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Enumeration of *Clostridium estertheticum* Spores in Samples from Meat Plant Conveyors and Silage Stacks by Conventional and Real-Time PCR Procedures

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Abstract: A real-time PCR procedure for enumeration of spores of *Clostridium estertheticum*, the organism that causes blown pack spoilage of vacuum packaged chilled raw meats was developed. The relationship between cycle threshold (Ct) values and log numbers of spores in samples from which DNA was extracted was linear in the range 3 to $3x10^5$ spores (R^2>0.99). Samples collected from conveyor belts for primal cuts at a beef packing plant and from silage stacks were processed for enumeration of *C. estertheticum* spores by cultivation and real-time PCR. The organism was not recovered from any samples using culture method; but PCR results indicated that spores were present in 35 of 63 samples from conveyor belts and 4 of 38 silage samples, at numbers between 3 and 108/sample and 5 and 119/g, respectively.
Key words: *Clostridium estertheticum*, spore enumeration, meat conveyors, silage

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Introduction

Spoilage of vacuum packaged raw meats by *Clostridium estertheticum*, *Clostridium gasigenes* or *Clostridium frigidicarnis* is accompanied by gas production which causes swelling of packs (Broda et al. 1999; 2000). However, only *C. estertheticum* has been found to cause gross swelling of vacuum packs at early times during storage at well maintained chiller temperatures (Moschonas et al. 2010). That is apparently the type of blown pack spoilage that is seen most commonly in vacuum packaged raw meats (Yang et al. 2009a).

Recovery of *C. estertheticum* from exudate or meat in blown packs, by direct plating or after enrichment of samples for psychrotolerant spore forming anaerobes is difficult and uncertain (Byrne et al. 2009). PCR methods have then been shown to be more reliable for detection of *C. estertheticum* in sample preparations or enrichment broths (Helps et al. 1999; Broda et al. 2003). Thus, in a study of the presence of *C. estertheticum* in samples obtained from carcasses and equipment at, and the environment of a meat packing plant, isolates were recovered from some faecal but few of other types of samples (Broda et al. 2002). In contrast, when enrichment cultures of samples from packing plants in New Zealand and Ireland were tested for the presence of *C. estertheticum* by PCR procedures the organism was detected in substantial fractions of the samples from faeces, hides, lairages and the carcass dressing facilities (Boerema et al. 2003; Broda et al. 2009; Moschonas et al. 2009). However, the organism was detected in few samples from carcass breaking facilities.

The time required for blown pack spoilage caused by *C. estertheticum* to become apparent is dependent on the storage temperature and the initial numbers of the organism on the product (Moschonas et al. 2010). PCR procedures for enumeration as well as detection of *C. estertheticum* in samples from meat plants are then desirable, to allow the risks of blown pack spoilage developing in product to be assessed from tests of samples obtained at packing plants.

Although a semiquantitative method for enumerating *C. estertheticum* spores from PCR products was described by Helps et al. (1999), such methods have been superseded by quantitative PCR. Therefore a quantitative PCR procedure for enumeration of *C. estertheticum* spores was developed.

The quantitative PCR and direct plating procedures were used for the enumeration of *C. estertheticum* spores recovered from beef packing plant conveyors and silage stacks. The conveyors were sampled at a plant that had previously experienced incidents of blown pack spoilage. The numbers of spores recovered from the conveyors could be expected to reflect the numbers present on the meat at the time of packaging (Gill et al. 2001). Samples from silage stacks were sampled because *C. estertheticum* ferments sugars and lactic acid similarly to *Clostridium tyrobutyricum*, the organism that causes late blowing of cheese (Le Bourhis et al. 2007; Yang et al. 2009b). The origins of the *C. estertheticum* spores found in the faeces and on the hides of slaughtered animals are not well understood. However, a major source of the *C. tyrobutyricum* found in milk is the silage fed to animals (Julien et al. 2008). It therefore seemed possible the silage might be similarly important as a source of *C. estertheticum* on vacuum packed beef derived from animals held in feed lots for fattening before slaughter.

