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ORIGINAL ARTICLE

Gases and volatile compounds associated with micro-organisms in blown pack spoilage of Brazilian vacuum-packed beef

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Significance and Impact of the Study: The data generated by this study provided useful information to correlate the microbial contamination of Enterobacteriaceae, lactic acid bacteria and Clostridium with the VOC and gaseous compound production to define, in a faster manner, not only the type of contamination, but also to prevent it.

Keywords
16S rRNA, beef, blown pack spoilage, volatile compounds.

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Abstract

This study correlated the composition of the spoilage bacterial flora with the main gaseous and volatile organic compounds (VOCs) found in the package headspace of spoiled, chilled, vacuum-packed meat. Fifteen chilled, vacuum-packed beef samples, suffering from blown pack spoilage, were studied using 16S rRNA clone sequencing. More than 50% of the bacteria were identified as lactic acid bacteria (LAB), followed by clostridia and enterobacteria. Fifty-one volatile compounds were detected in the spoiled samples. Although the major spoilage compounds were identified as alcohols and aldehydes, CO_2 was identified as the major gas in the spoiled samples by headspace technique. Different species of bacteria contribute to different volatile compounds during meat spoilage. LAB played an important role in blown pack deterioration of the Brazilian beef studied.

Introduction

Reports of incidents of early deterioration of chilled vacuum-packed meat were characterized by package distension caused by gases produced by spoilage micro-organisms (blown pack), have been reported and attributed to psychrophilic and psychrotrophic bacteria. This spoilage is characterized by the production of large amounts of gas, offensive and putrid odours, presence of exudates, extensive proteolysis and changes in pH and colour. These changes normally occur within 4–6 weeks of chilled storage (Broda *et al.* 1996), resulting in financial losses for the Brazilian meat industries. Despite Brazil being the second largest exporter of beef (ABIEC 2011), there are few studies concerning the micro-organisms involved in blown pack spoilage of Brazilian meat.

Several species of clostridia such as *Clostridium algidixylanolyticum*, *Cl. frigidicarnis*, *Cl. algidicarnis* and *Cl. gasigenes* were isolated from spoiled vacuum-packed meat from New Zealand. Moreover, *Cl. estertheticum* and *Cl. gasigenes* may also produce blown pack spoilage (Adam *et al.* 2010). Psychrotolerant enterobacteria have also been associated with blown pack spoilage of vacuum-packed meat (Brightwell *et al.* 2007; Pennacchia *et al.* 2011).

The disadvantages of using routine microbiological methods to detect and identify strict anaerobes, such as psychrotolerant clostridia, are the extended periods of time necessary for growth and the lack of species and strain specificity (Redemaker *et al.* 2006). Culture-independent methods to assess the microbial community, such as the rRNA gene clone libraries, have been used successfully in several studies identifying micro-organisms

in foods such as milk, cheese (Delbès *et al.* 2007), raw pork and beef (Olsson *et al.* 2003; Silva *et al.* 2011).

According to the study by Ercolini *et al.* (2009), volatile organic compound (VOC) profiles differ between vacuum-packed meat inoculated with *Serratia proteomaculans*, *Carnobacterium divergens*, *Pseudomonas fragi* and a culture mixed with these three bacteria observed in a 0, 15 and 30 storage days at 7°C.

The aim of this study was to identify the micro-organisms related to blown pack spoilage using a 16S rRNA clone library and relate them to the main gaseous and volatile compounds present in samples. The samples were grouped based on similarities in the composition of headspace (HS) gas using cluster analysis and principal component analysis (PCA).

Results and discussion

Bacteria identification using 16S rRNA clone library

The sequenced PCR products from the fifteen samples had between 97 and 100% sequence similarity with GenBank sequence of 16S rRNA genes. The most common were lactic acid bacteria (LAB) followed by enterobacteria and clostridia groups (Table 1). In the LAB group, *Lactobacillus*, *Lactococcus* and *Carnobacterium* genus were the frequently found; *Hafnia alvei* and *Serratia* genus were the main *Enterobacteriaceae*; and in the clostridia groups, *Cl. putrefaciens* and *Cl. algidicarnis* were the most frequent.

