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An investigation of the ecological niches and seasonal nature of *Clostridium estertheticum* and *Clostridium gasigenes* in the Irish beef farm environment

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Running head: Blown pack spoilage *Clostridium* spp. on beef farms.

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Significance and impact of the study

Blown pack spoilage (BPS) of vacuum packaged beef caused by *Clostridium estertheticum* and *Clostridium gasigenes* is a significant problem for the beef industry. At present control is reliant on regular disinfection of the abattoir and equipment with sporicidal agents but it has been suggested that preventing contamination of cattle at the source could be more effective. This study provides evidence that BPS *Clostridium* spp. are widely distributed on beef farms with multiple dissemination routes, prohibiting on-farm control.

Abstract

Blown pack spoilage (BPS) of vacuum packaged beef is caused by psychrotolerant and psychrophilic *Clostridium* species (PPC), primarily *Clostridium estertheticum* and *Clostridium gasigenes*. The aim of this study was to investigate the environmental niches and impact of season on these BPS *Clostridium* spp. on Irish beef farms. On each of 5 different beef farms, faecal (10), soil (5), silage (5), bedding straw (5), drinking water (5), puddle/ditch water (5) and air (5) samples were collected during Spring, Summer, Autumn and Winter and tested for *C. estertheticum* and *C. gasigenes* using culture (direct plating and enrichment) and molecular, (conventional PCR and quantitative PCR (qPCR)), based techniques. *C. estertheticum* and *C. gasigenes* were detected in all sample types, with qPCR detection rates ranging from 4% to 50% and at concentrations of up to 1.5 $\log_{10}$ cfu g^{-1} and 3.5 $\log_{10}$ cfu g^{-1}, respectively. The impact of season was not clear as the results were mixed depending on the detection method used. It was concluded that BPS causing *C. estertheticum* and *C. gasigenes* are widely distributed in the beef farm environment.

Keywords: Blown pack spoilage, *Clostridium estertheticum*, *Clostridium gasigenes,* beef farms, sources.

Introduction

Spoilage of beef is usually caused by the proliferation of bacteria, which contaminate the surface of the meat before packaging (Gribble *et al*. 2014). Psychrotrophic and psychrophilic *Clostridium spp.* (PPC) mainly *Clostridium estertheticum* and *Clostridium gasigenes* cause spoilage of vacuum-packed red meat at correctly stored temperatures resulting in 'blowing' of the pack, usually with a foul odour, and a metallic sheen on the surface of the meat. The gas responsible for the 'blown pack' is predominantly carbon dioxide but often contains low levels of hydrogen sulphide (Moschonas *et al.* 2010). Spores of these bacteria contaminate the beef prior to vacuum packaging and even low initial concentrations may proliferate resulting in blown pack spoilage as early as 3 to 4 weeks (Boerema *et al.* 2007).

Blown pack spoilage (BPS) is a worldwide issue for the beef industry (Bolton, *et al.* 2015). The first confirmed case occurred in Ireland in 2009 (Byrne *et al.* 2009) and research since then has shown that approximately 1.5% of beef primals are contaminated with *C. estertheticum* and/or *C. gasigenes* (Bolton *et al*, 2015). Although not a food safety hazard, meat spoiled in this way has no commercial value. Data on the prevalence of BPS in Ireland is considered to be commercially sensitive, however, given there are approximately 1.5 million primals produced per year, a contamination rate of 1.5% would represent a significant potential financial loss to the beef sector (Bolton et al., 2015).

There are no standardised methods for the detection of *C. estertheticum* or *C. gasigenes*. Indeed isolation methods tend to be species-specific (Broda et al. 1998a,b). This has, at least in part, contributed to the inconsistent results previously reported (Broda et al. 2003). In recent years molecular methods have been developed and validated for testing environmental, abattoir and meat samples for these bacteria, targeting specific 16S rRNA fragments, which are more sensitive than culture based methods but do not necessarily indicate the presence of viable cells (Moschonas

et al., 2009). Previous studies have therefore used a combination of both culture and molecular based testing methods.