Materials and Methods

Preparation of spore suspensions. *C. estertheticum* ATCC 51377 was grown in Hungate tubes, in media that were prereduced by flushing with an oxygen free mixture of 80% N_2, 10% CO_2 and 10% H_2 (Air Liquide Canada Inc., Red Deer, Alberta, Canada). Cultures grown in Reinforced Clostridial Medium (RCM; Oxoid, Mississauga, Ontario, Canada) at 10 ℃ for 3 days were used to inoculate Peptone Yeast Extract Glucose Starch broth (PYGS), prepared in accordance with Lund et al. (1990). PYGS cultures were incubated at 10 ℃ for up to 5 weeks. During the fourth week of incubation, wet mounts of the cultures were examined daily under phase contrast illumination. Spores were harvested when spores were observed in ≥ 50% of the cells.

To prepare spore suspensions, each sporulating culture was centrifuged at 7500 x g for 20 min. The pelleted cells were washed four times by resuspension in 10 ml of ice-cold 0.85% (w/v) NaCl and centrifugation as before. After washing the pellet was again suspended in 10 ml of NaCl solution, and the suspension was subject to three rounds of sonication for 5 min then washing, as before. Suspensions were sonicated using a Model 100 Sonic Dismembrator (Fisher Scientific, Ottawa, Ontario, Canada). The pellet obtained after the final sonication treatment was resuspended in 5 ml of NaCl solution, and the suspension was stored at -80 ℃ until used.

To determine the number of spores in each suspension, serial ten-fold dilutions of the suspension in NaCl solution to 10^{-6} were prepared. The numbers of spores in appropriate dilutions were counted by placing 10 µl of each dilution in a Petroff-Hausser counting chamber (Model 3900; Hausser Scientific, Horsham, PA, USA) and viewing the spores under phase contrast illumination with 400 x magnification. The spores in dilutions containing between 100 and 200 spores in 16 squares, each measuring 50 x 50 µm, were counted.

In addition, appropriate dilutions of spore suspensions were incubated at 80 ℃ for 10 min to activate spores. Triplicate plates of Columbia Blood Agar (CBA) were spread with 0.1 ml portions of the activated spore suspensions, and the plates were incubated at 10 ℃ for 3 weeks under anaerobic atmospheres generated using BBL GasPak™ anaerobic System Envelopes (Becton Dickinson, Sparks, MD, USA). Colonies on plates bearing between 25 and 250 colonies were counted.

Extraction of DNA from *C. estertheticum* spores. For removal of extraneous DNA, 1 ml portions of spore suspensions which contained about 10^9 spores/ml were centrifuged at 7500 x g for 20 min. Each pellet was resuspended in 500 µl of a lytic solution containing lysozyme (Sigma-Aldrich, Oakville, Ontario, Canada) at 10 mg/ml and DNase I (Sigma-Aldrich) at 2 mg/ml. After incubation of the suspension at 37 ℃ for 1 h, 10 mg of trypsin (Sigma-Aldrich) was added and the suspension was incubated for a further 1.5 h. The enzymes were inactivated by incubating the suspension at 80 ℃ for 10 min, then the spores were washed three times by resuspension in sterile distilled water and centrifugation.

The washed spores were resuspended, and incubated at 37 ℃ for 1 h in a solution containing 8 mol l^{-1} urea and 40% (w/v) sodium thioglycolate, then washed three times by centrifugation and resuspension in distilled water, to remove spore coats (Durban et al. 1974). DNA was extracted from the decoated spores using the DNeasy Blood and Tissue Kit (Qiagen, Mississauga, Ontario, Canada), by the method for Gram positive bacteria specified by the manufacturer, with some modification. Briefly, the spores were resuspended in 180 µl of lysis buffer which was comprised of 20 mmol l^{-1} Tris-HCl buffer, pH 8.0, supplemented with 2 mmol l^{-1} EDTA, 1.2% (v/v) Triton X-100 and lysozyme at 20 mg/ml. The suspension was incubated at 37 ℃ for 2 h, then 25 µl of the proteinase K solution from the kit was added. After incubation at 56 ℃ for 30 min, 200 µl of 99% ethanol was added and the resulting mixture was loaded to a mini spin column. After column cleaning according to the manufacturer's instructions, DNA was eluted with two 100 µl volumes of AE buffer containing 10 mmol l^{-1} Tris-HCl and 0.5 mmol l^{-1} EDTA, pH 9.0. The concentration and purity of the extracted DNA was determined from the absorbances at 260 nm and 280 nm measured using a spectrophotometer

(Model ND-1000; Nano Drop Technologies, Wilmington, DE, USA). DNA preparations were stored at -20 ºC.

