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A combination of modified atmosphere and antimicrobial packaging to extend the shelf-life of beefsteaks stored at chill temperature

Antonietta La Storia, Ilario Ferrocino [1], Elena Torrieri, Rossella Di Monaco, Gianluigi Mauriello, Francesco Villani, Danilo Ercolini *

Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli Federico II, Via Università 100, 80055 Portici, Italy

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ABSTRACT

An antimicrobial polyethylene (PE) film was obtained by coating a nisin-based antimicrobial solution. PE sheets were coated on both sides and were used for the packaging of beefsteaks to be stored in air or modified atmosphere packaging (MAP, 60% O_2–40% CO_2). Microbial populations, species diversity, headspace volatile organic compounds, colour and sensory properties were monitored after 0, 1, 7 and 12 days of storage at 4 °C. The viable counts showed that there was an effect of MAP and antimicrobial film on the development of all the spoilage associated microbial populations. *Carnobacterium* spp., *Brochothrix thermosphacta*, *Pseudomonas fragi* and *Rhanella aquatilis* were found in most of the samples. *C. maltaromaticum* was identified in MRS bulk cells from samples stored in air as well as MAP. Quantitative data of headspace-SPME–GC/MS analysis showed that during storage the production of volatile organic compounds (VOCs) was affected by the use of the treated film and the MAP storage. Compounds such as phenylethylalcohol, nonanal, decanal and ethylbutanoate were produced only from 7 to 12 day of storage and only in the samples stored in air. In agreement with the microbiological and VOCs data, the meat stored in active packaging scored the best rankings in the sensory evaluation. Principal component analysis of microbial, sensory and instrumental data showed that beefsteaks stored with the combination of MAP and active packaging for 12 days at 4 °C differed from the other samples that were more associated to high microbial loads, VOCs concentration and meat off odour perception.
In conclusion, the antimicrobial sheets in combination with MAP storage at 4 °C were effective for the storage of beefsteaks by retarding the growth of spoilage bacteria, determining lower concentration of VOCs and keeping acceptable levels of colour and other sensory parameters for more than 10 days.
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1. Introduction

Meat spoilage takes place when a certain amount of microorganisms develop in meat during storage causing release of volatile compounds or slime formation that render the product unacceptable for human consumption (Gram et al., 2002). The use of chill temperature, packaging and antimicrobials can influence the succession and metabolic activities of the 'Ephemeral/Specific Spoilage micro- Organisms (E/SSO)' (Nychas and Skandamis, 2004) that are members of the spoilage-associated microbial populations (Nychas et al., 2008). The organisms most commonly involved in meat spoilage are *Pseudomonas* spp., Enterobacteriaceae, *B. thermosphacta*, and lactic acid bacteria (LAB); their actual contribution to spoilage largely depends on the storage conditions (Borch et al., 1996; Dainty and Mackey, 1992; Gram et al., 2002; Koutsoumanis et al., 2008; Labadie, 1999; Stanbridge and Davies, 1998).

Nowadays, packaging of food performs beyond the conventional protection properties and provides many functions for the contained product (Han, 2005). Meat at retail can be packaged in air, modified atmosphere packaging (MAP) or vacuum packaging. The choice of the packaging type depends on the specific meat cut and the desired storage time. Beefsteaks are usually packed in plastic trays where, apart from an adsorbing pad used for draining meat exudates, there is no other part of the package in direct contact with the meat. In this kind of packaging, the steaks can be in contact with each other, the tray is covered with a plastic film (usually polyethylene) and the gas in the package is air. Such product has a commercial shelf life of no more than 5 days at 4 °C.

CO_2 is used in modified atmosphere packaging to retard the growth of Gram negative organisms responsible of aerobic spoilage such as *Psudomonas* spp. and Enterobacteriaceae, and a certain concentration of oxygen is employed for red meat MAP to preserve meat colour (Gill 2003; Jeremiah 2001). Recent advances in active antimicrobial packaging have shown that spoilage associated microbial populations can be efficiently inhibited by using bacteriocin activated plastic films for vacuum packaging of beef (Ercolini et al., 2010a, 2011). The contact between the antimicrobial surface of the plastic

* Corresponding author. Tel.: +39 081 2539449; fax: +39 081 2539407.
E-mail address: ercolini@unina.it (D. Ercolini).
[1] Present address: AGROINNOVA, Centro di competenza per l'innovazione in campo Agro-Ambientale, Università degli Studi di Torino, Via Leonardo da Vinci, 44, 10095 Grugliasco, Italy.

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film and the meat causes a direct killing of the microorganisms on the meat surface (Ercolini et al., 2006a). Moreover, if bacteriocins such as nisin are used as antimicrobials the efficacy of the active packaging is basically related to the Gram positive bacterial populations such as lactic acid bacteria (LAB) and *B. thermosphacta* while the effect on Gram negative populations is not always warranted although use of chelators in the antimicrobial soultion may help (Ercolini et al., 2010a, 2011).

Because MAP and bacteriocin activated packaging of meat can be effective against the above two different groups of target spoilage bacteria (i.e. MAP against Gram negative and bacteriocin activated packaging against Gram positive populations, respectively) the combination of the two storage systems appears promising for the extension of the microbiological shelf life of meat.

In this study we describe the development of a combination of MAP and antimicrobial packaging for the chill storage of beefsteaks. We evaluate the effect of the novel packaging system on the development of the spoilage-associated microbial populations, on the microbial species succession and volatile metabolites release during storage and also assess the sensory quality of the beefsteaks stored using the novel system versus traditional packaging technologies.

2. Materials and methods

2.1. Preparation of antimicrobial films

A nisin-based antimicrobial solution (NS) was prepared using a mix of organic acids and EDTA (Patent application MI2011A00805). NS was obtained as follows: 0.112 g/ml of Nisin (Nisin 2.5%, Sigma, Milano, Italy) was dissolved in a solution containing ascorbic acid 1% w/v, citric acid 1% w/v and $CaCl_2$ 1% w/v. The mixture was centrifuged at 6500 ×g for 10 min and then the pellet was suspended in the same volume of acid solution and centrifuged again at 6500 ×g for 10 min. Finally, the supernatant was added in 1:1 ratio of a solution containing 0.071 g/ml of EDTA. One milliliter of antimicrobial solution was coated manually on each side of a high density polyethylene film (HDPE, PO_2 2030 $cm^3\ m^{-2}$, PCO_2 8030 $cm^3\ m^{-2}$ in 24 h at 23 °C; density 0.92–0.95 g cm^{-3}) (21 × 38 cm^2) using a coating rod able to achieve 100 μm of coating thickness (Lee et al., 2004). The films were then treated with warm air in order to let the solution dry and promoting a homogenous distribution of the antimicrobial solution onto the surface of the plastic film. The antimicrobial activity of the plastic film was checked in agar assays as previously described (Mauriello et al. 2004) using *B. thermosphacta* 7R1 as indicator strain (La Storia et al., 2011).

