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Short communication

Possible involvement of psychrotolerant Enterobacteriaceae in blown pack spoilage of vacuum-packaged raw meats

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Abstract

Recent investigations of blown pack spoilage in New Zealand chilled vacuum-packaged meats have found moderate to high numbers of Enterobacteriaceae in the spoilage flora, but no clostridia, such as *C. estertheticum* and *C. gasigenes*, that are usually associated with blown pack spoilage. This study showed that pyschrotolerant Enterobacteriaceae produced gas in a lamb homogenate model under anaerobic conditions and that these organisms could cause blown pack spoilage of vacuum-packaged chilled meats. Significant gas production was observed with the majority of the psychrotolerant Enterobacteriaceae strains tested including presumptive species of *Enterobacter*, *Serratia*, *Hafnia* and *Rahnella*. However, no gas was produced in lamb homogenates inoculated with presumptive species of *Ewingella americana* or *Yersinia enterocolitica*. Gas production was also confirmed in vacuum-packaged lamb shoulders stored at 4 °C for 21 days after being inoculated with individual representative Enterobacteriaceae isolates. Biochemical characterisation proved to be more useful than genotype-based typing of 16S rRNA genes for discriminating different psychrotolerant Enterobacteriaceae from naturally contaminated meat microflora.
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Keywords: Psychrotolerant Enterobacteriaceae; Gas production; Vacuum-packaged; Blown pack spoilage

1. Introduction

Gas production in vacuum-packs of chilled meat leads to pack distension which is commonly referred to as pack blowing. Blown packs are rejected or downgraded with consequent economic losses. Psychrophilic *Clostridium* spp., particularly, *C. estertheticum* and *C. gasigenes*, have been established as causative agents of blown pack spoilage (Kalchayanand et al., 1989; Collins et al., 1994; Broda et al., 2002). This spoilage condition typically occurs in the absence of significant temperature abuse. Psychrotolerant (cold-tolerant) Enterobacteriaceae have also been implicated in incidents of spoilage of vacuum-packaged meats (Hanna et al., 1979; Gamage et al., 1997; Kang et al., 2002). However, spoilage by these organisms is usually due to off odours rather than gas production. The proliferation of Enterobacteriaceae in vacuum-packaged meats is usually limited to product with pH > 5.8 (Gill, 2004) and is more likely to occur when product has been temperature abused. The presence of Enterobacteriaceae in vacuum-packaged chilled meats is of particular significance due to their high spoilage potential and the food safety hazards from some members of this group.

Recent investigations of blown pack spoilage in New Zealand have detected moderate to high numbers (10^4–10^6 cfu/ml) of Enterobacteriaceae in the absence of organisms that are normally associated with blown pack spoilage. Subsequently, *Hafnia*, *Enterobacter*, *Serratia*, *Rahnella*, *Ewingella* and *Yersinia* were isolated from these samples. However, very limited data on gas production in vacuum-packs as a result of the growth of psychrotolerant Enterobacteriaceae are available (Hanna et al., 1979; Gamage et al., 1997). In this study, we demonstrated gas production by psychrotolerant Enterobacteriaceae in lamb meat homogenates under anaerobic conditions, and blown pack spoilage of chilled vacuum-packaged lamb shoulders stored at sub-optimal temperatures.

2. Materials and methods

2.1. Bacterial strains

A culture collection of 64 psychrotolerant Enterobacteriaceae isolates (Table 1) was established from investigations of

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Table 1
Gas production in lamb meat homogenates inoculated with psychrotolerant Enterobacteriaceae

