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Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings

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Abstract

'Blown pack' spoilage of vacuum-packed chilled beef, lamb and venison, and of a cooked meat product, chilled dog rolls packed in an oxygen-impermeable plastic casing, was characterised by sensory, chemical and microbiological analysis. Investigation of the probable causative agents led to the isolation of eight strains of psychrotrophic clostridia. Three strains have been provisionally identified as *C. difficile*, *C. beijerinckii* and *C. lituseburense;* the other five remain unidentified. In inoculation studies only one isolate produced significant amount of gas on meat, causing pack 'blowing'. It is, therefore, possible that 'blown pack' spoilage involves a synergism with one or more other organisms.

Keywords: Vacuum-packaging; Gas distension; Cold-tolerant clostridia; Chilled storage; Microbial spoilage

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1. Introduction

'Blown packs', packs spoiled by gross gas distension, are a dramatic manifestation of microbial spoilage of chilled vacuum-packed meats. Commercial episodes of 'blown' vacuum-packed red meats are usually due to temperature abuse, which allows proliferation of gas-producing organisms. Three species of the family *Enterobacteriaceae*: *Serratia liquefaciens*, *Enterobacter aerogenes* and *Hafnia alvei* have been isolated in significant numbers from vacuum-packages blown at abusive temperatures, and have been confirmed as gas-producers (Hanna et al., 1979; DeLacy and Cook, unpublished results).

There has, however, been a number of product failures where records indicate that temperature abuse has not occurred. Products affected have included primal cuts of beef, lamb and venison, as well as dog rolls cooked within a gas-barrier plastic wrap. Storage temperatures were consistently within the −1.5°C to 2°C range, well below the lower growth temperature limit of the three species just discussed. Gas-blowing occurred within 4–6 weeks of storage. Routine microbiological analysis of the meat from blown packages did not show significant departures from the expected norm, with members of the genus *Lactobacillus* dominating the floras. *Enterobacteriaceae* and other known gas-blowing spoilage organisms were either not detected or present in low numbers, confirming that temperature abuse was not the underlying cause of the problem.

Review of the literature suggested that psychrophilic *Clostridium* spp. could be the causative agents of this 'blown' pack spoilage. The occurrence of the psychrophilic and psychrotrophic *Clostridium* spp. in foods and non-food sources have been previously reported. Several species of these organisms have ben isolated from soil, mud and sewage (Sinclair and Stokes, 1964), marine sediments (Liston et al., 1969; Finne and Matches, 1974), potatoes (Brocklehurst and Lund, 1982), milk (Bhadsavle et al., 1972) and more recently from blown vacuum-packaged ready-to-eat roast beef meals (USA), and beef primal cuts (USA, UK)(Dainty et al., 1989; Kalchayanand et al., 1989). Reports by Collins et al. (1992) and Kalchayanand et al. (1993) indicate that the psychrophilic clostridia isolated from 'blown' vacuum-packed meat may be two separate species. *Clostridium estertheticum* (Collins et al., 1992) produces large amounts of carbon dioxide and hydrogen, together with significant amounts of butanol, butyric and acetic acids and, uniquely, a range of butyl esters. It also produces volatile sulfur compounds (Dainty et al., 1989). Similarly, *C. laramie* growing on meat generates copious amounts of gas causing severe pack distension. The organisms can proliferate and produce gas at refrigeration temperatures below 0°C and their maximum growth temperature is below 25°C. Both species could not be recovered using conventional techniques and routine enumeration media (Dainty et al., 1989; Kalchayanand et al., 1989). When the organisms were eventually isolated in pure culture, very few or no colonies developed when the isolates were transferred to solid media.

In the present study commercially produced vacuum-packed raw meats and pasteurised dog rolls showing gas distension were obtained from commercial sources. A series of investigations was conducted to characterise the spoilage and to isolate and identify probable causative agents. An attempt was made to confirm the ability of the

isolates to reproduce the characteristics of the original spoilage under experimental conditions.

2. Materials and methods

2.1. Spoiled commercial product

2.1.1. Raw vacuum-packed meat

'Blown' commercial vacuum-packs (Fig. 1) of a variety of chilled raw meats, including lamb legs, venison striploins and frenched racks and beef striploins, were received either as unsolicited samples for investigation or in response to requests for such material. Each meat species was represented by up to five samples.

2.1.2. Cooked dog roll samples

In association with the manufacturer's request for assistance, spoiled gas-distended dog rolls were made available. Dog roll manufacture involved blending the raw ingredients, principally fresh and frozen meat and offal, cereal meal and proprietary additives, filling the mixture into plastic, oxygen-impermeable casings (Chub-packed)

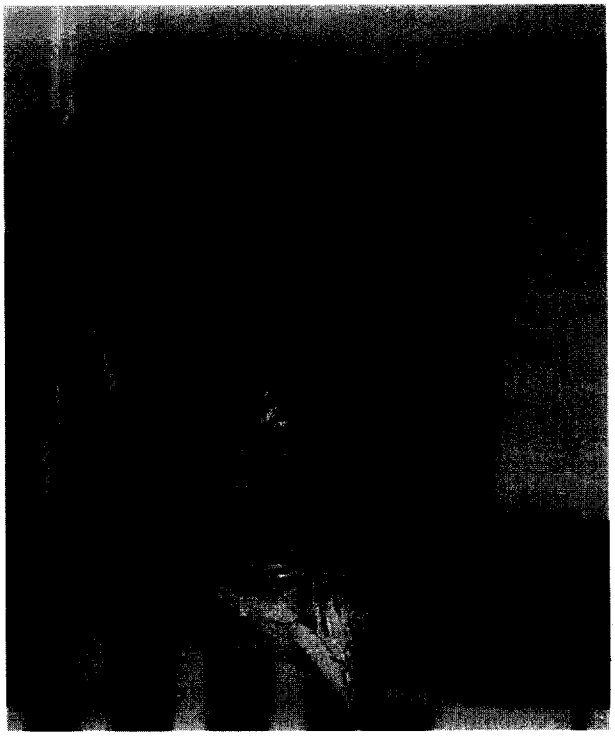

Fig. 1. Commercial vacuum-packed venison sample showing gross pack distension ('blowing').

