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Short communication

Association of psychrotrophic *Clostridium* spp. with deep tissue spoilage of chilled vacuum-packed lamb

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Abstract

Early spoilage of commercial vacuum-packed chilled lamb legs was manifested as an objectionable 'cheesy', deep tissue odour that became evident when a cut was made into the stifle joint. Investigation of the probable causative agents led to the isolation of two psychrotrophic strains of clostridia. The isolates could not be identified using traditional identification schemes. One isolate was able to produce strong, objectionable 'cheesy' odours in deep tissues of artificially inoculated beef.

Keywords: Vacuum-packaging; Psychrotrophic clostridia; Chilled storage; Microbial spoilage; 'Bone-taint'

1. Introduction

Early spoilage of chilled, vacuum-packed meats can usually be traced to temperature abuse or pack failure. Increasingly reported, however, are incidents of early spoilage of normal pH, chilled, vacuum-packed product where temperature abuse or pack failure has not occurred.

In a recent incident, deep tissue spoilage was reported in vacuum-packed chilled lamb legs. An off odour became noticeable when a cut was made into the stifle joint of the

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legs. The odour was usually described as 'dairy', 'milky' or 'cheesy' and was reported to dissipate within an hour on exposing the cut joint area to air. Temperature records obtained from loggers that had been placed with product suggested that the meat had not suffered temperature abuse during chilled holding and routine laboratory examination failed to detect the presence of any microorganisms that might be responsible for the spoilage. Consequently, a two carton sample of the product was sent to the Meat Industry Research Institute of New Zealand (MIRINZ) to determine if the off odour condition was likely to be of microbial aetiology.

Traditional routine examination was repeated at MIRINZ using standard microbiological procedures and failed to identify a microbial cause of spoilage. Two legs (one from each carton) were retained and frozen, pending further investigation.

Subsequent investigations of vacuum-packed meat spoilage characterised by gas distention of the pack ('blown pack' spoilage) pointed to psychrophilic or psychrotrophic *Clostridium* spp. as causative agents. It was therefore postulated that this group of organisms may have also cause the deep tissue spoilage.

The aim of the present study was to determine whether psychrotrophic clostridia were involved in the deep tissue spoilage of vacuum-packed chilled lamb legs. A series of investigations was conducted to isolate and identify the probable causative agents, and an attempt was made to confirm these isolates as the spoilage agents by evaluating their ability to reproduce the characteristics of the original spoilage under experimental conditions.

2. Materials and methods

2.1. Samples

Commercial vacuum-packed chilled lamb legs with objectionable 'cheesy' deep tissue odour.

2.2. Microbiological examination

2.2.1. Sampling procedure

The two frozen legs in their packaging were thawed overnight at 1 to 2°C, then transferred to a Forma anaerobic chamber (Forma Scientific, Marietta, OH, USA) operating at 15°C, for microbiological sampling. The packaging surface was sterilised with absolute ethanol and then aseptically opened. One 1 ml sample of the drip was withdrawn from each pack using a sterile pipette and transferred into universal bottles containing 9 ml of dilution fluid (0.1% peptone, 0.85% NaCl).

The meat surface was swabbed with absolute ethanol and cut aseptically, transverse to the long axis of the leg, to expose the stifle joint. The joint was then broken open by bending the shank end against the direction of normal joint movement. Exposed synovial surface, approximately 5 cm^2 in area, was swab sampled with a wet then a dry swab, and the swabs were broken into a universal bottle containing 9 ml of dilution fluid. A section of the articular cartilage, approximately 2 cm^2 in area, was then aseptically

removed and placed into 9 ml of pre-reduced Reinforced Clostridial Medium (RCM; Oxoid CM149).

Following microbiological sampling, the leg was removed from the chamber and the odour of the freshly opened joint assessed by three members of an in-house panel.

2.2.2. Enumeration of general spoilage microflora

Counts of aerobic bacteria (APC), *Enterobacteriaceae*, lactic acid bacteria and *Brochothrix thermosphacta* were obtained by plating 0.1 ml volumes of serial decimal dilutions of the drip and swab samples prepared in the dilution fluid onto appropriate growth media, as previously described (Broda et al., 1996). The plates were incubated and enumerated following the standard procedures of the New Zealand meat industry (Cook, 1991).

2.2.3. Enumeration of cold-tolerant anaerobes

All sample dilution, plating and incubation procedures were conducted within the Forma anaerobic chamber. Appropriate dilutions of drip and swab samples 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.

The media used for counting of anaerobes were those previously described (Broda et al., 1996). Briefly, counts of anaerobic bacteria were obtained on a modified RCM agar supplemented with sheep blood, glucose and catalase. Sporeforming anaerobes were enumerated, after heat or ethanol treatment of the samples, on Tryptone Sulfite Polymyxin Agar (SFP, Oxoid CM587) containing egg-yolk and glucose, and sulfite-reducing anaerobes, after the same treatments, on Tryptone Sulfite Cycloserine Agar (TSC, Oxoid CM587).

