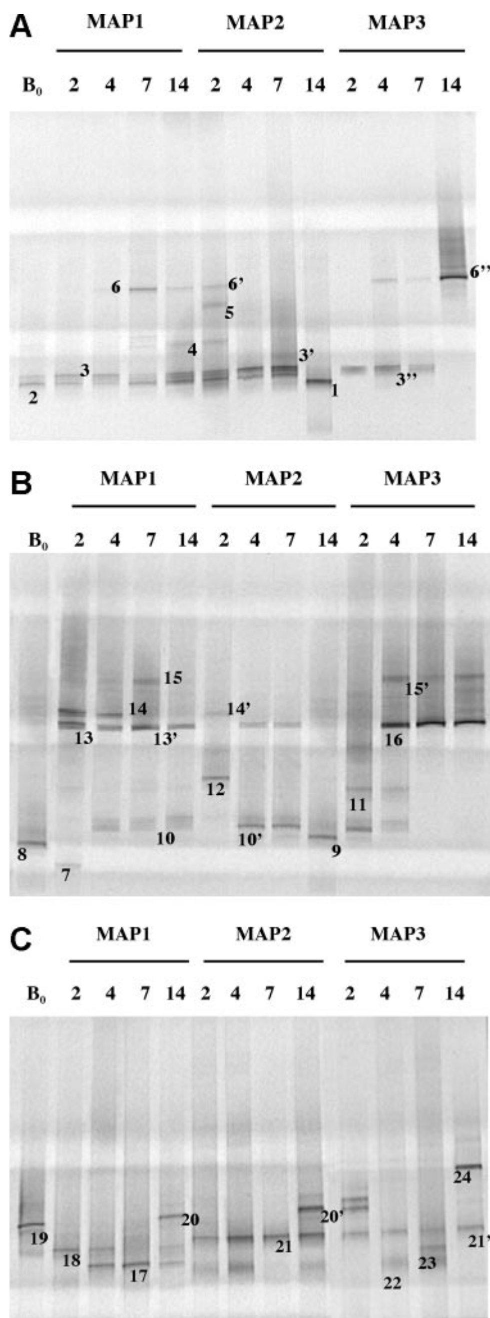

FIG. 3. PCR-DGGE profiles of bulk cells of (A) the *Pseudomonas* population monitored using Pseudomonas agar, (B) the population of *Enterobacteriaceae* monitored using VRBGA, and (C) the population of LAB monitored using MRS agar after 2, 4, 7, and 14 days. The packaging conditions are as follows: air packaging (MAP1), 60% O_2–40% CO_2 (MAP2), and 20% O_2–40% CO_2–40% N_2 (MAP3). B_0, bulk cells from medium plates for each group at time zero, before packaging. The sampling times (days) during storage are indicated at the top of each lane. The numbers indicate the sequenced bands, and fragments labeled with the same number showed identical sequences. The identifications are reported in Table 3.

stored under MAP3 conditions showed the presence of *Pseudomonas* spp. after 2 days of storage; however, the fragment identified as *Rahnella* spp. was also found after 4 days of storage, and it was the only entity occurring after 14 days (Fig. 3A).

The bulk cells of *Enterobacteriaceae* counted on VRBGA showed that at time zero, the population was ascribable to *Enterobacter agglomerans*, as this was the only species found in the DGGE profile. However, this species was not found in any other DGGE profile, indicating that it was probably outcompeted during storage under all the packaging conditions (Fig. 3B). During storage in air, *Pantoea ananatis* was found on VRBGA after 2 days, along with *Rahnella aquatilis* and *Serratia* spp. (Fig. 3B and Table 3). The latter species was also found in the further storage phases in air together with *Pseudomonas* spp. (Fig. 3B). Different *Pseudomonas* spp. alternated during storage under MAP2 conditions (Fig. 3B), but the only species occurring after 14 days of storage was found to be *Serratia grimesii* (Fig. 3B). *Pseudomonas* spp. and *S. proteamaculans* were found for up to 4 days under MAP3 conditions, while *Rahnella aquatilis* and *Rahnella* spp. were detected from day 4 and were the only species found after 7 days of storage (Fig. 3B).