and cooking the rolls in a water vat for not less than 2 h to a core temperature of at least 75°C. Following cooking, the rolls were cooled to less than 4°C within 12 h and stored chilled at 2°C. The rolls studied had spoiled by gas distension during chilled storage within 6 weeks of their manufacture.

2.2. Sample handling

Suppliers packed the spoiled product in chilled insulated containers, which were forwarded to the laboratory by express courier. On receipt, all samples were visually inspected, then a silicone rubber gas sampling septum was applied to the surface of each pack using silicone rubber adhesive (Silaflex MS; Fosroc Expandite, Petone, NZ). This adhesive was selected because it does not introduce contaminating volatiles into the pack headspace. The packs, with septa attached, were placed in a storage chiller operating at −1.5°C to await examination the next morning.

All blown commercial packs were then examined using the following sequence: visual inspection of the unopened pack; withdrawal of headspace gas for analysis; opening of packs within an anaerobic chamber (Forma Scientific, Marietta, OH, USA) for microbiological sampling and, finally, assessment of pack odour and product colour on removal of the opened packs from the anaerobic chamber.

2.3. Headspace volatiles

2.3.1. Sample collection

Samples of headspace volatiles were concentrated using a 'trap' technique. Gas traps consisted of 160 mm × 4.0 mm i.d. glass tubes, each containing 200 mg of 60/80 mesh Tenax TA (Alltech Associates, Deerfield, IL, USA) held in place with silanised glass wool plugs. The traps were pre-conditioned at 325°C under high purity nitrogen to remove absorbed volatiles. Immediately before use the pre-conditioned gas traps were conditioned under helium flow at 250°C for 15 min, cooled, sealed at both ends with PTFE tape and placed into a nitrogen-flushed gas-tight glass test tube. Conditioned gas traps were used within 5 min of their preparation.

For use, gas-tight Swagelok unions were fitted to both ends of the gas trap. A 16-gauge stainless steel hypodermic needle was then attached to one end and a 1500 ml syringe to the other. To obtain a headspace sample, the septum attached to the 'blown' pack was sterilised with ethanol, then the gas trap assembly needle was inserted through it. Headspace gas (400 ml) from the spoiled pack was withdrawn through the trap at a rate of 50 ml per min using a motorised device to pull out the syringe plunger. After sampling, the gas traps were resealed with PTFE tape and returned to nitrogen-flushed storage tubes for immediate transport for gas chromatographic analysis.

To obtain samples of 'headspace gas' from uninoculated control packs, which did not contain gas, 400 ml of high purity nitrogen was introduced through the septum. After 30 min equilibration at room temperature, a gas sample was taken using the previously described procedure.

Attempts to obtain headspace gas samples from gas-distended dog rolls proved unsuccessful. On insertion of the sampling needle, the fragile gas-strained casing burst, releasing a foul-smelling headspace gas into the laboratory.

2.3.2. Thermal desorption–gas chromatography

Headspace volatiles were analysed on a Fisons 8000 series gas chromatograph (GC) fitted with a Fisons MD800 mass spectral detector (Fisons Instruments, Manchester, UK). The column used was a 30 m × 0.25 mm, 0.25 μm film FFAP capillary column (J&W Scientific, Folsom, CA, USA). The gas chromatographic conditions for separation of headspace volatiles were as follows: injector temperature 260°C; column head pressure, 15 psi; split vent flow of helium carrier gas, 26 ml min^{-1}; column flow calculated at 60°C and under vacuum, 1.68 ml min^{-1}; the resultant split ratio, 15:1.

For thermal desorption of headspace volatiles into the gas chromatograph, the normal carrier gas flow was altered so that gas flowed through the Tenax trap and into the injector of the gas chromatograph. The Tenax trap with redirected carrier gas flowing at 28 ml min^{-1} was rapidly heated to 250°C over 1 min and maintained at that temperature for a further 10 min. Desorbed volatile compounds were carried into the split/splitless injector of the gas chromatograph by the redirected helium carrier gas. The desorbed volatiles were then cryofocused at the head the FFAP capillary column by cooling the gas chromatograph oven to −10°C with liquid carbon dioxide during the desorption period. After cryofocusing was completed, normal carrier gas flow was restored in preparation for analysis. The column oven was then programmed from −10°C to 60°C at a rate of 50°C per min, held at that temperature for 5 min before being raised to a final temperature of 240°C at a rate of 5°C per min.

Mass spectra of unknown compounds were identified by comparison with those of known compounds in the National Institute of Standards and Technology (Gaithesburg, MD, USA) library and retention times of authentic compounds. Semi-quantitative analysis was done by comparing peak areas of unknown compounds with peak areas produced from 1 μl liquid injections of appropriate dilutions (in pentane) of standards (Analytical Standards; PolyScience, Niles, IL, USA) under similar conditions.