2.2. Beef samples, storage conditions and microbial analysis

The different storage conditions used in this study are reported in Table 1 where sample abbreviations are listed. Thin beefsteaks (about 100 g each) from the same meat piece (chuck tender beef) were: (i) singly covered on both faces with HDPE films; singly covered on both faces with NS activated HDPE films (Patent application MI2011A00805) and (ii) not covered with any film (control). Three steaks were used for each condition; they were placed one on the top of the other and were separated or not by a HDPE film depending on the above described conditions. The steaks were placed in polystyrene trays (volume 500 ml, CoopBox, Bologna, Italy), and packed in air or MAP. The trays for air packaging were covered with low density polyethylene film OPP/LDPE (PO_2 < 2500 $cm^3\ m^{-2}$, PH_2O 7 g m^{-2} in 24 h at 23 °C) used as a sealing top. The MAP samples were prepared using a packaging machine (TSM, 105 Minipack Torre; Cava dei Tirreni [SA], Italy). The ratio between the headspace and weight of product was 2.5:1 ($cm^3\ g^{-1}$), 60% O_2–40% CO_2 was set as initial atmosphere and a barrier polyethylene film (PO_2 1.3 $cm^3\ m^{-2}$ in 24 h at 23 °C) was used as sealing top. All the packages were stored at 4 °C and samples for analyses were taken after 0, 1, 7 and 12 days of storage. The microbial populations monitored were total viable counts (TVC), Enterobacteriaceae, *B. thermosphacta*, LAB, and *Pseudomonas* spp. by using selective media and conditions previously described (Ercolini et al., 2011).

Table 1
Packaging conditions of the beef slices stored at 4 °C for 12 days.

Samples	Conditions	
	Initial atmosphere	Inter-slice film[1]
A	Air	–
AF	Air	HDPE film
AAF	Air	Antimicrobial HDPE film
MAP	60% O_2–40% CO_2	–
MAPF	60% O_2–40% CO_2	HDPE film
MAPAF	60% O_2–40% CO_2	Antimicrobial HDPE film

[1] The high density polyethylene (HDPE) film had the following characteristics: 0.95 g/cm^3 density, PO_2 2650 cm^3/m^2/24 h/atm at 23 °C.

2.3. Gas analysis, pH and colourimetric measurement

O_2 and CO_2 concentrations (% v/v) in the package headspace were monitored using a portable PBI Dansensor A/S (Check Mate 9900 O_2/CO_2; Ringsted, Denmark) analyser (accuracy ±0.1%) by sampling 3 ml of gas from the package headspace with a medical type needle (0.8 mm, 40 mm) (Thermo Europe N.V., 3001 Leuven, Belgium).

pH was measured by using a pH meter (Waterproof pHTestr, Thermo Scientific, Nijkerk, the Netherlands). Meat surface colour (CIE L*, a* and b* values) was measured using a colourimeter tristimolous (Minolta Chroma Meter, model CR-300, Osaka 541, Japan) having a circular measurement area (D = 8 mm), after removing the films from the meat surface. The colourimeter was calibrated using a white standard plate (L = 100). Ten readings were carried out on each beefsteak. Only the lean colour was measured, and precaution was taken to avoid any intramuscular fat. Total colour changes (ΔE) were calculated as follows:

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

2.4. Determination of volatile organic compounds (VOC) by GC/MS

The headspace (HS) solid-phase microextraction (SPME) analysis was carried out on single beefsteaks transferred from the packages into bottles of 250 ml. The fiber (CAR/DVB/PDMS; Supelco Sigma-Aldrich, Bornem, Belgium) was immersed in the HS for 1 h at room temperature. Thermal desorption of the analytes from the fiber inside the GC injection port was carried out in the split mode (1/10) at a desorption temperature of 250 °C for 2 min. All samples were analysed with a 7890A gas chromatograph coupled to a 5975 C quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). The gas chromatograph was equipped with an HP-5 ms capillary column (30 m, 0.25 mm [inner diameter]), and the carrier gas used was helium (1 ml min^{-1}). For the analysis of volatile compounds the GC oven temperature was programmed from 40 °C (held for 7 min) to 180 °C at 5 °C/min. The masses were scanned on *m/z* range of 45 to 350 amu. For the identification of volatile components, the NIST library and comparison with spectra and retention times of standards were used. The quantitative determinations were carried out through calibration in the matrix in the range of verified linearity; multiple replicates (n = 3 to 6) of the samples were analysed.

2.5. Sensory evaluation

The meat samples were submitted to sensory evaluation in order to ascertain whether there were differences between MAP, MAPF and MAPAF samples over the storage time. Samples packed in air were not included in the sensory evaluation because in preliminary experiments they showed too early spoilage symptoms. Eight judges, consisting of 4 females and 4 males, formed the panel for the evaluation of the samples. They were selected for their sensory ability and their previous experience in performing odour sensory profiling of spoiled meat (Ercolini et al., 2010b; Casaburi et al., 2011). They were trained through six preliminary sessions, in order to let them familiarise with samples object of investigation. Two visual (colour, fat colour) and two odour (fresh meat odour, off-odour) attributes, established during the first two training sessions, were evaluated by using continuous unstructured scales (10 cm) anchored both to the left end and right end: colour (0 = bright red; 10 = dark red); fat colour (0 = white; 10 = yellow); odours (0 = absent, 10 = very intense). Samples were equilibrated for fifteen minutes at room temperature and were served monadically, identified by three random digit codes. During each session six samples were evaluated; tasted in a randomised design with 3 replications. In order to avoid any stimulus error, the sample MAP was analysed at the end of the experimentation. In order to avoid any logical error, the odour attributes were evaluated under red light. All the sessions were conducted at 20–22 °C in an eight booth sensory panel room. Panel data were collected by means of “FIZZ Acquisition” software (Biosystèmes, Couternon, France).

2.6. DNA extraction and PCR–DGGE analysis

After microbial counts, the plates were used for the collection of bulk cells as previously described (Ercolini et al., 2006b). For DNA extraction from beef and bulk cells, the protocol described by the manufacturer of the Wizard DNA purification kit (Promega, Madison, Wiscon) was applied (Ercolini et al. 2006b). The extraction was performed directly from meat using 1 ml of the first decimal dilution prepared for the viable counts and from bulk cells by using 100 µl of the bulk suspension. DNA was quantified by using the Nanodrop 1000 (Thermo Scientific, Milano, Italy) and standardised at 50 ng/µl.