A large variety of LAB species was identified from the plates of MRS agar (Table 3). At time zero, the only species found was *Weissella hellenica* (Fig. 3C), which was also found to be dominant in the last spoilage phases (14 days) under MAP1 and MAP2 conditions but was not detected in any of the samples stored under MAP3 conditions (Fig. 3C). *Lactobacillus graminis* was found from the first 2 days of storage; however, *Carnobacterium divergens* was also detected at time 4 (Fig. 3C and Table 3) and occurred during the whole storage period in air packaging (MAP1) (Fig. 3C). During the storage of beef under MAP2 and MAP3 conditions, the last two species were not found at all (Fig. 3C). However, *Lactobacillus sakei* was always present in beef stored under both MAP conditions at any time of sampling, even though in some cases, leuconostocs such as *Leuconostoc kimchii* and *Leuconostoc carnosum* were also found (Fig. 3C and Table 3). Surprisingly, after 14 days of storage under MAP3 conditions, *Rahnella* spp. could also be identified from MRS agar plates.

Finally, bulk cells from STAA plates from beef at time zero, as well as all the storage times under any packaging conditions, showed simple PCR-DGGE profiles displaying only one band (data not shown) that was found to be *B. thermosphacta* after sequencing (Table 3).

The PCR-DGGE of the V6-V8 region of the16S rRNA gene was also applied to DNA directly extracted from meat samples during storage. An example of DGGE fingerprints of meat before packaging and after storage under any of the packaging conditions is shown in Fig. 4. Before packaging, the beef displayed a fingerprint containing two bands identified as *Pseudomonas* spp. and *Hafnia alvei* (Fig. 4 and Table 3). The profiles of beef samples after storage under the different packaging conditions were not different from each other at any time (data not shown). In addition to the above-mentioned species, *Carnobacterium divergens*, *B. thermosphacta*, and *Staphylococcus* spp. could be identified after sequencing of the DGGE fragments (Table 3).

DISCUSSION

The aim of this study was to monitor the changes of the spoilage-related microbial flora during the storage of beef under different packaging conditions. The large variation of the gas composition of the control samples was due to microbiological growth, which, on the contrary, is inhibited by the high

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TABLE 3. Microbial species identification after sequencing of the variable V6-V8 region of the 16S rRNA genes purified from PCR-DGGE profiles

Band[a]	Medium	Closest relative(s)	Identity (%)	GenBank accession no. of closest relative(s)	GenBank accession no. of sequence
1	Pseudomonas agar	*Pseudomonas* spp.[b]	100	AY900171	DQ405232
2	Pseudomonas agar	*Pseudomonas putida/P. fragi/P. syringae*	98	AY450557/AF094733/AJ576247	DQ405233
3	Pseudomonas agar	*Pseudomonas putida/P. fragi/P. syringae*	100	AY450557/AF094733/AJ576247	DQ405234
4	Pseudomonas agar	*Serratia proteamaculans*	99	AJ233435	DQ405235
5	Pseudomonas agar	*Pseudomonas putida/P. fragi/P. syringae*	100	AY450557/AF094733/AJ576247	DQ405236
6	Pseudomonas agar	*Rahnella* spp.	99	U88439	DQ405237
7	VRBGA	*Pantoea ananatis*	98	DQ133546	DQ405238
8	VRBGA	*Enterobacter agglomerans*	99	AF130952	DQ405239
9	VRBGA	*Serratia grimesii*	100	AY789460	DQ405240
10	VRBGA	*Pseudomonas* spp.	99	AY689082	DQ405241
11	VRBGA	*Serratia proteamaculans*	99	AY902209	DQ405242
12	VRBGA	*Pseudomonas* spp.	99	AY625608	DQ405243
13	VRBGA	Uncultured *Serratia*	100	DQ279304	DQ405244
14	VRBGA	*Rahnella aquatilis*	99	AY253920	DQ405245
15	VRBGA	*Rahnella* spp.	99	U88439	DQ405246
16	VRBGA	*Rahnella* spp.	99	U88434	DQ405247
17	MRS agar	*Carnobacterium divergens*	99	AY543037	DQ405248
18	MRS agar	*Lactobacillus graminis*	99	AM113778	DQ405249
19	MRS agar	*Weissella hellenica*	100	UB023240	DQ405250
20	MRS agar	*Weissella hellenica*	100	UB023240	DQ405251
21	MRS agar	*Lactobacillus sakei*	99	AM113784	DQ405252
22	MRS agar	*Leuconostoc kimchii*	100	AF173986	DQ405253
23	MRS agar	*Leuconostoc carnosum*	100	UB022925	DQ405254
24	MRS agar	*Rahnella* spp.	99	U88439	DQ405255
25	STAA	*Brochothrix thermosphacta*	99	AY543029	DQ405256
26	Beef	*Pseudomonas putida/P. fragi/P. syringae*	97	AY450557/AF094733/AJ576247	DQ405257
27	Beef	*Carnobacterium divergens*	98	AY543037	DQ405258
28	Beef	*Brochothrix thermosphacta*	98	AY543029	DQ405259
29	Beef	*Hafnia alvei*	99	M59155	DQ405260
30	Beef	*Staphylococcus xylosus/S. succinus/ S. saprophyticus/S. cohnii*	98	AF515587/AY748916/AP008934/AY161046	DQ405261