2.4. Headspace gases

To confirm anoxic conditions and preclude the possibility that gas generation had resulted from aerobic microbial metabolism, the oxygen content of all packs was measured with an oxygenmeter (Gaspace Systech Instruments, Thame, Oxfordshire, UK) equipped with an automatic sampler. The gas sample was withdrawn from the packs by means of the sampler syringe needle inserted through the attached pack septum. The presence of carbon dioxide and hydrogen, the most usual gaseous products of anaerobic metabolism, and of sulfur compounds in the headspace gas was confirmed by an independent laboratory using gas chromatography. Because of logistical and analytical limitations, carbon dioxide/hydrogen analysis was performed on only a single pack that was not subjected to the headspace and microbiological sampling procedures.

2.5. Microbiological examination

2.5.1. Sampling procedure

All vacuum-packs were opened within the anaerobic chamber operating at 15°C. Two 1 ml samples of the purge within each pack were withdrawn using a sterile pipette. One

sample, for general spoilage microflora determination, was transferred into a universal bottle containing 9 ml of dilution fluid (0.1% peptone, 0.85% NaCl). The other, for the isolation and enumeration of anaerobes, was transferred into a bottle containing 9 ml of pre-reduced Reinforced Clostridial Medium (RCM; Oxoid CM149). A smear of purge was prepared on a microscope slide, heat-fixed and stained by Gram's method.

With dog rolls, a solid sample was taken because there was no purge. Also, it was not possible to complete full sample preparation within the anaerobic chamber, so sampling was conducted in the laboratory. Following the unsuccessful attempts to obtain gas samples, each roll was gripped through the broken casing and aseptically broken in half. A 25 g sample was removed from the centre of the broken surface (a core sample) using sterile instruments and placed into a sterile stomacher bag. Dilution fluid (225 ml) was added and the sample homogenised for 2 min in a stomacher (Colworth BA 6020; A.J. Seward & Co., London, UK). For general spoilage flora determination, a 1 ml volume of this initial homogenate was transferred to 9 ml of dilution fluid. For isolation and enumeration of anaerobes, 1 ml of the initial homogenate was transferred into 9 ml of pre-reduced RCM.

2.5.2. Enumeration of general spoilage microflora

To obtain counts of total aerobic bacteria, *Enterobacteriaceae*, lactic acid bacteria and *Brochothrix thermosphacta*, serial decimal dilutions of purge or initial homogenate were prepared in dilution fluid. Volumes (0.1 ml) of appropriate dilutions were spread onto Plate Count Agar (PCA; Oxoid CM326), Violet Red Bile Glucose Agar (VRBG; Difco 0012-01-5), de Man, Rogosa, Sharpe Agar (MRS; Oxoid CM361) and Streptomycin Sulfate-Thallous Acetate-Actidione Agar (STAA; Oxoid CM881). Plates were incubated and enumerated following standard procedures of the New Zealand meat industry (Cook, 1991).

2.5.3. Enumeration of psychrotrophic and cold-tolerant anaerobes

All sample dilution, plating and incubation procedures were conducted within the anaerobic chamber. Appropriate dilutions of purge samples and homogenate were first prepared in pre-reduced RCM and 0.1 ml volumes spread onto the surface of various media that had been pre-reduced at 15°C during 24 h storage within the anaerobic chamber. All plates were incubated at 15°C within the anaerobic chamber for 3 weeks before the developing colonies were enumerated.

For total anaerobic count (Total AnO_2PC), a modified Reinforced Clostridial Agar (RCM containing 5% sterile defibrinated sheep blood, 0.5% glucose and 1.5% agar) was used. Immediately before use, 2000 IU of filter sterilised bovine catalase (Sigma, C-30) was spread over the surface of each plate and allowed to dry before the sample dilution was applied.

For counts of sporeforming anaerobes, an alcohol or heat treatment was applied to destroy vegetative cells in the purge and homogenised samples. In the alcohol treatment an equal volume of absolute ethanol was added to appropriately diluted samples and allowed to act for 30 min before those dilutions were spread onto Tryptone Sulfite Polymyxin Agar (SFP; Oxoid CM587) containing 10% egg-yolk solution (Oxoid SR047C) and 10% glucose. The heat treatment, 80°C for 10 min, was applied to the

initial serial dilutions of both purge and homogenised samples before further dilutions were prepared and spread onto SFP plates.

To enumerate sulfite-reducing sporeformers, the heat and alcohol treated purge and homogenate samples prepared for counting sporeforming anaerobes were spread onto Tryptone Sulfite Cycloserine Agar (TSC; Oxoid CM587).

2.5.4. Anaerobe enrichment

After use for the preparation of serial dilutions, the remainder of the initial purge or homogenate sample in RCM, approximately 9 ml, was incubated at 15°C within the anaerobic chamber for 5 days. Each enrichment was then streaked onto pre-reduced single plates of the three anaerobe enumeration media so as to obtain isolated colonies. These inoculated plates were incubated at 15°C within the anaerobic chamber for 3 weeks.

2.5.5. Selection of psychrotrophic and cold-tolerant clostridia

From the enumeration plates and from the enrichment plates isolates representing every colony morphotype (one per type) were streaked to confirm purity onto Columbia Blood Agar (CBA; Oxoid CM331) and incubated within the anaerobic chamber at 15°C until colonies became visible, usually 7–10 days. The isolates were examined under microscope for morphology, Gram reaction and the presence of spores (Holdeman et al., 1977) and were then subjected to the following screening tests: growth in the presence of oxygen, cell morphology, presence of catalase, presence of oxidase and sensitivity to metronidazole (Oxoid, MZDD) (Cook, 1991; Weenk et al., 1991). Seven of the eight clostridial isolates selected for further study were obligately anaerobic, catalase negative, oxidase negative, metronidazole sensitive, Gram-positive large rods with terminal or subterminal spores, whereas the eighth showed some degree of aerotolerance.

