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ORIGINAL ARTICLE

The effect of storage temperature and inoculum level on the time of onset of 'blown pack' spoilage

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Abstract

Aims: To examine the effect of storage temperature and inoculum level on the time of onset of 'blown pack' spoilage (BPS) caused by psychrotolerant bacteria in vacuum-packed (VP) meats.
Methods and Results: Gas-producing species and strains (n = 11), recovered in our laboratory or reported as associated with BPS, were inoculated onto beef or lamb meat pieces at final levels of <10, 10, 10^2 and 10^3 CFU cm^{-2}, VP and stored at −1·5, 1 or 4°C. Six strains produced observable amounts of gas within 42 days and a further four strains produced gas within 100 days. BPS was observed earliest in VP meats inoculated with *Clostridium estertheticum* ssp. *estertheticum* at all inoculum levels/storage temperature combinations examined. Storage temperature and inoculum level significantly affected ($P < 0{\cdot}001$ and $P < 0{\cdot}05$ respectively) the onset of BPS in all cases.
Conclusions: Controlling contamination levels and lowering the storage temperature delay the onset of BPS.
Significance and Impact of the Study: The study demonstrates the positive effects of low contamination–low temperature as control interventions preventing/delaying BPS in VP chilled meats and identifies some of the contaminants most likely to cause BPS in chilled stored VP meat products.

Introduction

A number of species of *Clostridium* have been implicated in the spoilage of both raw and cooked vacuum-packed (VP) beef (Dainty *et al.* 1989; Kalchayanand *et al.* 1993; Broda *et al.* 1996a), VP cooked pork (Lawson *et al.* 1994), VP lamb (Broda *et al.* 1996b, 1999), other cooked meat and poultry products (Kalinowski and Tompkin 1999), cheese (Liu *et al.* 2006; Lycken and Borch 2006; Le Bourhis *et al.* 2007) and potatoes (Brocklehurst and Lund 1982).

'Blown pack' spoilage (BPS), a particular form of spoilage associated with the outgrowth of *Clostridium* in VP fresh meats stored under refrigeration, is characterized by the accumulation of amounts of carbon dioxide and hydrogen, leading to significant pack distension. BPS usually includes the production of undesirable volatiles such as butanol, butanoic acid, ethanol, acetic acid and esters derived from these compounds and a range of sulfur-containing compounds associated with a putrid smell and a metallic sheen on the spoiled meat (Dainty and Mackey 1992). *Clostridium estertheticum* ssp. *estertheticum*, *Cl. estertheticum* ssp. *laramiense* and *Clostridium gasigenes* have been most frequently identified as the causative agents of this kind of spoilage (Collins *et al.* 1992; Kalchayanand *et al.* 1993; Broda *et al.* 2000). However, PCR analysis of some blown pack spoiled meats has failed to detect the above strains/species of clostridia, suggesting that other psychrophilic/psychrotrophic bacteria may also induce BPS.

Nonproteolytic strains of *Clostridium botulinum* type E are able to grow at 3·3°C (Lund *et al.* 1990) and produce

gas (Schmidt *et al.* 1961; Cockey and Tatro 1974). Other studies have shown that other psychrophilic clostridia and members of the family *Enterobacteriaceae* may also produce significant volumes of gases associated with BPS at temperatures as low as 4°C (Spring *et al.* 2003; Brightwell *et al.* 2007).

Low storage temperature is the most significant factor in delaying the microbiological spoilage of packaged meat products. A number of studies have demonstrated the relationship between storage temperature and the time of onset of overt BPS associated with *Cl. estertheticum* (Bell *et al.* 2001; Boerema *et al.* 2007), but little is known about the influence of temperature on the onset of BPS associated with other species.

As well as being influenced by storage temperature and the strain/species of contaminating BPS associated organisms, the time of onset of overt spoilage is influenced by the level of contaminating BPS agent(s). This inoculum effect (Bidlas *et al.* 2008) has been examined by many investigators (Koutsoumanis and Sofos 2005; Ahn and Balasubramaniam 2007; Bidlas *et al.* 2008) and has been demonstrated to dictate the onset of *Cl. estertheticum*-associated BPS of VP chilled beef (Boerema *et al.* 2007).