Real-time PCR procedure. The primers 16SEF (forward) 5'-TCG GAA TTT CAC TTT GAG-3' and 16SER (reverse) 5'-AAG GAC TTC ACT CAT CTC TG-3' described by Broda et al (2003), which yield a 790 bp amplicon were used for the assay. The PAGE purified primers from Sigma-Aldrich were suspended in sterile, Milli-Q water to make a 100 µmol l^{-1} stock solution. Primer concentration was optimized by varying both forward and reverse primer concentrations from 50 nmol l^{-1} to 600 nmol l^{-1}. The real-time PCR mix contained 12.5 µl of 2 x Brilliant II SYBR green master mix (Stratagene, La Jolla, CA, USA), appropriate amounts of the forward and reverse primers, 0.375 µl of the 5 µmol l^{-1} reference dye solution (Stratagene), and 5 µl of a *C. estertheticum* spore genomic DNA preparation with DNA at an appropriate concentration. For determination of the efficacy of removal of extraneous *C. estertheticum* DNA from spore preparations, 5 µl of an undiluted or diluted spore preparation was substituted for the DNA preparation. The volume of each reaction mixture was adjusted to 25 µl by adding sterilized DNA-free Milli-Q water. Real-time PCR was performed on an Mx3000P QPCR system (Stratagene) with one cycle of initial denaturation of template DNA and activation of Taq DNA polymerase at 95 ºC for 10 min, followed by 40 cycles of denaturation at 95 ºC for 30 s, primer annealing at 55 ºC for 1 min, and extension at 72 ºC for 1 min. Data acquisition was performed by MxPro™ software (Stratagene) at the end of each amplification step. To verify that formation of non-specific products or primer dimers had not occurred, the dissociation curves of final PCR products were analyzed from 55 to 95 ºC at 1 ºC intervals, and the products were subjected to electrophoresis on 1.5% (w/v) agarose gel at 90 V for 1.5 h. Purified *C. estertheticum* genomic DNA extracted from spores was used to determine the sensitivity and linearity of detection. Ten-fold serial dilutions of the DNA were prepared to obtain DNA preparations at concentrations that would give amounts of DNA in reaction mixtures that ranged from 0.0025 pg to 25 ng, inclusive. PCRs were performed in triplicate with each dilution.

Enumeration of spores in inoculated Meat Juice medium. Meat Juice Medium (MJM) was prepared by pummeling 100 g of extra lean ground beef with 100 ml of distilled water, in a stomacher bag fitted with a filter (Yang et al. 2009a). The fluid was withdrawn from the filter sleeve of the bag and further filtered through four layers of cheese cloth.

Ten-fold serial dilutions of a spore suspension were prepared to obtain suspensions containing spores at numbers ranging from 6 to 6 x 10^5 spores/ml. Six 500 µl volumes of each dilution were centrifuged at 16,000 x g for 2 min. Each pellet was resuspended in 5 ml of MJM. The MJM suspensions were centrifuged at 7,500 x g for 20 min. Each pellet was resuspended in 1 ml of distilled water and transferred to a 1.5 ml Eppendorf tube. The pellet was washed three times by centrifugation at 16,000 x g for 2 min and resuspension in water. DNA was extracted from the final pellets by the procedure described above.

Quantification of *C. estertheticum* spores in samples from conveyor belts and silage stacks. Samples from conveyor belts for primal cuts were collected at a beef packing plant where about 2000 carcasses are processed each day. Incidents of blown pack spoilage of product from the plant had occurred sporadically in some previous years. In years when blown pack spoilage had occurred, it had appeared in product that had been packed in the late summer and autumn. Therefore, samples were collected at the plant during the months of August, September and October.

The plant was visited once each month. Three samples were collected from each of seven conveyor belts in the carcass breaking facility during each visit. The surfaces of belts used in the preparation of primal cuts were sampled by swabbing areas of approximately 1000 cm^2 during breaks in periods of routine processing. Each sample was obtained by swabbing a belt with a 5 x 5 cm^2 cellulose acetate sponge (SpeciSponge; VWR Canlab, Mississauga, Ontario, Canada) that had been moistened with 10 ml of 0.1% (w/v) peptone water (Difco; Becton Dickinson). The sponges in separate plastic bags were stored on ice and processed within 24 h of being collected.