2.5.6. Determination of growth temperature range

To ensure that biochemical tests used in species identification were performed under optimum growth conditions, growth temperature ranges of each isolate were determined. Isolates were grown at 15°C in pre-reduced Peptone Yeast Extract Glucose Broth (PYG; Holdeman et al., 1977) in the anaerobic chamber until the optical density of the suspension reached 0.5 AU at 590 nm. These cultures served as a source of inoculum for determining the optimum growth temperature. Sixty subcultures of each isolate ($3 \times$ tubes $\times$ 5 temperatures $\times$ 4 incubation times) were prepared by transferring 0.1 ml of the respective inoculum suspensions into 5 ml of pre-reduced PYG broth in disposable plastic screw-top tubes. Growth at five temperatures: 15°C , 20°C, 25°C, 30°C and 37°C was determined. For each incubation temperature four sets of three inoculated PYG broth tubes of each isolate, equilibrated to their respective temperature, were placed into BBL GasPak Pouches (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). After closure and establishment of anaerobic conditions, the pouches were transferred to a conventional incubator operating at a test temperature. A pouch was removed after 12, 24, 36 and 48 h incubation. Growth was determined by measuring the optical density at 590 nm on a NovaspecII (Pharmacia, Uppsala, Sweden) spectrophotometer. Densities reached after 12, 24, 36 and 48 h of incubation were then plotted to show the growth curves of each isolate at each of the test temperatures.

For determination of growth temperatures in the refrigeration range (−1.5°C or 4°C) the pouches prepared as previously described were incubated in the conventional incubators for 1 week. An increase in the broth density of 0.5 AU or more was recorded as positive growth.

2.5.7. Biochemical characterisation of clostridial isolates

Clostridium isolates and the following reference organisms: *C. perfringens* (ATCC 13124), *C. sporogenes* (ATCC 19404), *C. acetobutylicum* (ATCC 824) and *C. tetanomorphum* (ATCC 15920), were grown anaerobically for 24–48 h in Peptone Yeast Extract Broth (PY; Holdeman et al., 1977) at temperatures close to their growth optima. Subsequently, tests for the biochemical properties of each isolate were performed at these temperatures. The procedures of Holdeman et al. (1977) were followed to determine: motility, gas production, gelatin hydrolysis, carbohydrate fermentation, lipase and lecithinase production, starch and esculin hydrolysis, nitrate reduction, indole production, meat digestion and milk reaction. The ability of each isolate to grow on RCMA, CBA, BHIA (Brain Heart Infusion Agar; Holdeman et al., 1977), TSC and SFP was assessed. Growth on CBA at −1.5°C, 4.0°C, 25.0°C, 30.0°C and 37.0°C was also determined. Additionally, the Rapid ID 32A system (bioMérieux, Marcy l'Etoile, France) was used in an attempt to identify those isolates shown to be mesophiles (see Section 3).

Following the procedure of Holdeman et al. (1977), isolates were further characterised by identification of the metabolic products they produced during growth in PYG broth. Isolates were grown at their optimum growth temperature until the cultures had reached their stationary phase, after 5–7 days. One ml of each stationary phase culture, uninoculated PYG broth and each standard solution (Matreup, Cat. No. 1075, 1076) was first acidified with 0.2 ml of 16 M H_2SO_4 (concentrated H_2SO_4 diluted 1:1 (v/v) in water) in the presence of 0.4 g NaCl per ml of culture, after which volatile fatty acids and alcohols were extracted with 1 ml diethyl ether. After separation of the ether layer from the culture broth, $CaCl_2$ was added to absorb water in preparation for gas chromatographic analysis. Culture extracts and extracts of uninoculated PYG broth and standard solutions were analysed on a Hewlett Packard 5890/II GC (Hewlett Packard, Avondale, PA, USA) fitted with a flame ionization detector (FID) on a 30 m × 0.53 mm, 1.0 μm FFAP capillary column (J&W Scientific) operating under the following conditions: column head pressure, 7 psi; split vent flow of He carrier gas, 50 ml min^{-1}; column flow measured at 60°C, 9.9 ml min^{-1}; the resultant split ratio, 5:1; the split/splitless injector and FID detector temperature, 260°C. Run conditions were: initial temperature 60°C for 2 min, raised to 180°C at 5°C per min, final temperature of 240°C held for 2 min after raising at a rate of 20°C per min. The area and retention time of each peak were recorded by a Maxima integration software package (Waters, Millipore Corp., Milford, MA). Unknown peaks were identified against retention times of known standards.

2.5.8. Assessment of the spoilage potential of the clostridial isolates

Fresh beef rumps were obtained from a local abattoir and trimmed of fat. The surface was sterilised by searing with a hot iron, then the meat was transferred to a laminar flow

cabinet, where the charred surface layer was removed using sterile instruments. Steaks, approximately 10 × 5 × 2 cm thick, were then cut and placed individually into PVDC laminate bags (Cryovac™ BB4L, W.R. Grace, Porirua, New Zealand) with a stated oxygen transmission rate of less than 50 ml m^{-2} atm^{-1} 24 h^{-1} at 25°C and 75% r.h. The bags, containing the meat samples, were moved to the anaerobic chamber in preparation for inoculation.