To date, the Irish beef industry has suffered serious financial losses as a result of BPS in its VP meat products. In a previous work (Moschonas *et al.* 2009), a number of gas-producing anaerobic strains were isolated from Irish commercial beef abattoirs and their environs. This investigation was carried out to assess the ability of a range of gas-producing isolates ($n = 11$) to cause BPS in VP chilled meats and to determine the effect of storage temperature and initial inoculum level on the time(s) to the onset of BPS.

Materials and methods

Micro-organisms

The bacteria used are listed in Table 1. Local strains were isolated from Irish commercial beef abattoirs and their environs using nonspecific media and extended anaerobic enrichments (*c.* 3 weeks) at 4°C, as previously described (Moschonas *et al.* 2009).

Preparation of inocula

All strains were recovered from protect beads (Technical Service Consultants Ltd, Lancashire, UK) in pre-reduced peptone yeast extract glucose starch (PYGS) broth (Lund *et al.* 1990) and incubated anaerobically at 4°C for 3 weeks.

Each *Clostridium* culture produced spore concentrates in a two-phase sporulation medium (Peck *et al.* 1992) as modified by Bell *et al.* (2001). Briefly, the medium was prepared in Durham bottles (two bottles per isolate). The initial (lower) phase was cooked meat medium (CMM) (Oxoid, Basingstoke, UK), supplemented with 4·5 g agar and 0·3 g glucose per 300 ml of CMM. After sterilization (121°C for 15 min), the media were pre-reduced in an atmosphere-controlled cabinet (Don Whitley Scientific, Shipley, UK) under an atmosphere of 100% carbon dioxide at 10°C for 24 h. Following this period, the media were supplemented with 40 ml of deoxygenated sterile water per bottle to form the (liquid) upper phase. Bottles of this medium (CMM+) were inoculated with 5 ml of the above PYGS cultures and incubated anaerobically at 4°C for 3 months. Spore suspensions were recovered

Table 1 Bacteria studied

a/a	Strain	Source	Strain number
1	*Clostridium estertheticum* ssp. *estertheticum*	Blown pack*	DSMZ 8809^T
2	*Cl. estertheticum* ssp. *laramiense*	Blown pack†	DSMZ 14864^T
3	*Clostridium gasigenes*	Blown pack‡	DSMZ 12272^T
4	TC1	Cattle hide	Isolate TC1
5	*Bacteroides propionicifaciens*	Bleeding area	Isolate B300
6	*Clostridium* sp. CYP7	Cattle faeces	Isolate C307
7	*Clostridium thiosulfatireducens* strain MG-2	Cattle faeces	Isolate C304
8	*Clostridium botulinum* type E	Cattle hide	Isolate C315
9	*Clostridium* sp. V13	Cattle faeces	Isolate C318
10	*Cl. estertheticum* ssp. *laramiense*	Hide puller	Isolate E324
11	*Clostridium bowmanii*	Soil	Isolate C325

1–3, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany); 4–11, Teagasc, Ashtown Food Research Centre, Ireland (local isolates); ^{T}, type strain.
*Dainty *et al.* (1989).
†Kalchayanand *et al.* (1989).
‡Broda *et al.* (2000).

from the resultant cultures by centrifugation (15 000 ***g***, 4°C) and washed (five centrifugation/wash cycles) with saline. The washed spore suspensions were sonicated (40 kHz, 15 min) in an ultrasonic waterbath (Algasan, Sevenoaks, UK) and centrifuged/washed as described above (three sonication/centrifugation/washing cycles), suspended in 10 ml saline and stored at −20°C. The number of spores in this final inoculum was estimated by plating heat-treated samples (60°C for 10 min) on Columbia blood agar (CBA) (Oxoid) supplemented with 5% defibrinated horse blood and incubated anaerobically at 4°C for 3 weeks.