Enterobacteriaceae species, particularly *H. alvei* and *S. liquefaciens*, were present in 60% of the samples. They are able to grow at low temperatures and under vacuum-packed conditions that may provide a selective advantage over other meat spoilage bacteria (Ray and Bhunia 2008). These bacteria species were detected on meat in modified atmosphere packaging (Doulgeraki *et al.* 2010) and in chilled vacuum-packed meats (Brightwell *et al.* 2007), confirming the potential of *Enterobacteriaceae* genera to cause blown pack spoilage corroborating our results.

Clostridia were identified in 66% of samples. The most common species were *Cl. putrefaciens* and *Cl. algidicarnis*. These bacteria have been mainly associated with deep-tissue spoilage and also blown pack spoilage (Boerema *et al.* 2003; Broda *et al.* 2009). Temperature abuse may have increased growth of *Cl. algidicarnis* on the sample (De Lacy *et al.* 1998) because of the use of temperatures up to 10°C in some meat shops. Recently, *Cl. algidicarnis* has been identified in Brazilian blown pack, beef samples (Silva *et al.* 2011), showing that this bacterium would be part of the Brazilian microbiota of blown pack spoilage. *Cl. algidicarnis*/*Cl. putrefaciens* (Table 1) are considered contaminants in the inner tissue (Broda *et al.* 2009),

Table 1 Micro-organisms detected by 16S rRNA gene sequencing in fifteen blown pack beef samples

Sample	Closest relative	% Identity	Bacteria groups
S1	*Enterococcus* sp.	97	LAB
	Enterococcus termitis	97	LAB
	Hafnia alvei	99	E
	Serratia liquefaciens	98	E
S2	*Carnobacterium divergens*	97	LAB
	Carnobacterium maltaromaticum	97	LAB
	Clostridium algidicarnis	97	C
S3	*Enterococcus faecium*	97	LAB
	Hafnia alvei	98	E
	Lactococcus lactis subsp. cremoris	97	LAB
	Lactococcus piscium	97	LAB
	Carnobacterium divergens	99	LAB
S4	*Citrobacter freundii*	97	E
	Hafnia alvei	98	E
	Lactococcus piscium	97	LAB
	Carnobacterium maltaromaticum	99	LAB
S5	*Clostridium algidicarnis*	99	C
	Hafnia alvei	99	E
	Lactobacillus curvatus	98	LAB
	Lactobacillus sakei	100	LAB
	Paenibacillus sp	99	*
S6	*Clostridium algidicarnis*	99	C
	Clostridium putrefaciens	98	C
	Hafnia alvei	99	E
	Lactobacillus fuchuensis	100	LAB
	Lactobacillus sakei subsp. sakei	100	LAB
	Lactococcus piscium	100	LAB
S7	*Hafnia alvei*	98	E
	Rahnella aquatilis	97	E
S8	*Clostridium algidicarnis*	97	C
	Clostridium putrefaciens	97	C
S9	*Clostridium sp.*	99	C
	Lactobacillus sakei	97	LAB
S10	*Clostridium perfringens*	97	C
	Serratia grimesii	97	E
S11	*Serratia liquefaciens*	97	E
	Shewanella baltica	98	*
	Uncultured *Rahnella* sp.	98	E
	Vagococcus fessus	99	LAB
	Carnobacterium divergens	99	LAB
S12	*Clostridium algidicarnis*	100	C
	Clostridium putrefaciens	100	C
	Lactobacillus curvatus	97	LAB
	Lactobacillus sakei	100	LAB
S13	*Carnobacterium divergens* isolate MF 66	100	LAB
	Clostridium putrefaciens	100	C
	Lactobacillus sakei	97	LAB
	Lactococcus piscium	100	LAB
	Serratia liquefaciens	97	E

(*continued*)

Table 1 (*continued*)

Sample	Closest relative	% Identity	Bacteria groups
S14	*Lactobacillus algidus*	98	LAB
	Clostridium algidicarnis	97	C
S15	*Clostridium putrefaciens*	97	C
	Lactobacillus sakei	99	LAB
	Lactococcus piscium	100	LAB
	Serratia liquefaciens	97	E

LAB, lactic acid bacteria; E, enterobacteria; C, clostridia; *, another group.

suggesting that both micro-organisms may be responsible by blown pack.