The primers U968 and L1401 were used (Zoetendal et al. 1998) amplifying the variable V6-V8 region of the 16S rRNA gene and giving PCR products of about 450 bp were used for the amplifications. PCR and DGGE analyses were carried out as previously reported (Ercolini et al. 2006b). DGGE bands to be sequenced were purified in water according to Ampe et al. (1999). One µl of the eluted DNA of each DGGE band was re-amplified by using the primers and the conditions described above. PCR products that gave a single band co-migrating with the original band were then purified by QIaex PCR purification kit (Qiagen, Milano, Italy) according to the manufacturer's instructions and sequenced. Sequencing was performed by Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems) using 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 (http://www.ncbi.nlm.nih.gov/blast/).

2.7. Multiplex PCR amplification of Pseudomonas spp. and real-time PCR assay for the specific detection of B. thermosphacta

DNA directly extracted from bulk cells collected from Pseudomonas Agar were used as template in a multiplex PCR assays for the identification of *P. fragi*, *P. lundensis* and *P. putida* by targeting the *carA* gene (Ercolini et al. 2007). A RTi-PCR assay was performed employing the species-specific primers for *B. thermosphacta* on the basis of the 16S rRNA gene (Pennacchia et al., 2009). DNA extracted directly from bulk cells from STAA plates were used as template. A response was considered positive if the amplification curve of two from three replicates crossed the fluorescence threshold line, which was positioned by a background-based algorithm calculated by the software.

2.8. Data analysis

To study the effect of sample and time on colour and microbial loads, a factorial design with two factors (time and sample) was used. Four were the levels of storage time (0, 1, 7 and 12 days) and six the levels of sample (A, AF, AAF, MAP, MAPF, MAPAF, see Table 1). Three replicates were performed for each experiment for a total of 72 samples. To study the effect of sample and time on sensory attributes change, a factorial design with two factors (time and sample) was used. Four were the levels of storage time (0, 1, 7 and 12 days) and three the levels of sample (MAP, MAPF, MAPAF). Three replicates were performed for each experiment for a total of 36 samples. ANOVA analysis was performed on the data to evaluate the effect of time (A), sample (B) and the interaction effect (AxB) on the colour, microbial loads and sensory attributes. Tukey test was performed to find out the source of the significant differences within samples. Significance of differences was defined at $p \leq 0.05$. Statistical analyses were performed using the SPSS computer package (SPSS Inc. 11.5, Chicago, 2002).

In order to analyse the similarity between the different packaging types and storage times, chemical, microbiological, sensory and physical data were subjected to a Principal Component Analysis (PCA) model by using all the samples as observations. PCA was carried out by using SIMCA-P 10 statistical software (Umetrics, Sweden).

3. Results

3.1. Viable counts of microbial populations during the storage of beefsteaks

The results of the viable counts of spoilage-related microbial groups during the storage of beefsteaks in all the conditions used are shown in Table 2. Microbiological data were significantly affected by the use of MAP ($p<0.01$) and by the antimicrobial film ($p<0.05$). The total viable counts showed loads more than 3 log cycles lower by using MAP if compared to the same type of samples stored in air for 12 days (Table 2); moreover, a further reduction was obtained by using antimicrobial packaging. *B. thermosphacta* was significantly affected ($p<0.05$) by the use of the antimicrobial packaging and was not countable in samples stored in MAPAF conditions for the first week of storage. By contrast, it reached peak loads of more than 10^6 CFU g^{-1} in samples stored in air for 12 days. The loads of LAB were never higher than 10^5 CFU g^{-1} in any of the conditions used; in addition, their growth was significantly ($p<0.05$) inhibited when the combination of MAP and active packaging was used (Table 2). The beefsteaks used showed a negligible contamination by Enterobacteriaceae that grew only after 12 days of storage and only in aerobic conditions. *Pseudomonas* spp. reached values of 10^7 CFU g^{-1} after 1 week of storage in air and were unaffected ($p>0.05$) by the use of the antimicrobial film. In MAP condition the load of *Pseudomonas* spp. was significantly lower than in air and the population was affected by the use of the active films after 7 days of storage ($p<0.05$, Table 2).

3.2. Identification of microbial species

The results of the band sequencing from the PCR–DGGE fingerprints obtained from DNA directly extracted from meat are shown in Table 3. The PCR–DGGE analysis of meat stored in aerobic conditions showed that the samples were contaminated by *Pseudomonas* spp., *Carnobacterium* spp. and *Rahnella aquatilis* (Table 3). *Pseudomonas* spp., *S. proteamaculans* and *R. aquatilis* were identified among the bands in the fingerprints of bulk cells from VRBGA and occurred after 12 days of storage in air

Table 2
Viable counts of different spoilage-associated microbial groups in beefsteaks during storage at 4 °C for 12 days.