Bacteroides culture in PYGS broth was washed five times with ice-cold saline (0·85% NaCl), resuspended in saline and used as inocula. Numbers in this final inoculum were estimated by plating on CBA supplemented with 5% defibrinated horse blood and incubated anaerobically at 4°C for 3 weeks.

Meat samples, inoculation and packing

Portions of beef (silverside) and lamb (leg) were purchased from local abattoirs, transferred to the laboratory immediately after boning and cut to form 10 × 10 × 1 cm samples (660 beef, 660 lamb). Serial dilutions of each strain were prepared in saline, and both 10 × 10 cm surfaces of each meat sample were spread inoculated with 0·1 ml serial dilutions of the prepared inocula to final levels of <10, 10, 10^2 or 10^3 CFU cm^{-2}. The inoculated meat samples were allowed to dry for 30 min at room temperature and placed in individual vacuum bags (BB325, Cryovac; SealedAir Ltd, St Neots, UK) containing a hydrogen sulfide test strip (Fluka, Buchs, Switzerland) within each pack. Samples were VP using Vac Star S220 (Sugiez, Switzerland). In line with industrial practice, vacuum packs were shrunk by dipping in a waterbath at 90°C for 2–3 s. Treated samples were stored at −1·5 (±1), 1 (±1), or 4 (±1)°C for up to 100 days. Storage temperatures were monitored using Grant Squirrel 1000 series data loggers (Grant Instruments, Shepreth, UK).

Five samples of each meat type (beef and lamb) were inoculated with each strain ($n = 11$) at four different spore levels and incubated at three storage temperatures. Uninoculated control samples (30) (five for each meat type and storage temperature) were processed in parallel with the 1320 test samples.

Packs were visually examined every 4 days for up to 100 days for the presence of gas and scored against the following criteria (Boerema *et al.* 2007): score 0, no gas production; score 1, small bubbles in drip; score 2, loss of vacuum; score 3, blown packs; score 4, fully distended, without tightly stretching the pack; score 5, overblown, tightly stretched packs/packs leaking.

Headspace gas analysis

Packs that reached score 5 within 100 days, or (in the case of packs that did not reach this score) packs that scored ≥3 within 100 days, were transferred to the laboratory where production/nonproduction of H_2S was determined using hydrogen sulfide test strips. Concentrations of CO_2, H_2 and N_2 in the headspace were determined semiquantitatively. Gas samples (50 ml) were withdrawn from individual packs using a 60-ml gas-tight syringe fitted with a 23-gauge hypodermic needle (Becton Dickinson, Franklin Lakes, NJ, USA) inserted into an area of the pack that was covered with a self-sealing 'patch'. Recovered gas samples were analysed using a gas chromatograph (Gow-Mac Spectra 250; Gow-Mac Instrument Co., Bethlehem, PA, USA), fitted with a Hayesep column (Varian Inc., Palo Alto, CA, USA), with a carrier gas (helium) flow rate of 50 ml min^{-1} and column, detector and injection port temperatures of 25, 100 and 50°C respectively and a detector current of 150 mA. Integration of peaks was carried out using Borwin™ software 1.0 (JMBS Developments, Le Fontanil, France). The gas chromatograph was calibrated daily using reference gases.

Detection of volatile compounds in the headspace

The presence of volatile alcoholic compounds in the headspace of blown packs was verified using headspace solid-phase microextraction (HS-SPME) coupled to GC-MS.