Another *Clostridium* species detected was *Cl. botulinum* type E (S3 and S4 samples) with identity of 96% (no showed in the Table 1), although this is not considered as significant blown pack spoilage bacteria, it can grow at 3·3°C and produce gas (Cockey and Tatro 1974; Lund *et al.* 1990). This species causes botulism, but its presence in food is not necessarily involves the production of botulinum toxin, once toxin production occurs in conditions such as high pH of medium (Haim and Timothy 2001), so it is possible that S3 and S4 have no toxin because its pH be, approximately, 5·0 (data not shown) and the neurotoxin detected in food samples occurs above pH 5·5 (Graham and Lund 1987). On the other hand, high concentrations of CO_2 in the samples could contribute to the synthesis of botulinum toxin (Lövenklev *et al.* 2004); however, Artin *et al.* (2010) concluded that there is no correlation between the toxin synthesis and gas concentration. Considering the statements aforementioned, more conclusive studies are necessary.

Although clostridial species have been detected in this study, the *Clostridium estertheticum*, involved in reported cases of blown pack spoilage of vacuum-packed beef in several countries (Helps *et al.* 1999; Broda *et al.* 2003; Byrne *et al.* 2009), was not detected. Similar results were obtained by Silva *et al.* (2011) in Brazilian meat samples, where they detected the presence of *Cl. gasigenes* and *Cl. algidicarnis*. Cavill *et al.* (2011) and others have suggested that cold-tolerant species of *Clostridium* could be involved in spoilage of vacuum-packed meat. However, inhibition of growth of *Cl. estertheticum* can also be explained by LAB-dominant presence in the samples, as reported to Jones *et al.* (2009) and Yang *et al.* (2011).

All samples analysed, except S7, S8 and S10, showed the presence of LAB species. Psychrotrophic LAB often compose the dominant part of the microbiota in chilled, modified atmosphere-packed (MAP) and vacuum-packed meat (Sakala *et al.* 2002; Rahkila *et al.* 2011), and their ability to cause spoilage depends on the product type and the storage conditions. The main LAB species detected in this study were *Lactobacillus sakei*, *Lactococcus piscium*, *Carnobacterium divergens* and *C. maltaromaticum*, which have been reported as occurring in vacuum-packed meat (Jiang *et al.* 2010) and produce gas at low temperatures (Monnet *et al.* 2002; Sakala *et al.* 2002).

Lactobacillus sakei is a relevant lactic acid bacterium in food microbiology mainly because of its ability to ferment meat proteins and is widely used by produce bacteriocins (sakacin) (Axelsson *et al.* 1993; Riley and Wertz 2002). Studies have shown that *L. sakei* bacteriocins can inhibit growth of *Brochothrix thermosphacta*, *Listeria monocytogenes* and *Cl. estertheticum* (Jones *et al.* 2009), species not detected in our analyses.

The presence of LAB on the samples suggests that these bacteria can play an important role in blown pack spoilage, complementing studies that identify *Clostridium* and *Enterobacteriaceae* and some of the bacteria responsible for blown pack spoilage of vacuum-packed chilled meat.

Package headspace gases and volatiles

The main functional chemical groups from 51 VOCs analysed were alcohols, followed by aldehydes and sulphur compounds (Table 2). The alcohol group was detected in 80% of the samples; aldehydes and sulphur compounds were detected in 47 and 33%, respectively. The same groups of volatiles have been detected in the studies of vacuum-packed beef (Ercolini *et al.* 2009; Casaburi *et al.* 2011), showing the relationship of these chemical groups with blown pack spoilage.

There was a predominance of CO_2 in all samples analysed, followed by H_2 (Table 3). The interaction between volatile compounds, gases and micro-organisms is shown in Figs 1 and 2. The PCA analysis (Fig. 1) showed three distinct groups: the first (S5, S6 and S11) with 40% CO_2 and 20% H_2, the second (S8, S9, S12 and S13) with more of 90% CO_2 production and the remaining samples in the third group. On the other hand, the cluster analysis (Fig. 2) grouped samples S3/S4, S5/S6, S8/S9, S12/S1 and S10/S15 together because of similar concentrations of H_2 and CO_2.