[a] Bands 1 to 30 are indicated in Fig. 3.
[b] When the sequence showed the same homology with more than four species of *Pseudomonas*, the result was reported as *Pseudomonas* spp.

level of carbon dioxide under the MAP conditions. The headspace gas composition is dynamic, with CO_2 dissolving in the meat and being formed by tissue and bacterial respiration with the consumption of O_2 (22). Similar results were reported previously by Kennedy et al. (33), who found that the oxygen concentration decreased and the carbon dioxide concentration increased during the storage of lamb under MAP conditions. However, carbon dioxide dissolution into the tissue liquid leads to an acidification of the medium and the subsequent formation of carbonic acid. Hence, the water-holding capacity decreases and the texture attributes deteriorate (47). Accordingly, beefsteaks packed with high carbon dioxide concentrations (MAP2 and MAP3) lost more water than samples stored with no carbon dioxide (MAP1).

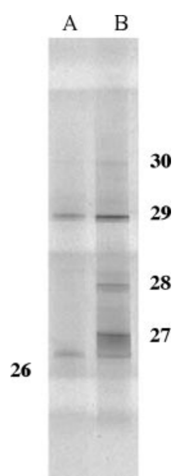

FIG. 4. PCR-DGGE profiles of the 16S V6-V8 amplicons from microbial DNA directly extracted from meat samples at (A) time zero, before packaging, and (B) after 14 days of storage under MAP1 conditions.

The color of muscle in red meat depends on the chemical state of the muscle pigment myoglobin. According to our results, the oxidation of myoglobin, monitored by means of ΔE, occurred faster when samples were packed in air than when samples were packed with high oxygen concentrations, and this finding is in agreement with those of previous studies (2, 22, 33). Once in the package, oxygen is consumed, and metmyoglobin can be reduced to the deoxy form, causing a color change (37). This explains the difference in color observed after 14 days of storage between samples packed with air as the initial gas composition and those packed with high oxygen concentrations. In fact, according to headspace gas analysis, the samples stored under MAP1 conditions were in anaerobic conditions after 7 days.

The results of the viable counts showed that the spoilage-

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related microbial groups had different trends depending on the packaging conditions. When the beef was packed in air (MAP1), all the microbial groups showed viable counts higher than those of the other packaging conditions. Particularly, as reported in the literature (13, 34, 35, 48), the pseudomonads were the dominant population after 4 days of storage, although all the other groups grew to unacceptable levels in 2 weeks. Under these packaging conditions, the pH did not show a strong variation, but the carbon dioxide increased while the oxygen level decreased proportionally with the microbial growth; particularly, with the establishment of anaerobic conditions after 7 days of storage, the LAB loads increased by 3 log units. The beef stored under MAP2 conditions (60% O_2–40% CO_2) kept the best microbiological quality for at least 7 days. The protective effect of MAP3 conditions (20% O_2–40% CO_2) was effective for the first 7 days of storage; thereafter, *Pseudomonas*, *Enterobacteriaceae*, and *B. thermosphacta* increased by almost 3 log units, causing a sudden drop of oxygen and an increase of carbon dioxide inside the package. Overall, MAP2 conditions were shown to have the best protective effect, keeping the microbial loads and color change to acceptable levels in the first 7 days of refrigerated storage.

