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# **Germination of psychrotolerant clostridia responsible for red meat spoilage**

A thesis presented in partial fulfilment of the requirements for the degree of  
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## Abstract

Psychrotolerant clostridia are responsible for spoilage of fresh chilled vacuum-packed red meat (beef, lamb and venison). Red meat is one of New Zealand's primary exports, and spoilage results in financial loss. Spoilage by psychrotolerant clostridia is difficult to control due to the ability of these bacteria to grow at cold temperatures, down to -1.5 °C. They can also form spores that have increased resistance to heat, chemicals, oxygen and desiccation compared to vegetative bacterial cells. As clostridia are strict anaerobes, it is considered highly likely that initial contamination of meat is primarily with spores. The main objective of this work was to determine the triggers of germination of spores, of those psychrophilic and psychrotrophic clostridia, associated with spoilage of New Zealand red meat.

Germination of psychrotolerant clostridia was studied using a range of techniques including molecular, *in vitro*, and on meat methods. In this study *in vitro* germinant systems were identified for *Clostridium frigidicarnis*, and a New Zealand species designated LA1, consisting of lactate in combination with an amino acid. Some of the amino acids identified, including valine and cysteine, are naturally present on the surface of red meat. Failure to chill to, or maintain meat at, the recommended temperature, of -1.5 °C and a pH of above 5.5 were identified as being important factors leading to spoilage by *Cl. frigidicarnis*. Germination in *Clostridium estertheticum* was extremely poor in media, compared with meat slurry or fresh meat, preventing the identification of a specific germinant system(s), and indicating a non-nutrient factor may be involved. Two distinct nonchemical interventions, hot water wash (HWW) and cold water wash (CWW), were found to reduce spoilage of vacuum-packed chilled lamb inoculated with spores of *Cl. estertheticum*. Vegetative cells of psychrotolerant clostridia survived exposure to air longer than expected, upwards of seven days in the case of *Cl. estertheticum* subsp. *estertheticum*, suggesting that they play a greater role in initial contamination of meat than originally thought. From an industry point of view the results highlight the importance of preventing initial contamination and proper chilling, as well as the need for further investigation of HWW and CWW interventions.



## List of Publications

**Adam, K.H.,** Flint, S.H. and Brightwell, G. (2010). Psychrophilic and psychrotrophic clostridia: sporulation and germination processes and their role in the spoilage of chilled, vacuum-packaged beef, lamb and venison. *International Journal of Food Science & Technology*. **45**:1539-1544.

**Adam, K.H.,** Brunt, J., Brightwell, G., Flint, S.H. and Peck, M.W. (2011). Spore germination of the psychrotolerant, red meat spoiler, *Clostridium frigidicarnis*. *Letters in Applied Microbiology*. **53**:92-97

**Adam, K.H.,** Flint, S.H. and Brightwell, G. (2012). Mechanisms and reduction of spoilage of chilled vacuum-packed lamb by psychrotolerant clostridia. *Meat Science*. In Press.



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## List of Abbreviations

|                                 |   |
|---------------------------------|---|
| APC                             | aerobic plate count   |
| AFR*                            | L-alanine, L-phenylalanine, L-arginine and NaHCO <sub>3</sub> |
| AGFK                            | L-asparagine, D-fructose, D-glucose and potassium ions        |
| bCMM                            | biphasic cooked meat medium (recipe section 3.4)              |
| BHI                             | brain heart infusion  |
| BLAST                           | basic logical alignment search tool                           |
| bp                              | base pair   |
| °C                              | degrees Celsius   |
| CBA                             | Columbia Blood Agar   |
| CFU                             | colony forming units  |
| CLEs                            | cortex lytic enzymes  |
| dCMM                            | dried cooked meat medium                                      |
| fCMM                            | fresh cooked meat medium (recipe section 3.4)                 |
| CWW                             | cold water wash   |
| DIG-                            | dioxigenin-   |
| DNA                             | deoxyribonucleic acid   |
| dNTP                            | deoxynucleotide triphosphate                                  |
| DPA                             | pyridine-2, 6-dicarboxylic acid (dipicolinic acid)            |
| F                               | variance of the means   |
| <i>g</i>                        | acceleration due to gravity                                   |
| <i>g</i>                        | gram(s)   |
| GR                              | germinant receptor  |
| GMP                             | good management practice                                      |
| h                               | hour  |
| mH <sub>2</sub> O               | Milli-Q water   |
| uH <sub>2</sub> O               | Gibco™ UltraPure™ distilled, DNase and RNase free water       |
| HCl                             | hydrochloric acid   |
| HWW                             | hot water wash  |
| ITS                             | internal transcribed spacer                                   |
| KH <sub>2</sub> PO <sub>4</sub> | monobasic potassium phosphate                                 |

|                    |   |
|--------------------|---|
| kmh <sup>-1</sup>  | kilometre per hour  |
| L                  | litre   |
| log                | logarithm   |
| MgCl <sub>2</sub>  | magnesium chloride  |
| mg                 | milligram   |
| ml                 | millilitre  |
| mM                 | millimolar  |
| min                | minute  |
| mol                | molar   |
| NaCl               | sodium chloride   |
| NaHCO <sub>3</sub> | sodium bicarbonate  |
| ng                 | nanogram  |
| O <sub>2</sub>     | oxygen  |
| OD                 | optical density   |
| PCR                | polymerase chain reaction   |
| POAA               | peroxyacetic acid   |
| PYGS               | peptone, yeast extract, glucose, starch medium (recipe section 3.4) |
| rpm                | revolutions per minute  |
| s                  | second  |
| SASP               | small, acid-soluble, spore proteins                                 |
| SDS                | sodium dodecyl sulphate   |
| SSC                | saline-sodium citrate   |
| sp.                | species   |
| subsp.             | sub species   |
| Tris               | tris(hydroxymethyl)aminomethane                                     |
| µg                 | microgram   |
| µl                 | microliter  |
| µmol               | micromolar  |
| µm                 | micrometre  |
| UV                 | ultraviolet   |
| %                  | percentage  |

### Nucleotides

|   |           |
|---|-----------|
| A | adenine   |
| C | cytidine  |
| G | guanosine |
| T | thymadine |

### Bases in degenerate primers

|   |               |
|---|---------------|
| R | A + G         |
| Y | C + T         |
| M | A + C         |
| K | G + T         |
| S | G + C         |
| W | A + T         |
| H | A + T + C     |
| B | G + T + C     |
| D | G + A + T     |
| V | G + A + C     |
| N | A + C + G + T |





# Chapter 1

## Introduction and project objectives

### 1.1 Introduction

New Zealand red meat (beef, lamb and venison), exported chilled and vacuum-packed, spends several weeks in transit during shipping to overseas markets such as Asia and Europe. During this time meat can potentially spoil. The chances of spoilage are increased when meat is exposed to temperature abuse prior to or during shipping. Of particular concern are psychrophilic and psychrotrophic clostridia, as they are able to grow in anaerobic vacuum-packs where it has been identified that temperature abuse has not occurred or was minimal (Broda et al. 1997). One such organism, *Clostridium estertheticum*, has been identified as a cause of blown-pack spoilage and is able to grow at -1.5°C the optimal storage temperature for chilled red meat (Spring et al. 2003).

Spoilage of chilled vacuum-packed red meat, caused by psychrotolerant clostridia at non-abusive temperatures, was first reported in 1989. Two groups, one working in the United States (Kalchayanand et al. 1989) and the other working in the United Kingdom (Dainty et al. 1989), identified *Clostridium* sp. as the causative agents. Both incidents involved fresh chilled vacuum-packed beef. Spoiled packs were described as containing “large quantities of foul smelling gas and purge”, and with meat that had suffered a “loss of colour and texture” (Kalchayanand et al. 1989). The bacterium responsible was later described and named *Cl. estertheticum* (Collins et al. 1992).

Psychrophilic and psychrotrophic clostridia are strictly anaerobic, and it is thought that spoilage occurs as a result of contamination of meat with resistant endospores. Spores enter the processing plant on the hides and in the faeces of animals, and are transferred to carcasses during slaughter and processing (Boerema et al. 2003). To date, the best means of controlling spoilage, by psychrophilic and psychrotrophic

clostridia, have been through maintaining best practices during slaughter, processing and shipping.

Spores are highly resistant to heat, UV radiation, desiccation and toxic chemicals that rapidly kill growing bacteria, making deactivating them, without affecting meat quality, difficult. It is cells, not spores, which cause spoilage. Prior to entering the growth phase, and causing spoilage, spores must germinate. The triggers for spore germination vary among species and strains, and were not known for psychrophilic and psychrotrophic red meat spoilage related clostridia prior to this study.

The focus of this study was to determine the triggers of germination of psychrophilic and psychrotrophic clostridia. Increased understanding of the spoilage process could potentially lead to novel methods to decrease or eliminate spoilage. The research hypothesis was that spoilage, by psychrotolerant clostridia, can be reduced by a two part intervention strategy, involving inducing spores to germinate thereby reducing their resistance properties, then treating them to kill or inactivate.

## 1.2 *Project objectives*

The main aim of the research, presented in this thesis, was to determine the triggers (germinants) of spore germination in psychrophilic and psychrotrophic clostridia associated with spoilage of New Zealand red meat.

Initially a literature review was carried out to determine current findings on the process and triggers of germination in spore formers, especially closely related *Clostridium botulinum*. The results of the literature review are presented in Chapter 2.

The first objective, of this project, was to determine which strains, of psychrophilic and psychrotrophic clostridia, sporulated well under laboratory conditions, and to produce sufficient quantities to screen a long list of potential germinants. These were *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense*, *Clostridium frigidicarnis* and LA1.

The second objective of this project, was to identify an appropriate method of measuring germination in *Cl. estertheticum* and *Cl. frigidicarnis*, to determine which of a list of potential germinants, identified during the literature review, trigger germination.

Following identification of germinants, the effect of environmental conditions, such as temperature, salt concentration, atmosphere and pH, on germination was determined, and some preliminary work was carried out on inactivating germinated spores, under conditions that could potentially be applied to red meat.

Finally the selected psychrophilic and psychrotrophic clostridia were screened for the presence of germinant receptor genes using molecular techniques.

It is hoped that, through this research, the understanding of the triggers of germination in psychrophilic and psychrotrophic clostridia can be increased ultimately leading to a germination based intervention for the reduction of blown-pack spoilage, in New Zealand red meat, during export.



## Chapter 2

### Literature review

#### 2.1 Spoilage of vacuum-packed chilled red meat

##### 2.1.1 Characteristics of spoilage of vacuum-packed chilled red meat

A vacuum-pack suffering blown-pack spoilage is typically grossly distended, to the point where meat would be considered “off” prior to opening (**Figure 2.1**). On opening the pack, strong offensive ‘off’ odours, of a sulphurous or cheesy nature, and copious quantities of drip are present (Dainty et al. 1989; Helps et al. 1999). Historically gas production, in meat packs was associated with temperature abuse, resulted from the growth of non clostridial spoilers, such as *Serratia liquefaciens*, *Enterobacter aerogenes* and *Hafnia alvei*, from the *Enterobacteriaceae* family (Broda et al. 1997). Following the failure of product, where temperature records indicated abuse had not occurred, several strains of psychrotolerant clostridia were isolated, described and subsequently shown to cause blown-pack spoilage (Broda et al. 1996; Broda et al. 2009). These included *Cl. estertheticum* (subspecies *estertheticum* and subspecies *laramiense*, formerly *Cl. laramiense*) and *Clostridium gasigenes* (Collins et al. 1992; Broda et al. 2000b; Spring et al. 2003). Several non gas producing, spoilage strains have also been isolated from meat including *Clostridium algidicarnis*, *Clostridium algidixylanolyticum* *Cl. frigidicarnis* (Lawson et al. 1994; Broda et al. 1999; Broda et al. 2000a) and LA1.



**Figure 2.1** Vacuum-packed beef displaying typical symptoms of blown-pack spoilage.

### 2.1.2 Spore forming clostridia associated with spoilage of chilled vacuum-packed red meat

Historically, the inclusion of an organism in the genus *Clostridium* was based on four morphological characteristics: the ability to form endospores, strict anaerobic metabolism, an inability to carry out dissimilatory reduction of sulphate and a Gram-positive cell wall (Andreesen et al. 1989). There is considerable diversity amongst clostridial isolates. Analysis of 16S rDNA resulted in 19 or 20 designated clusters (Collins et al. 1994). The spoilage strains of interest, to chilled meat producers, are all psychrophilic or psychrotrophic, and fall into a diverse range of clusters, including I and XIVa (Broda et al. 2003b). Historically the definitions of psychrophilic, psychrotrophic and psychrotolerant have varied among reports in the scientific literature. Here the following definitions have been adopted: psychrophilic: having optimum growth at or below 15 °C, a maximum growth temperature of 15 to 20 °C, and minimum growth

temperature of -5 to 5 °C, psychrotrophic: having optimum growth at 25 to 30 °C, a maximum growth temperature of 30 to 35 °C and a minimum growth temperature at -5 to 5 °C, and psychrotolerant: capable of growing at less than 5 °C i.e. covers psychrophilic, psychrotrophic and mesophilic strains with low minimum growth temperatures (Broda et al. 1997).