HS-SPME analysis used a 85-μm polyacrylate-coated fibre (Supelco, Bellefonte, PA, USA), preconditioned (1 h 300°C^{-1}) in the GC injection port. A 10-mm PTFE/silicone septum (Supelco) was attached to each blown pack to be examined, and a sampling port pierced through the septum using a 0·9 × 25 mm needle (Becton Dickinson). The fibre was inserted through the sampling port, allowed to equilibrate for 5 min and exposed to the pack headspace for 1 h at room temperature. Volatile components were subsequently separated on a 30 m ZB-XLB capillary column (0·25 mm [i.d.], 0·25 μm [film thickness]) installed in a Saturn 2000 GC-MS (Varian Associates, Inc., Walnut Creek, CA, USA) equipped with a split/splitless injector. Saturn workstation 5.5 software was used for analyte detection and integration. The temperature programme was: 30°C (hold 5 min), 30°C min^{-1}, 200°C (hold 10 min). The split vent was opened after 7 min to give a split ratio of 100 and closed again after a further 3 min. Carrier gas flow rate was as follows: carrier He, 1·0 ml min^{-1} at a head pressure of 25 psi. MS transfer line temperature was 260°C. The mass spectrometer was tuned using the autotune procedure,

and masses from *m/z* 33 to 400 were recorded after electron impact ionization under EI auto mode. Thermal desorption of all samples in splitless mode took place in the injector port at 250°C for 1 min. Authenticated standards (>98% purity) were purchased from Sigma-Aldrich (St Louis, MO, USA). The alcohols tested were 1-butanol, 1-propanol, isobutanol, butyric acid and 2-methyl-1-butanol. A standard solution of a mixture of the above alcohols in milliQ water was prepared, in which the final concentration of each volatile compound was 7 mg l^{-1}. A volume of 2 ml of the mixture was put in a 4-ml vial with a PTFE/silicone screw-top cap (Supelco) and absorption of the volatiles was performed as described above. Analytes were identified by comparison of their mass spectra against a commercial spectral database (NIST98) and the authenticated standards.

Statistical analyses

The time of onset of gas production was defined as the day the packs had score distension status of >1. In the case of packs that did not acquire score >1 over the course of the experiment, the time of onset of gas production was given as 100 days. The time of onset of gas production (days) from five replicates of each meat type/strain/inoculum level/storage temperature was analysed using GenStat ver. 11.1 (VSN International Ltd, Hemel Hempstead, UK). All individual and pooled data failed the normality tests and were analysed using non-parametric methods. In particular, meat-type data (beef and lamb) were analysed using the Mann–Whitney *U* (Wilcoxon rank-sum) test. The Kruskal–Wallis one-way analysis of variance was used to determine the effect of inoculum levels (the null hypothesis being that inoculum levels were equal) and the storage temperatures (the null hypothesis being that storage temperatures were equal) on the time of onset of gas production. Whenever the null hypothesis was rejected, factors (inoculum levels and storage temperatures) were subjected to multiple comparison tests.

Results

Uninoculated controls were negative for gas production throughout the experiment with a score of ≤1. There was no significant difference on the times of onset of gas production between beef and lamb samples ($P > 0{\cdot}05$), for any of the strains. Therefore, data obtained from beef and lamb samples were pooled to give 10 replicates (five beef and five lamb) for each strain.

The time of onset of gas production (days) for each strain, spore/cell level and storage temperature, is shown in Table 2.

Table 2 The time of onset of gas production (days) for each *Clostridium* spp./*Bacteroides* sp. at various spore/cell levels and storage temperatures