The farm environment is often the initial source of spoilage bacteria. *Carnobacterium spp*, for example, cause spoilage of lamb products, originate in soil, faeces and water troughs on sheep farms (Mills *et al.* 2018). While previous studies have investigated sources of BPS *Clostridium* spp. in beef abattoirs, similar data for beef farms is lacking (Broda *et al.* 1997; Broda *et al* .2003; Moschonas *et al.* 2009). The aim of this study was to investigate the environmental niches and impact of season on *Clostridium estertheticum* and *Clostridium gasigenes* on Irish beef farms.

Results and discussion

This study investigated the presence of *C. estertheticum* and *C. gasigenes* in faecal, soil, silage, bedding straw, drinking water, puddle and drinking water on Irish beef farms over the course of a year. Of the 800 samples tested only 1% (2 silage, 3 air, 2 bedding straw and 1 drinking water) were positive for *C. estertheticum* using peptone yeast extract glucose starch (PYGS) enrichment followed by conventional PCR (Table 1). In contrast, 10% of samples tested positive by direct qPCR (17 faeces, 15 soil, 9 silage, 8 air, 9 bedding straw, 8 drinking water and 14 puddle water samples), reflecting the increased sensitivity of the qPCR method (Reid et al., 2017). Approximately 1% of samples were positive by direct plating including 3 faecal, 1 soil, 1 silage and 4 puddle water samples and counts ranged from 1 to 1.5 $\log_{10}$ cfu g^{-1}.

C. gasigenes was more frequently detected with 7.6% (61/800) of samples testing positive by enrichment plus PCR (1 faecal, 3 soil, 1 silage, 15 air, 4 bedding straw, 18 drinking water and 19 puddle water samples), 39.6% (317/800) by direct qPCR (90 faeces, 36 soil, 38 silage, 37 air, 26 straw, 46 drinking water and 44 puddle water) and direct counts were obtained for 184 samples (42 faecal, 50 soil, 17 silage, 12 air, 36 straw, 6 drinking water and 42 puddle water samples) (Table 1). The counts ranged from 2.3 $\log_{10}$ cfu ml^{-1} in drinking water to 3.5 $\log_{10}$ cfu g^{-1} in soil and silage.

Up to 8.5% of faecal and 50% of soil samples were positive for *C. estertheticum* and *C. gasigenes*, respectively. Both have been previously reported to be sources of pathogenic and food spoilage bacteria on beef farms (Boerema *et al*, 2003; Brightwell *et al*, 2009). Soil in particular is a primary

reservoir for *Clostridium* spp., where the microflora and presence of water create an oxygen deprived environment that encourages the survival of *Clostridium* spores (Brightwell *et al*. 2009). Moreover, maintaining a low metabolic acitvity, dormant *Clostridium* spores, can exist in the soil microecosystem for an extended period of time (Heyndrickx, 2011). From a beef industry perspective, faeces and soil are important contaminants on cattle hides, as BPS *Clostridium* spores are readily transferred from soiled hides to beef carcasses during dehiding (Bell, 1997; Boerema *et al*. 2003; Moschonas *et al*. 2009).

C. *estertheticum* was detected in 2 to 9% of silage samples and 1 to 38% for *C. gasigenes* with counts of 1.5 $\log_{10}$ cfu g^{-1} for the former and 3.5 $\log_{10}$ cfu g^{-1} for the latter. *Clostridium* spores have previously been isolated from leaves on horticultural plants (Ercolani, 1997) and grass may be contaminated with *Clostridium* spores from the soil before harvesting and ensiling, an anaerobic process that creates an ideal environment conducive to the germination and outgrowth of *Clostridium* spores (Pahlow *et al*. 2015), especially if the fermentation process is delayed or reduced (Rammer, 1996).

The presence of *C. estertheticum* and *C. gasigenes* in up to 8% and up to 37% of air samples, respectively, at concentrations of up to 3.1 $\log_{10}$ cfu m^{-3}, suggests spores may be readily disseminated around the farm environment by air movements. Although little is known about air dispersal of *Clostridium* spores, air has been recognised as a source of contamination in the poultry farm environment (Skóra *et al*. 2016). The bedding straw was also contaminated with 2 to 9% (*C. estertheticum*) and 4 to 26% (*C. gasigenes*) of samples positive and counts of up to 3.2 $\log_{10}$ cfu g^{-1}. Although previous studies for *Clostridium* spp. are lacking, Magnusson *et al*. (2007) reported *Bacillus cereus* spores in bedding material on dairy farms. Almost half of water samples were positive for *C. estertheticum* and/or *C. gasigenes* by qPCR at direct plating counts of up to 2.9 $\log_{10}$ cfu ml^{-1} were obtained. Water has previously been reported as a source of *Clostridium* spores on farms (Broda *et al*. 2009).