The two strains of *Cl. estertheticum*, were originally isolated from chilled vacuum-packed beef, and are true psychrophiles (**Table 2.1**). The two sub species, *estertheticum* and *laramiense*, have optimum growth temperatures of 6-8 and 15°C respectively (Collins et al. 1992; Kalchayanand et al. 1993; Spring et al. 2003). The gas produced by *Cl. estertheticum* sub sp. *estertheticum* has been identified as primarily hydrogen (H<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>). High levels of butanol and butanoic acid together with a complex range of esters and volatile sulphur containing compounds are also produced (Dainty et al. 1989). The second gas producer, *Cl. gasigenes*, was originally isolated from vacuum-packed spoiled chilled lamb that was spoiled and also primarily produces H<sub>2</sub> and CO<sub>2</sub> (Broda et al. 2000b). *Cl. gasigenes* is a psychrotroph with an optimum growth temperature of 20 to 22°C. The remaining strains of interest: *Cl. algidicarnis*, *Cl. algidixylanolyticum*, *Cl. frigidicarnis*, and LA1 do not produce large quantities of gas, and are all psychrotrophs (Lawson et al. 1994; Broda et al. 1999; Broda et al. 2000a). LA1 was isolated locally, from spoiled vacuum-packed beef.



**Table 2.1** Psychrotolerant clostridial strains associated with spoilage of chilled, vacuum-packaged beef lamb and venison.

| Strain   | Collection number       | Source                                       | optimal growth temp (°C) | growth range (°C) | optimal pH | type of spoilage                | Reference (s)                                    |
|--|-------------------------|--|--------------------------|-------------------|------------|---------------------------------|--|
| <i>Cl. aligidicarnis</i>                             | NCFB 2931 <sup>T</sup>  | vacuum-packed, cooked pork                   | 25-30                    | 4 -37             | not stated | surface-spoilage/<br>bone taint | Lawson et al. (1994)                             |
| <i>Cl. algidixylanolyticum</i>                       | DSM 12273 <sup>T</sup>  | temperature-abused, vacuum-packed raw lamb   | 25.5-30                  | 2.5-32.5          | 6.8-7.0    | surface-spoilage                | Broda et al. (2000a)                             |
| <i>Cl. estertheticum</i> subsp. <i>estertheticum</i> | DSM 8809 <sup>T</sup>   | spoiled, chilled, stored, vacuum-packed beef | 6-8                      | 1-15              | 6.5-7.2    | blown-pack                      | Collins et al. (1992), Spring et al. (2003)      |
| <i>Cl. estertheticum</i> subsp. <i>laramiense</i>    | ATCC 51254 <sup>T</sup> | vacuum-packed, fresh and roast beef          | 15                       | -3-21             | 6.5        | blown-pack                      | Kalchayanand et al. (1993), Spring et al. (2003) |
| <i>Cl. frigidicarnis</i>                             | DSM 12271 <sup>T</sup>  | temperature-abused, vacuum-packed beef       | 30-38.5                  | 3.8-40.5          | 6.4-7.2    | surface-spoilage                | Broda et al. (1999)                              |
| <i>Cl. gasigenes</i>                                 | DSM 12272 <sup>T</sup>  | blown-packs of chilled lamb                  | 20-22                    | -1.5-26           | 6.2-8.6    | blown-pack                      | Broda et al. (2000b)                             |
| LA1  |                         | spoiled, vacuum-packed beef                  |                          |                   |            |                                 | locally isolated                                 |

### 2.1.3 Prevalence of psychrotolerant clostridia associated with spoilage of vacuum-packed, chilled red meat

Products suffering psychrotolerant clostridia related spoilage include primal cuts of beef, lamb and venison, cooked dog rolls, precooked turkey, roast beef and sous-vide product (food heat processed under vacuum in oxygen-impermeable barrier bags, and stored at refrigeration temperature) (Broda et al. 1996; Kalinowski and Tompkin 1999). Blown-pack spoilage, where *Cl. estertheticum* was confirmed as the causative agent, has occurred in packs originating from the United States (Kalchayanand et al. 1989), New Zealand (Broda et al. 1996), Northern Europe, and South Africa (Helps et al. 1999). *Cl. estertheticum* subsp. *laramiense* was isolated in North America. *Cl. gasigenes* has been isolated from meat originating from Ireland, the United Kingdom and New Zealand (Cavill et al. 2011; Broda et al. 2000a). *Cl. frigidicarnis* and *Cl. algidixylanolyticum* isolation, from spoiled vacuum-packed meat, has been reported only for product originating from New Zealand (Broda et al. 1999; Yang et al. 2009). *Cl. algidicarnis* was originally isolated from cooked, vacuum-packed pork in Northern Europe (Lawson et al. 1994), and has since been isolated from vacuum-packed, chilled meats from New Zealand (Boerema et al. 2002). In a relatively small survey of export quality, vacuum-packed beef striploins and lamb legs, from nine New Zealand meat processing plants, a greater number of psychrotolerant clostridia were isolated from lamb than from beef (Broda et al. 1997). This may be a reflection of the greater level of contamination generally associated with lamb legs than beef striploins (Broda et al. 1997).

### 2.1.4 Sources of psychrotolerant clostridia associated with spoilage of vacuum-packed, chilled red meat

The results of two surveys, carried out in New Zealand meat processing plants, on abattoir sources of clostridial meat spoilers, suggest that they are brought into plants on the hides and in the faeces of animals, and transferred to carcasses during slaughter and processing (Broda et al. 2002; Boerema et al. 2003). *Cl. estertheticum* and *Cl. gasigenes* were detected in soil, faecal and pelt samples, but not in the boning

room or chillers, and only to a limited extent on the slaughter floor. A wider survey, including samples from ten South Island farms supplying stock to meat processing plants, detected the presence of *Cl. estertheticum* and *Cl. gasigenes* in soil, mud, vegetation and creek water. Similar results were seen in a survey of four beef abattoirs in Ireland (Moschonas et al. 2009). Soil and mud can provide the moist anoxic conditions conducive to growth of mesophilic clostridia. It is suspected that this extends to psychrotolerant clostridia (Broda et al. 2009).

#### 2.1.5 Triggers of spoilage of vacuum-packed, chilled, red meat by psychrotolerant clostridia

For spoilage to occur conditions must be appropriate for growth. Firstly spores must be triggered to germinate. Temperature, pH, atmosphere and nutrient availability must meet the subsequent growth requirements of the vegetative cells of the organism in question. Finally, conditions must be sufficiently favourable for the spoilage organism(s) to avoid being out competed by other bacteria present. Although the germinants required to trigger germination, in cold tolerant red meat spoilage clostridia, are unknown the ability of spores to spoil vacuum-packed red meat indicates germinants are present on the surface of meat. Temperature requirements vary between strains. With an increase in temperature above the optimum for storage (-1.5 °C) growth of clostridial spoilers increases. However, the number and growth speed of competing microflora also increases. Observations of past spoilage incidences indicate that at pH of <6.0 *Cl. estertheticum* is usually present, and at a pH of a >6.0 there is an increase in the presence of other spoilers (Cavill et al. 2011). The ultimate pH of red meat varies between animals and muscles within the carcass. Ultimate pH values are 5.5 to 6.7, in young bulls, 5.4 to 7 in beef, 5.65 to 6.38 in reindeer and 5.5 to 6.38 in lamb (Tarrant and Sherington 1980; Fjellkner-Modig and Rudérus 1983; Devine et al. 1993; Wiklund et al. 1997). The vacuum-packaging process results in the anaerobic environment suitable for growth of the cold tolerant clostridia discussed here.

### 2.1.6 Current strategies for the prevention of spoilage of vacuum-packed chilled, red meat by psychrotolerant clostridia

Currently good manufacturing practice (GMP) is the primary strategy for preventing spoilage of red meat, by psychrotolerant clostridia, during transport from New Zealand to the market place (Adam et al. 2010). GMP relates to the running of processes, in this case those employed by meat processing plants. It covers areas such as plant cleaning regimes and dressing technique, for example, preventing the hide from rolling back on to the carcass while making the opening cut. By following GMP the risk of spoilage is reduced through ensuring initial microbial loading is minimised, boxed meat is chilled evenly prior to load out, and the temperature is maintained at -1.5 °C during transport and storage. Peroxyacetic acid (POAA)-based sanitizer has been shown to inactivate spores of *Cl. estertheticum* *in vitro* (Broda 2007). POAA-based sanitizer has also been shown to delay the onset of spoilage by *Cl. estertheticum* spores on beef (Boerema et al. 2007). The commercial use of POAA-based sanitizer, on carcasses, is limited as chemically treated meat is not acceptable to the European market ([http://www.fsis.usda.gov/regulations/European\\_Union\\_Requirements/index.asp#VII](http://www.fsis.usda.gov/regulations/European_Union_Requirements/index.asp#VII) 2012). A preliminary study has shown that spoilage of vacuum-packed beef steaks, inoculated with small numbers of *Cl. estertheticum* spores, can be reduced by the presence of *Leuconostoc mesenteroides* (Yang et al. 2011). The viability of using *L. mesenteroides*, as a biopreservation agent, on a commercial scale remains to be seen. Other intervention strategies, that have been shown to be effective in reducing microbiological spoilage of meat, include lactic acid wash, hot water wash (HWW), steam vacuuming and trimming. However, hot water washing and steam vacuuming risk activating spores, if heating is insufficient to kill spores, and trimming only removes visible contamination (Adam et al. 2010).

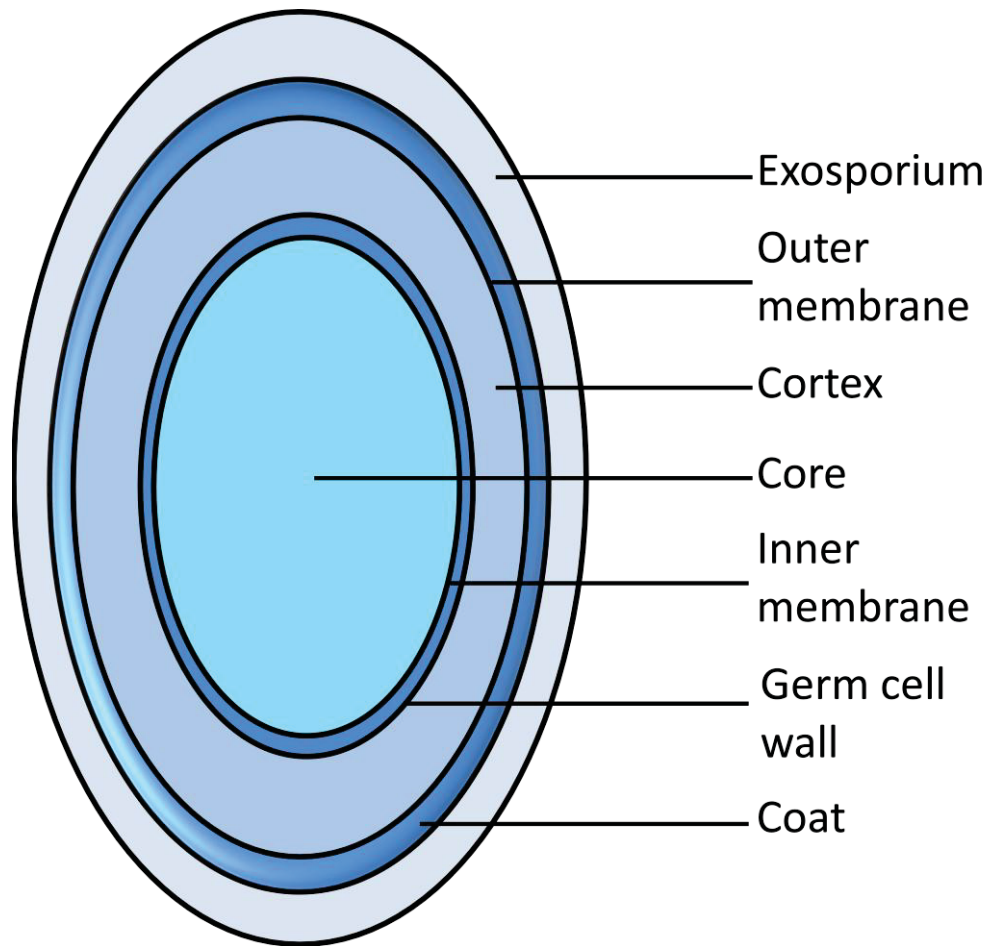
## 2.2 Spores of meat spoilage bacteria

### 2.2.1 Clostridial spores role in red meat spoilage

Due to the strictly anaerobic nature of the spoilage strains discussed here it is thought that initial contamination of meat with spores plays a significant role in spoilage. The structure of bacterial spores renders them highly resistant to environmental insults such as heat, radiation, chemicals and desiccation (Setlow 2007). Spores' extreme resistance allows them to persist in the environment for extended periods of time. Twenty five to forty million-year-old spores, thought to be closely related to *Bacillus sphaericus*, originating from the gut contents of extinct bees preserved in Dominican amber, have been successfully revived (Cano and Borucki 1995). Central to bacterial spore resistance is the protection of DNA (Setlow 2007). The vegetative stage, of a spore forming bacterium's life cycle, mirrors that of non spore formers. In good growth medium, the population increases by the usual route of central division, following elongation of the cell and replication of DNA (Errington 1993). Under appropriate conditions, the standard lag phase, log or growth phase and stationary phases can be observed. The life cycle diverges, from that of a non spore former, when limiting factors trigger the formation of spores. This is usually during the stationary phase. *Cl. frigidicarnis* forms spores early in stationary phase, but *Cl. algidixylanolyticum* and *Cl. gasigenes* form spores late in growth phase (Broda et al. 1999; Broda et al. 2000a; Broda et al. 2000b). Once cells have formed spores they remain inactive until they sense environmental conditions are favourable for growth. Triggered spores germinate and undergo outgrowth before resuming a vegetative state (Setlow 2003).