Source	Isolate	Microbact 24E identification	I.D. % probability	Gas production score		
				Day 7	Day 14	Day 21
Processed pork product storage trial (3 weeks/1 °C)	1	*Enterobacter amnigenus* biogrp 1	96.55	1	3	3
	2	*Hafnia alvei*	99.4	1	1	1
	3	*Enterobacter amnigenus* biogrp 1	86.8	1	3	3
	4	*Enterobacter amnigenus* biogrp 1	86.83	0	2	3
	5	*Enterobacter amnigenus* biogrp 1	99.34	1	2	2
	6	*Hafnia alvei*	81.71	0	0	0
	7	*Enterobacter amnigenus*	86.8	1	2	3
	8	*Enterobacter amnigenus*	81.56	1	3	3
	9	*Enterobacter agglomerans*	98.96	1	1	2
	10	*Serratia marcescens* biogrp 1	80.58	0	1	1
	11	*Enterobacter agglomerans*	98.8	1	2	2
'Blown' vacuum-packaged venison	12	*Hafnia alvei*	95.15	0	1	1
	13	*Hafnia alvei*	80.27	0	1	1
	14	*Hafnia alvei*	44.5	1	2	2
	15	*Hafnia alvei* biogp 1	90.3	1	1	3
	16	*Hafnia alvei*	95.1	1	2	2
	17	*Hafnia alvei*	95.1	1	2	2
	18	*Hafnia alvei* biogp 1	96.96	1	3	3
	19	*Hafnia alvei*	95.15	1	1	1
	20	*Hafnia alvei*	90.76	0	1	2
	21	*Hafnia alvei*	99.6	1	2	2
Spoiled vacuum-packaged beef returned from overseas	22	*Enterobacter agglomerans*	42.4	1	2	3
	23	*Enterobacter agglomerans*	97.05	1	2	3
	24	*Enterobacter agglomerans*	42.4	1	1	2
	25	*Enterobacter agglomerans*	99.99	1	2	2
	26	*Serratia liquefaciens*	95.4	1	2	2
	27	Enteric Grp 45	70.45	0	0	0
	28	*Enterobacter agglomerans*	83.55	1	2	3
	29	*Serratia plymuthica*	98.63	2	2	3
Vacuum-packaged lamb from investigation of lamb products 'blown' in the marketplace	30	*Enterobacter intermedium*	47.35	1	1	3
	31	*Enterobacter intermedium*	47.35	1	1	2
	32	*Enterobacter intermedium*	47.35	1	2	3
	33	*Enterobacter intermedium*	47.35	1	2	3
	34	*Enterobacter intermedium*	47.35	1	2	3
	35	*Serratia liquefaciens*	91.79	1	2	3
	36	*Serratia liquefaciens*	91.79	1	2	3
	37	*Enterobacter intermedium*	47.35	1	2	3
	38	*Hafnia alvei*	97.3	1	2	3
	39	*Enterobacter cloacae*	68.64	1	1	2
	40	*Enterobacter amnigenus* biogrp 1	96.55	1	2	3
	41	*Rahnella aquatilis*	89.06	1	2	3
	42	*Enterobacter cloacae*	68.64	1	2	3
	43	*Enterobacter agglomerans*	42.4	1	2	2
	44	*Enterobacter agglomerans*	42.4	1	2	3
	45	*Enterobacter agglomerans*	42.4	1	2	3
	46	*Ewingella americana*	99.57	0	0	0
	47	*Rahnella aquatilis*	89.06	1	2	2
	48	*Enterobacter cloacae*	66.43	1	2	3
	49	*Rahnella aquatilis*	89.06	1	2	2
	50	*Ewingella americana*	93.86	0	0	0
	51	*Ewingella americana*	99.66	0	0	0
Vacuum-packed lamb tested during investigation of elevated Enterobacteriaceae counts	52	*Serratia liquefaciens*	68.73	1	2	2
	53	*Serratia liquefaciens*	99.94	0	2	2
	54	*Serratia marcescens* Biogrp 1	99	0	1	3
	55	*Yersinia enterocolitica*	90.79	0	0	0
	56	*Yersinia enterocolitica*	90.79	0	0	0
Lamb processing (boning room) environment	57	*Serratia marcescens*	99.69	0	0	0
	58	*Serratia liquefaciens*	99.68	1	2	2
	59	*Serratia liquefaciens*	94.84	1	2	3
	60	*Serratia liquefaciens*	97.19	1	2	2
	61	*Enterobacter amnigenus* Biogrp 1	96.55	1	3	3
	62	*Serratia liquefaciens*	94.84	0	2	3
NZRM 109 *H. alvei*	63		99.9	1	2	2
NZRM 869 *H. alvei*	64		99.9	0	2	3

spoilage incidents involving chilled vacuum-packaged meats (pork, beef, venison and lamb), meat plant environment or from reference strains obtained from the New Zealand Institute of Environmental Science & Research (ESR).