For inoculating, a culture of each isolate was grown in pre-reduced RCM broth at 15°C until the broth became turbid (5–7 days) after which it was diluted 1 to 100 in fresh RCM broth to give a cell concentration of approximately 6.0 log CFU ml^{-1}. A 1 ml volume of this inoculum was pipetted onto both sides of a steak and distributed evenly on the surface by gentle massage. The inoculated meat packs were immediately removed from the anaerobic chamber, evacuated and sealed on a prototype Captron 1 controlled atmosphere/vacuum packaging machine (Securefresh Pacific, Auckland, New Zealand). The inoculated steaks, together with two uninoculated controls, were stored at 1 to 2°C and examined weekly for gas distension. When significant gas production was evident, the packs were subjected to the headspace volatiles analysis described previously.

3. Results

3.1. Characteristics of the spoilage

Spoilage characteristics associated with the 'blown' packs are summarised in Table 1. With beef and venison, spoiled product was darker, often with green discoloration, and softer than unspoiled product; with lamb, spoiled product was reddish-green. Large amounts of purge accumulated in all 'blown' vacuum packs (Fig. 1). Meat odours 15 min after pack opening, although described as being 'strong dairy' or 'cheesy', were decidedly offensive. Although distended dogroll packs did not contain purge, they

Table 1
Spoilage characteristics of 'blown' vacuum-packed meat and dog rolls

Sample No.	Sample type	Spoilage characteristics
1	Vacuum-packed lamb legs	Gross pack distension, reddish-green colour, large amount of purge,'sulfurous' odour on opening, becoming 'cheesy' on exposure to air
2	Vacuum-packed striploin of venison	Gross pack distension, extensive meat proteolysis, large amount of purge, 'strong dairy' odour
3	Vacuum-packed striploin of beef	Gross pack distension, meat proteolysis with green discoloration, large amount of purge, 'putrid' odour
4	Dog rolls	Gross pack distension, localised meat blackening, 'sour', 'off-milk' odour, crumbly texture

Table 2
Concentrations of major headspace volatiles in commercial samples

	Concentration (ng ml^{-1}) in sample packs		
	1	2	3
Headspace volatiles			
Acetic acid	2.0	6.2	1.9
Butyric acid	13.2	9.0	2.3
Butanol	70.1	84.5	66.5
Butyl acetate	4.0	2.5	1.0
Butyl butyrate	16.2	10.2	9.9
Benzaldehyde	P	P	P
Ethyl butyrate	P	P	P
Hexanal	P	–	–
Octanol	P	–	–
Propanol	P	–	–
Headspace gases			
Carbon dioxide	approx. 70%		
Hydrogen	approx. 30%		
Oxygen (in ppb)	< 0.5	< 0.5	< 0.5

Sample packs: 1, lamb leg; 2, venison; 3, beef. For dog rolls no gas sample could be taken due to explosive failure of the plastic wrap on sampling. P, present; –, not detected.

exhibited similar strong 'sour', 'off-milk' odours as were observed with vacuum packs. Spoiled dog rolls were dry and crumbly, and had patches of blackening on the surface and distinct zones of blackening within the product.

3.2. Headspace volatiles and gases

Headspace volatiles in spoiled beef, lamb and venison packs were similar (Table 2) but differed in their relative proportions. Quantitative analysis showed that the major volatiles were 1-butanol, butyl butyrate, butyl acetate and butyric and acetic acid (Fig. 2). Ethyl butyrate and benzaldehyde were present in all spoiled commercial samples. Hexanal and the alcohols propanol and octanol were also detected in the packs of 'blown' lamb legs.

Oxygen was not detected in any pack. Carbon dioxide and hydrogen were the only major gaseous species found within the single distended pack examined for gas composition. The gas sample from the single pack of lamb analysed for headspace gases (carbon dioxide, hydrogen and nitrogen) was also analysed on an FSOT column and significant amounts of sulfur compounds (dimethyl sulfide and dimethyl disulfide) were detected.

3.3. Microbiological examination

Microscopic examination of Gram stained purge smears from all 'blown' vacuum packs showed that the material contained significant proportion of large sporeforming rods.

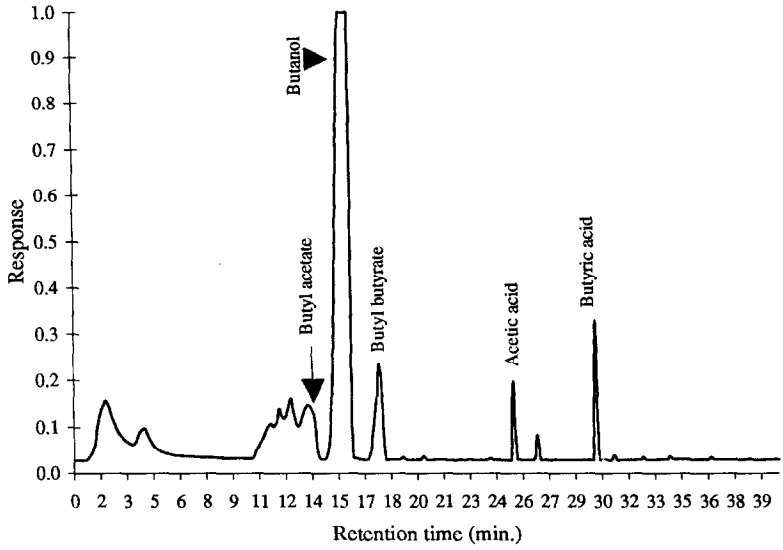

Fig. 2. Gas chromatography (FID) profile of headspace volatiles collected from 'blown' vacuum-packed venison.