The study of the cultivable community by PCR-DGGE showed that the microbial species enumerated in the above-described viable counts were not always the same in each spoilage-related microbial group and often changed according to packaging conditions and time of storage. PCR-DGGE analysis of bulk colonies from selective media has been recognized as a useful tool (17), and it has proven to be effective for a satisfactory description of microbial diversity in several habitats (14–16, 40, 46). In our study, this approach was used to monitor the cultivable microbial community of beef and to identify the microbial colonies in bulk from countable plates of selective media during storage. We chose to examine the countable plates in order to identify the species of each group, leading to the viable counts reported in Table 2. It is clear from Fig. 3 that the countable species for each spoilage-related microbial group were different according to packaging conditions and time of storage. The approach used allowed us to understand that similar viable counts found under different packaging conditions can be represented by different spoilage-related bacterial species. Overall, 40 DGGE fragments were sequenced, leading to an unequivocal identification of at least 17 different species and 13 different genera. It is interesting that so many different microbial species can cohabitate in the same food matrix. The spoilage occurred between 7 and 14 days of storage, and the microbial species found in the spoiled meat in the last phases of the storage varied according to the initial atmosphere used. *Rahnella aquatilis*, *Rahnella* spp., *Pseudomonas* spp., and *Carnobacterium divergens* were identified as acting during beef storage in air (MAP1). *Pseudomonas* spp. and *Lactobacillus sakei* were found in beef stored under MAP conditions with high oxygen content (MAP2), while *Rahnella* spp. and *L. sakei* were the main species found during storage under MAP3 conditions.

As shown in Fig. 3A, a few species could be detected from the countable Pseudomonas agar plates, and fragments identified as *Rahnella* spp. were detected at the end of the storage in air and were the only species present after 14 days of storage under MAP3 conditions. Identification of *Pseudomonas* at the species level could not be achieved in this study. In fact, the sequences analyzed were equally homologous to sequences belonging to many species of the genus, and it was not possible to obtain univocal identifications for most of the DGGE fragments. The 16S V6-V8 regions used in this study are probably not variable enough among the species of *Pseudomonas*, and this represents a limit in the use of a 16S-based PCR-DGGE approach for the identification of *Pseudomonas* in the meat ecosystem. As a matter of fact, several other authors involved in studies of *Pseudomonas* identification and systematics previously found that the 16S rRNA gene is not always sufficiently discriminating within the genus and that the analysis of other target genes such as *carA*, *recA*, *gyrB*, *fliC*, and *rpoD* may be supportive for *Pseudomonas* species differentiation (3, 26, 54).