2.2. Isolation and Microbact identification of pure cultures

Isolates were identified as Enterobacteriaceae following isolation of red colonies with yellow zones and/or red colonies with gas bubbles with or without yellow zones on Petrifilm™ Enterobacteriaceae plate count films (Biolab Scientific, Auckland, New Zealand). Pure cultures were confirmed as psychrotolerant by their ability to grow at 4 °C. Pure cultures that were confirmed as oxidase-negative, Gram negative rods, capable of nitrate reduction (AgResearch, Meat Industry Microbiological Methods, Edition 4)http://www.agresearch.co.nz/micromanual, were identified using the Microbact 24E (Oxoid Ltd, Basingstoke, Hants, UK) Gram negative identification scheme (12A+12B). Procedures recommended by the manufacturer of the Microbact 24E scheme were followed, except that test strips were incubated at 30 °C for 24 h. Results were interpreted using the Microbact 24E computer aided identification package with a percent probability being assigned for each possible identity of an isolate. Each isolate was presumed to be the species to which the highest probability was assigned.

Pure cultures of Enterobacteriaceae isolates were maintained on Columbia Blood Agar (CBA) containing 5% sterile sheep blood (Fort Richard Laboratories, Auckland, New Zealand). For the inoculation studies, each pure culture was sub-cultured into Tryptic Soy Broth (Fort Richard Laboratories) and incubated overnight (16–18 h) at 30 °C to obtain a culture to be used as an inoculum. Numbers of bacteria in each inocula were determined as Aerobic Plate Counts, on Plate Count Agar (Fort Richard Laboratories) incubated at 30 °C for 48 h.

2.3. Typing Enterobacteriaceae by restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes

Total genomic DNA was isolated from each psychrotolerant Enterobacteriaceae using a High Pure PCR Template Preparation kit (Roche Diagnostics, Wellington, New Zealand) according to the manufacturer's instructions. Amplification and RFLP analysis of 16S rDNA was performed using previously described procedures (Brightwell et al., 2006). Enterobacteriaceae isolates were assigned to RFLP groups using banding patterns obtained from *Cfo* I and *Hae* III (Roche Diagnostics) restriction enzymes.

2.4. Sequence analysis of 16S rDNA

Seventeen Enterobacteriaceae isolates were selected to represent at least one strain of each species of Enterobacteriaceae identified by Microbact 24E. For each of the 17 Enterobacteriaceae, PCR amplification of 16S rDNA from genomic DNA was carried out using primers, pA 5′-AGAGTTTGATCCTGGCTCAG-3′ (8–28 *E. coli* numbering system) (Hutson et al., 1993) and pH* 5′-AAGGAGGTGATCCAGCCGCA-3′ (1542–1522) (Boddinghaus et al., 1990). PCR reactions were performed in 50 µl of reaction mixture containing 1× Roche PCR reaction buffer (Roche Diagnostics), 1 µM of each primer (Invitrogen, Groningen, Netherlands), 200 µM of dNTPs (Roche Diagnostics), 1.25 U of Taq Polymerase (Roche Diagnostics) and 5 µl of DNA template. PCR was carried out using a PT100 thermal cycler (MJ Research, Waltham, MA USA) and the following conditions: 93 °C for 3 min; 92 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min for 30 cycles; with a final extension at 72 °C for 3 min. 16S rDNA PCR products were then purified using the Qiaquick PCR purification kit (Biolab Scientific) according to the manufacturer's instructions. Approximately 500 nucleotides were sequenced from the 5′ end of each purified PCR product using the forward primer, pA (Waikato University Sequencing Facility, Hamilton, NZ). Inspection and editing of electropherograms was performed using the Chromas software (Technelysium Pty Ltd, Tewantin QLD, Australia), sequence homology was identified with the GenBank Basic Logical Alignment Search Tool (Altschul et al., 1990), and CHECK_CHIMERA software (Larsen et al., 1997) was used to scan for potential chimeric sequences.