3.3.1. Enumeration of general spoilage microflora

Results of the microbiological analyses are shown in Table 3. The aerobic plate counts (APCs) were similar for all distended vacuum packs. The dominant organisms were lactic acid bacteria with counts approaching log 7 CFU ml^{-1} of purge fluid. *Enterobacteriaceae* and *Brochothrix thermosphacta* were only minor elements of the

Table 3
Microbiological counts in commercial samples

	Sample pack			
	1	2	3	4
	(log CFU ml^{-1})			(log CFU g^{-1})
General spoilage microflora				
APC	6.91	8.21	7.35	2.82
Enterobacteriaceae	3.30	2.49	3.90	–
Lactic acid bacteria	5.69	5.90	7.24	*
Brochothrix thermosphacta	3.70	–	–	–
Psychrotrophic and cold-tolerant anaerobes				
Total AnO_2PC	6.95	7.56	8.22	6.38
Sporeformers I [a]	5.52	–	5.61	*
Sporeformers II [b]	–	–	4.65	*
Sulfite-reducing anaerobes I [a]	–	–	5.20	6.20
Sulfite-reducing anaerobes II [b]	–	–	4.05	5.54

Sample packs: 1, lamb leg; 2, venison; 3, beef; and 4, dog rolls; –, not detected (limit of detection 2.00 log CFU); *, not done; [a] ethanol-treated purge; [b] heat-treated purge.

total flora. Very low numbers of non-sporeforming spoilage organisms were present in the spoiled cooked dogrolls.

3.3.2. Enumeration of psychrotrophic and cold-tolerant anaerobes

Total anaerobic counts on RCM agar were high in all samples and reached log 8 CFU ml^{-1} of purge fluid. Apart from two distended beef packs, no colonies grew from heat-treated purge on any medium for sporeformers. Sulfite-reducing sporeformers were enumerated in both heat and ethanol-treated purge of distended beef samples and dog rolls. On all selective media, alcohol-treated purge fluid gave higher sporeforming anaerobe counts than heat-treated purge.

3.3.3. Selection of psychrotrophic and cold-tolerant clostridia

Of the 46 isolates picked from enumeration and enrichment plates, initial screening tests showed 11 to be members of the genus *Clostridium*. Most of the rejected isolates were lactic acid bacteria. However, two isolates selected from sulfite-reducing anaerobe plates for dog rolls were subsequently excluded from further analysis as they were unable to grow at 4°C and consequently could not have caused spoilage at chilled temperatures. Isolates designated DB1 and DB2 were obtained from lamb packs; KDL26 from venison; DB9, DB58 and DB77 from beef; and KDL361, KDL365 and a third isolate subsequently shown to be identical to DB9, from dog rolls.

All isolates were obtained through enrichment except DB1 and DB77. Strain DB1 was present only in ethanol-treated purge on SFP medium (corresponding sporeforming anaerobe count of 5.5 log CFU ml^{-1}) but was not detected on TSC medium (sulfite-reducing anaerobes). Strain DB77, from a distended pack of beef, was enumerated on both SFP and TSC medium, with higher recoveries on the former. This isolate was the only one recovered from heat-treated purge and capable on initial isolation of producing black colonies on TSC medium.

3.3.4. Determination of growth temperature ranges

Four of the eight strains: DB1, DB2, KDL361 and KDL365, did not grow at temperatures above 25°C, but grew well at −1.5°C in both liquid and solid media. The strains grew only weakly at 25°C with the best growth rates obtained between 15°C and 20°C. Two other strains, DB9 and DB58, grew well at both −1.5°C and 37°C and their optimum growth temperature fell within 25°C to 30°C. Strain KDL26 grew weakly at −1.5°C, and strain DB77 failed to grow at this temperature but did grow at 4°C. Both strains grew best at 37°C.

3.3.5. Biochemical characterisation of the clostridial isolates

The nine isolates contained eight strains different in their morphological and biochemical characteristics (Table 4). Cells of all isolates were medium to large (6–10 μm), sometimes grainy, Gram-positive, motile rods with oval, subterminal to terminal or terminal (strain DB58) spores (Fig. 3). Cells were generally single, sometimes in short chains or palisades. Colonies of DB1, DB2 and KDL365 on CBA were usually gray-white, small, smooth and convex. Colonies of strains DB9, KDL26 and DB58 were cream to yellow, moist, smooth and convex. Colonies of strain DB77 were large, cream

Table 4
Characteristics of eight strains of psychrotrophic *Clostridium* spp. isolated from spoiled meat

	Isolates							
	DB1	DB2	KDL361	KDL365	DB58	DB9	KDL26	DB77
Cell shape	lgr	lgr	lgr	mr	lfr	lgr	mr	lgr
Cell size (μm)	3×10	2×8	5×13	3×7	2×10	3×10	2×6	5×10
Spores	ST-T	ST	ST	ST	T	ST	ST	ST
Haemolysis in sheep-blood	w	β	–	β	–	–	–	β
Growth at: – 1.5°C	+	+	+	+	+	+	w	–
4.0°C	+	+	+	+	+	+	+	+
25°C	w	w	w	w	+	+	+	+
30°C	–	–	–	–	+	+	+	+
37.0°C	–	–	–	–	+	+	+	+
Growth on: RCM	+	+	+	+	+	+	+	+
CBA	+	+	+	+	+	+	+	+
BHIA	–	–	–	–	+	–	+	+
SFP	+	+	+	+	+	+	+	+
TSC	–	–	+	+	+	–	+	+
Products from PYG	AB 2,4	BA	ABivib 2,4	ABivibpc 2,4	ABiVib 2,4	ABibiv 2,4	ABicivib	BAivib 2,4
Acid from glucose	a	a	a	a	a	a	a	a
Gelatin hydrolysed	–	–	–	+	–	–	w	+
Motility	+	+	+	+	+	+	–	+
Abundant gas produced	+	+	+	+	+	+	+	+
Indole produced	–	–	–	–	–	–	–	–
Lecithinase produced	+	–	–	–	–	–	–	+
Lipase produced	–	–	–	–	–	–	–	–
Esculin hydrolysed	+	+	+	+	+	+	+	+
Starch hydrolysed	+	+	–	–	+	–	–	–
Nitrate reduced	–	–	–	–	–	–	–	–
Acid produced from:								
Arabinose	–	–	–	–	–	–	–	–
Cellobiose	–	a	–	–	–	–	a	–
Fructose	a	a	a	a	a	a	a	a
Lactose	a	a	–	–	w	a	–	–
Maltose	a	a	w	a	a	a	–	a
Mannitol	–	a	–	–	–	a	–	–
Mannose	a	a	a	a	a	a	a	a
Melibiose	a	–	–	w	–	a	–	–
Raffinose	a	a	–	a	–	–	–	–
Ribose	–	–	–	–	–	w	w	a
Salicin	a	a	–	–	–	–	–	–
Sorbitol	a	a	a	–	a	–	–	–
Sucrose	a	a	a	a	a	–	–	a
Xylose	w	a	–	–	–	–	–	–
Milk reaction	–	c	c	–	–	–	–	d
Meat digestion	+	w	+	+	+	+	–	–