Samples[b]	Log CFU/g ± SD at 1, 7 and 12 days of storage[a] Total viable counts (PCA)			*B. thermosphacta* (STAA)			*LAB* (MRS agar)			Enterobacteriaceae (VRBGA)			*Pseudomonas* spp. (CFC)		
	1	7	12	1	7	12	1	7	12	1	7	12	1	7	12
A	2.51 ± 1.08^{a}	8.16 ± 0.71^{ab}	7.68 ± 1.07^{a}	1.07 ± 0.41^{a}	6.19 ± 0.72^{a}	6.39 ± 1.37^{a}	1.49 ± 0.60^{a}	4.95 ± 0.29^{a}	4.19 ± 1.01^{a}	<1.00	<1.00	1.89 ± 0.55^{a}	2.16 ± 1.00^{a}	7.85 ± 0.93^{a}	7.21 ± 1.22^{a}
AF	2.81 ± 0.41^{a}	7.25 ± 0.95^{a}	7.06 ± 0.90^{a}	1.60 ± 0.91^{a}	6.49 ± 0.48^{a}	5.47 ± 1.74^{a}	1.37 ± 0.74^{a}	4.72 ± 0.34^{a}	4.69 ± 1.36^{a}	<1.00	<1.00	2.03 ± 1.11^{a}	1.80 ± 0.80^{a}	6.30 ± 1.92^{a}	7.14 ± 0.60^{a}
AAF	1.81 ± 0.97^{a}	6.28 ± 1.94^{b}	6.71 ± 0.52^{a}	$<1.00^{b}$	3.85 ± 1.06^{b}	4.36 ± 2.79^{b}	$<1.00^{b}$	4.19 ± 0.22^{a}	4.00 ± 0.36^{a}	<1.00	<1.00	1.77 ± 0.77^{a}	1.72 ± 0.76^{a}	6.33 ± 1.83^{a}	7.77 ± 0.54^{a}
MAP	2.08 ± 0.76^{A}	4.04 ± 0.79^{A}	4.17 ± 1.22^{A}	1.14 ± 0.13^{A}	3.68 ± 0.31^{A}	3.03 ± 1.51^{A}	1.58 ± 0.76^{A}	3.22 ± 0.92^{A}	4.70 ± 1.89^{A}	<1.00	<1.00	<1.00	1.79 ± 0.91^{A}	3.23 ± 1.57^{A}	1.58 ± 0.47^{A}
MAPF	2.43 ± 0.24^{A}	4.45 ± 1.42^{A}	4.83 ± 1.99^{A}	1.85 ± 0.90^{A}	3.05 ± 1.01^{A}	3.64 ± 2.93^{A}	1.37 ± 0.39^{A}	3.51 ± 1.38^{A}	4.02 ± 1.89^{B}	<1.00	<1.00	<1.00	1.36 ± 0.63^{A}	2.27 ± 0.46^{AB}	3.05 ± 1.14^{A}
MAPAF	2.73 ± 0.18^{A}	2.69 ± 0.93^{A}	3.67 ± 1.38^{B}	$<1.00^{B}$	$<1.00^{B}$	1.11 ± 1.19^{B}	$<1.00^{B}$	1.00 ± 0.00^{B}	1.82 ± 0.49^{B}	<1.00	<1.00	<1.00	1.53 ± 0.66^{A}	1.69 ± 0.80^{B}	1.77 ± 0.62^{A}

[a] The microbial loads at time zero were: PCA: 3.22 ± 0.49; STAA: 1.64 ± 0.61; MRS: 1.83 ± 1.01; VRBGA: <1.00; PSA: 1.66 ± 0.79. Values with different superscripts corresponding to the same time of storage differ significantly ($P < 0.05$).
[b] Sample labelling is described in Table 1.

Table 3
Microbial species identification after sequencing of the variable V6-V8 region of the 16S rRNA gene purified from PCR–DGGE profiles of samples of meat and bulk cells from selective media.

Source	Storage conditions (days of storage)	Closest match	Identity (%)	Closest match (accession no.)
Beef	A (7, 12); AF (7, 12)	*Pseudomonas* spp.	100	DQ405241
Beef	A (0, 1, 7, 12); AF (7, 12); AAF (12)	*Pseudomonas* spp.	99	DQ405236
Beef	A (0, 1, 7, 12); AF (1, 7); AAF (7, 12); MAP (1, 7); MAPF (1, 7)	*Rahnella aquatilis*	99	FJ811859
Beef	A (0, 1, 7, 12); AF (1, 7); AAF (1, 7); MAP (1, 7, 12); MAPF (1, 7, 12)	*Carnobacterium* spp.	99	DQ405248
VRBGA	A (12); AAF (12)	*Pseudomonas* spp.	99	DQ405236
VRBGA	AAF (12)	*Pseudomonas* spp.	100	DQ405224
VRBGA	A (12); AF (12)	*Rahnella aquatilis*	100	DQ440548
VRBGA	AAF (12)	*Serratia proteamaculans*	99	EU834352
MRS	A (0, 1, 7, 12); AF (1, 7, 12); AAF (7, 12); MAP (1, 7, 12); MAPF (1, 7, 12); MAPF (7, 12)	*Carnobacterium maltaromaticum*	100	GQ304931
MRS	MAPF (12)	*Staphylococcus saprophyticus*	98	GU722234

conditions. Only *C. maltaromaticum* was identified from bulk cells from MRS plates occurring in all the samples regardless of the packaging conditions; however, it grew only after 7 days of storage when the activated films were used both in air and MAP conditions (Table 3).

Multiplex PCR amplification of the *carA* gene for the identification of *Pseudomonas putida*, *P. fragi* and *P. lundensis* performed on DNA extracted from bulk cells showed a contamination from *P. fragi* in all the samples stored in air at any time of sampling and in the MAP stored samples up to 7 days of storage (Table 4). *P. lundensis* was mostly detected in steaks stored in air with active and non active plastic films (Table 4). The results of the RTi-PCR assay showed that *B. thermosphacta* occurred in all the meat samples where the antimicrobial packaging was not employed (Table 4).

3.3. pH, gas composition and colour change

The initial pH of meat was 5.4 ± 0.1 and did not show significant changes ($p < 0.05$) during storage in any of the packaging conditions except in A samples where it reached values of 6.1 ± 0.1 at the end of storage. In all the air stored beefsteaks O_2 concentration varied from initial 21% to about 18% after 1 week of storage and did not change until the end ($p < 0.05$) whereas CO_2 showed an increase from 3 to 23% after 1 week of storage only in A samples while it did not change during storage in AF and AAF samples ($p < 0.05$). No significant ($p < 0.05$) variation in gas composition was found in MAP stored samples (data not shown).

The effect of sample and time on colour change was studied by monitoring the colourimetric parameters L*, a*, b* and ΔE in meat samples during the time of storage as reported in Fig. 1. ANOVA results highlighted a significant effect of the time ($p < 0.001$) and

Table 4
Detection of *Pseudomonas fragi*, *P. lundensis*, *P. putida* by using multiplex PCR amplification of the *carA* gene (Ercolini et al., 2007) and of *B. thermosphacta* by RT-PCR amplification of the 16S rRNA gene (Pennacchia et al., 2009).