Overall, it is easy to envisage the contamination cycle for *C. estertheticum* and *C. gasigenes* on beef farms. *Clostridium* spp. survive well in soil (Heyndrickx, 2001) which serves as a reservoir for BPS *Clostridium* spp. Silage (and the grass from which it is produced) and water are readily contaminated by soil bacteria (Ercolani, 1997; Mills et al., 2018) including *C. estertheticum* and *C. gasigenes*, at relatively high concentrations (3.0 $\log_{10}$ cfu g^{-1}/ml^{-1}). When ingested by cattle, these

survive passage through the gastrointestinal tract, are excreted in the faeces (Vissers et al., 2007), contaminating the environment (soil, grass, water, etc.) and completing the cycle. Furthermore, dissemination by air ensures these bacteria are not just widely disseminated within a given farm but are also spread from farm to farm.

Examination of the season distribution yielded mixed results. There was no significant difference in the prevalence of *C. estertheticum* obtained using enrichment plus PCR, regardless of season (Table 2). In contrast, direct qPCR provided significantly ($P < 0.05$) higher prevalence in Spring than in Autumn which using direct plating suggested there was significantly higher prevalence of *C. estertheticum* in Winter as compared to the other seasons. Season did not influence the prevalence of *C. gasigenes* when enrichment plus PCR or direct plating was used (Table 3). However, significantly higher prevalence was obtained in Summer versus Spring, Autumn versus Spring and Summer versus Winter when direct qPCR was used as the detection method. A higher Summer prevalence has been reported for both organisms in beef primals and farm samples (Jones *et al*. 2008; Moschonas *et al*. 2009) although Bolton *et al*. (2015) did not observe any seasonal effect in the prevalence on Irish beef primals. Regardless, the ubiquitous nature (soil, air and water) of *C. estertheticum* and *C. gasigenes* on beef farms and the extreme resistance of the spores to environmental stress (Vepachedu and Setlow, 2006) will ensure exposure of beef animals to these bacteria throughout the year.

It was concluded that BPS causing *C. estertheticum* and *C. gasigenes* are widely distributed in the beef farm environment throughout the year prohibiting on-farm control. High levels of BPS *Clostridium* in faeces and soil will ensure a steady source of these bacteria on carcasses if cross-contamination from the hide to the carcass is not carefully controlled in the abattoir, which, based on the information in this study, is the first line of defence in minimising the risk of BPS in the beef sector.

Materials and methods

Sample collection

Five beef farms in the northeast of Ireland were randomly selected from the Teagasc client farmers list and each farm was visited for sample collection once per season. A total of 40 samples were

collected per farm per visit as follows; faecal (10), soil (5), silage (5), bedding straw (5), drinking water (5), puddle/ditch water (5) and air (5) samples.

Fresh voided faecal samples were obtained from 10 different animals either in the field or the cattle shed, depending on the time of the year. Soil samples were taken from 5 different locations within a given field (entrance, beside the ditch and in the middle of the field) using sterile scoops. Silage samples (when available) were extracted from the silage pit or bags by hand using sterile gloves. As with the faecal and soil samples, these were stored in sterile 250 ml jars (VWR International, Dublin, Ireland). Bedding straw was collected, preferably from sheds with beef animals but from storage when this was not available. Drinking water samples (2 L) were collected directly from the source supplying the animals, while puddle (or if unavailable ditch) water samples of approximately 1 L were obtained throughout the farm. The air was sampled using Air Ideal 3P, supplied by BioMerieux (Craponne, France). A plate count agar (PCA) (Oxoid Ltd, Basingstoke, UK) plate was placed in the sampling holder and air drawn over this at a rate of 100 L per minute for 90 s. All samples were transported to the laboratory in a cool box at approximately 2°C for no more than 2 h followed by immediate processing.