Samples from the first group produced the same gas concentrations. These bacterial species, such as *C. maltaromaticum*, *L. sakei* and *Cl. algidicarnis* found in S5 and S6, were associated with vacuum-packed meat and blown pack spoilage because of its ability to produce CO_2 and H_2 gas (Ercolini *et al.* 2009; Casaburi *et al.* 2011). In addition, *Clostridium* sp. produce alcohol and acetate in fermentative process (Broda *et al.* 2000), compounds detected in the first group samples.

The LAB, *Carnobacterium divergens*, *Cl. algidicarnis* and *Cl. putrefaciens* are grouped together, in the second

Table 2 Headspace volatiles and gas composition in fifteen blown pack beef samples

	Blown pack meat samples															
	Compound	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
Alcohols	2,3-Butanediol							+								
	1-Butanol		+	+								+		+		
	3-Methyl-1-butanol	+		+												
	Ethanol														+	+
	1-Hexanol										+		+			
	1-Heptanol															
	1-Pentanol						+									
	1-Propanol	+				+										
Aldehydes	Benzaldehyde										+		+	+	+	+
	3-Methylbutanal	+														
	Heptanal										+		+	+		
	Hexanal		+													
Hydrocarbons	Cyclooctane										+	+	+			
	Ethylbenzene														+	+
	Xylene														+	+
Ketones	2-Butanone					+	+									+
	Propanone								+	+						
Organic acids	Acetic Acid						+	+			+		+			
	Butanoic acid (butyric)										+					
	2-Methyl-ethyl ester-butanoic acid												+	+		
Sulphur compounds	Dimethyl disulphide	+														
	Dimethyl sulphide				+							+	+			
	Dimethyl trisulphide	+									+	+				
	S-Methyl thioacetate	+														

Table 3 Gas composition in fifteen blown pack beef samples

	Blown pack meat samples															
	Compound	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
Gas composition (% v/v)	CO_2	75·0	85·2	78·6	79·8	37·5	44·0	71·6	97·3	96·4	78·4	45·3	91·1	91·3	73·5	76·7
	H_2	5·3	24·1	30·2	26·5	20·5	19·1	20·7	0·4	1·2	18·8	24·4	0·1	0·1	0·0	13·5
	N_2	7·1	7·3	5·2	0·4	3·4	4·9	21·5	1·4	1·7	10·9	21·6	7·8	8·6	24·7	4·4

group, because of high production of CO_2. These bacterial species were already associated with blown pack spoilage (Broda *et al.* 2009; Casaburi *et al.* 2011; Silva *et al.* 2011). *C. divergens* besides producing CO_2 also synthesizes propanone (Leisner *et al.* 2007), a compound detected in second group of samples, showing their association with blown pack spoilage.

The VOCs and gases correlated with the microbiota found in the samples, indicating that they are volatile metabolites generated from organic compounds used as carbon sources by bacteria. The presence of 3-methyl-1-butanol, dimethyl sulphide and dimethyl trisulphide in the samples may have resulted from the proteolytic activity of the micro-organisms and from leucine catabolism. The detection of phenylethyl alcohol and benzaldehyde indicates that it was derived from phenylalanine catabolism (Smit *et al.* 2005; Gänzle *et al.* 2007), and compounds such as hexanal, nonanal, decanal, 1-octen-3-ol and acetic acid were derived from both hydrolysis of triglycerides and amino acid degradation (Montel *et al.* 1998).

In all samples, except S7, S8 and S10, at least one of the LAB species, *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Vagococcus* and *Carnobacterium*, was present. The VOCs associated with these species were dimethyldisulphide, hexanal, 1-propanol, 1-pentanol, 1-butanol, propanone, butanone, benzaldehyde and butanoic acid. These compounds are associated with fermentation by LAB (Drake *et al.* 1999; Nordvi *et al.* 2007). Recently, Casaburi *et al.* (2011) detected hexanal, dimethyl sulphide, heptanal and benzaldehyde as VOCs produced by *Carnobacterium* in vacuum-packed beef. These same compounds were