		Temperature (°C)		
Micro-organism	CFU cm^{-2}	−1·5	1	4
Clostridium	<10	52 ± 7	34 ± 6	28 ± 7
estertheticum ssp.	10	47 ± 2	27 ± 8	22 ± 5
estertheticum	10^2	40 ± 5	23 ± 7	20 ± 3
	10^3	35 ± 5	20 ± 6	14 ± 3
Cl. estertheticum ssp.	<10	63 ± 6	46 ± 5	36 ± 2
laramiense	10	58 ± 3	38 ± 5	33 ± 3
	10^2	52 ± 4	34 ± 6	32 ± 5
	10^3	47 ± 6	31 ± 6	23 ± 4
Clostridium gasigenes	<10	>100	72 ± 11	56 ± 4
	10	>100	49 ± 22	50 ± 3
	10^2	>100	49 ± 20	42 ± 10
	10^3	>100	36 ± 6	35 ± 6
TC1	<10	99 ± 3	71 ± 10	69 ± 19
	10^1	98 ± 5	63 ± 15	66 ± 20
	10^2	98 ± 5	50 ± 14	44 ± 10
	10^3	88 ± 15	42 ± 9	38 ± 7
Bacteroides	<10	>100	74 ± 15	81 ± 6
propionicifaciens	10	>100	61 ± 13	63 ± 13
	10^2	98 ± 5	53 ± 7	42 ± 4
	10^3	92 ± 12	47 ± 7	39 ± 5
Clostridium sp. CYP7	<10	>100	95 ± 12	75 ± 17
	10	>100	92 ± 15	65 ± 14
	10^2	>100	82 ± 12	61 ± 16
	10^3	>100	77 ± 14	54 ± 12
Clostridium	<10	>100	94 ± 13	95 ± 10
thiosulfatireducens	10	>100	94 ± 13	94 ± 13
MG-2	10^2	>100	89 ± 23	81 ± 25
	10^3	>100	82 ± 24	70 ± 23
Clostridium botulinum	<10	>100	>100	88 ± 25
type E	10	>100	>100	88 ± 25
	10^2	>100	>100	85 ± 23
	10^3	99 ± 4	94 ± 14	61 ± 23
Clostridium sp. V13	<10	>100	91 ± 19	92 ± 14
	10	>100	90 ± 22	82 ± 24
	10^2	98 ± 8	76 ± 19	77 ± 26
	10^3	89 ± 23	61 ± 22	73 ± 29
Cl. estertheticum ssp.	<10	57 ± 1	48 ± 6	42 ± 8
*laramiense**	10	54 ± 2	44 ± 8	37 ± 5
	10^2	49 ± 1	36 ± 8	34 ± 5
	10^3	43 ± 3	32 ± 7	27 ± 10

Meat samples were inoculated with either spore suspensions of clostridial species or vegetative cells of *Bact. propionicifaciens*.
*Teagasc, isolate E324. All data generated are by means of 10 replicates.

Regardless of the storage temperature and inoculum level, the fastest gas producer was *Cl. estertheticum* ssp. *estertheticum*, followed by *Cl. estertheticum* ssp. *laramiense* DSMZ 14864^T and E324. It was generally noted that these strains produced large volumes of gas (e.g. score ≥4). On

the other hand, *Cl. gasigenes*, TC1 and *Bacteroides propionicifaciens* produced smaller volumes of gas and never reached the tightly blown status (i.e. score 4 or above). The rest of the bacteria did not produce gas within the commercial holding period (42 days) (Table 2). *Clostridium bowmanii* produced no gas (score ≤ 1) throughout the experiment.

Regardless of the inoculum levels, the effect of storage temperature on the time of onset of gas production was significant ($P < 0{\cdot}001$) for all strains examined. At −1·5°C, only packs inoculated with *Cl. estertheticum* ssp. *estertheticum* scored >1 within 42 days (the expected arrival time at the overseas marketplace). At 1°C, *Cl. estertheticum* ssp. *estertheticum*, *Cl. estertheticum* ssp. *laramiense* DSMZ 14864, E324, *Cl. gasigenes* and TC1 scored >1 within 42 days. At 4°C, *Cl. estertheticum* ssp. *estertheticum*, *Cl. estertheticum* ssp. *laramiense* DSMZ 14864, E324, *Cl. gasigenes*, TC1 and *Bact. propionicifaciens* scored >1 within 42 days. The onset of gas production for all strains (apart for *Cl. botulinum* type E) was significantly faster when samples were stored at 1°C rather than −1·5°C ($P < 0{\cdot}01$), and significantly faster for all microorganisms when stored at 4°C, rather than −1·5°C ($P < 0{\cdot}001$). The onset of gas production was significantly faster at 4°C, rather than 1°C for *Cl. estertheticum* ssp. *estertheticum*, *Cl. estertheticum* ssp. *laramiense* (DSMZ 14864^T and E324), *Clostridium* sp. CYP7 and *Cl. botulinum* type E ($P < 0{\cdot}05$).