Enrichment and direct plating

All sample processing was undertaken in an anaerobic chamber (Don Whitley Scientific Ltd, Shipley, UK). Exactly 10 g of faeces was added to 90 ml PYGS (Fannin Oxoid Ltd, Basingstoke, UK) enrichment broth. Exactly 25 g of soil, silage or bedding straw was added to 225 ml PYGS enrichment broth. Drinking water and puddle water samples were divided into 50 ml aliquots and centrifuged at 5000x g for 10 min. The resultant pellet was re-suspended in 10ml maximum recovery diluent (MRD) (Oxoid Ltd, Basingstoke, UK) before adding it to 90 ml PYGS. For air samples, agar from PCA plates were aseptically added to 90 ml PYGS. All samples supplemented with PYGS enrichment broth were thoroughly mixed. Immediately before incubation 5 ml of the PYGS suspension was transferred to 20 ml sterile polystyrene tubes (Sarstedt Ltd, Nümbrecht, Germany) to which 5 ml absolute ethanol (Emprove, Merck Production Chemicals, Frankfurt, Germany) was added and incubated at 4°C for 1 h to eliminate competitive microflora. Thereafter, 0.1 ml of the resultant suspension was plated on Columbia blood agar (CBA, Oxoid Ltd, Basingstoke, UK) and incubated anaerobically at 4°C for a further 3 weeks. A further 1 ml was

collected from the PYGS enrichment broth and transferred to eppendorf tubes for direct qPCR analysis. The remaining PYGS enrichment broths were also incubated anaerobically at the same temperature for a similar time after which 0.1 ml samples were plated on CBA and incubated as before. *C. estertheticum* colonies were round with often coarsely granulated margins, smooth, slightly raised, cream-white to greyish and semi-transparent to opaque and non-haemolytic, while, *C. gasigenes* colonies appeared as grey-white and opaque, circular, raised, convex, shiny, smooth and β-haemolytic colonies on CBA.

DNA extraction

DNA was extracted from the pre-incubated PYGS for qPCR and from both the post-incubation enrichment cultures and CBA plates streaked from these broths, for conventional PCR. Either 1ml of the enrichment mixture and/or 5 isolated colonies (as described above) on the CBA plates, were suspended in 1 ml phosphate buffered saline (PBS) (Oxoid Ltd, Basingstoke, UK) and centrifuged at 5000x g for 10 min. The pellet washed with 1 ml PBS and 10 mg ml^{-1} lysozyme was added before incubation for 30 min to 1 h at 37°C to lyse the cells. DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen Ltd, Crawley, UK) according to manufacturer's protocol for the isolation of Gram positive bacteria.

PCR

Type strains *Clostridium estertheticum subsp. estertheticum* (strain DSMZ 8809T, T = type strain) and *Clostridium gasigenes* (DSMZ 12272T) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and used as a positive control for PCR based methods. These reference strains were cultured anaerobically in pre-reduced peptone yeast extract glucose starch (PYGS) broth. All sterilized media were cooled and stored inside an anaerobic cabinet (Don Whitley Scientific Ltd, Shipley, UK), under an atmosphere of mixed gas, CO_2 and N_2 at 37°C and used within 48 h.

16SF and 16SER primers for the detection of 16s rRNA gene fragments of *C. estertheticum* and 16SDBF and 16DBR primers for the detection of 16s rRNA gene fragments of *C. gasigenes*

(Brightwell *et al*. 2009) were purchased from Tib Molbiol, Berlin, Germany. The PCR mix (Qiagen Ltd, Crawley, UK) consisted of 25 µl of Taq master mix, 0.5 µmol l^{-1} of each primer, 19 µl molecular-grade water and 5 µl template DNA making a final reaction volume of 50 µl. Amplification was performed in the Gradient Cycler DNA engine (MJ Research, Waltham, MA). Initial denaturation was done for 3 min at 93°C followed by denaturation for 1 min at 92°C, annealing for 1 min at 55°C and extension for 2 min at 72°C. A 7 µl aliquot of the PCR products was examined by electrophoresis on a 1.5% (w/v) agarose gel containing 0.5 mg ml^{-1} SYBR Safe (Invitrogen, Ireland) at 90 V for 1.5 h and visualised on an ultraviolet transilluminator. A 100 bp DNA ladder (Promega, Southampton, UK) was used to determine amplified product size.