Species	Meat Packaging conditions	Time of storage
P. fragi	A, AF, AAF	0, 1, 7, 12
P. fragi	MAP, MAPF, MAPAF	0, 1, 7
P. lundensis	AF, AAF	0, 1, 7, 12
B. thermosphacta	A, AF, MAP, MAPF	1, 7, 12
B. thermosphacta	AAF	7, 12

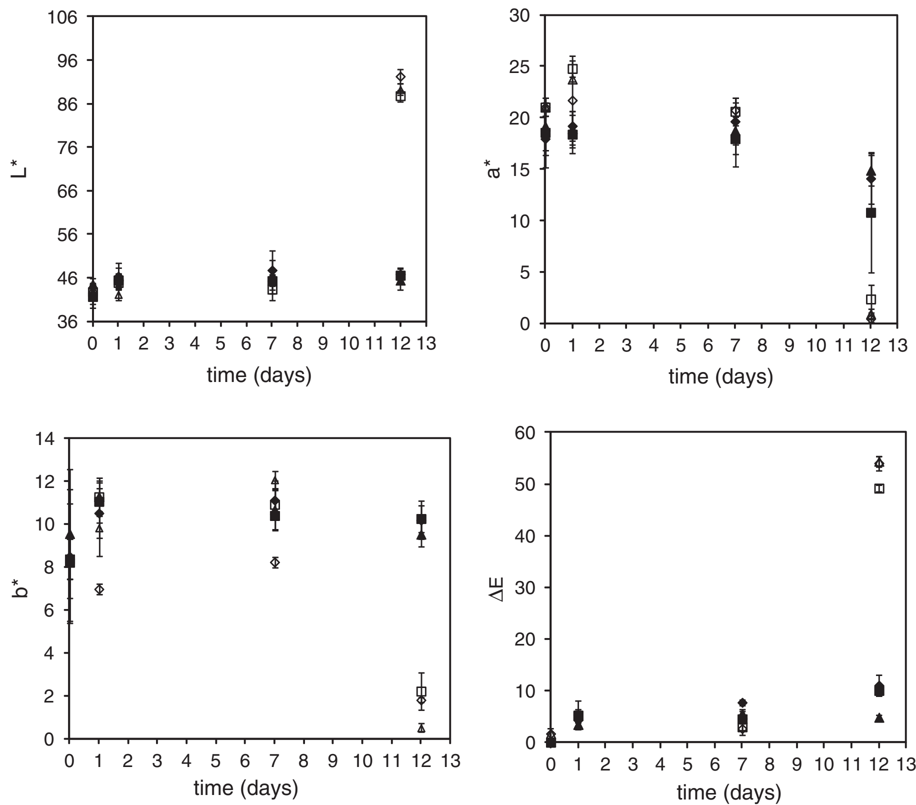

Fig. 1. Colour changes of packed beefsteaks during storage at 4 °C. Samples: A (□), AF (◊), AAF (△), MAP (■), MAPF (♦), MAPAF (▲).

samples ($p<0.001$) on all colourimetric parameters with a significant effect of the interaction between time and sample ($p<0.001$). In particular, in samples packed in air, all parameters remained constant up to 7 days of storage at 4 °C whereas the effect of the time was significant only after 12 days (Fig. 1). In particular, a* decreased from an average value of 22 ± 2 to a value of 2 ± 1 after 12 days at 4 °C, a decrease of a* corresponding to a change of the colour of the meat from red to brown. For samples packed in MAP, a* decreased from an initial value of 18 ± 2 to an average value of 11 ± 6 after 12 days at 4 °C with the first significant difference from fresh samples observed after 12 days. The parameter b* increased from an initial value of 8 ± 3 to an average value of 10 ± 1 after 12 days with the first significant difference after 7 days ($p<0.05$). By using the parameter ΔE in order to describe the colour change during the storage time, it was possible to show same differences among samples after 12 days of storage ($p=0.019$). In particular, samples packed with active film (MAPAF) showed the lowest ΔE value compared to the others. Overall, the results showed that MAP had a protective effect on colour change of the meat surface and that MAPAF showed the least change of colour during time.

3.4. Analysis of VOCs by GC/MS

In order to determine the presence of spoilage-associated molecules in the headspace of the beefsteaks stored in all the different conditions tested, HS–SPME–GC/MS analyses were carried out following the analytical conditions described in Materials and methods. The different molecules detected are reported in Table 5. The amount of volatile metabolites in the headspace of samples ranged from 10^{-3} mg up to some mg, with odour threshold from some units of ppb up to 10 ppm.

The total concentration of VOCs released generally increased during storage regardless of the packaging conditions (Table 5). Acetoin was detected after 24 h of storage regardless of the storage conditions. A group of molecules including phenylethylalcohol, ethylbutanoate, ethylhexanoate and ethylheptanoate were associated to the storage in air and were mainly detected at the end of the storage time (Table 5). The 3-methyl-1-butanol showed the highest quantities after 12 days of storage in A and MAP conditions. Overall, the storage in air was characterised by the release of the highest diversity of VOCs. On the other hand, the beefsteaks stored in MAP combined with antimicrobial films (MAPAF) showed the lowest contamination levels both in terms of type and quantities of molecules detected (Table 5).

3.5. Sensory evaluation

The changes of sensory attributes of beefsteaks during storage in MAP conditions are shown in Fig. 2. Both time ($p=0.001$) and sample ($p \ll 0.001$) significantly affected the perceived off odour and the interaction effect (time x sample) was also significant ($p \ll 0.001$). In particular, off odour intensity varied from about 4 to 9 during storage only in MAPF samples, while no differences were found for the other two types of package (Fig. 2A). The sample effect was not significant on fresh meat odour ($p=0.2$), whereas both the storage time ($p=0.02$) and interaction (time × sample) ($p=0.006$) had a significant effect. The intensity of fresh meat odour decreased from about 7 to 5 on the 10 cm scale (Fig. 2B); however, this did not occur in MAPAF samples that kept their fresh meat odour unaltered ($p=0.1$) during all the storage time. Meat red colour was significantly affected from both time ($p<0.001$) and sample ($p=0.04$). In particular, red colour changed from about 4.7 to 6.8 on the 10 cm scale during the storage. After 12 days, MAP samples were darker than MAPF and MAPAF samples (Fig. 2C). Storage time did not modify fat colour of the meat ($p=0.8$), whereas sample effect was significant ($p=0.01$) only after 7 days when the fat colour showed some yellowing in MAP stored meat (Fig. 2D).

3.6. Principal component analysis

The similarity between the different packaging types and times of storage was further analysed by PCA and the results are reported in

Table 5
VOCs and their mean relative quantities (µg) detected in beefsteaks stored for 12 days at 4 °C in different packaging conditions.