2.2.4. Anaerobe enrichment

After being used for preparing serial dilutions, the remaining portions of the initial drip and swab samples were each added to 9 ml of pre-reduced RCM and incubated at 15°C within the anaerobic chamber for 5 days. Similarly, the articular cartilage tissue in RCM was incubated under the same conditions. 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.

2.2.5. Selection of psychrotrophic and cold-tolerant clostridia

One isolate representing each colony morphotype on each type of enumeration plate and on the enrichment plates was streaked to confirm its purity onto Columbia Blood Agar (CBA; Oxoid CM331) and incubated within the anaerobic chamber at 15°C until colonies became visible, usually after 7–10 days. The isolates were examined under the 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).

2.2.6. Biochemical characterisation of clostridial isolates

Clostridium isolates and the reference organisms *C. perfringens* (ATCC 13124) and *C. sporogenes* (ATCC 19404) were grown anaerobically in Peptone Yeast Extract Broth (PY; Holdeman et al., 1977) at their optimum growth temperatures for 24–48 h. Optimum growth temperatures were determined as described previously (Broda et al., 1996). Subsequently, the biochemical properties of each isolate were determined at its optimum temperature. 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. Growth on CBA at −1.5°C, 4.0°C and 37.0°C was also determined. The procedure of Holdeman et al. (1977) was used to further characterise the isolates by identifying the metabolic products they produced during growth in PYG (PY + glucose; Holdeman et al., 1977). Additionally, the Rapid ID 32A system (Bio Merieux SA) was used in an attempt to identify the isolates.

2.3. Assessment of the spoilage potential of the clostridial isolates

Hot-deboned fresh beef rumps were obtained from a local abattoir within 30 min of slaughter and trimmed of fat. The surface was sterilised by searing with a hot iron and then the meat was transferred to a laminar flow cabinet, where the charred surface layer was removed using sterile instruments. Cubes, approximately $8 \times 8 \times 8$ cm, and steaks, $10 \times 5 \times 2$ cm, 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.

A culture of each isolate was grown in pre-reduced RCM broth at 20°C for 24 h, then 0.2 ml of these cultures was injected into the middle of each of two meat cubes or spread onto the surface of two steaks and distributed evenly by gentle massaging. The inoculated meat packs were immediately vacuum-packed on a prototype Captron 1 controlled atmosphere/vacuum-packaging machine (Securefresh Pacific, Auckland, New Zealand). The still-warm inoculated meat cubes, together with uninoculated controls, were passed through a marginally abusive cooling regime for beef carcasses (temperature reduction from 37°C to 20°C within 20 h) and examined for the presence of offensive deep tissue odours. The inoculated steaks and uninoculated controls were stored at 1 to 2°C for 8 weeks and examined for gas distension of the pack and the presence of offensive odours.

3. Results and discussion

The appearance of two vacuum-packed chilled lamb legs was typical of unspoiled product. The meat had the normal anoxic colour of vacuum-packed lamb. Packaging was tightly applied around the meat and very little drip had accumulated in the packs.

Table 1
Microbiological counts in the drip and a stifle joint swab samples from spoiled chilled vacuum-packed lamb

Microbiological count	Drip sample (log CFU ml^{-1})	Swab sample (log CFU cm^{-2})
Aerobic plate count	6.24	2.60
Enterobacteriaceae	2.71	–
Lactic acid bacteria	7.05	2.12
Brochothrix thermosphacta	–	–
Anaerobic plate count	7.29	2.72
Sporeformers I [a]	–	–
Sporeformers II [b]	–	–
Sulfite – reducing anaerobes I [a]	–	–
Sulfite-reducing anaerobes II [b]	–	–

[a] Heat treated sample; [b] ethanol treated sample; –, not detected (< 2.00 log CFU per g of samples).

The odour of the opened stifle joint of one leg, assessed 15 min after pack opening, was described as 'strong cheesy', 'dairy' and was decidedly offensive. The opened joint of the second leg did not exhibit an offensive odour.

Microbiological counts obtained from samples of both legs were similar. Consequently, only the results for the pack showing stifle joint odour are reported (Table 1). The dominant organisms in the drip samples were lactic acid bacteria. *Enterobacteriaceae* were also detected, but in very low numbers. Low numbers of general spoilage bacteria were found in the synovial surface swab samples.

High numbers of anaerobes, up to 7.3 log CFU ml^{-1}, were present in the drip samples. In the swab samples, however, only low numbers of these organisms were detected. Sporeforming bacteria were not enumerated in either drip or swab samples. All isolates picked from anaerobic enumeration plates were found to be non-sporeforming, facultative anaerobes, mainly lactic acid bacteria.

Screening of enrichment plates (SFP and TSC) revealed the presence of two types of obligately anaerobic, sporeforming, sulfite-reducing microorganisms (DB4 and DB7) in the swab and articular cartilage samples from the leg showing stifle joint odour. These organisms, members of the genus *Clostridium*, were not found in the RCM enrichment of the drip sample. Isolates, despite similar colonial morphology, differed slightly in their biochemical characteristics (Table 2). The biochemical characteristics of isolate DB9, obtained from 'blown' beef vacuum-packs and chilled dog rolls have been included in Table 2 for comparative purposes. Both deep tissue isolates had optimum growth temperatures between 25 to 30°C and also grew well at −1.5°C and 4°C (results not shown). According to definition proposed by Eddy (1960) both isolated organisms can be considered to be psychrotrophic. The isolates could not be identified by criteria of Cato et al. (1986) or using the Rapid ID 32A system.