lgr, large rods; mr, medium rods; lfr, long, fine rods; ST, subterminal; T, terminal; w, weak reaction; a, acid; c, curd; d, digestion; –, no reaction. Products from PYG as per Cato et al. (1986): a, acetic; b, butyric; c, caproic; ib, isobutyric; ic, isocaproic; iv, isovaleric; p, propionic; capital letters indicate at least 1 meq/100 ml of culture. Alcohols: 2, ethanol; 4, butanol; bold, abundant.

Fig. 3. Electron micrograph of swollen clostridial cell (terminal spore) in the purge of a 'blown' venison pack.

to yellow, flat and dry, and colonies of strain KDL361 were gray-white, moist and spreading even on media with an increased amount of agar (RCM with 2% agar). All but DB58 were obligately anaerobic. This strain was aerotolerant and grew slowly on CBA in the presence of oxygen. All strains were sensitive to metronidazole, with a growth inhibition zone of 4 cm diameter around each disk.

Applying the differential criteria of Cato et al. (1986), three of the isolated strains matched closely the morphological and biochemical characteristics of *C. difficile* (DB26), *C. beijerinckii* (DB2) and *C. lituseburense* (DB77). The aerotolerant strain DB58 had morphological and biochemical characteristics similar to *C. carnis*, but digested meat and did not fully conform to the fatty acid profile normally produced by *C. carnis* in PYG. In an attempt to confirm isolate identification the Rapid ID 32A anaerobic system was used, but proved unsatisfactory except for identifying KDL26 as *C. difficile*.

3.4. Inoculation studies

Only one of the nine experimental packs that were inoculated with DB1 showed pack distension after storage at 1 to 2°C for 8 weeks. Microbiological analysis of the DB1 inoculated pack (Table 5) found no significant aerobic microflora and an anaerobic microflora comparable in size to that found in the commercial lamb pack that yielded isolate DB1. This observation suggests that the anaerobic flora in the inoculated pack was responsible for gas production. Examination of this anaerobic microflora revealed it to be composed entirely of strain DB1.

The headspace volatiles in this experimental pack were similar in composition to the major headspace volatiles found in the commercial pack from which DB1 had been

Table 5
Microbiological counts and concentrations of major headspace volatiles in a commercial sample of 'blown' lamb legs and in a 'blown' experimental pack inoculated with isolate DB1 and an uninoculated control, after storage for 8 weeks at −1.5°C

	Commercial lamb pack	Experimental packs	
	1	DB1	Control
Microbiological counts		Counts (log CFU ml^{-1})	
APC	6.91	3.56	–
Enterobacteriaceae	3.30	*	*
Lactic acid bacteria	5.69	*	*
Brochothrix thermosphacta	3.70	*	*
Total AnO_2PC	6.95	6.45	*
Sporeformers I [a]	5.52	4.45	*
Sporeformers II [b]	–	–	*
Sulfite-reducing anaerobes I [a]	–	–	*
Sulfite-reducing anaerobes II [b]	–	–	*
Headspace volatiles		Concentration (ng ml^{-1})	
Acetic acid	2.0	1.3	0.6
Butyric acid	13.2	0.2	–
Butanol	70.1	17.5	t
Butyl acetate	4.0	t	–
Butyl butyrate	16.2	0.5	–
Benzaldehyde	P	–	–
Ethyl butyrate	P	P	
Hexanal	P	–	–
Octanol	P	–	–
Propanol	P	–	–
Headspace gases			
Carbon dioxide	approx. 70%		
Hydrogen	approx. 30%		
Oxygen (in ppb)	< 0.5	< 0.5	< 0.5

–, not detected; [a], ethanol-treated purge; [b], heat-treated purge; P, present; *, not done; t, trace.

isolated (Table 5). However, the concentrations of compounds in this pack were much lower than those in the original commercial pack. Of the compounds found in the spoiled packs, only acetic acid and traces of butanol were detected in the uninoculated control.