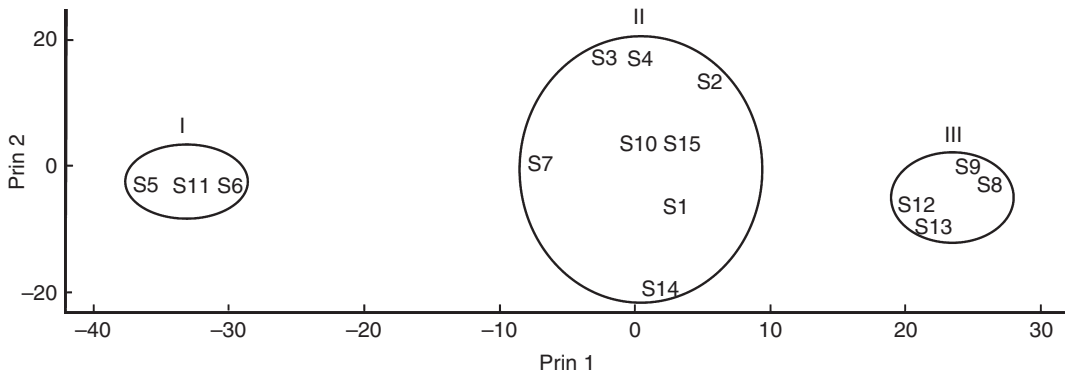

Figure 1 principal component analysis from the headspace gas, volatiles composition and number of bacteria in fifteen blown pack beef samples using the SAS 9.2 program. (I) first group, (II) second group and (III) third group.

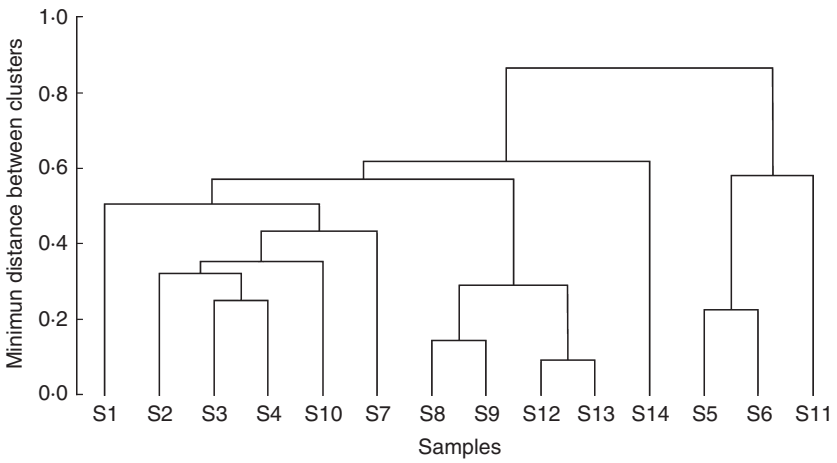

Figure 2 Cluster analysis from the headspace gas, volatiles composition and number of bacteria in the fifteen blown pack beef samples using the SAS 9.2 program.

identified in the samples containing by *Carnobacterium*, suggesting a possible relationship of this micro-organism species with these volatile compounds.

Hafnia alvei and *Serratia* were the most common *Enterobacteriaceae* found in the samples (Table 1). *Hafnia*, facultative anaerobes (Rodriguez *et al.* 1999), can produce acid with or without gas release from the metabolism of D-glucose and can metabolize amino acids to produce amines, ammonia, dimethyl sulphide and mercaptans (Ray 2005). These characteristics may favour the growth of this bacterium in the environment of vacuum-packed samples analysed in this study.

The volatile compounds dimethyl sufide, 1-propanol, 3-methyl-1-butanol, 1-butanol, 1-pentanol and acetic acid are related to *H. alvei* in the S1, S3, S5, S6 and S7 samples; similar results were observed in cheese inoculated with this micro-organism (Morales *et al.* 2004), suggesting that these compounds are associated with proteolytic metabolism by the bacteria. On the other hand, compounds such as 1-propanol, dimethyl disulphide, 3-methyl-1-butanol and S-methyl thioacetate were detected in beef samples containing *Serratia* (Ercolini *et al.* 2009). These volatiles were present in headspace of S1, S11, S13 and S15, showing that these VOCs may be associated with *Serratia* metabolism. However, studies using meat beef inoculated with *H. alvei* and *Serratia* are required to better correlate VOCs with these micro-organisms where a mixture of different bacteria was present in samples.