	A*			AF			AAF			MAP			MAPF			MAPAF		
	1	7	12	1	7	12	1	7	12	1	7	12	1	7	12	1	7	12
Acetoin	0	227 ± 21^a	484 ± 87^a	576 ± 44^a	59 ± 48^b	23 ± 2^b	630 ± 55^a	421 ± 49^c	197 ± 21^c	206 ± 23^b	15 ± 21^b	0	755 ± 81^c	11 ± 2^b	0	23 ± 4^d	295 ± 33^a	87 ± 9^d
3-methyl-1-butanol	0	0	3140 ± 403^a	0	0	40 ± 21^b	0	0	95 ± 11^c	0	0	286 ± 29^d	0	0	195 ± 29^d	0	0	0
1-hexanol	0	0	91 ± 12^a	0	0	1 ± 0.08^b	0	0	6 ± 0.4^b	0	0	35 ± 3^c	0	0	18 ± 2^d	0	0	0
1-octen-3-ol	0	0	117 ± 23^a	0	0	2 ± 0.09^b	0	0	16 ± 3^c	0	0	27 ± 3^c	0	0	24 ± 3^c	0	0	6 ± 0.9^b
Phenylethylalcohol	0	0	71 ± 11^a	0	0	714 ± 87^b	0	0	626 ± 77^b	0	0	0	0	0	0	0	0	0
Ethylbutanoate	0	0	369 ± 38^a	0	31 ± 2	80 ± 7^b	0	0	33 ± 2^c	0	0	0	0	0	0	0	0	0
Ethylhexanoate	0	0	5901 ± 459^a	0	0	1064 ± 134^b	0	0	586 ± 71^c	0	0	9 ± 0.9^d	0	0	12 ± 2^d	0	0	0
Ethyl 2-hexenoate	0	0	18 ± 2^a	0	0	6 ± 0.7^b	0	0	4 ± 0.09^b	0	0	0	0	0	0	0	0	0
Ethylheptanoate	0	632 ± 55^a	208 ± 21^a	0	28 ± 3^b	43 ± 5^b	0	14 ± 2^c	22 ± 2^c	11 ± 2^a	10 ± 0.7^c	0	0	6 ± 0.4^d	0	17 ± 2^a	18 ± 3^c	0
Ethyloctanoate	0	48 ± 3^a	533 ± 66^a	0	4 ± 0.7^b	67 ± 7^b	0	12 ± 2^c	45 ± 5^b	7 ± 1^a	0	1 ± 0.05^c	10 ± 0.7^a	2 ± 0.3^b	1 ± 0.2^c	0	164 ± 27^d	14 ± 2^d
Ethyldecanoate	0	3 ± 04^a	214 ± 22^a	0	0	8 ± 0.9^b	0	0	1 ± 0.08^c	0	0	1 ± 0.07^c	0	0	0	0	8 ± 1^a	0

* Comparing meat samples at the same time of sampling between the different storage conditions, values labelled with different letters differ significantly ($p<0.05$).

Figs. 3 and 4. The PCA plots obtained considering all the meat samples (as observations), concentration of gases, VOCs, microbial loads, pH and colour parameters (as variables) are reported in Fig. 3. The sample A12 was excluded from the PCA as it overcame the critical distance from the model. The samples were distributed in the scores plot according to packaging type and time of storage; the samples stored in air for 12 days were in the left part of the plot (Fig. 3A) and characterised by the highest microbial loads, colour changes (ΔE) and concentrations of several VOCs (Fig. 3B). Decreasing microbial loads and VOC concentrations determined another group of samples including air stored beefsteaks after 7 days (Fig. 3A) to form in the central part of the plot. MAP samples stored for 12 days without active films grouped in the top part of the plot (Fig. 3A) and were correlated to higher concentrations of 3-methyl-1-butanol, 1-octen-3-ol and 1-hexanol (Fig. 3B). Finally, the air stored samples at the beginning of the storage, the MAP samples stored for 7 days and the sample MAPAF12 stored in MAP with antimicrobial packaging for 12 days were included in a different group opposite to the group of the samples stored in air for 12 days, characterised by positive PC1 coordinates and positively related to high values of the cromatic coordinates a* and b* (Fig. 3A). By analysing the MAP samples separately and including the results of the sensory analysis, the plots shown in Fig. 4 were obtained. The samples stored for 12 days without antimicrobial films were grouped in the left part of the scores plot (Fig. 4A); it was possible to notice that MAPF12 was positively related to off odour intensity and microbial loads, MAP12 was positively related to VOCs concentration and red colour, both samples were characterised by high colour change (ΔE) (Fig. 4B). By contrast, the beefsteaks stored in MAP with antimicrobial films for 12 days (MAPAF12) were in the central part of the plot and closer to the other MAP samples stored for less days (Fig. 4A). It was also possible to observe that increased microbial loads and VOCs concentrations corresponded to increasing off odour intensity as well as increased ΔE corresponded to increasing red colour intensity (Fig. 4B).

4. Discussion

Modified atmosphere packaging (MAP) is recognised as one of the most effective applications for shelf life extension of fresh meat products (Gill and Gill, 2005). Composition of the atmosphere determines to a large degree the extent and type of spoilage that can develop during storage. Efficacy of MAP is based on the antimicrobial activity of CO_2 present in the headspace of meat packages, affecting bacterial cells permeability, pH changes and enzymatic inhibition (King and Nagel, 1967; Blakistone 1999). The inhibitory efficiency of CO_2 is increased at lower temperature, because the solubility of the gas increases with decreasing temperature. This condition could inhibit aerobic Gram-negative bacteria such as *Pseudomonas* spp. and Enterobacteriaceae (Church and Parsons, 1995) and allow the growth of Gram-positive bacteria such as LAB and *B. thermosphacta* (Koutsoumanis et al. 2006). We only used one type of MAP in this study (60%O_2–40%CO_2); this gas composition was chosen because it proved effective in retarding the growth of spoilage bacteria in beef in a previous study (Ercolini et al., 2006b). In addition, we also used only a single chill temperature for the storage (4 °C), which represents a limit of this study. Since the chill temperature during distribution can fluctuate, the effect of the developed MAP and antimicrobial packaging storage should be examined under a range of other temperatures.

The results of the viable counts confirmed that *Pseudomonas* spp. and Enterobacteriaceae were inhibited only by using MAP. The use of antimicrobial films showed an antimicrobial power from the beginning by inhibiting the growth of *B. thermosphacta* and LAB populations. The microbiological results suggested that the choice to combine MAP and antimicrobial packaging assured an effect against both Gram positive and Gram negative spoilage-associated populations.