Each sponge was squeezed within its bag to expel fluid. All the expelled fluid was collected and centrifuged at 7,500 x g for 10 min. The pellet was resuspended in 1 ml of 0.85% NaCl solution. A 0.3 ml portion of the suspension was used for extraction of DNA from spores and quantification of *C. estertheticum* spores by real-time PCR as described above. The remaining suspension was incubated at 80 ºC for 10 min, then 0.1 ml portions of the heat treated suspension were spread on triplicate plates of CBA for recovery of psychrotolerant, spore forming anaerobes, as before but with incubation for 4 weeks.

Twenty samples, each of about 25 g, were collected from a silage stack at a feed lot about 2 km from the packing plant. The samples were collected from dry parts of the stack that remained from the year before. A further 18 samples were similarly collected from a silage stack at a feed lot remote from the plant. A 10 g portion of each sample was pummeled with 90 ml of 0.1% peptone water, using a stomacher. All the stomacher fluid was collected and centrifuged at 7,500 x g for 10 min. The resultant pellet was washed three times by resuspension in peptone water and centrifugation at 7,500 x g for 10 min. The final pellet was resuspended in 10 ml of peptone water, and 1 ml of the suspension was used for extraction of DNA from spores, as described above. The DNA preparation was treated for removal of PCR inhibitors using the Fast DNA Spin Kit for

Soil (MP Medicals, Solon, OH, USA) according to the manufacturer's instructions.

Psychrotolerant, anaerobic, spore forming bacteria from the final suspensions of pellets obtained from silage samples were recovered on triplicate plates of CBA by the methods described above, but with incubation of the plates at 4℃ for 4 weeks.

A colony that was grey, opaque, smooth, slightly raised and 1 to 2 mm in diameter, as are colonies of *C. estertheticum* on CBA (Spring et al. 2003) was picked from each plate bearing such colonies that had been spread with material from a silage sample. The colonies were each streaked on a plate of CBA that was incubated as before, to obtain isolated colonies. An isolate from each plate was inoculated into a Hungate tube containing RCM and a Durham tube, and a second Hungate tube containing PYGS. The tubes were incubated at 10 ℃ for three weeks. The tubes containing RCM were examined weekly for the appearance of gas in the Durham tubes. DNA was extracted from 1 ml of each PYGS culture, and the extracted DNA was tested for the presence of *C. estertheticum* DNA by real-time PCR, as before.

Data analysis. Regression lines were fitted to plots of PCR cycle threshold (Ct) values against log values for the picogram amounts of *C. estertheticum* DNA in reaction mixes or the log numbers of *C. estertheticum* spore from which DNA was extracted for inclusion in PCR reaction mixes. The correlation coefficients for the regression lines were calculated. A coefficient of variation for the Ct values obtained for PCRs performed with the same amount of *C. estertheticum* DNA or with DNA from the same number of spores was also calculated for each amount of the DNA or DNA from each number of spores used in reaction mixes. All calculations were performed using Microsoft Windows XP 5.1 statistical functions (Microsoft Corp., Redmond, WA, USA).

Table 1 Sensitivity, linearity and reproducibility of detection of spore DNA from *Clostridium estertheticum* spores using real-time PCR with SYBR green dye, as determined from three fold replications

DNA (pg/PCR)	Ct values								Coefficient of variation
	Rep 1		Rep 2		Rep 3		Overall		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
NTC	ND[a]		ND		ND		ND		-[b]
0.0025	ND		ND		ND		ND		-
0.025	33.24	0.12	33.26	0.11	32.85	0.08	33.11	0.23	0.70
0.25	29.86	0.09	30.10	0.10	29.73	0.07	29.90	0.18	0.63
2.5	26.08	0.08	26.13	0.03	26.02	0.06	26.07	0.06	0.21
25	22.78	0.13	22.67	0.09	22.94	0.13	22.80	0.14	0.60
250	18.86	0.2	18.78	0.06	18.85	0.02	18.83	0.04	0.23
2500	15.33	0.14	15.16	0.05	16.01	0.10	15.50	0.45	2.90
25000	12.46	0.09	12.50	0.05	12.60	0.05	12.52	0.07	0.58
	Slope		Slope		Slope		Slope		
	-3.522		-3.554		-3.405		-3.494		
	Intercept		Intercept		Intercept		Intercept		
	27.58		27.63		27.476		27.56		
	R^2 0.999		R^2 0.998		R^2 0.999		R^2 0.999		

[a] Ct values>40.
[b] Not determined.