Green discoloration of the meat and malodorous diamines (putrescine and cadaverine) were observed in samples with *Hafnia alvei* and *Serratia* in this study. Similar characteristics were associated with the growth of both organisms by Stanbridge and Davies (1998); therefore, the presence of these bacteria in many samples of meat cuts is of commercial importance.

Among clostridial groups, *Cl. putrefaciens* and *Cl. algidicarnis* were the most frequently detected in samples and the VOCs related them were 1-butanol, acetic acid, 1-propanol, dimethyl sulphide, butanoic acid and 2-methyl-ethyl ester-butanoic acid. Studies show that the main volatile compounds detected in vacuum-packed meat inoculated with *Clostridium* are butanoic acid, 1-butanol, 3-methylbutanal, 1-butyl butyrate, dimethyl disulphide, acetic acid, 1-butyl acetate, 1-propanol, isobutanol and 2-methyl-1-butanol (Broda *et al.* 2000; Moschonas *et al.* 2010), and this indicates that butanoic acid, 1-butanol and dimethyl disulphide may be possible markers for the *Clostridium* genus.

The headspace gas composition of the sample consisted mainly of carbon dioxide, nitrogen and hydrogen. The microbiota dominant in S2 was LAB with 85% CO_2, 24·1% H_2 and 7·3% N_2; in S7 was enterobacteria with 71% CO_2, 20% H_2 and 21% N_2 and in S8 was clostridia with 97% CO_2, 0·4% H_2 and 1·4% N_2. Previously, both *Enterobacteriaceae* and clostridia have been shown to be associated with blown pack spoilage (Broda *et al.* 2000; Brightwell *et al.* 2007); however, we have shown that LAB could also be associated with such deterioration. The CO_2, H_2 and N_2 presence on the samples can be attributed to metabolic butyric fermentation by *Clostridium* (Moschonas *et al.* 2011), as well as to LAB and *Enterobacteriaceae* metabolism resulting in characteristic blown pack spoilage.

The highest concentration of CO_2, and the compounds butanoic acid, 1-butanol, dimethyldisulphide, acetic acid and 3-methyl-1-butanol may serve as a marker for spoilage species associated with blown pack.

The presence of LAB on the samples demonstrated that it can play an important role in blown pack spoilage complementing studies that appointed *Clostridium* and *Enterobacteriaceae* as responsible for vacuum chilled meat deterioration. Therefore, the results of this study would be of interest and value to the meat industry in its efforts to better understand the causes of spoilage in of chilled red meat. However, further studies are necessary to better establish a relationship of the volatile compounds produced by LAB with blown pack spoilage.

Materials and methods

Meat sample

Fifteen vacuum-packed chilled beef samples of cuts *thin skirt* (S1), *sirloin* (S2, S3, S5, S6, S8 and S9), *PAD rump heart* (S4), *hump* (S7), *strip loin* (S10, S12 and S13), *rump cap* (S14) and *shank* (S11 and S15) were obtained by donation from meat shops. Although those cuts were within expiration date, they had blown pack spoilage characteristics and would be discarded. The samples were transported under refrigeration to the laboratory and stored at 1°C by 5 days.

Gas analysis and VOC determination

The CO_2, H_2 and N_2 concentrations (per cent, volume/volume) in the headspace of blown pack samples were determined using a gas chromatography (CG Instrumentos Centificos model 2527, Sao Paulo, Brazil), equipped with a thermal conductivity detector (TCD) operated at 140°C, Porapak Q and 5A molecular sieves columns (Supelco, Milwaukee, WI, USA) at 82°C, and an injector set at 84°C. Argon was used as the carrier gas (Aga, Campinas, Brazil). From each package, an aliquot of 0·3 ml of headspace gas was hermetically withdrawn with a gas tight syringe through a septum adhered to the package surface. The headspace gases were identified and quantified based on calibration curves of analytical gas standards (Praxair, Campinas, Brazil).