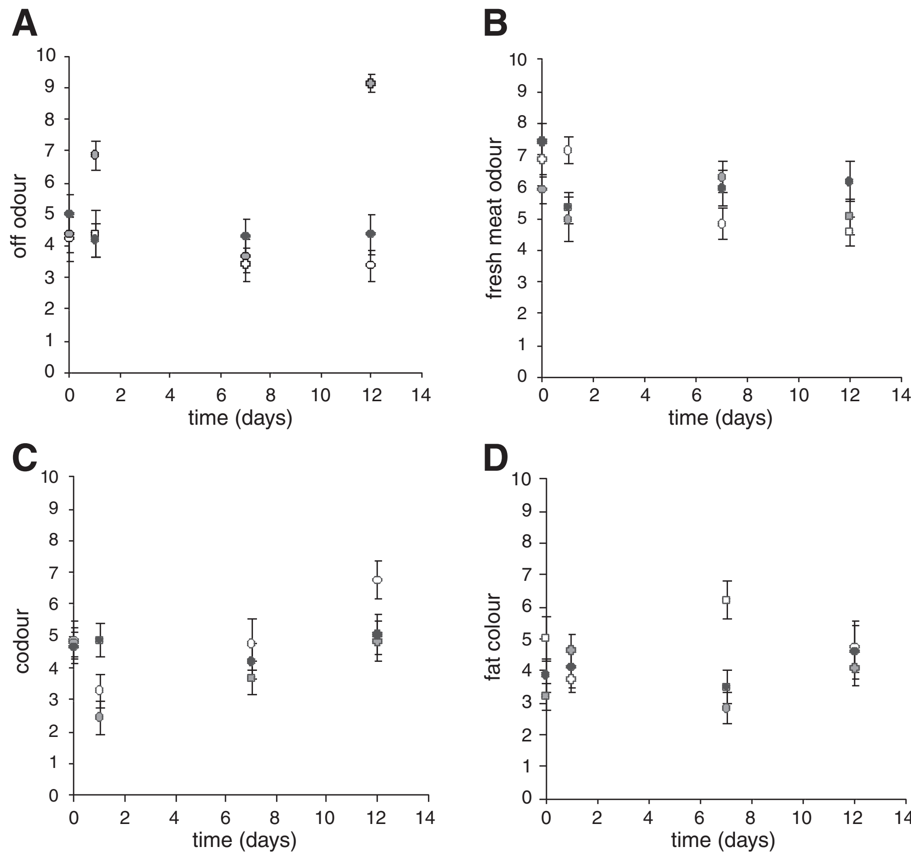

Fig. 2. Changes of sensory attributes of beef steaks stored in MAP at 4 °C for 12 days. Samples: ○ MAP; ● MAPF; ● MAPAF.

The identification of microbial species obtained by species-specific PCR indicated that *P. fragi* was the species most commonly occurring during storage in both air and MAP while *P. lundensis* was only found in samples stored in air with active and non active films. Although the load of *Pseudomonas* spp. was affected by the use of MAP conditions, it was possible to find *P. fragi* in MAP samples. It was reported that this microorganism can grow at low oxygen concentrations (Economou et al. 2009, Ercolini et al., 2010a,b, Hasapidou and Savvaidis 2011) and that a certain tolerance to CO_2 has been found (Gill & Jeremiah, 1991). *Rahnella* spp. is an emerging member of Enterobacteriaceae in meat storage (Ercolini et al., 2006b,a; Pennacchia et al., 2011); although the counts on VRBGA of MAP samples were below the detection limit (Table 2), *R. aquatilis* was found in most of MAP samples by analyzing DNA directly extracted from meat by PCR–DGGE. *C. maltaromaticum* has been recently shown to give a negligible contribution to meat spoilage in absence of competing microbiota (Casaburi et al., 2011); however, it was identified in almost all the samples both in air and MAP conditions.

The presence of the microorganisms and their specific loads in the various samples influenced the quality of the beefsteaks as represented by the results of the PCA analyses (Figs. 3 and 4). The loads of the different spoilage groups, along with the concentration of volatile metabolites, allowed a defined separation of the samples according to the storage time where the highest counts corresponded to the end of the storage. In particular, the loads of the above species and the concentration of some VOCs were associated to increasing off odours of MAP and MAPF samples stored for 12 days (Fig. 4), while the sensory quality of the beefsteaks packed in MAP with antimicrobial films kept higher scores during storage.

The detected volatile metabolites (alcohols, ketones and ethylesters) derived from the main microbial catabolic pathways of lipids, carbohydrates and amino acids and could contribute to sensory spoilage because they have been associated to off-flavour (Montel et al., 1998; Ercolini et al., 2010b, 2011). The trend of VOCs production in the differently packed beefsteaks followed the changes in microbial loads and diversity. In fact, ethyl esters where much more diverse and abundant in air than MAP stored samples in agreement with higher counts of *Pseudomonas* spp. and the presence of *P. fragi*. In a recent study, it was shown that ethylhexanoate, ethyloctanoate and ethyldecanoate were the most commonly detected molecules in air stored meat inoculated with strains of *P. fragi* and that the ester's release was associated to the sensory perception of fruity off odours of meat (Ercolini et al., 2010b).

The presence of 1-octen-3-ol is related to an unpleasant sensory answer (Calkins and Hodgen, 2007) and it was recently associated with MAP storage (Ercolini et al., 2011). In this study, phenylethyl alcohol was associated to air storage in agreement with what recently reported (Ercolini et al., 2011). Acetoin was frequently found in the analysed beefsteaks, it is considered an important flavour compound related to a creamy dairy odour (Soncin et al., 2007) and Dainty et al. (1989) reported that the accumulation of acetoin was not unpleasant for the meat to be regarded as spoiled, but rather as not fresh. Unaccountably, acetoin was found at the initial stage of storage although this was not associated to increase of any microbial count.

The colour of muscle in red meat depends on the chemical state of the muscle pigment myoglobin. During storage, in presence of oxygen the colour of meat change from a red bright colour to a brown colour for effect of myoglobin oxidation with production of metmyoglobin (Blakistone, 1999; Gill, 2003; Gill and Gill, 2005; Mancini and Hunt, 2005). Colour alteration makes products unattractive, affecting consumer preference at purchase (Carpenter et al., 2001). High oxygen concentration in the package headspace help to decrease the oxidation kinetics, allowing an extension of product shelf life of 3–5 days at refrigeration temperature (Gill, 1996; Martinez et al., 2006). The presence of an active film on the product surface can change the