Quantitative real time PCR (qPCR)

All real-time PCR assays were performed on the LightCycler 480 platform (LC480; Roche Diagnostics GmbH) using the method of Reid et al. (2017). TMF (forward primer), TMR (reverse primer) and Probe were used for the detection of *C. estertheticum*. 16SDB_for (forward primer), 16SDB-A (reverse primer) and 16sDB_TM probe was used for the detection of *C. gasigenes*. All primers and probes used in this method were purchased from Tib Molbiol, Berlin, Germany. For the *C. estertheticum* assay, qPCR was performed in a 10 µl reaction volume containing 0.3 µmol l^{-1} primer and 0.1 µmol l^{-1} probe, 2.8 µl H2O, 5 µl LightCycler 480 Probe master mix (2X) (Roche Diagnostics) and 1 µl of DNA to be tested. A positive and negative DNA control and no template control (NTC) were included in each qPCR run. The cycling protocol included a hot start of 95°C for 10 min, followed by 45 cycles (95°C for 10 s, 65°C for 30 s and 72°C for 1 s). For *C. gasigenes* the qPCR was performed in a 10 µl reaction volume containing 0.5 µmol l^{-1} primer and 0.2 µmol l^{-1} probe, 2.8 µl H2O, 5 µl LightCycler 480 Probe master mix (2X) (Roche Diagnostics) and 1 µl of DNA to be tested. A positive and negative DNA control and no template control (NTC) were included in each run. The cycling protocol included a hot start of 95°C for 10 min, followed by 45 cycles (95°C for 10 s, 62°C for 30 s and 72°C for 1 s).

Data analysis

Data on the number of positive samples using the different testing approaches were collated per sample type and per organisms (*C. estertheticum* and *C. gasigenes*). The prevalence (as provided in Table 1), were calculated as the number of positive samples over the total number of samples tested expressed as a percentage. To investigate if there was a seasonal effect, the mean number of positive samples for each sample type for a given combination of detection method and organism were analysed using a one-way analysis of variance (ANOVA). When statistical differences were detected, Tukey's post-hoc comparison test was used to measure differences between means. Differences were considered significant at the 5% ($P < 0.05$) level. The statistical package used was GraphPad Prism 7.02 (Graphpad Software Incorporated, San Diego, California, USA).

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Statement of contribution

Dr. Declan Bolton obtained the funding for this study. Ms. Eden Esteves undertook the laboratory work. All of the other authors made a substantial contribution to the conception and design of the study, analysis of the data and the preparation and reviewing of the manuscript. All of the authors have approved the final (submitted) version.

Conflict of interest

No conflict of interest declared.

References

Barnes, E. M. (1985) Isolation methods for anaerobes in foods. *Int J Food Microbiol* **2**, 81-87.

Bell, R. G. (1997) Distribution and sources of microbial contamination on beef carcasses. *J Appl*

Microbiol **82**, 292-300.

Boerema, J. A., Broda, D. M. and Bell, R. G. (2003) Abattoir sources of psychrophilic clostridia causing blown pack spoilage of vacuum-packed chilled meats determined by culture-based and molecular detection procedures. *Lett Appl Microbiol* **36**, 406-411.

Boerema, J. A., Broda, D., Penney, N. and Brightwell, G. (2007) Influence of peroxyacetic acid-based carcass rinse on the onset of "blown pack" spoilage in artificially inoculated vacuum-packed chilled beef. *J Food Prot* **70**, 1434-1239.

Bolton, D. J., Carroll, J. and Walsh, D. (2015) A four-year survey of blown pack spoilage *Clostridium estertheticum* and *Clostridium gasigenes* on beef primal cuts. *Lett Appl Microbiol* **61**, 153–157.

Brightwell, G., Broda, D. M. and Boerema, J. A. (2009) Sources of psychrophilic and psychrotolerant clostridia causing spoilage of vacuum-packed chilled meats, as determined by PCR amplification procedure. *J Appl Microbiol* **107**, 178-186.

Broda, D. M., De Lacy, K., Bell, R., Braggins, T. and Cook, R. (1996) Psychrotrophic *Clostridium* spp. associated with "blown pack" spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. *Int J Food Microbiol* **29**, 335-352.

Broda, D.M., De Lacy, K.M. and Bell, R.G. (1998a) Efficacy of heat and ethanol spore treatments for the isolation of psychrotrophic *Clostridium* spp. associated with the spoil-age of chilled vacuum-packed meats. *Int J Food Microbiol* **39**, 61–68.