Volatile organic compound determination was performed using GC/MS. A headspace solid-phase microextraction (SPME) analysis was carried out on 5 g of meat from the chopped samples. The meat was homogenized in water (100 ml) and NaCl (30 g) and placed in a hermetically sealed vial (150 ml). The fibre (CAR/DVB/PDMS; Supelco Sigma-Aldrich, Bornem, Belgium) was immersed in the HS for 30 min at room temperature. Thermal desorption of the analytes from the fibre inside the GC injection port was carried out in splitless mode at a desorption temperature of 200°C for 20 min.

All samples were analysed with an HP 6890 gas chromatograph (GC) coupled with a 5973N quadrupole HP mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The GC was equipped with a fused-silica capillary column HP-5MS (Agilent Technologies, Palo Alto, CA: 5% phenyl, 95% methylpolysiloxane, 30 m × 0·25 mm i.d., 0·25 μm film thickness). GC operating conditions were as follows: injection and detector temperatures, 200 and 280°C, respectively; the GC oven temperature was 50°C for 15 min, increased to 200°C at 10°C min^{-1} and held at 200°C for 10 min. Helium with a purity of 99·99% was used as the carrier gas at a flow rate of 1 ml min^{-1}, maintained by an electronic pressure controller, and split ratio 22 : 1. The electronic impact (EI)-MS conditions were the following: ion source temperature, 230°C; ionizing voltage, 70 V. Mass range was m/z 45–450 amu.

Identification was made by comparing the retention times of the components of interest with the retention time of the analytical standard and/or mass spectra of compounds in the National Institute of Standards and Technology (NIST) library (*Mass Spectra Database*). The retention time used to compare the samples was 13 min.

DNA extraction

Exudates (1 ml) and swabs were collected from the surface and inside the beef samples, after chopped the meat up. An aliquot (1 ml) of 0·8% NaCl solution was used to release the micro-organisms attached to the swab. Genomic DNA was isolated using a HighPure PCR template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. After DNA extraction, the material was resolved by electrophoresis in 1·5% agarose gel and quantified. Eluted DNA was stored at −20°C pending PCR amplification.

16S rRNA clone library

Amplification of 16S rDNA gene was carried out using primer sets FD1 5′ AGA GTT TGA TCC TGG CTC AG 3′ and RD1 5′ AAG GAG GTG ATC CAG CC 3′, these amplify nearly the full length of the 16S ribosomal DNA (rDNA) from many bacterial genera (Bresler *et al.* 2000). Each 25-μl reaction mixture contained 50 ng of template DNA, 1X PCR buffer (Invitrogen, Carlsbad, CA, USA), 5·0 mmol l^{-1} $MgCl_2$, 0·8 mmol l^{-1} dNTP, 0·3 μmol l^{-1} of each forward and reverse primer and 1·25 U of *Taq* DNA polymerase (Invitrogen). The PCR was performed under the following conditions: 5 min initial denaturation at 95°C; 30 cycles of denaturation (30 s at 94°C), annealing (1 min at 55°C) and extension (45 s at 59°C); and a final extension at 72°C for 15 min in GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA). The PCR products were purified with GFX^{TM} PCR DNA and a Gel Band Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ) and analysed by gel electrophoresis. The purified PCR products were linked into a pGEM-T easy vector (Promega, Madison, WI, USA) and were transformed into competent *E. coli* DH5α cells. About 480 clones were randomly selected and sequenced. Sequencing was performed using sp6 and T7 vector primers, DyEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) and an ABI PRIME 3100 Genetic Analyser capillary sequencer (Applied Biosystems). All sequences were compared with those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis

The interaction between volatile compounds and gases found in the packaging was evaluated by clustering according to similarity and PCA. The volatile compounds were analysed according to the sum of the compounds in each chemical function (alcohols, aldehydes, hydrocarbons, ketones, organic acids and sulphur compounds), while the concentrations of H_2, N_2 and CO_2 gases were analysed as a percentage of total headspace gas volume. The analysis of the bacteria was based on the sum of the same bacterial group (*Enterobacteriaceae*, *Clostridium* and lactic acid bacteria). The statistical analyses of the standardized values were performed with the statistical package SAS 9.2.

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