### 2.2.2 Bacterial spore structure

The dormant spore performs little or no cellular processes so is dependent on its structure to maintain its resistance. Dormant spores are relatively dehydrated, in comparison to vegetative cells, and are comprised of several layers assembled during sporulation (**Figure 2.2**) (Makino and Moriyama 2002). Spore structure has been most studied in *Bacillus subtilis*. However, basic spore structure is conserved among all the bacterial endospore formers (Atrih and Foster 2002; Driks 2002). Starting from the centre and progressing out, a bacterial spore consists of a core, inner membrane, germ cell wall, cortex, outer membrane, a coat or coats and, in some cases, an exosporium.



**Figure 2.2** Diagrammatic representation of a generalised bacterial spore, adapted from (Foster and Johnstone 1990; Paredes-Sabja et al. 2011). The exosporium is not present in all spores.

The inner-most compartment, the spore core (cytoplasm), contains the cellular components (e.g. DNA, RNA and metabolic enzymes) necessary for establishment of a vegetative cell cytoplasm, following spore germination and outgrowth (Foster and Johnstone 1990). The core has a low water content (25-50 % wet weight), low physiological pH (around 6.5, approximately 1 pH unit lower than in growing cells) and contains large amounts of pyridine-2,6-dicarboxylic acid (dipicolinic acid; DPA), which results in the mineralisation of the core (Setlow 2007). Another unique feature, of the core, is the saturation and protection of spore DNA with  $\alpha/\beta$ -type small, acid-soluble, spore proteins (SASP), which comprise 5-10 % of total core protein (Setlow 2007). Surrounding the core is the spore inner membrane, a layer of vegetative cell-type peptidoglycan or lipid bilayer (Foster and Johnstone 1990; Moir 2006). The inner membrane has low permeability increasing spore resistance (Setlow 2007). Alteration of the inner membrane affects germination properties, and it is likely that, in *B.*

*subtilis*, the components of the nutrient receptor complex, consisting of GerAA, GerAB and GerAC, span the inner membrane (Atrih and Foster 2002; Moir et al. 2002). The germ cell wall is also composed of peptidoglycan, and becomes the cell wall of the outgrowing spore preventing loss of cellular integrity, and serving as a template for peptidoglycan biosynthesis during outgrowth (Atrih and Foster 2002; Setlow 2007).

The cortex has a unique cage-like structure, of peptidoglycan, formed around the core and inner membrane, and characteristics that are broadly conserved in a number of spore forming bacteria (Foster and Johnstone 1990; Makino and Moriyama 2002). These include the occurrence of  $\delta$ -lactam predominantly at every alternate muramic acid residue, and a low cross-linking index, which occurs at only 2.9 % of muramic acid, in *B. subtilis* spores (Atrih and Foster 2002). The muramic  $\delta$ -lactam moiety is not required for heat resistance, but is necessary for substrate recognition by germination specific lytic enzymes (Atrih and Foster 2002). The cortex is involved in the maintenance, but not the establishment, of the heat-resistant dormant state (Atrih and Foster 2002).

The outer membrane surrounds the spore cortex which is, in turn, enclosed by the complex proteinaceous spore coats (Foster and Johnstone 1990). The outer spore membrane is essential in spore formation, but might not be a permeability barrier in mature spores (Setlow 2007). The coat protects spores against predatory eukaryotic microbes and reactive chemicals, while allowing small nutrient molecules to access germination receptors beneath the coat (Setlow 2007). A *B. subtilis* mutant, completely lacking the spore coat layers, is highly sensitive to lysozyme, but shows normal heat resistance (Atrih and Foster 2002). In species of *Bacillus* the proteinaceous coat layer contains >40 different proteins, almost all being spore specific (Setlow 2007). The coat appears to house enzymes with direct germination roles, such as the cortex-lytic enzyme CwlJ, allowing direct contact with the cortex, on which it acts during germination (Driks 2002; Setlow 2003). Finally, some species, including *Cl. botulinum* 78A, are enveloped by an exosporium, a balloon-like structure composed of proteins and carbohydrates (Foster and Johnstone 1990; Stevenson and Vaughn 1972).

The function of exosporium is unknown, although it might be involved in resistance, adhesion to inert surfaces, and interactions with epithelial cells (Severson et al. 2009).

## 2.3 Sporulation and germination

### 2.3.1 The process of sporulation

Actively growing cells of *B. subtilis* are induced to sporulate by starvation for carbon, nitrogen, or, in some cases, a phosphorus source (Piggot and Hilbert 2004). In contrast, to *Bacillus* species, clostridial species commonly require complex sporulation medium, and the presence of a slowly fermentable carbon source, at the end of the log phase of growth, to allow continuation of energy supply during the early stages of the sporulation process (Peck et al. 2004). Initiation of sporulation may also be affected by cell signalling, when cells become crowded, resulting in a rise in the concentration of specific signal compounds (Peck et al. 2004).

Physiologically, the first observable sign of bacterial sporulation is the formation of the forespore septum (Fitz-James and Young 1969). Unlike the septum in a vegetative cell this forms asymmetrically, nearer one end of the cell, with a copy of the genome partitioned into each half (Henriques and Moran Jr 2007). The smaller of the two halves, the forespore (sometimes called the prespore), becomes the spore while the larger, called the mother cell, supports the spore's formation. The mother cell engulfs the smaller forespore protecting it and producing components necessary for spore development. Following engulfment, expression of sporulation genes is controlled by sigma factors,  $\sigma^E$  and  $\sigma^K$  in the mother cell, and  $\sigma^F$  and  $\sigma^G$  in the forespore in *B. subtilis* (Losick and Stragier 1992). Two types of peptidoglycans are layered between the inner and outer membranes, surrounding the forespore, resulting in the formation of the germ cell wall and the cortex. In addition, a protein coat is deposited around the outer surface of the outer membrane (Henriques and Moran Jr 2007). Synthesis of the cortex, coat, and exosporium are a function mainly, if not exclusively, of the mother cell. This is accompanied by a large decrease in the volume, and water content of the forespore protoplast. There is also a decrease in forespore pH. Later, the forespore



takes up large amounts of DPA that have been synthesised, by the mother cell, in the forespore. In some species, a large, external, balloon-like exosporium is added. Finally the mother cell lyses, releasing the spore into the environment (Piggot and Hilbert 2004; Setlow 2007). During this process the mother cell and forespore participate in extensive cross talk, to ensure gene expression in both compartments remains coordinated (Setlow 2007). For example, in *B. subtilis*  $\sigma^E$ , in the mother cell, activates expression of SpoIIIA which releases  $\sigma^G$ , in the prespore, from inhibition (Piggot and Hilbert 2004).

### 2.3.2 The process of germination

The process, by which a spore changes from a dormant, highly resistant state to a vegetative cell, able to actively divide, can be separated into three sequential stages: activation, germination and outgrowth. Activation is not always necessary for spore germination, is reversible and is often the result of heat treatment (Keynan and Evenchik 1969). Following activation spores will, with appropriate stimulation, commit themselves to germination. Germination is a series of inter-related degradative events, such as the breakdown of the peptidoglycan making up the spore cortex (Foster and Johnstone 1990). Following germination the spore is physiologically different from a vegetative cell despite having lost many spore specific properties. Before a cell can be considered vegetative it must undergo outgrowth, following which division can proceed (Keynan and Evenchik 1969). Commitment to germinate is triggered by germinants, which fall into three broad categories: physiological, chemical and mechanical (Gould 1969). Germination is non-reversible and results in the loss of refractility and resistance to a wide range of environmental assaults including heat, UV and solvents (Foster and Johnstone 1990; Atrih and Foster 2002). Outgrowth transitions the germinated spore to a growing, dividing, vegetative cell.

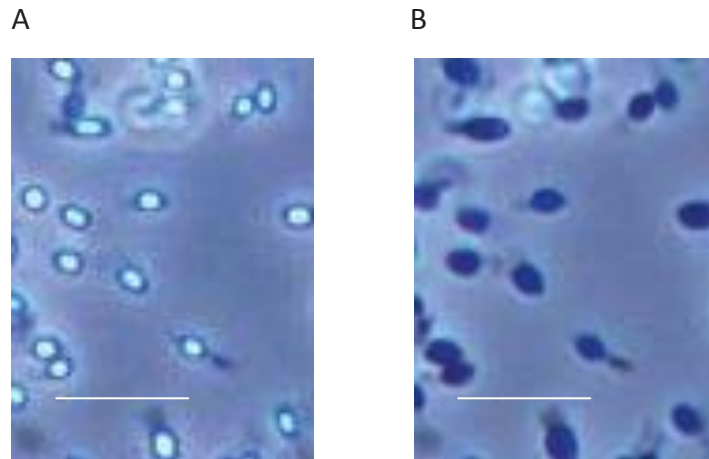
Activation enhances the rate and extent of germination. Activated spores retain most of the important properties of the dormant state, such as resistance to heat and radiation, non-stainability and refractility (Keynan and Evenchik 1969). Activation agents include sub-lethal heat, pH, reducing agents, ionizing (gamma) radiation and

ageing. Variations in activation parameters (for example the temperature and time of heating) have been shown to affect the speed with which germination occurs, and the extent of germination within a population of spores. In addition, activation influences the spores qualitative and quantitative requirements for germinants (Keynan and Evenchik 1969). Unlike germination, activation is a reversible state. A specific example of reversibility was shown by Powell (1950) who found that the heat induced activated state of *Bacillus coagulans* and *Bacillus calidolactis* wore off after storage, for a few hours, under anaerobic conditions. Spores of many strains of *Cl. perfringens* require activation to enhance the germination process. Activation by sublethal heating is routinely used as a prerequisite for studies of spore germination, and for enumeration of spore populations on plating media.

The specific requirements for activation vary between species and strain. Within the group of psychrotolerant spore forming clostridia there are two sub groups: low optimum growth temperature (heat sensitive), and high optimum growth temperature (heat tolerant). Temperatures which activate heat tolerant strains are often lethal to heat sensitive strains (Broda et al. 1998). Activation appears to function by altering the molecular interactions on the surface of the spores increasing the ability of germinants to interact with germinant receptors (GRs). Treatments that enhance activation of spores of *Clostridium perfringens* NCTC 8679 (increased temperature, higher pH and presence of chaotropic ions [ions responsible for destabilising hydrogen bonding and hydrophobic interactions], alcohols, or urea) are all known to disrupt or weaken hydrophobic interactions (Craven 1988). Treatments that retard spore activation (decreased temperature, lower pH and presence of antichaotropic ions) strengthen hydrophobic attractions (Craven 1988).

When a bacterial spore enters the germination stage of its life cycle it becomes committed to the process and can no longer return to dormancy, this distinguishes an activated spore from a germinating spore. Changes that occur during germination include loss of resistance, breaking of dormancy, increase in permeability, increase in volume, rehydration of the spore core, hydrolysis of peptidoglycan in the spore cortex, excretion of spore constituents (for example DPA), cytological changes, phase

darkening (**Figure 2.3**) and onset of stainability (Gould 1969; Johnstone 1994; Okamura et al. 2000). Germination is divided into two stages. Stage I involves cation and DPA release and partial core hydration. During Stage II the cortex hydrolyses, the core expands and dormancy is lost (Setlow 2003).



**Figure 2.3** *Cl. frigidicarnis* spores: Dormant, phase bright (A) and germinated, phase dark (B). Bar = 10  $\mu\text{m}$ .

Germination is generally triggered by the interaction of a germinant or group of germinants with the spore's GRs. Germinants are small molecules, often required by the cell for growth and division. They are highly specific to given strains, but vary between strains. After penetrating the outer layers of the spore coat and cortex, germinants interact with GRs (Moir 2006), which are present at the inner membrane. Experimentally, the microscopically observable change of a spore from phase light to phase dark, or loss of  $\text{OD}_{600}$  of a spore suspension, is often used as an indicator of germination (Sorg and Sonenshein 2008). Once triggered, the germination process proceeds without the continued presence of the germinant, or synthesis of macromolecules. This indicates that germination is a process controlled by the sequential activation of a set of pre-existing germination-related enzymes already present in the mature spore (Johnstone 1994; Okamura et al. 2000; Moir et al. 2002).

Following the triggering of germinant receptors, in *B. subtilis* spores, DPA and monovalent cations, hydrogen ( $\text{H}^+$ ), potassium ( $\text{K}^+$ ) and sodium ( $\text{Na}^+$ ) are also released in exchange for water (Setlow 2003). This triggers downstream events in spore

germination, most importantly hydrolysis of the spore peptidoglycan cortex by one or more cortex-lytic enzymes, allowing the core to expand and take up water until hydration levels equivalent to that of growing cells are reached. The latter event restores protein movement, ion fluxes and enzyme action, in the spore core, leading to the resumption of energy metabolism and macromolecular synthesis (Atrih and Foster 2002; Paredes-Sabja et al. 2008a). The peptidoglycan of the spore cortex is degraded (essential for outgrowth), and the coat layers are partially degraded. ATP synthesis and oxidative metabolism resume. Any DNA damage is repaired, and the SASP's are degraded by a specific protease, releasing amino acids for use during outgrowth (Moir et al. 2002).