Results

Dissociation curve analysis of amplicons and gel electrophoresis of PCR products did not show any formation of primer dimers or non-specific amplification of DNA in the real-time PCR reactions (data not shown). When the threshold Ct values obtained with different primer concentrations were compared, Ct values were found to be reduced by between 3 and 4 cycles when primer concentrations were increased from 50 to 100 nmol l^{-1}, and by between 1 and 2 cycles when primer concentrations were increased from 100 to 200 nmol l^{-1}. Further increases in primer concentrations had no effect on Ct values. Therefore, a concentration of each primer of 200 nmol l^{-1} was used for all reaction mixes.

The detection limit for *C. estertheticum* spore DNA in the real-time PCR was 0.025 pg (Table 1). The relationship between log values for the amounts of DNA in reaction mixes and the Ct values obtained with real-time PCRs were

linear for amounts of DNA in the range 0.025 pg to 25 ng ($R^2 > 0.99$). Coefficients of variation for Ct values obtained in replicated PCRs were all < 3 and mostly ≤ 0.7.

The numbers of spores in suspensions as determined from counts of spores were each < 0.3 log units more than the corresponding numbers determined from counts of colonies recovered on CBA plates. For calibration of the real-time PCR with respect to the numbers of spores from which DNA was extracted for quantification, the number of spores in each suspension was taken as being the mean of the numbers determined from the spore and colony counts (Herman et al. 1995).

Table 2 Sensitivity, linearity and reproducibility of detection by real-time PCR with SYBR green dye, of DNA extracted from spores of *Clostridium estertheticum* suspended in water or a meat juice medium, as determined from six fold replications

Log number of spores	Water			Meat Juice		
	Ct values		Coefficient of variation	Ct values		Coefficient of variation
	Mean	SD		Mean	SD	
0.48	35.17	0.01	0.02	35.58	0.20	0.58
1.48	33.06	0.04	0.11	33.43	0.13	0.38
2.48	29.79	0.22	0.74	29.30	0.30	1.01
3.48	25.56	0.63	2.46	25.58	0.08	0.31
4.48	21.90	0.61	2.77	22.04	0.29	1.29
5.48	17.94	0.68	3.78	18.47	0.66	3.54
	Slope				Slope	
	-3.538				-3.527	
	Intercept				Intercept	
	37.765				37.898	
	R^2 0.991				R^2 0.996	

No Ct value≤40 was obtained when 5 µl volumes of spore suspensions containing ≤ $3x10^6$ spores/ml were substituted for DNA preparation in PCR reactions. The detection limit for *C. estertheticum* spores suspended in either water or MJM, by PCR with extracted DNA, was three spores (Table 2). The relationship between the log number of spores from which DNA was extracted and the Ct values obtained with real-time PCR was linear for numbers of spores in the range 3 to 3 x 10^5 ($R^2 > 0.99$). The coefficients of variation for six fold replications of PCRs with DNA extracted from the same number of spores ranged from 0.1 to 3.8.

Spores of *C. estertheticum* were detected by real-time PCR in about half the samples collected each month from conveyor belts (Table 3). The Ct values indicated that, with one exception, the numbers of spores recovered from the samples were < 100 spores 1000 cm^{-2}. No colonies were obtained on the CBA plates spread with heat treated fluid from the samples and incubated for recovery of psychrotolerant anaerobes.

C. estertheticum spores were detected by real-time PCR in only four of the 38 silage samples, at numbers of 5, 6, 19 and 119 spores g^{-1}. However, colonies of psychrotolerant anaerobes were found on CBA plates spread with heat treated fluids from 35 of the samples. The numbers of colonies on the plates showed that the numbers of psychrotolerant, spore-forming anaerobes in the silage samples that yielded such organisms ranged from 10 to > 10^5 cfu g^{-1}. None of the 26 isolates tested by PCR were *C. estertheticum.* However, 15 of the isolates produced gas. Three isolates grew without gas production, and eight did not grow in PYGS.