Offensive 'cheesy' odours were detected on cutting through the middle of the meat cubes inoculated with the isolate DB4 after 20 h cooling to 20°C. The same isolate, inoculated onto steaks, produced 'dairy', 'cheesy' odours during 8 weeks storage at 1 to 2°C. These odours, although stronger than those normally produced by lactic acid bacteria on chill-stored meat, were not offensive. Isolate DB7 produced only a mild

Table 2
Comparison of the biochemical properties of the psychrotrophic *Clostridium* spp. DB4 and DB7, isolated from deep tissue spoiled vacuum-packed lamb, and DB9, isolated from 'blown' vacuum-packed beef and chilled dog rolls (Broda et al., 1996)

Property	DB4	DB7	DB9
Cell shape	lgr	lgr	lgr
Cell size (μm)	3×10	3×10	3×10
Spore position	ST	ST	ST
Hemolysis in sheep blood	−	−	−
Growth at −1.5°C	+	+	+
4.0°C +	+	+	
37.0°C	+	+	+
Acid from glucose	a	a	a
Gelatin hydrolysed	−	−	−
Motility	+	+	+
Indole produced	−	−	−
Lecithinase produced	−	−	−
Lipase produced	−	−	−
Esculin hydrolysed	+	+	+
Starch hydrolysed	−	−	+
Nitrate reduced	−	−	−
Products from PYG	ABibiv 2,4	ABibiv 2,4	ABibiv 2,4
Acid produced from			
Arabinose	−	−	−
Cellobiose	a	−	−
Fructose	a	a	a
Lactose	a	a	a
Maltose	−	a	−
Mannitol	a	a	a
Mannose	a	a	a
Melibiose	a	a	−
Raffinose	a	−	−
Ribose	−	w	w
Salicin	a	a	a
Sorbitol	a	a	a
Sucrose	a	a	a
Xylose	−	a	w
Milk reaction	−	−	−
Meat digestion	+	+	+

lgr, large rods; ST, subterminal; a, acid; w, weak; −, 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.

'dairy' odour in the artificially inoculated samples subjected to either cooling to 20°C in 20 h or during prolonged storage at 1 to 2°C.

The psychrotrophic clostridia isolated in this study, DB4 and DB7, like the psychrotrophic isolates obtained from 'blown pack' spoilage (Broda et al., 1996), belong to the butanol-producing group of saccharolytic clostridia. Indeed, isolate DB7 showed morphological and biochemical characteristics almost identical with those of isolate DB9, isolated from 'blown' beef packs and dog rolls. The three isolates may belong to

the same species, although isolate DB4 has a slightly different carbohydrate fermentation pattern to isolates DB7 and DB9 and its growth kinetic data suggest that it is more psychrotrophic than DB7 and DB9.

Gas-producing psychrophilic clostridia have been isolated from 'blown' vacuum-packaged ready-to-eat roasted beef meals (USA), and beef primal cuts (USA, UK) (Dainty et al., 1989, Kalchayanand et al., 1989). More recently, Lawson et al. (1994) isolated a non-gas-producing psychrotrophic species, *Clostridium algidicarnis*, that causes offensive, sickly spoilage odours on chilled, vacuum-packed, cooked pork. The significance of the presence of psychrotrophic *Clostridium* spp. in both the deep tissue spoilage reported here and 'blown pack' spoilage reported from this laboratory and elsewhere remains unexplained.

Reproduction of the offensive 'cheesy' odour in the artificially inoculated meat cubes was obtained only under conditions simulating mild temperature abuse during carcass cooling and not under chilled storage conditions. This finding indicates that the deep tissue spoilage may actually occur within a relatively short time after slaughter. The failure to isolate psychrotrophic clostridia from drip suggests that these organisms were already present in the deep tissue at slaughter rather than being introduced as a surface contaminant during dressing and packaging procedures. It is speculated that the organisms have gained their entry through small injuries/abrasions of the lower part of the hind leg and were subsequently transported via the lymphatic system to lymph nodes in the vicinity of the stifle joint. The fact that spoilage could not be reproduced in surface-inoculated samples may provide additional evidence that the observed spoilage condition was limited to the deep tissues. In other words the spoilage condition can reasonably be described as being a 'bone-taint'. This condition has been frequently reported in beef or pork (Ingram, 1952; Roberts and Mead, 1986) but not in ovine carcasses.

The full economic importance of ovine bone-taint caused by the growth of psychrotrophic clostridia has not been established. The deep tissue spoilage investigated in this study appears to have been restricted to product derived from a particular batch of early season animals. This suggests that a combination of animal age and production location may play an important part in predisposing the legs of early season lamb to deep tissue spoilage.

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