Broda, D.M., De Lacy, K.M. and Graham Bell, R. (1998b) influence of culture media on the recovery of psychro-trophic *Clostridium* spp. associated with the spoilage of vacuum-packed chilled meats. *Int J Food Microbiol* **39**, 69–78.

Broda, D. M., De Lacy, K., Cook, R. and Bell, R. (1997) Prevalence of cold-tolerant clostridia associated with vacuum-packed beef and lamb stored at abusive and chill temperatures. *N Z J Agric Res* **39**, 61-68.

Broda, D.M., Boerema, J.A. and Bell, R.G. (2003) PCR detection of psychrophilic Clostridium spp. causing 'blown pack'spoilage of vacuum-packed chilled meats. *J Appl Microbiol* **94**, 515–522.

Broda, D., Boerema, J. and Brightwell, G. (2009) Sources of psychrophilic and psychrotolerant clostridia causing spoilage of vacuum-packed chilled meats, as determined by PCR amplification procedure. *J Appl Microbiol* **107**, 178-186.

Byrne, B., Monaghan, A., Lyng, J., Sheridan, J. and Bolton, D. (2009) A case of "blown pack" meat linked to *Clostridium Estertheticum* in Ireland. *J Food Saf* **29**, 629-635.

Ercolani, G. (1997) Note: Occurrence and persistence of culturable clostridial spores on the leaves of horticultural plants. *J Appl Microbiol* **82**, 137-140.

Gribble, A., Mills, J. and Brightwell, G. (2014) The spoilage characteristics of *Brochothrix thermosphacta* and two psychrotolerant *Enterobacteriacae* in vacuum packed lamb and the comparison between high and low pH cuts. *Meat Sci* **97**, 83-92.

Heyndrickx, M. (2011) The Importance of Endospore-Forming Bacteria Originating from Soil for Contamination of Industrial Food Processing. *Appl Environ Soil Sci* **2011**, 1-11.

Jones, R., Hussein, H., Zagorec, M., Brightwell, G. and Tagg, J. (2008) Isolation of Lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. *Food Microbiol* **25**, 228-234.

Magnusson, M., Christiansson, A. and Svensson, B. (2007) *Bacillus cereus* spores during housing of dairy cows: factors affecting contamination of raw milk. *J Dairy Sci* **90**, 2745-2754.

Mills, J., Horváth, K., Reynolds, A. and Brightwell, G. (2018) Farm and abattoir sources of *Carnobacterium* species and implications for lamb meat spoilage. *J Appl Microbiol* **125**, 142-147.

Moschonas, G., Bolton, D., Sheridan, J. and McDowell, D. (2009) Isolation and sources of blown pack spoilage clostridia in beef abattoirs. *J Appl Microbiol* **107**, 616–624.

Moschonas, G., Bolton, D., Sheridan, J. and McDowell, D. (2010) The effect of storage temperature and inoculum level on the time of onset of "blown pack" spoilage. *J Appl Microbiol* **108**, 532-539.

Pahlow, G., Muck, R.E., Driehuis, F., Elferink, S.J.W.H.O. and Spoelstra, S.F. (2015) Microbiology of Ensiling. In Silage Science and Technology ed. D.R. Buxton, R.E. Muck and J.H. Harrison. pp.31-93.

Rammer, C. (1996) Quality of grass silage infected with spores of *Clostridium tyrobutyricum*. *Grass Forage Sci* **51**, 88-95.

Reid, R., Burgess, C., McCabe, E., Fanning, S., Whyte, P., Kerry, J. and Bolton, D. (2017) Real-time PCR methods for the detection of blown pack spoilage causing *Clostridium* species; *C. estertheticum*, *C. gasigenes* and *C. ruminantium*. *Meat Sci* **133**, 56-60.

Skóra, J., Matusiak, K., Wojewódzki, P., Nowak, A., Sulyok, M., Ligocka, A., Okrasa, M., Hermann, J. and Gutarowska, B. (2016) Evaluation of microbiological and chemical contaminants in poultry farms. *Int J Environ Res Public Health* **13**, 192.

Vepachedu, V. and Setlow, P. (2006) Role of SpoVA proteins in release of dipicolinic acid during germination of *Bacillus subtilis* spores triggered by dodecylamine or lysozyme. *J Bacteriol* **189**, 1565-1572.