Table 3 Numbers of spores, as determined by real-time PCR, in 21 swab samples of 1000 cm^2 areas of conveyor belts used with primal cuts at a beef packing plant, collected during one day in each of three months

Month	Number of samples				Total No. of spores
	<3 spores	>3-9 spores	10-99 spores	≥ 100 spores	
August	8	4	8	1	366
September	9	2	10	0	348
October	11	2	8	0	282

Discussion

The primers used for the PCR were shown to be specific for *C. estertheticum* by Broda et al. (2003), and have been used by those and other workers to confirm isolates as *C. estertheticum*, or to detect the organism in enrichment cultures (Boerema et al., 2003; Moschonas et al., 2009). The primers are for amplification of sequence of the 16S rRNA gene of *C. estertheticum*. The detection of DNA from as few as three spores was therefore not wholly surprising, because copy numbers of the 16S rRNA gene in other Clostridium spp. listed in the ribosomal RNA operon copy number data base (http://ribosome.mmg.msu.edu/rrndb/search.php) are most between 7 and 14. The procedures identified in this study for removal of extraneous DNA, extraction of DNA from spores of *C. estertheticum* and quantification of the extracted DNA by real-time PCR gave results that were highly reproducible. Because the vegetative cells of *C. estertheticum* are highly sensitive to oxygen, viable *C. estertheticum* that contaminate the surface of meat processing equipment and meat before packaging must necessarily be in the form of spores. Therefore the procedures used in the study can be used to quantify the contamination with *C. estertheticum* of meat plant environments and meat being prepared for vacuum packaging.

In four previous studies, in New Zealand and Ireland, of *C. estertheticum* contamination at venison, lamb and beef packing plants, samples were enriched for psychrotolerant clostridia. The enrichment broths were used for recovery of psychrotolerant clostridia and were tested for the presence of the organisms by PCR. In two such studies *C. estertheticum* was detected in faeces and on hides of slaughtered animals, but not in samples from carcass breaking facilities (Broda et al. 2002; Boerema et al. 2003). In two recent studies *C. estertheticum* was detected in samples from carcass breaking facilities, but in only 2% and 6% of the samples that were tested (Broda et al. 2009; Moschonas et al. 2009). The failure to recover *C. estertheticum* from conveyor belts in this study is agreeable with such findings. However, the detection of *C. estertheticum* by PCR in more than half the samples from conveyor belts for beef primals is obviously at variance with previous findings. The difference between the findings with cultivation or PCR evidently reflects the difficulties with recovery of *C. estertheticum* from meat and environmental samples.

The frequent presence of *C. estertheticum* on the conveyor belts and thus on product might suggest that blown pack spoilage of some vacuum packed product might develop relatively often. In fact, only one instance of blown pack spoilage is known to have occurred in the months during or after the sampling period. The spoiled product was packed at the end of August. The facility examined in this study might then have been no more heavily contaminated with *C. estertheticum* than facilities elsewhere. Instead it is possible that the method of testing used in this study is more sensitive than testing of enrichment cultures. That possibility will have to be investigated. Whether or not greater test sensitivity explains the findings of this study, it is evident that the contamination of equipment and meat with *C. estertheticum* at the low levels ($\leq 1/10$ cm^2) found in this study is not generally predictive for the development of blown pack spoilage in vacuum packaged product. However, the procedures used in this study might be applied for monitoring meat plant environments or meat being prepared for vacuum packaging, to detect higher levels of *C. estertheticum* that could indicate the possibility of extensive losses due to blown pack spoilage. Early recognition of such levels of contamination would allow initiation of corrective actions, and diversion of compromised product to freezing or use before blown pack spoilage could develop.

Although *C. estertheticum* was found in silage samples it was present as only a small fraction of the population of psychrotolerant, spore forming anaerobes. This would suggest that silage is not an important source of the *C. estertheticum* that can contaminate meat. However, silage stacks do not provide a uniform environment for bacteria, and numbers of clostridia can vary greatly within as well as between stacks (Julien et al. 2008; Vissers et al. 2007). As yet no study of the types and numbers of psychrotolerant clostridia found in silage has been reported. As some of the isolates from silage that were not *C. estertheticum* evidently produced gas, they may play some role in blown pack spoilage (Broda et al., 2002; Moschonas et al., 2009), further examination of the matter would seem to be required before conclusion can be drawn about whether or not silage is an important source of *C. estertheticum* or other psychrotolerant clostridia that may be involved in the spoilage of meat.

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