That part of the growth cycle following germination and ending in the formation of the first mature vegetative cell is referred to as outgrowth (Strange and Hunter 1969). In suitable conditions, metabolism is initiated and germinated spores begin macromolecular synthesis and start to grow. A germinated spore, although sensitive and metabolically active, differs from a vegetative cell cytologically, and does not possess a full complement of typical vegetative macromolecules and enzymatic activities (Gould 1969). Eventually, spore coats are shed, absorbed, or fragmented and young cells emerge (Strange and Hunter 1969). Within cell synthesis continues until cells obtain the full complement of molecules found in adult cells. Mature cells continue to increase in size and proceed to cell division, completing the life cycle of spore forming bacteria (Stringer et al. 2005).

A substrate which triggers germination of bacterial spores does not necessarily support division and growth of the germinated spores. Unlike the process of germination, which is driven by preformed enzymes and does not utilize exogenous nutrients, outgrowth cannot occur without access to appropriate external nutritional sources (Strange and Hunter 1969). Spores of *Bacillus megaterium* germinated in glucose, but did not undergo post germinative development unless sources of sulphur and phosphate were added (Levinson and Hyatt 1960). *Bacillus cereus* and *B. megaterium* spores have been shown to undergo microcycle sporogenesis or resporulation, without intervening cell division, where germination has been initiated but nutritional

requirements for growth and division are lacking (Vinter and Slepecky 1965; Holmes and Levinson 1967).

Germination within a population of spores varies. The distribution of growth times from individual spores of non-proteolytic *Cl. botulinum* has been studied in relation to predicting time until growth in food (Stringer et al. 2005). Spore populations contain a small percentage of spores that germinate extremely slowly under ideal conditions; these have been termed superdormant spores (Wei et al. 2009). Superdormant spores pose a challenge when attempting to reduce red meat spoilage.

### 2.3.3 Germination specific proteins

Of the spore formers, the most detailed information on the process of germination, and the proteins involved, relates to *B. subtilis*. A number of spore specific proteins have been identified in *B. subtilis*, many of which appear to be conserved within spore formers (Paredes-Sabja et al. 2011). The two major groups of spore specific proteins are GRs and cortex lytic enzymes (CLEs). Low numbers (tens) of GRs are located in the inner membrane of the spore (Paidhungat and Setlow 2001; Alberto et al. 2005). Germinants bind to specific ligand binding sites on GRs triggering germination. The specific location(s) of binding site(s) on the GR, or the means by which the message to germinate is passed to downstream effectors, has yet to be identified (Paredes-Sabja et al. 2011). Spores of most spore formers contain multiple proteins from the GR family, with different specificities for different germinants (Paredes-Sabja et al. 2011; Ross and Abel-Santos 2010). In *B. subtilis*, the GRs are GerA, which responds to L-alanine, and GerB and GerK, which collaboratively respond to L-asparagine, D-fructose, D-glucose and potassium ions (AGFK) (Paidhungat and Setlow 2000; Cabrera-Martinex et al. 2003). The GRs consist of three subunits, A and B proteins, which are integral transmembrane proteins, and C, which is a peripheral membrane protein (Paredes-Sabja et al. 2011). In *B. subtilis*, and many other cases, the subunits are encoded by homologous tricistronic *gerA* family operons (Moir and Smith 1990). Analysis of whole genome sequences has identified the presence of GR operons in numerous species from the clostridial genus (with the exception of *Cl. difficile* and *Clostridium bartlettii*).

However, studies using knock out mutants to link specific germinants with specific GRs are limited (Paredes-Sabja et al. 2011). Analysis of whole genomes of *Clostridium* species revealed lower numbers of genes encoding germination receptors than in *Bacillus* species, and, in addition, many incomplete operons or operons with unusual structures (Xiao et al. 2010).

Recently, a GR independent germination pathway has been identified in several *Bacillus* species. Muropeptides derived from the breakdown of peptidoglycan, from the same or closely related species are recognised by PrkC, an inner membrane associated eukaryotic-like protein kinase (Shah et al. 2008). Most species have *spoVA* operons (Paredes-Sabja et al. 2011). In *B. subtilis*, SpoVA proteins are involved in transport of chelated DPA into the core of the developing spore during sporulation and in DPA release during germination (Vepachedu and Setlow 2007).

Hydrolysis of spore cortex peptidoglycan is an essential step in germination, allowing outgrowth and formation of a new vegetative cell (Kumazawa et al. 2007). *Bacillus anthracis*, *B. megaterium* and *B. subtilis* possess two redundant CLEs, SleB and CwlJ, either of which is sufficient for complete spore germination (Setlow 2003). SleB and CwlJ require auxiliary proteins, YpeB and GerQ respectively, for correct functioning and localization (Paidhungat et al. 2001). In contrast *Cl. perfringens* possess two CLEs, SleC and SleM, with only SleC being essential for germination. Unlike CwlJ and SleB, which are synthesized in mature form during sporulation, SleC is synthesised with an N-terminal pre-region and a C-terminal pro-region, that are removed during sporulation, activating the enzyme (Paredes-Sabja et al. 2011). While CwlJ and SleB are present in most *Bacillus* species, there is less of a pattern amongst the clostridia. For example, *Cl. botulinum* A strain ATCC19397 carries homologs for CwlJ and SleB, but not SleC or SleM, while *Cl. botulinum* B strain Eklund 17B carries homologues for SleB, SleC and SleM but not for CwlJ (Paredes-Sabja et al. 2011).

#### 2.3.4 Triggers of bacterial spore germination

Germinants, the triggers of germination, can be grouped into three broad categories: nutrient, chemical and physical, examples of which are summarised in **Table 2.2**. Nutrient, or physiological, germinants are those substances that may play a role in germination in nature, and are metabolised by the resulting vegetative cell. Nutrient germinants include amino acids, sugars and nucleosides. Chemical germinants are substances such as lysozyme, salts and  $\text{Ca}^{2+}$ -DPA which are not metabolised by the spore (Setlow 2003). Mechanical germination can be achieved by abrasion of the outer layers of the spore, or exposure to high pressure (Rode and Foster 1960; Setlow 2003). The triggers of germination vary between species and strains. In some cases a single germinant will trigger germination. However, the most complete germination is often achieved by using combinations of germinants such as AGFK (Gould 1969). Germination is commonly measured by microscopic enumeration of phase darkened spores, or a reduction of optical density (OD) in a population of spores, and factors able to induce these changes are termed germinants. In the natural environment, exposure to nutrient germinants is a likely cause of germination and outgrowth. Spores exposed to simple germinant mixtures *in vitro* exhibit indications of germination, such as loss of refractility and a drop in OD, but fail to proceed to outgrowth in the absence of more complex growth media (Moir 2006).

**Table 2.2** Summary of bacterial spore formers with known germinants. For optimal germination pre-treatment (for example exposure to a short burst of heat), the presence of co-germinants, such as sodium thioglycolate or bicarbonate, or pH buffers may be required. Effectiveness of germinants is often strain dependent. AGFK is a combination of L-asparagine, D-fructose, D-glucose and potassium ions (usually from KCl). ARF\* is a combination of L-alanine, L-phenylalanine, L-arginine and NaHCO<sub>3</sub>.

| Organism                | Type        | Germinant                       | Reference  |
|-------------------------|-------------|---------------------------------|--|
| <i>B. anthracis</i>     | Amino acid  | L-alanine                       | Broussolle et al. (2008)                             |
| <i>B. cereus</i>        | Amino acid  | L-alanine                       | Broussolle et al. (2008)                             |
|                         | Nucleoside  | Inosine                         | Alberto et al. (2003); Broussolle et al. (2008)      |
|                         | Combination | L-alanine and inosine           | Hornstra et al. (2007)                               |
|                         | Combination | AGFK                            | Alberto et al. (2003)                                |
| <i>B. circulans</i>     | Amino acid  | L-alanine                       | Gould (1969)   |
| <i>B. coagulans</i>     | Amino acid  | L-alanine                       | Gould (1969)   |
| <i>B. licheniformis</i> | Amino acid  | L-alanine                       | Gould (1969)   |
| <i>B. megaterium</i>    | Amino acid  | L-alanine                       | Hyatt and Levinson (1962); Hyatt and Levinson (1964) |
|                         | Combination | L-alanine and inosine           | Rode and Foster (1962)                               |
|                         | Nutrient    | Glucose                         | Hyatt and Levinson (1962), Hyatt and Levinson (1964) |
|                         | Combination | L-alanine and glucose           | Levinson and Hyatt (1966)                            |
|                         | Chemical    | KI                              | Rode and Foster (1962); Paredes-Sabja et al. (2008b) |
|                         | Chemical    | KBr                             | Rode and Foster (1962); Paredes-Sabja et al. (2008b) |
|                         | Chemical    | KH <sub>2</sub> PO <sub>4</sub> | Rode and Foster (1962); Paredes-Sabja et al. (2008b) |
|                         | Chemical    | KCl                             | Rode and Foster (1962); Paredes-Sabja et al. (2008b) |
|                         | Chemical    | long chain alkyl primary amines | Rode and Foster (1961)                               |
|                         | Mechanical  | Abrasion                        | Rode and Foster (1960)                               |



| Organism                     | Type           | Germinant                      | Reference   |
|------------------------------|----------------|--------------------------------|---|
| <i>B. sphaericus</i>         | Complex        | TSA                            | Caijo et al. (2002)   |
|                              | Amino acid     | L-alanine                      | Gould (1969)  |
|                              | Amino acid     | L-alanine                      | Gould (1969)  |
| <i>B. stearothermophilus</i> | Amino acid     | L-alanine                      | Romick and Tharrington (1997); Hornstra et al. (2007); Kumazawa et al. (2007); Paredes-Sabja et al. (2008b) |
|                              | Amino acid     | L-valine                       | Paredes-Sabja et al. (2008b)  |
|                              | Nucleoside     | Inosine                        | Alberto et al. (2003)   |
|                              | Combination    | AGFK                           | Foster and Johnstone (1990); Alberto et al. (2003); Kumazawa et al. (2007); Paredes-Sabja et al. (2008b)    |
|                              | Physical       | high pressure                  | Black et al. (2005)   |
|                              | Chemical       | Ca <sup>2+</sup> -dipicolinate | Paidhungat et al. (2001)  |
|                              | Chemical       | Dodecylamine                   | Setlow et al. (2003)  |
| <i>B. thuringiensis</i>      | Chemical       | Bryostatins                    | Wei et al. (2010)   |
|                              | Cell fragments | peptidoglycan fragments        | Wei et al. (2010)   |
|                              | Amino acid     | L-alanine                      | Broussolle et al. (2008)  |
|                              | Nucleoside     | Inosine                        | Broussolle et al. (2008)  |
|                              | Amino acid     | L-arginine                     | Howerton et al. (2011)  |
|                              | Amino acid     | Glycine                        | Howerton et al. (2011)  |
|                              | Amino acid     | L-phenylalanine                | Howerton et al. (2011)  |
|                              | Bile salt      | Taurocholate                   | Howerton et al. (2011)  |
|                              | Complex        | brain heart infusion (BHI)     | Paredes-Sabja et al. (2008a)  |
|                              | Chemical       | KCl                            | Paredes-Sabja et al. (2008a)  |
| <i>Cl. difficile</i>         | Chemical       | Dodecylamine                   | Paredes-Sabja et al. (2008a)  |

| Organism                                      | Type        | Germinant                   | Reference                    |
|---|-------------|-----------------------------|------------------------------|
| <i>Cl. botulinum</i> type A (proteolytic)     | Chemical    | Ca <sup>2+</sup> -DPA       | Paredes-Sabja et al. (2008a) |
| <i>Cl. botulinum</i> type B (proteolytic)     | Nutrient    | L-alanine                   | Alberto et al. (2003)        |
| <i>Cl. botulinum</i> type B (non-proteolytic) | Nutrient    | L-alanine                   | Alberto et al. (2003)        |
| <i>Cl. botulinum</i> type E (non-proteolytic) | Combination | L-alanine, and L-lactate    | Plowman and Peck (2002)      |
| <i>Cl. botulinum</i> Type E (Irwanai)         | Combination | L-cysteine, and L-lactate   | Plowman and Peck (2002)      |
| <i>Cl. botulinum</i> Type E (Irwanai)         | Combination | L-alanine and glucose       | Ando (1971)                  |
| <i>Cl. botulinum</i> type F (proteolytic)     | Combination | L-alanine, L- and D-lactate | Ando (1971)                  |
| <i>Cl. botulinum</i> type F (proteolytic)     | Combination | L-alanine and inosine       | Ando (1971)                  |
| <i>Cl. botulinum</i> type F (non-proteolytic) | Nutrient    | L-alanine                   | Alberto et al. (2003)        |
| <i>Cl. botulinum</i> type F (non-proteolytic) | Combination | L-serine and L-lactate      | Plowman and Peck (2002)      |

| Organism  | Type        | Germinant                              | Reference                      |
|---|-------------|--|--------------------------------|
| <i>Cl. botulinum</i> type G                       | Combination | L-cysteine, L-lactate, and bicarbonate | Takeshi et al. (1988)          |
| <i>Cl. botulinum</i> Eklund 17B (non-proteolytic) | Complex     | PYGS                                   | Stringer et al. (2005)         |
| <i>Cl. sordellii</i>                              | Complex     | ARF*                                   | Ramirez and Abel-Santos (2010) |

The nutrient germinant L-alanine has been shown to trigger germination in many strains of *Bacillus* including: *B. anthracis*, *B. subtilis*, *B. cereus*, *Bacillus licheniformis*, *B. coagulans*, *Bacillus stearothermophilus*, *Bacillus circulans*, *B. sphaericus* and some strains of *B. megaterium* (Gould 1969). The spores of many clostridial species have also been shown to respond to L-alanine. Alberto et al. (2003) followed the fall in OD of spores of proteolytic *Cl. botulinum* types A (Eyemouth, 62A, 97A, NCTC 7272 and Hall A), B (B6, 231B, NCIB 4301 and Beans), and F (Langeland), and found that they germinated in response to L-alanine alone, as does *Clostridium sporogenes*. This is not true of all clostridia. Spores of *Clostridium difficile* did not respond to any of 20 individual amino acids including L-alanine (Paredes-Sabja et al. 2008a), and *Cl. perfringens* SM101 spores germinated only slightly with L-alanine and L-asparagine (Paredes-Sabja et al. 2008b). Strains that respond to a single amino acid or a single amino in combination with L-lactate often respond less optimally to other single amino acids. Non-proteolytic *Cl. botulinum* types B, E and F germinate optimally in response to L-lactate in combination with L-alanine, L-cysteine or L-serine, and sub-optimally in response to L-lactate in combination with L-valine or L-threonine (Plowman and Peck 2002). Other single amino acids act as germinants including L-proline, which triggers germination in *B. megaterium* GMB1551. L-Histidine and L-lysine, are variously germinative for the spores of clostridial species including *Clostridium acetobutylicum*, *Clostridium roseum*, *Clostridium bifermentans* and *Clostridium septicum* (Foster and Johnstone 1990; Peck et al. 2004).