The effect of inoculum level on the time of onset of gas production was significant ($P < 0{\cdot}05$) for all bacteria tested. Multiple comparison tests between pairs of spore/cell levels showed that differences between levels <10 and 10^2 were significant for all bacteria except *Clostridium thiosulfatireducens* MG-2 and *Cl. botulinum* type E ($P < 0{\cdot}05$). The time of onset of gas production was significantly different between levels <10 and 10^3 ($P < 0{\cdot}05$) or 10 and 10^3 ($P < 0{\cdot}05$) for all strains. For most strains, the time of onset of gas production was not significantly different, when pairs of inoculum levels that differed by one log (i.e. <10 and 10, 10 and 10^2 or 10^2 and 10^3) were compared.

The onset of BPS was not significantly different ($P > 0{\cdot}05$) between *Cl. estertheticum* ssp. *laramiense* DSMZ 14864 and *Cl. estertheticum* ssp. *laramiense* E324. However, combinations between *Cl. estertheticum* ssp. *estertheticum* DSMZ 8809 and *Cl. estertheticum* ssp. *laramiense* DSMZ 14864 or between *Cl. estertheticum* ssp. *estertheticum* DSMZ 8809 and *Cl. estertheticum* ssp. *laramiense* E324 showed that there was a significant difference between them ($P < 0{\cdot}001$).

The spoilage profile of *Cl. estertheticum* ssp. *estertheticum*, the fastest gas producer among the bacteria under investigation, is shown in Fig. 1. The distension status

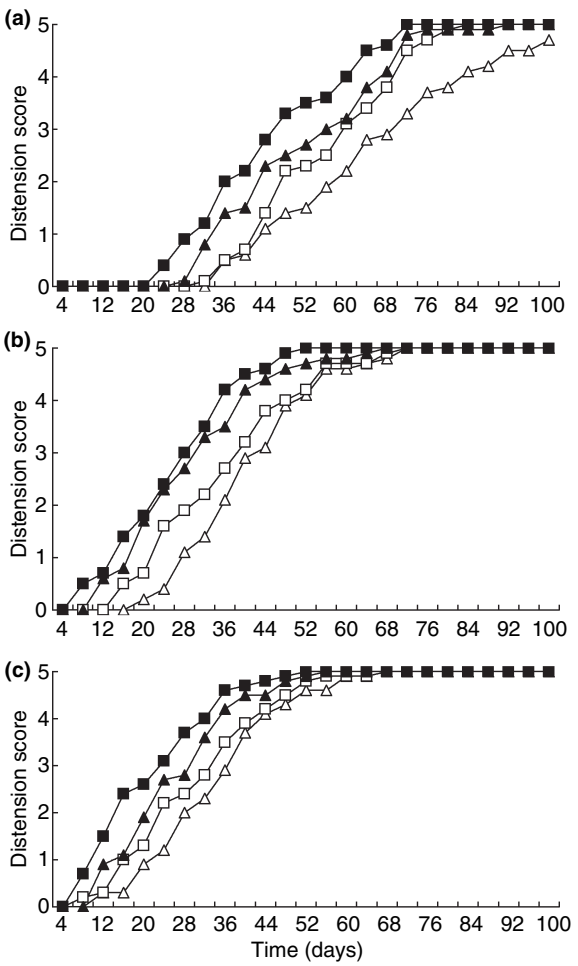

Figure 1 Distension status over time (days) of vacuum packs inoculated with spores of *Clostridium estertheticum* ssp. *estertheticum* (DSMZ 8809) and stored at −1·5°C (1a), 1°C (1b) and 4°C (1c). (■) 10^3 CFU cm^{-2}; (□) 10^2 CFU cm^{-2}; (▲) 10^1 CFU cm^{-2} and (Δ) <10^1 CFU cm^{-3}.

was calculated by means of 10 replicates (five beef and five lamb samples). The day of the initiation of the spoilage (i.e. score > 0) varied; packs maintained scores of 0 for periods of between 8 and 36 days, depending on the storage temperature and inoculum level. However, all packs except those inoculated with <10 spores stored at −1·5°C were scored at 5 (fully distended) within the maximum (100 day) storage period.