2.5. Preparation of sterile lamb meat homogenates

Preparation of sterile lamb meat homogenates was adapted from Roberts et al., (1981). Vacuum-packaged lamb legs obtained from a local lamb processing plant on the day following their fabrication were stored at −1.5 °C for 3 days. A surface area of approximately 15 cm × 10 cm on each lamb leg was sterilised by searing it with a superheated metal plate. Within a laminar flow cabinet, the seared surface was aseptically removed. Muscle pieces excised from the newly exposed tissue were transferred to a sterile Waring blender cup. The excised muscle was homogenised, at low speed, with the addition of 1 part meat to 1 part sterile 0.85% NaCl to obtain a lamb meat homogenate. The pH of the homogenate was determined using a pH probe (MeterLab PHM210, Radiometer, Copenhagen, Denmark) and adjusted to pH 5.8 using filter sterilised 1 M NaOH. The homogenate was then mixed, to ensure homogeneity of the lamb sample. Finally, 10 g aliquots of sterile homogenate were dispensed into sterile glass Universal bottles for use in gas production studies.

2.6. Determination of gas production in lamb meat homogenates

Each bacterial suspension was inoculated into the lamb homogenate to give approximately 10^7 cfu/g, and bacterial suspension mixtures were stirred with a sterile inoculating loop to ensure an even distribution of bacteria in the homogenate. To create anaerobic growth conditions, the Universal bottles containing inoculated homogenates were plugged with Vaspar, a 1:1 w/w mixture of petroleum jelly (PSM Healthcare, Auckland, New Zealand) and paraffin oil (BDH Chemicals, Poole, England). The plugged bottles were incubated at 4 °C for 21 days. Six uninoculated lamb meat homogenates served as negative controls. At 7, 14 and 21 days the homogenates were

removed from chilled storage for periods of no longer than 10 min, during which time the homogenates were assessed for accumulation of gas in the lamb meat homogenate and beneath the Vaspar plug. Tubes with no observable gas were recorded as 0. Scores of 1 (a few bubbles formed beneath the Vaspar plug), 2 (several bubbles beneath the Vaspar plug) or 3 (Vaspar plug lifted away from lamb homogenate) were assigned to tubes with minor, moderate or major gas accumulation, respectively.

2.7. Packaging and storage of vacuum-packed chilled lamb

Boneless lamb shoulders from a local lamb processing plant were obtained as soon after boning as possible, vacuum-packaged and stored overnight at −1.5 °C. After 24 h, packs were opened, the pH of the muscle and adipose tissue surfaces were determined, and the shoulders were each placed in an individual pre-labelled vacuum bag (30×39 cm, BB7L; Cryovac Food Packaging, Hamilton, New Zealand). Dilutions of the 17 cultures of Enterobacteriaceae, selected for 16S rDNA analysis were prepared and used to inoculate each lamb shoulder with an isolate at numbers of approximately 5 log cfu/cm^2. The experiment was conducted in triplicate for each Enterobacteriaceae isolate and three uninoculated lamb shoulders were included as negative controls.

Immediately following inoculation, each lamb shoulder was vacuum-packed using a Securepak 10 Controlled Atmosphere Packaging Machine (Securefresh Pacific, Auckland, New Zealand) and subjected to heat shrinking by total immersion in 82 °C water for 4 to 5 s. The sealed packs were placed into chilled storage at 4 °C for 3 weeks. The operating temperature of the storage chiller was monitored using KoolTrack data loggers (KoolTrack Inc., Southampton, PA, USA). Except for during defrosting cycles, temperature in the 4 °C chiller was maintained within 0.5 °C of the set point. Following defrosting cycles, the set point temperatures were re-established within 0.5 h of the start of each cycle.

Packs were examined twice weekly for 3 weeks. Gas bubbles in the drip and pack distension status were scored on a scale from 0 to 5. Where 0 = no gas bubbles in the drip; 1 = small numbers of gas bubbles in the drip; 2 = loss of vacuum; 3 = 'blown'; puffy packs; 4 = fully distended packs; and 5 = overblown packs. In commercial practice, packs with a distension score of 1 would not be regarded as abnormal. At the end of the trial representative packs of each Enterobacteriaceae inoculum were massaged thoroughly to obtain homogeneity, opened aseptically and a volume of exudate was collected for culture and enumeration of bacteria.