*B. cereus* has been shown to respond to the nucleoside germinant inosine at low levels. Guanosine and xanthine, functioning alone or synergistically with an amino acid, are often germinative for strains of *B. cereus*, *B. anthracis* and *B. megaterium* (Peck et al. 2004). Nucleosides are generally less effective germinants, than amino acids such as L-alanine. Spores of clostridial species have been found to be relatively unresponsive to ribosides such as adenosine, inosine and xanthoxine (Gould 1969; Peck et al. 2004).

In some cases sugars act as germinants. *B. megaterium* germinates in the presence of glucose (Hyatt and Levinson 1962). Clostridial species appear less responsive to sugars as germinants. Non-proteolytic *Cl. botulinum* types B, E and F failed to germinate in the

presence of glucose, fructose, lactose, galactose, xylose, ribose, mannose, or maltose (Plowman and Peck 2002). Sugars are also common co-germinants. Where a compound, such as L-lactate improves germination in combination with other germinants, but does not trigger germination singly, it is referred to as a co-germinant. Germination in *B. subtilis*, and most other *Bacillus* species, has been triggered by a mixture of: L-asparagine, fructose, glucose and KCl (AFGK), but not by the individual components (Foster and Johnstone 1990). There are also a number of non-nutrient chemicals that act as germinants or co-germinants including DPA, salts, lysozyme, hydrogen peroxide, and a variety of cationic and anionic surfactants (Rode and Foster 1961). By adding sodium bicarbonate or sodium thioglycolate, germination of *Cl. botulinum* 62A was enhanced (Rowley and Feeherry 1970). Sodium thioglycolate is sometimes included in anaerobic growth medium for bacteria as it lowers oxidation potential. When included in a solution, that also contains 11.9 mM sodium bicarbonate, 10 mM sodium phosphate and 7.4 mM L-cysteine, 4.4 mM sodium thioglycolate assisted in achieving germination of approximately 90 % of heat activated (80 °C, 10 min) spores of *Cl. botulinum* 62A (Rowley and Feeherry 1970). Germination of clostridia is also stimulated by CO<sub>2</sub> (Enfors and Molin 1978).

In *B. subtilis*, DPA release during stage I of germination, triggers stage II events of germination (Magge et al. 2008). *In vitro* exposure to DPA results in germination by triggering of CLEs. Spores generally require a critical concentration of ions, in the medium for, maximum germination (Gould 1969). Bicarbonate anions stimulated the germination of many types of clostridial spores, in rich media, including strains of *Cl. botulinum* (Gould 2006). *Clostridium cylindrosporium* spores germinated rapidly under reducing conditions when bicarbonate, uric acid and calcium were present (Smith and Sullivan 1989). The cationic surfactant dodecylamine has been shown to be highly germinative for spores of *B. subtilis* prepared in liquid but not solid medium. In liquid medium several indicators of germination were observed: almost all spore DPA was released, degradation of the spore's peptidoglycan cortex occurred, and the spore's pool of free adenine nucleotides was released. However, the spores did not initiate metabolism, did not degrade their pool of SASP's efficiently, and had a significantly lower level of water in the core than spores germinated by nutrients (Setlow et al.

2003). Spores of *B. megaterium*, that germinated as a result of exposure to surfactants, were unable to form colonies on an agar based medium (Rode and Foster 1961).

The germination of *Cl. difficile* has been studied in relation to its ability to cause disease in immune compromised people. Bile salts present in the digestive tract have been investigated as possible germinants. Conflicting results, as to the effectiveness of the bile salt taurocholate as a co-germinant, have been reported. Sorg and Sonenshein (2008) reported that inclusion of 0.1 % taurocholate in brain heart infusion (BHI) agar plates enhanced the recovery (growth) of *Cl. difficile* spores. Addition of 1 % taurocholate to brain BHI resulted in a rapid decrease in the OD, to about 85 %, indicating significant germination. Paredes-Sabja et al. (2008a) reported bile salts as having little effect on germination, either alone or in BHI.

Rode and Foster (1960) agitated spores of *B. megaterium* in the presence of glass particles. This resulted in the conversion of a sizable portion of the spores to a non-heat resistant, non-refractive and stainable state. Microscopic observation, of the abraded spores, showed they began growth and elongation significantly sooner than unabraded spores. Abrasion appears to function by activating CwlJ or SleB. Germination of *B. subtilis* spores has been induced with high pressure (150 MPa) which activates CwlJ or SleB. Higher pressure (500 to 800 MPa) releases the spore's  $\text{Ca}^{2+}$ -DPA depot (Black et al. 2005).

Many foods, of both plant and animal origin, have endogenous lysozyme activity, and may increase the recovery of heat-damaged spores (Stringer et al. 1999). Under some conditions lysozyme is able to induce germination. Heat treatment of spores of *Cl. perfringens*, sufficient to rupture disulphide bonds in the spore, prevented germination in the presence of nutrient germinants. Spores damaged in this manner germinated in response to exposure to lysozyme, which was able to attack the underlying muropeptide of the spore cortex (Duncan et al. 1972; Gould 2006).

Exposure of spores to muropeptide fragments (degraded peptidoglycan), from spores of the same or closely related strains, induced germination in *B. subtilis* (Shah et al.

2008). Muropeptides act on PrkC, a well conserved eukaryotic-like Ser/Thr membrane kinase. Bryostatin, a natural product synthesised by a marine bacterium, is also able to induce germination via PrkC (Shah et al. 2008). Putative *prkC* homologues are present in the genome of proteolytic *Cl. botulinum* and other clostridia, although it remains to be established whether the PrkC protein contributes to germination in these species (Paredes-Sabja et al. 2011; Peck et al. 2011).

## 2.4 Conclusion

A number of psychrotolerant clostridia have been identified as causing spoilage in fresh, chilled, vacuum-packed red meat. The vegetative cells of clostridia are oxygen sensitive suggesting that spores play a significant role in initial contamination of meat leading to spoilage. Before resuming a vegetative state multiplying and causing spoilage, spores must germinate. Germination pathways and germinants have been well studied in *B. subtilis*. Less is known about germination in *Clostridium* and, prior to this study, nothing had appeared in literature about germination in psychrotolerant meat spoilage clostridia. Germination is triggered by germinants, small molecules, usually nutrients interacting with specific proteins in spores called germinant receptors. Germinants are species and strains specific necessitating individual testing.





## Chapter 3

### General Materials and Methods

#### 3.1 *Water*

Two types of water were used during this study. For preparation of media and buffers, for general use, and cleaning of glassware, mH<sub>2</sub>O, water purified through a Millipore, Elix™ 10 water purification was used. For molecular applications, uH<sub>2</sub>O Gibco™ UltraPure™ distilled, DNase and RNase free water was obtained from Invitrogen (Auckland, New Zealand).

#### 3.2 *Culturing under anaerobic conditions*

Unless otherwise stated, all culturing was carried out under strict anaerobic conditions, in an anaerobic chamber containing H<sub>2</sub> / CO<sub>2</sub> / N<sub>2</sub> (5:10:85, Don Whitley, Shipley, UK). An indicator solution (0.3 g Tris base, 4 g glucose, 100 ml mH<sub>2</sub>O, 2 drops Löffler's methylene blue [0.5 g Methylene blue, 1 ml 1 % w/v KOH, 30 ml 95 % ethanol, 100 ml mH<sub>2</sub>O]), through which the chambers atmosphere was bubbled, was monitored visually to ensure the chamber remained oxygen free. Where necessary plates and bottles were incubated or maintained in anaerobic conditions in Anaerobic Jar GasPak 100 systems (Fort Richard Laboratories, Auckland, New Zealand) containing AnaeroGen sachets (Oxoid, Thermo Fisher Scientific, Auckland, New Zealand) and anaerobic indicator strips (BD, Thermo Fisher Scientific).

#### 3.3 *Strains*

The strains used in this study (**Table 3.1**) were either original New Zealand strains, isolated from spoiled red meat and deposited in culture collections, or obtained from NCFB, the National Collection of Food Bacteria (Reading, UK. CSIRO); DSMZ, the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), ATCC, the American Type Culture Collection (Manassas, VI, USA), or the New Zealand

Reference Culture Collection (NZRM). The isolates were maintained on Columbia blood agar (CBA; Oxoid CM331, Oxoid, Basingstoke, UK) containing 5 % sheep blood (venous supplies, Auckland, New Zealand) under strict anaerobic conditions.

**Table 3.1** Strains used in this study. Strains were obtained from NCFB the National Collection of Food Bacteria (Reading, UK. CSIRO), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), ATCC (American Type Culture Collection, Manassas, VA, USA), New Zealand Reference Culture Collection (NZRM).

| Strain   | Collection number       | Source                                     | Reference (s)                                    |
|--|-------------------------|--|--|
| <i>Cl. algidicarnis</i>                              | NCFB 2931 <sup>T</sup>  | vacuum-packed, cooked pork                 | Lawson et al. (1994)                             |
| <i>Cl. algidixylanolyticum</i>                       | DSM 12273 <sup>T</sup>  | temperature-abused, vacuum-packed raw lamb | Broda et al. (2000a)                             |
| <i>Cl. estertheticum</i> subsp. <i>estertheticum</i> | DSM 8809 <sup>T</sup>   | spoiled, chill-stored, vacuum-packed beef  | Collins et al. (1992); Spring et al. (2003)      |
| <i>Cl. estertheticum</i> subsp. <i>laramiense</i>    | ATCC 51254 <sup>T</sup> | vacuum-packed, fresh beef and roast beef   | Kalchayanand et al. (1993); Spring et al. (2003) |
| <i>Cl. frigidicarnis</i>                             | DSM 12271 <sup>T</sup>  | temperature-abused, vacuum-packed beef     | Broda et al. (1999)                              |
| <i>Cl. gasigenes</i>                                 | DSM 12272 <sup>T</sup>  | blown-packs of vacuum-packed chilled lamb  | Broda et al. (2000b)                             |
| LA1  | local isolate           | spoiled vacuum-packed beef                 |  |
| <i>Cl. perfringens</i> type B                        | NZRM 132                |  |  |
| <i>Cl. perfringens</i> type C                        | NZRM 133                |  |  |
| <i>Cl. sporogenes</i>                                | ATCC 19404              | ATCC                                       |  |
| <i>B. cereus</i>                                     | ATCC 10702              | ATCC                                       |  |

| Strain | Collection number | Source                             | Reference (s)                             |
|--------|-------------------|------------------------------------|---|
| K21    | local isolate     | blown-pack                         | Bell et al. (2001); Boerema et al. (2002) |
| V1     | local isolate     | meat processing plant respectively |   |
| L1     | local isolate     | venison hide sample                |   |

### 3.4 Recipes for media

#### *Biphasic cooked meat medium (bCMM)*

|  |        |
|--|--------|
| CMM pellets (Acumedia, Neogen, Lansing, Michigan, USA) | 15 g   |
| Glucose,   | 0.15 g |
| Agar   | 2.25 g |
| mH <sub>2</sub> O                                      | 150 ml |

All ingredients were autoclaved at 121 °C for 15 min in 500 ml culture bottles. Media was allowed to set, forming the solid phase, and deoxygenated in an anaerobic cabinet for at least 24 h prior to the addition of 40 ml of sterile, deoxygenated mH<sub>2</sub>O, forming the liquid phase (Adam et al. 2011).

#### *Cooked meat medium from fresh meat (fCMM)*

|  |       |
|--|-------|
| Fresh beef heart finely chopped (minced) | 424 g |
| Proteose peptone                         | 20 g  |
| NaCl                                     | 5.0 g |
| Glucose                                  | 2 g   |

The finely minced beef heart was covered with mH<sub>2</sub>O and boiled for 10 min then strained through muslin. Cooked beef heart mince (1.5 g portions) were added to 30 ml universal tubes. The peptone, NaCl and glucose were added to 1 L of broth (liquid collected from boiled mince), and dissolved. The pH was adjusted to 7.1 +/- 0.2 and the broth dispensed in 20 ml aliquots into the universal tubes containing the mince prior to autoclaving at 121 °C for 15 min.