The results of the headspace analyses are presented in Table 3. Gas analysis was not performed for *Clostridium* sp. V13 and *Cl. bowmanii* because they scored <3 for all spore levels/storage temperatures. All other packs contained CO_2, H_2N_2 and butyric acid. Butanol was detected in all packs except those inoculated with *Bact. propionicifaciens*. Various other volatile compounds were detected

Table 3 Detection of gases and volatile compounds inside the blown packs inoculated with *Clostridium* spp. and a *Bacteroides* sp. after storage at 1°C for a maximum of 100 days

Compound	1	2	3	4	5	6	7	8	9
1-butanol	+	+	+	+	−	NT	+	+	+
1-propanol	+	+	+	+	+	NT	+	−	+
Isobutanol	+	+	+	+	−	NT	+	−	+
Butyric acid	+	+	+	+	+	NT	+	+	+
2-methyl-1-butanol	+	+	+	+	−	NT	+	−	+
CO_2 (%)	60·3 ± 5	60	54·5	56·4 ± 1·9	63·4 ± 6·3	58	45·7	66·7	64
H_2 (%)	13·9 ± 1·1	18	19·5	15·3 ± 4·3	14 ± 3·3	17·5	15·8	13·2	15
N_2 (%)	1·6 ± 1·2	5	1·2	1·2 ± 0·3	1·6 ± 0·8	0·8	9·1	0·71	1·1
H_2S	+	+	+	v	v	+	+	−	+

1, *Clostridium estertheticum* ssp. *estertheticum*; 2, *Cl. estertheticum* ssp. *laramiense*; 3, *Clostridium gasigenes*; 4, TC1; 5, *Bacteroides propionicifaciens*; 6, *Clostridium* sp. CYP7; 7, *Clostridium thiosulfatireducens* MG-2; 8, *Clostridium botulinum* type E; 9, *Cl. estertheticum* ssp. *laramiense* (E324); +, present; −, absent or below 7 mg l^{-1}; v, variable; NT, not tested.

in packs with *Cl. estertheticum*, *Cl. gasigenes*, TC1 and *Cl. thiosulfatireducens*.

Discussion

Of the bacteria tested, *Cl. estertheticum* ssp. *estertheticum* was capable of causing BPS within the shortest time. The fact that this strain is a true psychrophile, with an optimum growth temperature as low as 6°C and unable to grow above 13°C, may explain its ability to grow and spoil meat, at chill temperatures, faster than the other bacteria. Alternatively, the other subspecies of the same organism, *Cl. estertheticum* ssp. *laramiense*, caused BPS significantly slowly. This finding indicates that based on the onset of BPS, these two organisms are different. This is in agreement with Spring *et al.* (2003) who differentiated them based on phenotypic and genotypic characterization. Other findings (Yang *et al.* 2009), however, suggest that the organisms are indistinguishable between each other and that the current classification needs to be re-assessed. Further investigation should be undertaken to define the true relationship between them at species or subspecies level.