Table 1. The percentage of samples positive and the concentrations of *C. estertheticum* and *C. gasigenes* in the different farm samples

Sample		Testing method				
Type	n[1]	Enrichment plus PCR (% positive)	Direct PCR (qPCR) (% positive)	Direct plating		
				% positive	Count (log_{10} cfu g^{-1} or ml^{-1} (water) or m^{-3} (air))	
					mean	SD[3]
C. estertheticum						
Bovine faeces	200	ND[2]	8.5	1.5	1.3	0.17
Soil	100	ND	15	1	1.5	0.16
Silage	100	2	9	1	1.5	0.20
Air	100	3	9	ND	ND	NA[4]
Bedding straw	100	2	10	ND	ND	NA
Drinking water	100	1	8	ND	ND	NA
Puddle water	100	ND	14	4	1.0	0.25
C. gasigenes						
Bovine faeces	200	0.5	45	21	3.3	1.0
Soil	100	3	37	50	3.6	0.85

Silage	100	1	38	17	3.5	1.02
Air	100	15	37	12	3.1	0.91
Bedding straw	100	4	41	36	3.6	0.97
Drinking water	100	18	46	6	2.4	1.13
Puddle water	100	18	38	45	3.1	0.97

[1]Number of samples tested

[2]Not detected. The limit of detection for enrichment plus PCR is theoretically 1 viable cell per 10g of faeces, per 25g of soil, silage or straw, per 50ml of the water sample and per 100L of air, but is reliant on this cell multiplying during the enrichment phase. The qPCR method does not require growth and may be able to detect as little as one copy of the target genes. The limits for direct plating are 1000 cells per gram of faeces, soil, silage and straw, 100 cells per L of air and 200 cells per ml of water sample.

[3]SD = standard deviation

[4]NA = not applicable

Table 2. Distribution of positive *C. estertheticum* samples per season.

Season/ sample	faeces	soil	silage	air	straw	drinking water	puddle water	Total
	Enrichment plus PCR – *C. estertheticum*							
Spring	ND	ND	2	2	2	ND	ND	6^{A}
Summer	ND	ND	ND	ND	ND	ND	ND	0^{A}
Autumn	ND	ND	ND	ND	ND	1	ND	1^{A}
Winter	ND	ND	ND	1	1	ND	ND	2^{A}
	Direct qPCR – *C. estertheticum*							
Spring	10	6	4	3	3	5	5	36^{B}
Summer	ND	2	3	5	7	2	3	22^{AB}
Autumn	2	3	ND	ND	ND	ND	ND	5^{A}
Winter	5	4	2	1	ND	1	6	17^{AB}
	Direct plating – *C. estertheticum*							
Spring	ND	ND	1	ND	ND	ND	ND	1^{A}
Summer	ND	ND	ND	ND	ND	ND	ND	0^{A}
Autumn	ND	ND	ND	ND	ND	ND	ND	0^{A}
Winter	3	1	ND	ND	ND	ND	4	8^{B}

^{A,B,} Different letters indicate statistical significant ($P < 0.05$)

Table 3. Distribution of positive *C. gasigenes* samples per season.

Season/ sample	faeces	soil	silage	air	straw	drinking water	puddle water	Total
	Enrichment plus PCR – *C. gasigenes*							
Spring	ND	1	ND	10	1	7	2	21^{A}
Summer	ND	2	1	2	2	5	8	20^{A}
Autumn	ND	ND	ND	1	ND	2	5	8^{A}
Winter	1	ND	ND	2	1	4	3	11^{A}
	Direct qPCR – *C. gasigenes*							
Spring	ND	2	ND	ND	ND	ND	1	3^{A}
Summer	50	20	23	16	17	24	20	170^{C}
Autumn	26	10	10	16	20	15	11	108^{BC}
Winter	14	5	5	5	4	7	6	46^{AB}
	Direct plating – *C. gasigenes*							
Spring	10	19	6	5	14	2	21	67 ^{A}
Summer	9	10	2	ND	3	1	1	26^{A}
Autumn	5	11	6	5	15	3	10	55^{A}
Winter	18	10	3	2	4	ND	13	50^{A}

^{A,B,C} Different letters indicate statistical significant ($P < 0.05$)