#### *Peptone, yeast extract, starch and glucose medium (PYGS)*

|                  |     |
|------------------|-----|
| Protease peptone | 5 g |
| Tryptone         | 5 g |

|                   |                             |
|-------------------|-----------------------------|
| Yeast extract     | 10 g                        |
| Lab lemco powder  | 10 g                        |
| Glucose           | 2 g                         |
| Resazurin         | 0.001 g                     |
| mH <sub>2</sub> O | 1 L                         |
| Salts solution -A | 40 ml                       |
| -B                | 40 ml                       |
| Agar              | 15 g (omit if making broth) |
| Cysteine HCl      | 0.2 g                       |

Salts solutions- Dissolve in mH<sub>2</sub>O and store in refrigerator.

|                         |  |         |
|-------------------------|--|---------|
| A- (g l <sup>-1</sup> ) | CaCl <sub>2</sub> .2H <sub>2</sub> O               | 0.256 g |
|                         | MgSO <sub>4</sub> .7H <sub>2</sub> O               | 0.48 g  |
|                         | NaCl   | 2.0 g   |
| B- (g l <sup>-1</sup> ) | KH <sub>2</sub> PO <sub>4</sub>                    | 1.0 g   |
|                         | K <sub>2</sub> HPO <sub>4</sub> .3H <sub>2</sub> O | 1.3 g   |
|                         | NaHCO <sub>2</sub>                                 | 10.0 g  |

All ingredients were mixed together except the cysteine, and heated to dissolve. The cysteine was added and the pH adjusted to 7.0 ± 0.2. Aliquots (500 ml) were autoclaved at 121 °C for 15 min (Stringer et al. 1999).

### 3.5 Spore production

Spores of *Cl. estertheticum* and *Cl. estertheticum* like strains were produced in peptone, yeast extract, glucose and starch (PYGS) broth. A volume (500 ml) of PYGS broth in a culture bottle was inoculated with an exponentially growing culture of *Cl. estertheticum* and incubated, anaerobically at 15 °C, for a minimum of 3 months to promote sporulation. A spore suspension was prepared when a typical spore appearance, presence of refractive spheres, was observed with phase contrast

microscopy, in the sporulating culture. The culture was centrifuged, washed twice with ice-cold saline (0.85 % NaCl), and resuspended in 5 ml of ice-cold saline.

Spores of *Cl. frigidicarnis* and LA1 were produced in bCMM. Batches of the medium, in 500 ml bottles, were inoculated with 1 ml each of exponentially growing culture in PYGS broth. After three weeks of incubation at 30 °C spores were harvested by collection of the liquid phase and washing, by centrifugation (2400 g for 10 min), in ice-cold saline (0.85 % NaCl), four times.

### 3.6 *Cleaning spores*

Spores were then separated, from vegetative cells and debris, by centrifugation through a Urografin 370 (Schering, Germany) density gradient as described by Plowman and Peck (2002). Pellets were resuspended in 0.85 % saline layered on top of 50 % Urografin and centrifuged at 6000 g for 45 min. The resulting pellet was washed 4 times, resuspended in 0.85 % saline and stored at -18 °C until needed. When necessary spore clumps were dispersed by 10 s bursts with a Soniprep 150 ultrasonic disintegrator (Sanyo, Gallenkamp, Leicester, UK).

### 3.7 *Measuring germination using microscopy*

Spores were centrifuged and resuspended in sterile mH<sub>2</sub>O at a concentration of approximately 10<sup>7</sup> spores ml<sup>-1</sup>. Aliquots (100 µl) were transferred to Superfrost Plus slides (Thermo Fisher Scientific) and air dried. The slides were washed with mH<sub>2</sub>O and dried a second time before being transferred to an anaerobic cabinet to deoxygenate for at least 24 h prior to use. To measure germination 10 µl of potential germinant solution was transferred to the slide covered with a cover slip and sealed in place with aluminium tape. Following incubation the spores were observed, under phase contrast, using a Nikon eclipse 50i microscope (Coherent Scientific, Auckland, New Zealand). Five unique images containing a total of at least 200 spores were captured for enumeration using a Nikon DSFi1 camera and DS camera control unit (Coherent Scientific).



### 3.8 *Measuring germination based on change in optical density*

The rate and extent of germination were assessed by measuring the fall in optical density at 600 nm ( $OD_{600}$ ) using a Bioscreen C analyser system (Labsystems, Basingstoke, UK) in an anaerobic cabinet as described previously (Plowman and Peck 2002). Potential germinants and buffer were dispensed into honeycomb plates (Labsystems) in 100  $\mu$ l aliquots in triplicate. Spores were diluted to approximately  $5 \times 10^8$  CFU  $ml^{-1}$ , deoxygenated overnight in an anaerobic cabinet and (where necessary) heat shocked at 60 °C for 10 min prior to being dispensed (100  $\mu$ l) into honeycomb plates. The plates were then placed in the analyser, which was maintained at a constant temperature.

### 3.9 *Flow cytometry*

Flow cytometry was performed using a BactiFlow™ analyser (Chemunex SA, Paris, France). The sheath fluid ChemSol S/1 (Chemunex) was prepared daily according to the manufacturer's instructions. Spore and vegetative cell samples were prepared in 1 ml aliquots in proprietary 3 ml sample vials, diluted to approximately  $10^4$  cells  $ml^{-1}$ , containing 12  $\mu$ mol propidium iodide and 0.5  $\mu$ mol SYTO 16 (Invitrogen), mixed vigorously and incubated in the dark at room temperature for 20 minutes prior to being presented to the BactiFlow™ for analysis.

### 3.10 *Nucleic acid extraction (kit)*

Nucleic acid was extracted using a High Pure PCR template Preparation Kit (Roche, Auckland, New Zealand). One ml of drip, or broth culture, was centrifuged and resuspended in 200  $\mu$ l of lysozyme (10 mg  $ml^{-1}$  in 10 mM Tris [pH 8.0] solution). Where an RNA free extraction was required 0.5  $\mu$ l of RNase I (100 units  $\mu$ l<sup>-1</sup>, Invitrogen) was added. The samples were incubated at 65 °C for 30 min. Steps 3 to 5 of section 2.6: Isolation of nucleic acids from bacteria or yeast and steps 2 to 6 of section 2.8:

protocol for washing and elution were followed (June 2007 version of manufacturer's instructions).

### 3.11 16s and *Cl. estertheticum* specific PCR

PCR was performed, in a Techne TC-512 thermocycler (Total Lab Systems, Auckland, New Zealand) gradient thermocycler, or a PTC100 programmable thermal controller (BioRad, Auckland, New Zealand). All reagents and primers were obtained from Invitrogen. The 16S rRNA primers used were pA (forward) 5'-AGA GTT TGA TCC TGG CTC AG-3' and pH\* (reverse) 5'-AAG GAG GTG ATC CAG CCG CA-3' (Hutson et al. 1993). The *Cl. estertheticum* specific primers employed amplified a section of 16S rRNA and were 16SEF (forward) 5'-TCG GAA TTT CAC TTT GAG-3' and 16SER (reverse) 5'-AAG GAC TTC ACT CAT CTC TG-3' (Broda et al. 2003a). Each 50 µl reaction contained 1 µmol of each primer, 1.5 mM MgCl<sub>2</sub>, 1 x buffer, 200 µmol dNTPs and 1 unit of Platinum® *Taq* DNA Polymerase. The amplification programme consisted of 3 min at 93 °C, thirty cycles of 1 min at 92 °C, 1 min at 55 °C and 2 min at 72 °C, followed by 3 min at 72 °C.

### 3.12 Agarose gel electrophoresis

Agarose gels were prepared using Agarose UltraPure™ (Invitrogen) at 1.0 or 1.5 % (w/v) in 1 x TBE buffer (Invitrogen). Agarose electrophoresis was performed at 100 V for 1 to 2 h. DNA molecular weight marker 1 Kb Plus (Invitrogen) was included to determine the size of the PCR products. Loading buffer was combined with samples using either 1 µl loading buffer and 5 µl of sample or 3 µl loading buffer and 18 µl of sample. Bands were visualized with ethidium bromide by UV transillumination and photographed.

Sample loading buffer:

|                               |       |
|-------------------------------|-------|
| 30 % Glycerol                 | 3 ml  |
| 0.25 % Bromophenol blue (w/v) | 25 mg |
| 0.25 % Xylene cyanol FF (w/v) | 25 mg |
| uH <sub>2</sub> O             | 7 ml  |

### 3.13 Purification of PCR product

PCR product was purified either directly, using a QiaGen QuiaQuick® PCR purification kit (Thermo Fisher Scientific), or from bands excised from agarose gels, using a QiaGen QuiaQuick® gel extraction kit (Thermo Fisher Scientific), following the manufacturer's instructions.

### 3.14 Southern blot analysis

#### 3.14.1 Blot preparation

Agarose gels for Southern blotting analysis were prepared as described above, with the inclusion of a digoxigenin- (DIG-) labelled DNA molecular weight marker VI (Roche). Following visualisation, the gel was bathed for 15 min in solution 1 (1.5 M NaCl, 500 mM NaOH) to denature DNA. This step was repeated and the gel rinsed in mH<sub>2</sub>O. The gel was bathed twice in solution 2 for 15 min (1.5 M NaCl, 500 mM Tris-HCl pH 7.4) to neutralise the gel, and rinsed with distilled H<sub>2</sub>O. The gel, Hybond-N<sup>+</sup> membrane (Amersham Pharmacia Biotech, Auckland New Zealand) and 2 x 3MM filter papers (Whatman), cut exactly to the gel size, were soaked in 20 x SSC (175.3 g NaCl, 88.2 g sodium citrate in 1 L mH<sub>2</sub>O pH 7.4) for 10 to 30 min, to increase the salt content and facilitate DNA transfer to the membrane. The gel was placed on cling film, and covered with the membrane, two soaked pieces of filter paper and three further pieces of dry 3MM filter paper. Bubbles were removed from between the gel and the membrane to improve DNA transfer. Absorbent paper, cut to the size of the gel was stacked on top of the filter paper, and a 500 g weight placed above. The assembly was left overnight at room temperature. The membrane was air-dried and DNA cross-linked to the membrane by exposure to UV light for 3 min on each side of the membrane.

### 3.14.2 Probe synthesis

Southern blotting was carried out using DIG-labelled probes produced by PCR. PRC reactions were optimised using unlabelled dNTP's. The optimised reaction was repeated replacing the unlabelled dNTP's with PCR DIG labelling mix (Roche). Just prior to use, 50  $\mu$ l of uH<sub>2</sub>O and the labelled probe (2  $\mu$ l per 1 ml hybridisation buffer) were boiled for 5 min, to denature and chilled on ice for at least 5 min.

### 3.14.3 Hybridisation

Hybridisation was carried out using the DIG wash and block buffer set and Anti-Digoxigenin-AP, Fab fragments (Roche). Working solutions and hybridization buffers were prepared as per the manufacturer's instructions. The hybridization oven, hybridization tubes (Amersham Pharmacia Biotech), and pre-hybridisation buffer (10 ml buffer containing 1  $\mu$ l probe per 100 cm<sup>2</sup> of membrane) were pre-heated to 55 °C. The membrane was placed the tube, and incubated for at least 30 min, with gentle agitation. The pre-hybridisation buffer was removed from the hybridisation tube. The probe was added to the pre-warmed hybridisation buffer (10 ml per 100 cm<sup>2</sup> of membrane, 55 °C), which was transferred to the hybridisation tube and incubated overnight with gentle agitation.

### 3.14.4 Washing and detection

The membrane was washed twice with high stringency buffer (0.1 x SSC, 0.1 % [w/v] sodium dodecyl sulphate [SDS]) heated to 55 °C for 15 min then washed twice with low stringency buffer (2 x SSC, 0.1 % [w/v] SDS). The membrane was transferred to a tray containing 150 ml of washing buffer, and agitated for 2 min at room temperature. Blocking solution, 150 ml was added and the tray agitated for a further 30 min. The solution was discarded, 30 ml of antibody solution (containing Anti-Digoxigenin-AP, Fab fragments) was added and the tray agitated for 30 min. The antibody solution was discarded and the membrane agitated twice in 150 ml of washing buffer for 15 min and room temperature. Excess buffer was drained from the membrane prior to

transfer to cling film. The membrane was equilibrated in 30 ml of detection buffer prior to addition of 1 ml per cm<sup>2</sup> CDP-star (Roche), drop wise. The membrane was covered with the cling film, excluding bubbles and incubated at room temperature for 5 min. The membrane was visualised using a GelDoc™ XR+ and band size determined using Image Lab™ software (BioRad).

## Chapter 4

### Spore germination in psychrotolerant *Cl. frigidicarnis*

#### 4.1 Introduction

Although germinant systems had been identified in a number of spore forming bacteria, including psychrotolerant non-proteolytic *Cl. botulinum* (Plowman and Peck 2002), prior to this study nothing had been reported about the triggers of germination in any of the psychrotolerant spore forming clostridia associated with spoilage of vacuum-packed, New Zealand, red meat (beef, lamb and venison). Germination of bacterial spores is generally triggered by germinants that signal to the spore that the environment is suitable for growth (Setlow 2003). Inoculation of beef, with spores of *Cl. estertheticum*, prior to vacuum-packaging resulted in spoilage (Bell et al. 2001; Boerema et al. 2007). Due to the strictly anaerobic nature of psychrotolerant clostridia, it is thought that contamination of red meat with spores is responsible for a significant portion of spoilage by psychrotolerant clostridia. It is also thought that, the trigger(s) of germination, in psychrotolerant clostridia, are present in vacuum-packed red meat.