4. Discussion

Spoilage produced by psychrotrophic clostridia in the absence of temperature abuse is characterised by copious gas production causing gross pack distension. On pack opening, an offensive cheesy/dairy odour with or without sulfurous overtones is apparent. This type of spoilage is not restricted to a single meat species and its occurrence in cooked products suggests that the causative organism is introduced as a spore. The absence of oxygen and the presence of carbon dioxide and hydrogen in the headspace is entirely consistent with fermentative metabolism of the butyric type (Jones and Woods, 1986). However, the relative proportion of carbon dioxide and hydrogen

may have changed from that in which they were produced as a result of diffusion of hydrogen through the packaging and absorption of carbon dioxide by the meat. The presence of butyl esters, butyric acid and butanol in the headspace gas of all samples would account for the distinctive cheesy odour characteristic of this type of clostridial spoilage (Dainty et al., 1989). However, the odour of other 'blown' packs examined in this laboratory has been described as being 'sweet faecal' rather than 'cheesy' or 'dairy'. Where the headspace gas also has a sulfurous component, the surface of vacuum packaged meat may assume a green hue as a result of the formation of sulfmyoglobin.

The low number of *Enterobacteriaceae* detected in all 'blown' packs suggests that this group of organisms was not responsible for the abundant gas production and provides evidence that spoilage had occurred at non-abusive storage temperatures. Similar aerobic (APC) and anaerobic (Total AnO_2) counts obtained in all commercial packs were probably due to growth of the numerically dominant lactic acid bacteria. The total anaerobic count may thus be misleading and not represent *Clostridium* spp. in the spoilage flora. Poor recovery of psychrophilic clostridia from the 'blown' packs on solid media has been previously reported (Dainty et al., 1989; Kalchayanand et al., 1989). Difficulties with enumeration and isolation of these organisms on direct plating could be due to a possible antagonistic effect of lactic acid bacteria on solid media. This effect may also occur naturally in meat during storage at low temperatures, causing inhibition of clostridial species by bacteriocin-producing lactic acid bacteria (Crandall and Montville, 1993). Suppression of the recoverable organisms may also be an effect of butanol toxicity. When the carbon source becomes limiting after prolonged storage of meat, some clostridia may show an increased sensitivity to butanol in concentrations that are normally non-toxic (Cortinas et al., 1994). Similarly, cell or spore injury often results in an increased sensitivity to reducing or selective agents in solid culture media (Mossel and Van Netten, 1984). Counts of sporeforming anaerobes from ethanol-treated purge were consistently higher than those obtained from heat-treated purge, suggesting that spores of psychrotrophic clostridia may be less heat-resistant than spores of mesophilic species (Hatheway, 1992).

The psychrotrophic clostridia associated with 'blown pack' meat spoilage appear to be a very heterogeneous group of dominantly saccharolytic, butanol-producing organisms. The studied strains may be easily distinguished from other butanol-producing *Clostridium* spp. by their ability to grow at low temperatures, with minimum growth temperatures well below those of named mesophilic butanol-producing clostridia. Major physiological groups of low-temperature bacteria can be separated into psychrophiles and psychrotrophs (Olson and Nottingham, 1980). The term 'psychrophile' is applied to organisms with an optimum growth temperature at about 15°C or lower, a maximum temperature at about 20°C or lower, and a minimum temperature at 0°C or lower. Clearly, no isolates obtained in the present study can be regarded as being psychrophilic. According to Morita (1975) other organisms capable of growing between -5°C and $+5$°C but not meeting the other requirements for psychrophiles are called 'psychrotrophs'. All eight isolates can therefore, be considered to be psychrotrophs as defined by Morita (1975). Olson and Nottingham (1980), on the other hand, defined the optimum growth temperature for psychrotrophs in range 25 to 30°C, and maximum growth temperature in range 30 to 35°C. Using this definition the two isolates with 37°C

growth optima, KDL26 and DB77, cannot be regarded as psychrotrophs. Furthermore, they are also excluded from these authors' definition of mesophiles by virtue of their ability to grow below +5°C. We, however, feel it is appropriate to regard our isolates that are able to grow at, or below, +5°C, as psychrotrophs, irrespective of their optimum growth temperatures (Eddy, 1960).

The production of isovaleric acid, a product of leucine metabolism, appears to be a property common to many psychrotrophic and cold-tolerant clostridia (Cato et al., 1986; Roberts and Mead, 1986). *Clostridium botulinum*, *C. hastiforme*, *C. sordelli*, *C. glycolicum* and other clostridia are all known to utilise the leucine fermentation pathway but unlike isolates obtained in this study are rarely saccharolytic.

The taxonomic status of the majority of meat spoilage related psychrotrophic clostridia remains uncertain. The isolates obtained in the present study appear to be no more closely related to each other than to *C. esthertheticum*, *C. laramie* and *C. algidicarnis*, isolated in the Northern Hemisphere (Collins et al., 1992; Kalchayanand et al., 1993; Lawson et al., 1994). The Rapid ID system designed for medically important anaerobes proved unsatisfactory with psychrotrophic isolates. This ID system is designed to be used at an incubation temperature of 37°C. Its use at lower temperatures introduced increased variability that, compounded with a database that contains few if any organisms not associated with humans, conspired to confound meaningful identification. Detailed taxonomic investigations of isolates will be conducted in the future.

Only partial reproduction of the original spoilage characteristics was obtained in the inoculation studies. Thus, the possibility that the organisms used for inoculation studies may not have been the organisms responsible for 'blowing' of commercial packs of venison, beef and dog rolls cannot be precluded. However, a more likely explanation is that the full effect may require the presence of more than one type of organism (synergistic effect) on the meat.

The results of the present and other studies (Dainty et al., 1989; Kalchayanand et al., 1989) indicate a probable wide distribution psychrotrophic clostridial species capable of causing 'blown' pack spoilage. As yet the source of these organisms remains uncertain, although a soil habitat appears most likely (Newton et al., 1978). Until the origins of these meat contaminants can be established, control of 'blown' pack spoilage under non-temperature abuse conditions remains elusive.

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