Appropriate 10 fold dilutions of meat exudate were prepared and 1 ml of each dilution was spread onto a Petrifilm™ Enterobacteriaceae Count Plate (Biolab Scientific). Petrifilms were incubated at 30 °C for 24 to 48 h and colonies were enumerated following incubation. To verify that gas production observed in this trial was in fact caused by the original inoculated Enterobacteriaceae, the inoculated Enterobacteriaceae species were compared, using Microbact 24E identification, to the numerically dominant Enterobacteriaceae species recovered from the meat packs at day 21.

3. Results

3.1. Identification of psychrotolerant Enterobacteriaceae

Among the 62 psychrotolerant Enterobacteriaceae isolated from spoiled meat and lamb boning rooms, members of six genera were provisionally identified by Microbact 24E, with 12 distinct species, including 1 identified only as Enteric Grp 45 (Table 1). The isolates were identified as the species assigned the highest probability value. However, the range of the probabilities of identification was wide for some of the genera, and therefore the identification of isolates should be viewed as presumptive.

Only 1 genus of psychrotolerant Enterobacteriaceae, *Hafnia*, had been recovered from blown vacuum-packaged venison, but several different genera were recovered from spoiled vacuum-packaged lamb and beef. *Yersinia enterocolitica*, *S. liquefaciens* and *Serratia marcescens* were isolated from incidents of vacuum-packaged lamb rejected at the market place due to high levels of Enterobacteriaceae. *S. liquefaciens*, *S. marcescens* and *Enterobacter amnigenus* Biogrp 1 were isolated from lamb processing boning rooms and 3 *Enterobacter*, 2 *Hafnia* and 1 *Serratia* species were isolated from processed pork samples that had been stored at 1 °C for 3 weeks (Table 1).

Results from the 16S rDNA analysis were less informative. Digestion of 16S rRNA gene products with *Hae* III and *Cfo* I restriction enzymes, yielded 2 (A and B) and 7 (1–7) distinct RFLP pattern types, respectively. Consideration of patterns obtained with both enzymes resulted in assignment of isolates to 8 RFLP groups (designated A1–A7 and B1). No specific species banding pattern differences were evident for a number of species and genera. To give examples, isolates identified as *Hafnia alvei*, *Enterobacter agglomerans*, *Enterobacter intermedium*, *E. amnigenus*, *Enterobacter cloacae* and *Rahnella aquatilis* gave identical RFLP patterns that placed them in RFLP Group A1; and RFLP group A5 was represented by isolates identified as *Y. enterocolitica* and *S. liquefaciens*. The 16S rDNA sequence analysis of the 17 representative Enterobacteriaceae isolates also failed to discriminate between the isolates to the same level as the Microbact analysis. Ten of the 17 isolates shared homology with *R. aquatilis* (98% similarity), of which 9 also shared the same RFLP pattern. However, Microbact analysis further subdivided these 10 isolates into 8 different species. Only isolates 15, 35, 38, 41, 54, 59 and 64 were consistently identified by both 16S sequence and Microbact analysis.

3.2. Gas production by psychrotolerant Enterobacteriaceae in lamb homogenates

Results of gas production by 64 psychrotolerant Enterobacteriaceae strains in inoculated lamb meat homogenates are presented in Table 1. No gas production was observed with any of the 6 uninoculated controls. Gas was produced by the majority of psychrotolerant Enterobacteriaceae. Major gas production was detected with all 4 isolates identified as *Enterobacter*. Moderate or major gas production scores were

obtained with presumptive *Serratia*, *Rahnella*and *Hafnia* spp. However, no gas was observed with presumptive *Ewingella americana*, *Y. enterocolitica* and the single isolate identified by the Microbact 24E system as Enteric Group 45.

3.3. Gas production by psychrotolerant Enterobacteriaceae in vacuum-packaged lamb

The pH of adipose tissue and meat from each lamb leg used in this study ranged from 5.76 to 6.18 and 5.77 to 6.26 respectively.