The most common methods for measuring germination are, monitoring changes in OD<sub>600</sub> of spore suspensions, or by counting phase bright (dormant) and phase dark (germinated) spores observed using phase contrast microscopy (Plowman and Peck 2002). The drop in OD<sub>600</sub> occurs as a result of DPA being released from the spore (Setlow et al. 2003). Germination can also be monitored by measuring the level of DPA released into supernatant (Cabrera-Martinez et al. 2003). Flow cytometry, following staining with SYTO 16, was used to determine the percentage of *B. subtilis* spores that germinated following pressure treatment (150 MPa) (Black et al. 2005). Comparisons were made of SYTO 9, SYTO 11, SYTO 12, SYTO 13, SYTO 14, SYTO 15 and SYTO 16, with SYTO 16 giving the largest separation in fluorescence between dormant and germinated *B. subtilis*. Germination has also been measured in spores of *B. subtilis*, modified with a luxAB gene (originally from *Vibrio harveyi*), to specifically induce bioluminescence upon germination (Ciarciaglini et al. 2000). Light production was

almost instantaneous following germination, and, as a measure of germination, correlated well with results using other methods (Hill et al. 1994). This method requires understanding of the genetic basis of germination, of the strain in question, and aerobic conditions for bioluminescence to occur. Where samples can be heat treated, such that vegetative cells are inactivated while spores are left intact or activated, comparison of treated and untreated counts can give an indication of the extent of germination. Braconnier et al. (2003) used this method to determine germination of proteolytic *Cl. botulinum* in vegetable-based media.

Specific germinants vary between strains, and are often nutrients necessary for vegetative growth (Setlow 2003). Germination has been most widely studied in *Bacillus* species, where the nutrient germinants L-alanine and inosine are each reported to induce spore germination, as is AGFK (Moir and Smith 1990; Gounina-Allouane et al. 2008). The germination of spores in species of *Clostridium* often requires complex mixtures of germinants (Peck 2009). For example, germination of spores of *Clostridium sordellii* is triggered by the presence of L-alanine, L-phenylalanine, L-arginine and NaHCO<sub>3</sub> (AFR\*) (Ramirez and Abel-Santos 2010). Spores of proteolytic *Cl. botulinum* types A, B and F germinated in response to a three-component system, comprising of L-alanine, L-lactate and NaHCO<sub>3</sub> (Peck 2009; Peck et al. 2011), while spores of non-proteolytic *Cl. botulinum* types B, E and F responded to a combination of L-lactate and either L-alanine, L-cysteine, or L-serine (Plowman and Peck 2002). Freshly slaughtered red meat contains a wide range of amino acids and other potential germinants (Lawrie 1998).

Storage temperature, pH, sodium chloride (NaCl) concentration, oxygen levels and concentration of potential germinants vary in meat packs, and can affect germination of bacterial spores. Even under an optimal cooling regime, red meat experiences a range of temperatures during processing and shipping. Typically boning rooms are maintained at 10 °C, and product should ideally be chilled to -1.5 °C prior to loading into shipping containers. Considerable variation was observed in the effect of suboptimal temperature on germination of 10 strains of proteolytic *Cl. botulinum* highlights the need to test the responses of individual strains (Alberto et al. 2003). As

with growth of the vegetative form, spores have an optimal pH range for germination, 5.5-8.0 in non-proteolytic *Cl. botulinum* (Plowman and Peck 2002). Increasing the NaCl concentration, in combination with heat treatment, has been shown to prevent or slow growth from spores of non-proteolytic *Cl. botulinum* (Graham et al. 1996; Stringer and Peck 1997). However, increasing NaCl concentration to only 5 % was not sufficient to prevent growth of non-proteolytic *Cl. botulinum* (Graham et al. 1997). Although anaerobic conditions are necessary for the growth of clostridia some clostridial spores, such as non-proteolytic *Cl. botulinum*, are able to germinate under aerobic conditions (Plowman and Peck 2002). A shortage of germinants will reduce the extent of germination within a population of spores. Once germinants of psychrotolerant clostridia, and the concentration at which they induce germination, have been identified in the laboratory they can be compared with those present in red meat.

The purpose of the present study was to compare the effectiveness of different methods to measure germination in *Cl. frigidicarnis*. These methods were then used to identify the triggers of germination in *Cl. frigidicarnis*, and the effects of environmental factors such as atmosphere, pH, temperature and NaCl concentration on germination. Finally the optimum concentration of components of a simple germinant system consisting of L-valine and L-lactate was determined.



## 4.2 Methods

Prior to using the microscopy method for measuring the extent of germination, a trial was carried out to determine the shelf life of *Cl. frigidicarnis* spores dried onto Superfrost Plus slides. Spores and slides were prepared as per Section 3.5, 3.6 and 3.7. The extent of germination, following 3 h incubation, in PYGS containing chloramphenicol ( $100 \mu\text{g ml}^{-1}$ ) to prevent multiplication of cells, at  $25 \text{ }^\circ\text{C}$  was determined microscopically (Section 3.7) for slides at the time of production, after 1 week, 2 weeks and every fortnight thereafter until germination fell below 10 %.

Measurement of germination of *Cl. frigidicarnis* using the Bioscreen C method was compared to that using the microscopy method. Spores were prepared as per Section 3.5 and 3.6. Spore samples were exposed to a range of potential germinants for 3 days at  $10 \text{ }^\circ\text{C}$ , while being monitored in the Bioscreen C analyser as per Section 3.8. Subsamples were taken from samples representing a range of final  $\text{OD}_{600}$  values. Wet mounts of the subsamples were prepared, the slides photographed under phase contrast microscopy and the phase bright and phase dark spores enumerated as per Section 3.7.

### 4.2.1 Preparation of germinant solutions

All potential germinants were prepared under strict anaerobic conditions (Stringer et al. 1999), except when tests were to be carried out under aerobic conditions. All chemicals were obtained from Sigma-Aldrich (Auckland, New Zealand) unless otherwise stated. Two standard buffer solutions were prepared ( $\text{pH } 7.0 \pm 0.2$ ):  $100 \text{ mmol l}^{-1}$  sodium phosphate,  $100 \text{ mmol l}^{-1}$  sodium phosphate,  $100 \text{ mmol l}^{-1}$   $\text{NaHCO}_3$  and  $50 \text{ mmol l}^{-1}$  L-cysteine. Potential germinants were dissolved in one of the buffer solutions in an anaerobic cabinet and the pH readjusted using NaOH where necessary. All solutions were prepared within 24 h of use to prevent precipitation and pH changes. Potential germinants tested were L-valine, L-norvaline, glycine, L-threonine, L-alanine, L-serine and L-cysteine ( $50$  or  $100 \text{ mmol l}^{-1}$ ); L-lactate, fructose, glucose and sucrose ( $50 \text{ mmol l}^{-1}$ ); bryostatin ( $10 \text{ mmol l}^{-1}$ ) and taurocholate ( $10 \text{ mmol l}^{-1}$ ); hen egg

white lysozyme (625 units ml<sup>-1</sup>); AGFK mixture [L-asparagine (10 mmol l<sup>-1</sup>), D-glucose (10 mmol l<sup>-1</sup>), D-fructose (10 mmol l<sup>-1</sup>), KCl (50 mmol l<sup>-1</sup>); and AFR\* mixture [L-alanine (25 mmol l<sup>-1</sup>), L-phenylalanine (5 mmol l<sup>-1</sup>), L-arginine (10 mmol l<sup>-1</sup>), NaHCO<sub>3</sub> (50 mmol l<sup>-1</sup>)]. Germination was also evaluated in PYGS medium (described in Section 3.4), and filtered dCMM (Acumedia, Neogen, Lansing, Michigan, USA), each containing chloramphenicol (100 µg ml<sup>-1</sup>), to prevent multiplication of cells that could mask a fall in OD<sub>600</sub> caused by spore germination.

#### 4.2.2 Analysis of *Cl. frigidicarnis* cells with a flow cytometer

To determine if staining of vegetative cells and spores, by SYTO 16 and propidium iodide, was sufficiently different to be separated by flow cytometry, cells stained using the staining procedure described in Section 3.9 were examined under a fluorescence microscope. Samples were then analysed using a BactiFlow flow cytometer as described in Section 3.9. Samples analysed included *Cl. frigidicarnis* spores prepared as per Section 3.5 and 3.6, exponentially growing *Cl. frigidicarnis* vegetative cells (16 h culture grown at 25 °C), early stationary phase *Cl. frigidicarnis* vegetative cells (24 h culture grown at 25 °C in PYGS) and *Cl. frigidicarnis* spores exposed to PYGS for 2.5 hours to induce germination.

#### 4.2.3 Assessment of spore germination

Spore germination was assessed using either the microscopy method described in Section 3.7 or the optical density method using the Bioscreen C analyser described in Section 3.8.

#### 4.2.4 Effect of NaCl concentration, pH and temperature on germination of *Cl. frigidicarnis*

The effect of NaCl, pH and temperature, on germination of *Cl. frigidicarnis*, was determined, using microscopic methods described in Section 3.7. To test the effect of NaCl, aliquots of PYGS and dCMM supernatant containing 0, 0.5, 1, 1.5 and 2 % (v/w)

NaCl were prepared. To test the effect of pH, aliquots of PYGS and dCMM were adjusted, no more than 10 min before use, to a range of pHs between 3.5 and 10.0. Slides were inoculated in triplicate and incubated for 3 h at 25 °C prior to imaging and analysis. To determine the effect of temperature on germination, slides were inoculated in triplicate with either dCMM or PYGS, sealed in a BD GasPak™ anaerobic pouch (Becton Dickinson, Auckland, New Zealand) without anaerobic generators (as these produce heat) and transferred from the anaerobic chamber to a temperature controlled water bath for 3 h prior to imaging. The process was repeated for a range of temperatures from 0 to 45 °C. The effect of pre-exposure to PYGS at temperatures that inhibit germination was determined by exposing prepared slides to 0 °C and 45 °C for 3 h then returning to room temperature. The speed with which the samples heated and cooled, to the highest and lowest temperature tested, from room temperature, was measured using a thermocouple attached to a Squirrel logger (Model Number 1034KT, Grant by Eltek Ltd, Cambridge, UK).

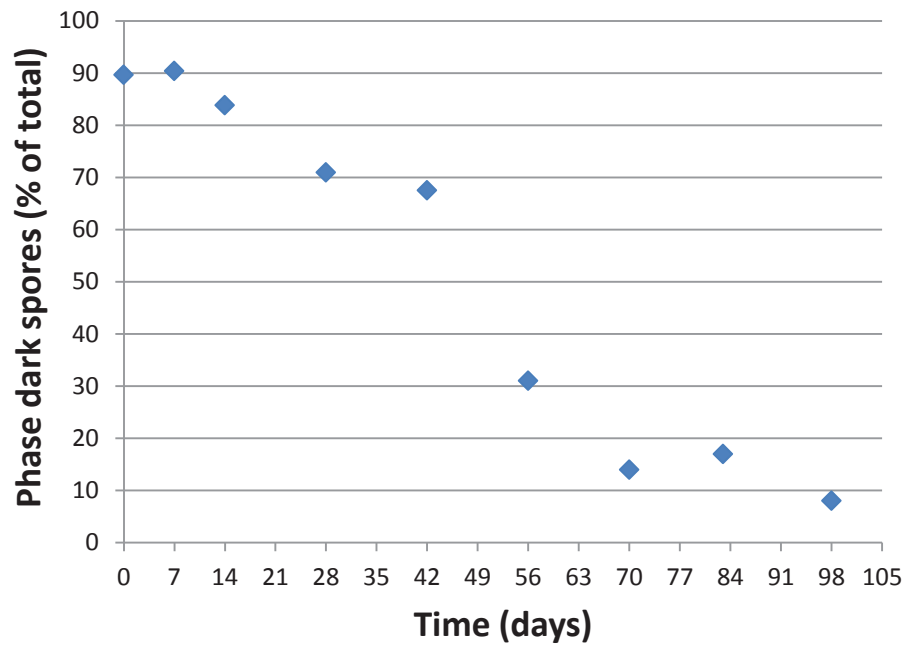
#### 4.2.5 Effect of concentration of co-germinants on germination of *Cl. frigidicarnis*

To determine the concentration of L-lactate and L-valine necessary to induce germination in *Cl. frigidicarnis* two dilution series were prepared. The first contained L-valine between 100 mmol l<sup>-1</sup> and 1 mmol l<sup>-1</sup> in L-lactate (50 mmol l<sup>-1</sup>) and sodium phosphate buffer (50 mmol l<sup>-1</sup>). The second contained L-lactate between 50 mmol l<sup>-1</sup> and 0.005 mmol l<sup>-1</sup> in L-valine (100 mmol l<sup>-1</sup>) and sodium phosphate buffer (50 mmol l<sup>-1</sup>). Variation was high resulting in each treatment being replicated five times.