The present study showed that apart from *Cl. estertheticum* and *Cl. gasigenes*, which are known BPS bacteria (Dainty *et al.* 1989; Kalchayanand *et al.* 1993; Broda *et al.* 2000), several other organisms are able to produce gas in VP chilled meats. However, spoilage by *Clostridium* sp. CYP7, *Cl. thiosulfatireducens* MG-2, *Cl. botulinum* type E and *Clostridium* sp. V13 occurred at later times (e.g. later than 54 days). Therefore, these strains are not considered as significant BPS bacteria. On the other hand, the study highlights the commercial importance of the BPS strain TC1, which causes BPS within the commercial holding period of VP meats, i.e. 42 days. The gas and volatile compound composition in packs inoculated with this organism resembled the typical mixtures of the clostridial BPS, containing mainly CO_2, H_2 and butanol (Dainty *et al.* 1989; Broda *et al.* 2000). Additionally, *Bact. propionicifaciens* (B300) caused spoilage at 1°C within 47 days and produced H_2S in some packs, which was unexpected because the type strain (DSM 19291) was not able to grow below 5°C or produce H_2S (Ueki *et al.* 2008). This indicates the need for further investigation of intraspecies differences between B300 and DSM 19291. Although *Bacteroides* can be found on cattle (Richards *et al.* 1980; Fogarty and Voytek 2005), there is no indication that they can reside on the derived meats and meat products. However, as demonstrated in the present study, the spoilage potential of *Bacteroides* on meats can be significant and requires further investigation.

In general, higher storage temperatures led to more rapid onset of BPS. For *Cl. estertheticum*, *Clostridium* sp. CYP7 and *Cl. botulinum* type E, differences between −1·5 and 1°C, −1·5 and 4°C, and 1 and 4°C were significant. Therefore, for these organisms, an increase in storage temperature by a few degrees above −1·5°C significantly accelerated the time of onset of gas production. In order to minimize BPS associated with these micro-organisms, the storage temperature should be kept as low as possible and the tolerance for temperature elevation above −1·5°C should be small. For the rest of the micro-organisms, differences between −1·5 and 1°C or −1·5 and 4°C were significant. However, an increase of the storage temperature from 1 to 4°C did not significantly affect the time to spoilage. This lack of a relationship between storage temperature and the time of onset of BPS for some micro-organisms has been previously reported (Bell *et al.* 2001; Boerema *et al.* 2007).

The inoculum level significantly affected the time of onset of gas production for all strains. Previous investigations on the effects of inoculum level on *Listeria* (Bidlas

et al. 2008), *Clostridium sporogenes* (Ahn and Balasubramaniam 2007) or nonproteolytic *Cl. botulinum* types B and E (Jensen *et al.* 1987) have suggested that inoculum level had a direct influence on growth. With regard to spoilage by *Cl. estertheticum*, Boerema *et al.* (2007) demonstrated that inoculum level significantly influenced the time of onset of BPS in VP chilled beef. In the present study, inoculum levels as low as <10 spores per cm^2 were sufficient to produce gas within 100 days at 1 or 4°C after inoculation with all gas-producing organisms. A one log increase in inoculum level (from <10 to 10, from 10 to 10^2, or from 10^2 to 10^3 CFU cm^{-2}) did not significantly change the time of onset of BPS for most of the organisms tested. This indicates that for each holding temperature, the effect of inoculum level on the time of onset of BPS could in future be assessed using only two inoculum levels, low and high, with more than one log difference between them.

This study demonstrated the potential of several gas-producing bacteria to spoil VP meat under commercial processing and storage conditions, i.e. heat shrinkage of packs after vacuum packing and chilled storage temperatures. The time of onset of BPS was related to initial contamination level on meat and the storage temperature, but not to meat type. In most cases, spoilage generally occurred at low levels (<10 or 10 CFU cm^{-2}). Storage of heavily contaminated meat at lowest possible temperatures can significantly delay the time of onset of spoilage and extend the shelf life of the products beyond the usual 42-day commercial holding period. However, this strategy is not applicable where product is heavily contaminated with *Cl. estertheticum* ssp. *estertheticum*. Even at −1·5°C, this organism causes BPS within 42 days and remains a major meat quality issue. Therefore, a better strategy should focus on minimizing contamination below the level of $10–10^2$ CFU cm^{-2} of meat surfaces. This could, for example, be achieved by cleaning the surfaces and equipment in boning halls and abattoirs with a sporicidal agent such as peroxyacetic acid (e.g. on a monthly basis). Such cleaning would minimize the contamination levels from both spore and nonspore forming bacteria, which gain access on meat and meat products during commercial processing.

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