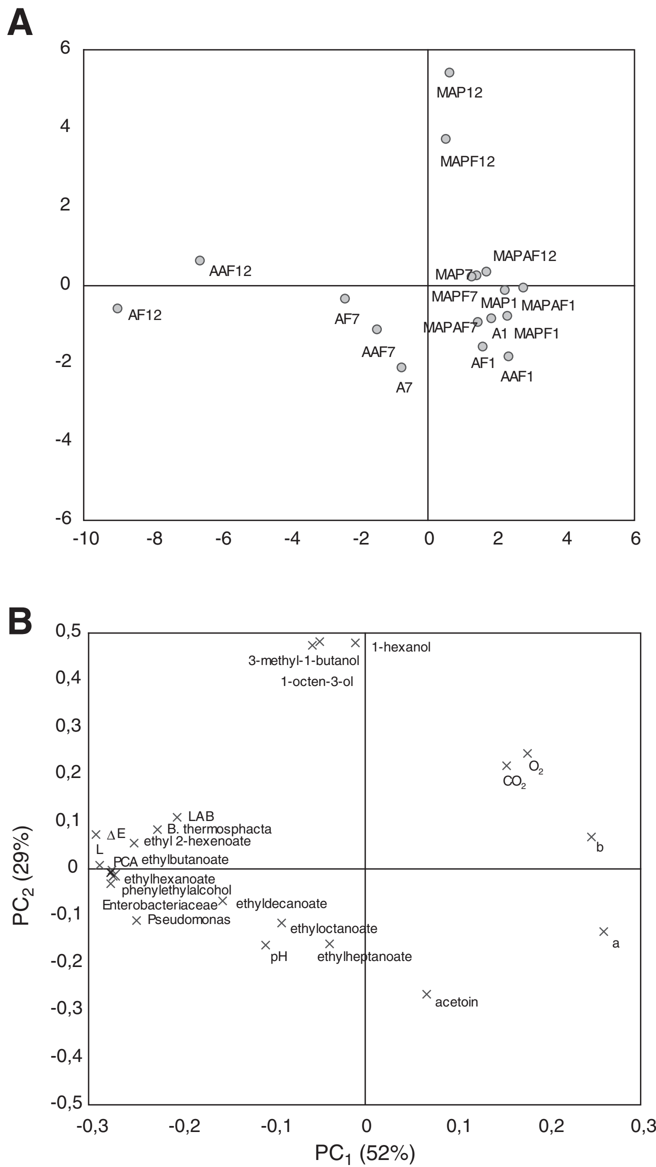

Fig. 3. Principal component analysis of instrumental data: A) scores plot, B) loadings plot.

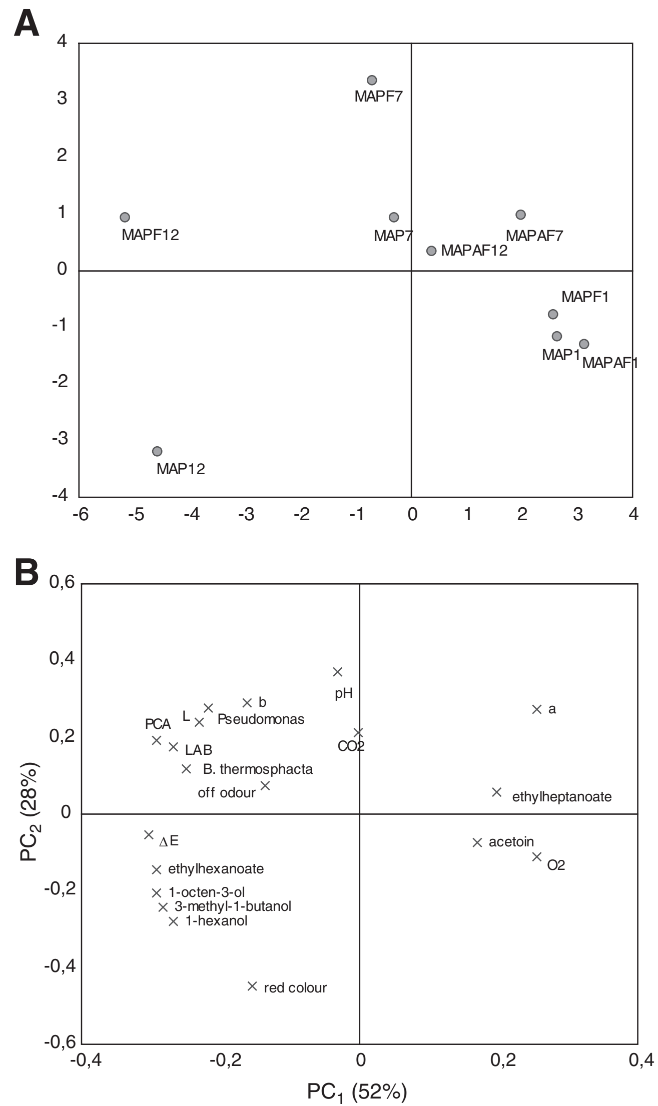

Fig. 4. Principal component analysis of instrumental and sensory data of MAP stored samples: A) scores plot, B) loadings plot.

micro-atmosphere surrounding the product. According to our results, the oxidation of myoglobin occurred faster when samples were packed in MAP without film than when samples were packed with film. The HDPE film was chosen for its high O_2 permeability; moreover, the ascorbic acid in the antimicrobial solution can preserve red meat colour owing to antioxidant properties (Lund et al. 2007); finally, the addition of a chelator such as citric acid, could improve ascorbic acids's efficacy (Mancini et al. 2007). Although few works are available on the effect of an antimicrobial film on colour change of beef, the changes registered in our work are similar to those reported by Lund et al. (2007), Park et al. (2010) and Ercolini et al. (2006b) who studied, respectively, the effect of a antioxidant film, chitosan-incorporated LPDE film and MAP on colour change of beefsteaks.

Instrumentally measured colour changes (ΔE) were confirmed by sensory results, which showed a browning of the meat colour after 12 days of storage in particular for sample packed with MAP. Sensory evaluations were useful to investigate the sensory changes of the meat in the different conditions; they were only applied to MAP samples that had better potential antimicrobial performance. No literature was found on the effect of antimicrobial films on the sensory properties of beef. Most studies investigate the effect of beef packaging on sensory attributes of the resulting cooked meat (Zakrys et al. 2008; O'Sullivan et al., 2011; Zakrys-Waliwander et al. 2011), whereas only few studies focus on the odours of raw beef (Montgomery et al. 2003; Stivarius et al. 2002, Houbena et al. 2000). The sensory analysis demonstrated that the antimicrobial packaging was able to preserve fresh odour of the meat and this result is in agreement with previous studies where the use of MAP in combination with addition of nisin–EDTA resulted in a shelf-life extension of refrigerated fresh chicken maintaining acceptable odour attributes up to 24 days of storage (Economou et al., 2009).

Antimicrobial packaging is an extremely challenging technology that could extend shelf-life and improve quality and safety of meat products. Combination of this application with MAP can provide improved methods of beef storage at retail allowing a prolonged shelf life of the products while preserving their quality.

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