At day 7, no packs had gas bubbles in the drip. After 14 days most packs had small numbers of gas bubbles in the drip, but two packs inoculated with presumptive *E. agglomerans* had lost vacuum. At 21 days, all packs showed loss of vacuum, except the negative controls, which had not accumulated gas bubbles by the end of the storage trial. None of the packs were obviously distended at day 21. In all cases, the bacterial species recovered in the highest numbers (ranging from 5.3 to 9.2 log cfu/ml) from a pack was the same as the organism with which the meat had been inoculated. *Yersinia frederiksenii* was recovered at numbers of 7.4 log cfu/ml from uninoculated control packs.

4. Discussion

Premature spoilage of vacuum-packed chilled meats as a result of gas production and gross pack distension is principally attributed to the growth of psychrotolerant *Clostridium* spp. (Dainty et al., 1989; Kalchayanand et al., 1989; Broda et al., 1996). However, at times we have seen high numbers of psychrotolerant Enterobacteriaceae in 'blown packs', in the absence of clostridia. Spoilage by these organisms is usually associated with putrid off odours and/or greening of the meat.

The accurate identification of Enterobacteriaceae has been the subject of many publications over the years. Continued changes in the taxonomy of the Enterobacteriaceae over the last few decades make attempts to classify these bacteria extremely problematic. The Microbact 24E system has so far been evaluated only for the identification of Enterobacteriaceae of medical origin grown under controlled conditions at 37 °C (Mugg and Hill, 1981). Many of the psychrotolerant Enterobacteriaceae used in this study will not grow at 37 °C, so we incubated the isolates at 30 °C, probably changing the biochemical profile of some or all the isolates with resultant loss of accurate identification. Therefore, all Microbact identification results obtained in this study are referred to as presumptive. Results from the Microbact 24E reactions were entered into the Microbact computerized identification package. However, the list of taxa includes only 29 genera, 109 species, 7 enteric groups of oxidase-negative organisms, and 12 genera and 31 species of oxidase-positive organisms, all mainly of clinical origin. Thus, identification of none clinical Enterobacteriaceae is extremely difficult as they may not be represented in the database. Many of the Enterobacteriaceae genera tested in this study showed a wide range of % I.D. probabilities, particularly *Hafnia* and *Enterobacter* species. For this reason we also included a molecular approach, based on RFLP and sequence analysis of PCR-amplified 16S rRNA genes. This approach has been successfully used to determine taxonomic relationships of other bacterial species (Heyndrickx et al., 1996; Pukall et al., 1998; Gurtler et al., 1991) including psychrotolerant and psychrophilic *Clostridium* spp. (Broda et al., 2000). Our results indicated that both RFLP and sequencing analysis of PCR-amplified 16S rDNA employed here were less discriminatory compared with Microbact 24E, for typing psychrotolerant Enterobacteriaceae. Although this lack of discriminatory ability may have been improved by selecting alternative restriction enzymes, our results are consistent with previous studies that found the utility of 16S rDNA analysis of Enterobacteriaceae to be questionable due to the high degree of intra-family DNA relatedness (Cilia et al., 1996; Janda and Abbott, 2006; Wertz et al., 2003). Unfortunately, none of the typing methods were definitive. Further taxonomical investigations of the psychrotolerant Enterobacteriaceae isolates tested in this study will be carried out.

Our results showed that species of *Hafnia*, *Enterobacter*, *Serratia*, *Rahnella* and, *Ewingella* when present in initial numbers of 10^5 cfu/cm^2 were able to produce blown pack spoilage of vacuum-packaged lamb stored at 4 °C.

All lamb shoulders used in this trial were from a commercial lamb processing plant, and were not sterile. It is reasonable to suspect that some spoilage could be due to previously established microflora. The *Y. frederiksenii* recovered from uninoculated control packs must have been part of the naturally occurring microflora and did not cause gas blown pack spoilage.

While the results of this study confirm the potential for a number of genera of Enterobacteriaceae to cause blown pack spoilage of chilled vacuum-packaged lamb when they are present in high initial numbers, and the meat is stored at a sub-optimal temperature, it is not known if that form of spoilage would occur under different conditions. Further work needs to be carried out to determine the relevance of storage temperatures, meat pH and initial numbers upon gas production by Enterobacteriaceae in vacuum-packaged meat systems.

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