Eight different microbial species could be identified by the analysis of bulk cells from VRBGA (Fig. 3B). *Enterobacter agglomerans* was the only species found on the beef at time zero, but several other species took over during storage. *Serratia* spp. and *Pseudomonas* spp. were the bacteria counted in the spoiled meat at the end of storage under MAP1 and MAP2 conditions, while members of the genus *Rahnella* were found exclusively after 4 days of storage under MAP3 conditions. In addition, *Rahnella aquatilis* and *Rahnella* spp. were also detected during the early phases of storage under MAP1 conditions. MAP1 and MAP3 were characterized by lower oxygen concentrations, especially in the last phases of the storage. Therefore, the presence of *Rahnella* at this time of storage may be explained by the fact that this bacterium is inhibited by high oxygen concentrations, or it is outcompeted by other spoilage-related bacteria under strongly aerobic conditions. It is important to underline the occurrence of *Rahnella* spp. in this study. This microorganism has been shown to play an important role in the spoilage of meat in this research, and it was always found as the dominant bacterium in the late phases of the refrigerated storage. *Rahnella* spp. and *Rahnella aquatilis*, in particular, are members of the *Enterobacteriaceae* that are seldom isolated from meat. They have been isolated from fish (36, 44, 53), vegetables (4), and milk (31, 55). *Rahnella aquatilis* was also isolated from minced meat by Lindberg et al. (36), and it was shown to harbor the heat-labile toxin genes of *Escherichia coli*. This microorganism has been shown to produce off-odors in milk (31, 55) and is suspected to produce histamine (53). In addition, *Rahnella aquatilis* can be associated with human infections (52). Two other *Rahnella* genomospecies that are not phenotypically distinguishable from *Rahnella aquatilis* were characterized previously by Brenner et al. (6). Remarkably, in that study, several DGGE bands identified as *Rahnella* spp. were found (Table 2), showing the closest homology to genomospecies 2, identified previously by Brenner et al. (6).

Among LAB, *Lactobacillus sakei* was always present in beef stored under MAP2 and MAP3 conditions at any time during storage, suggesting that it plays an important role in the development of microbial spoilage. *Weissella hellenica* was cultured from meat at time zero and was found among the dominant LAB population in the last spoilage phases under MAP1 and MAP2 conditions but was not detected in any of the samples stored under MAP3 conditions. However, it was never found in the later days of storage under any packaging conditions, probably due to the development of the other LAB. The

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benefit of LAB during the storage of raw meat is quite controversial. While they are recognized as causative agents of meat spoilage (34, 35, 48), they are also supposed to be important for preventing the growth of other spoilage-related groups under appropriate conditions through the production of organic acids and bacteriocins (5, 22, 25, 39).

The PCR-DGGE analysis of the DNA directly extracted from the beef during storage revealed the presence of microbial species not detected by an analysis of the cultivable community. Two DGGE fragments were identified as a *Staphylococcus* sp. and *Hafnia alvei*, which were not among the species identified from the analysis of bulk colonies. Active populations of *Staphylococcus* spp. were found previously by Cocolin et al. by 16S rRNA analysis of fresh pork sausages (9). Since uncultured microbial species were identified, the wide diversity observed in this study could be even greater, and the use of more culture media could have widened the number of species to be identified. A wider microbial diversity after cultivation was also observed in previous studies (9, 14, 15). By comparing the results obtained from the bulk and direct meat analyses, it can be concluded that based on the cultivation and thus enrichment of the bacteria occurring in meat, the bulk analysis is more satisfactory because it can potentially highlight a larger number of microbial species. However, the occurrence of uncultivated species in PCR-DGGE from meat makes the direct approach necessary to widen the quantity of data.

In conclusion, it is interesting that our approach to study the spoilage-related microbial groups during storage by DGGE analysis of bulk cells from selective media revealed that the species occurring for each spoilage-related microbial group changed during storage and according to the packaging conditions. Therefore, analysis of the spoilage-related microbial groups by viable counts may be not enough to highlight the shifts of the bacterial communities according to environmental changes. Behind each CFU/gram value may be a different microbial species, and this is important to know when the suitability of specific meat storage conditions needs to be evaluated. Certain conditions may be effective in controlling the growth of a particular microbial species but can be unsuccessful in inhibiting another species of the same spoilage-related microbial group, and this cannot be predicted by viable counts. As far as we know, this is the first study to report the changes of a spoilage-related microbial flora during storage of fresh meat. Appropriate molecular approaches are welcome in this field in order to widen the knowledge of spoilage-related bacterial succession during storage of foods.

In addition to the postpackaging sources of contamination, the microorganisms present on the beef during packaging can potentially be involved in meat deterioration. The relationships between spoilage-related bacterial populations under different storage conditions play an important role in the development of spoilage. The assessment of microbial species diversity occurring in meat during storage and the study of the response and adaptability of the species to different antimicrobial conditions will be fundamental for improving and implementing packaging systems aimed at prolonging the shelf life of meat products.

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