### 4.3 Results

#### 4.3.1 Storage of prepared slides

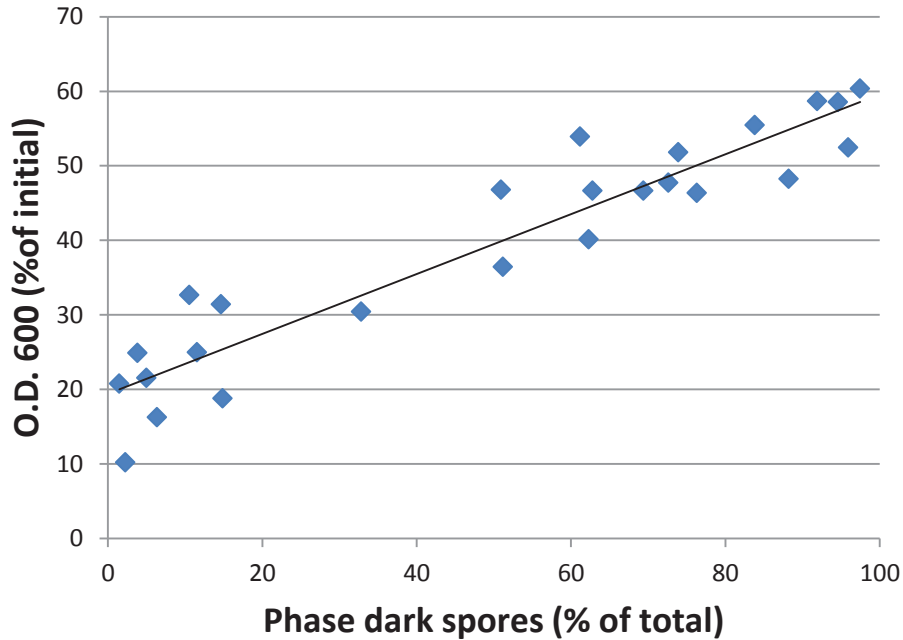
To determine the longevity of spores adhered to Superfrost Plus slides stored anaerobically, for use in subsequent trials, a storage trial was carried out. The extent of germination in nutrient rich PYGS began to decrease following two weeks of storage, and decreased steadily with increasing storage time thereafter (**Figure 4.1**). In all following work slides used were no more than one week old.



**Figure 4.1** Germination of *Cl. frigidicarnis* spores in PYGS following storage of prepared microscope slides. Germination was measured as the percentage of phase dark (germinated) spores by phase-contrast microscopy.

#### 4.3.2 Bioscreen C validation

The Bioscreen C method for measuring germination in populations of spores of *Cl. frigidicarnis* was validated prior to use. A linear relationship between the percentage of phase dark (germinated) spores and the decrease in  $OD_{600}$  was demonstrated (**Figure 4.2**).



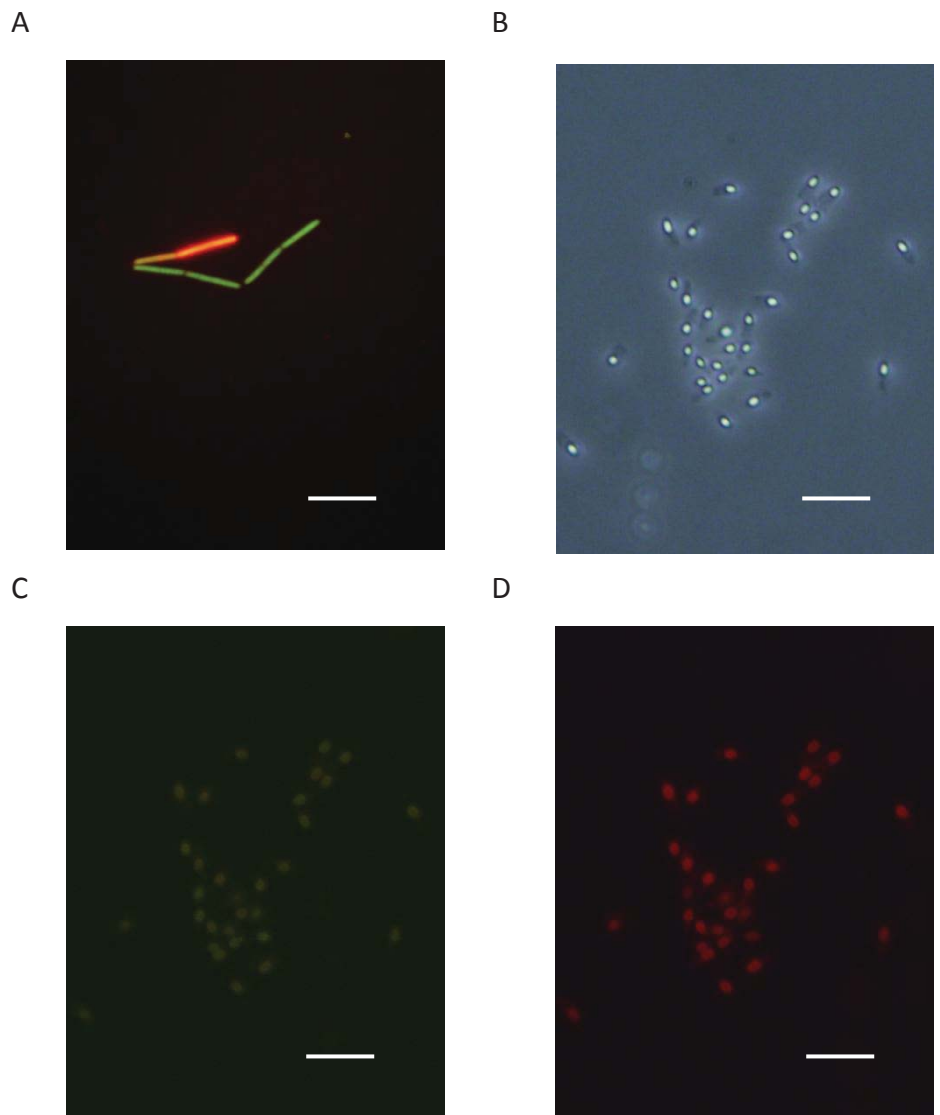
**Figure 4.2** Validation of the Bioscreen C method to measure germination of spores of *Cl. frigidicarnis*. Germination was measured as the percentage of phase dark (germinated) spores by phase-contrast microscopy and the decrease in OD<sub>600</sub>. A linear relationship between the decrease in OD<sub>600</sub> and the percentage of phase dark (germinated) spores was demonstrated. Adapted from Adam et al. (2011).

#### 4.3.3 Germination of *Cl. frigidicarnis* in complex media

Complex media known to support growth of *Cl. frigidicarnis* were chosen for initial testing as germinants are often required for growth. Germination of unheated spores and heat-shocked (60 °C for 10 min) spores of *Cl. frigidicarnis* was tested in PYGS and dCMM. After incubation for 72 h at 10 °C, a discernible drop in OD<sub>600</sub> was recorded in PYGS and in dCMM (data not shown). In both cases, microscopic counts of phase bright and phase dark spores confirmed that the extent of germination exceeded 95 %. By contrast, spores incubated for 72 h in 100 mmol l<sup>-1</sup> sodium phosphate showed no discernible germination (<10 % of the spores became phase dark). In all subsequent work, PYGS was included as a positive control and sodium phosphate as a negative control.

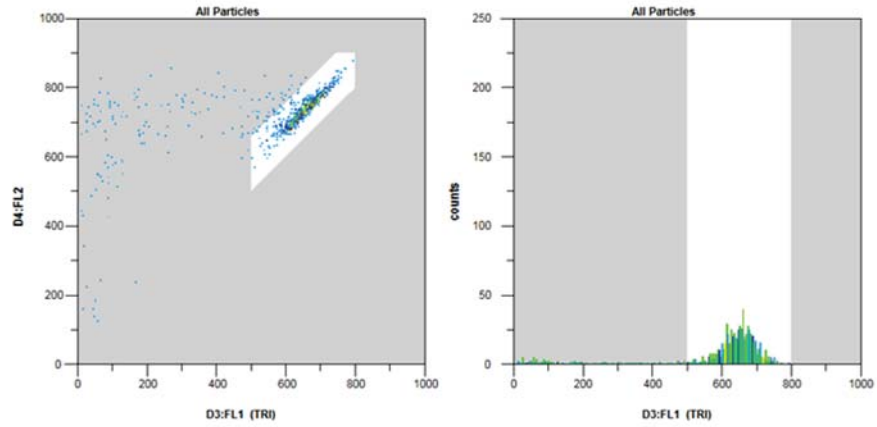
#### 4.3.4 Analysis of *Cl. frigidicarnis* cells with a flow cytometer

A BactiFlow flow cytometer was tested as an alternative tool for quantifying germination. To determine if fluorescence of *Cl. frigidicarnis* vegetative cells and spores, stained with SYTO 16 and propidium iodide, differed sufficiently to permit differentiation by flow cytometry, stained cells were visualised using a Leica DMRE microscope with I3 (BP 450-490, DM 510, LP 515) and N2.1 (BP 515-560, DM 580, LP 590) filter sets and an Olympus DP70 camera (**Figure 4.3**). Samples from a vegetative culture and a spore suspension, of *Cl. frigidicarnis*, were then analysed using a BactiFlow flow cytometer. The majority of events, recorded during sampling of spores and vegetative cells from a 16 hour culture, formed two distinct populations (**Figure 4.4**). Based on a visual assessment of the results the gate settings (describing the area for counting) for vegetative cells and spores were set as shown in **Appendix 1**. Analysis of a 24 h culture of vegetative *Cl. frigidicarnis* cells revealed two further populations within the sample.

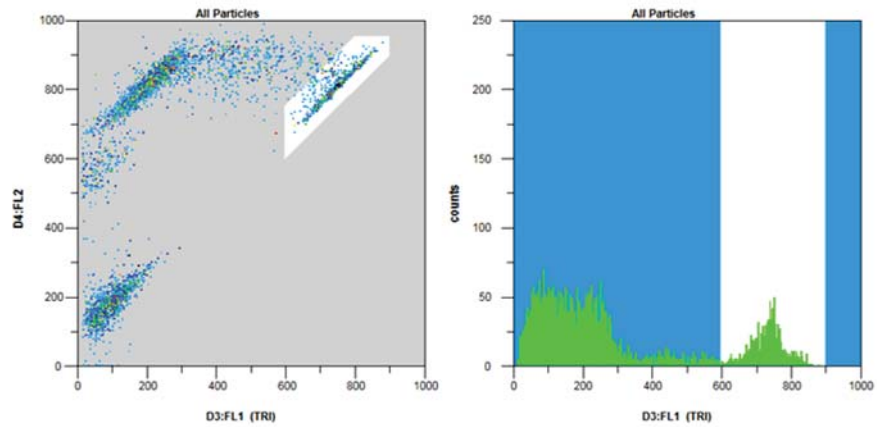


**Figure 4.3** *Cl. frigidicarnis* cells, stained with propidium iodide and SYTO 16, viewed under 100x objective. Vegetative cells from fresh culture, I3 filter set (A) spores, phase contrast (B).spores, I3 filter set (C) spores, N2.1 filter set (D). Bar = 10  $\mu$ m.

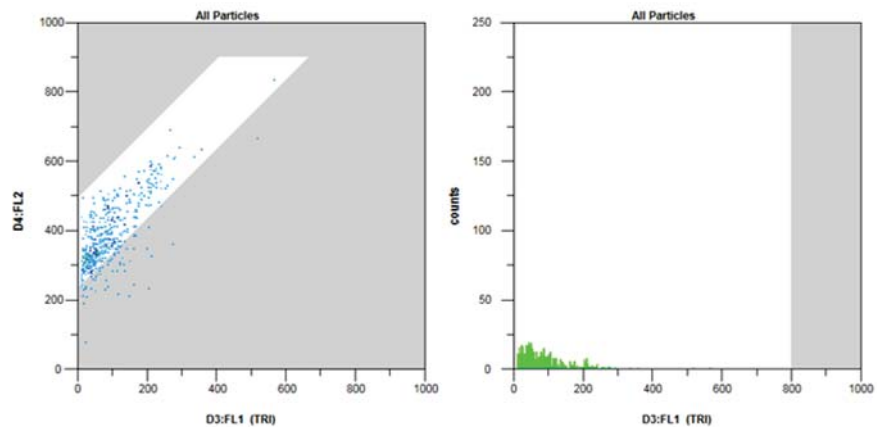
A



B



C



**Figure 4.4** Graphical representation of flow cytometry analysis of young vegetative cells (A, 16 hour culture,  $7.1 \times 10^3$  cells  $\text{ml}^{-1}$ ), old vegetative cells (B, 24 hour culture,  $8.5 \times 10^3$  cells  $\text{ml}^{-1}$ ) and spores (C,  $4.1 \times 10^3$  cells  $\text{ml}^{-1}$ ) of *Cl. frigidicarnis* stained with propidium iodide and SYTO 16.



To determine the accuracy of the flow cytometer counts, they were compared to plate counts on CBA (**Table 4.1**). In both cases the plate count was higher, in the case of the spore sample the plate count was approximately one log higher than the count from the flow cytometer.

**Table 4.1** Comparison of plate counts (CBA) and flow cytometry counts of *Cl. frigidicarnis* cells stained with propidium iodide and SYTO 16.

| Cell type                | Cell count (per ml) |                   |
|--------------------------|---------------------|-------------------|
|                          | Flow cytometry      | Plate count       |
| Vegetative (16h culture) | $7.1 \times 10^7$   | $1.2 \times 10^8$ |
| Spores                   | $4.1 \times 10^5$   | $3.6 \times 10^6$ |

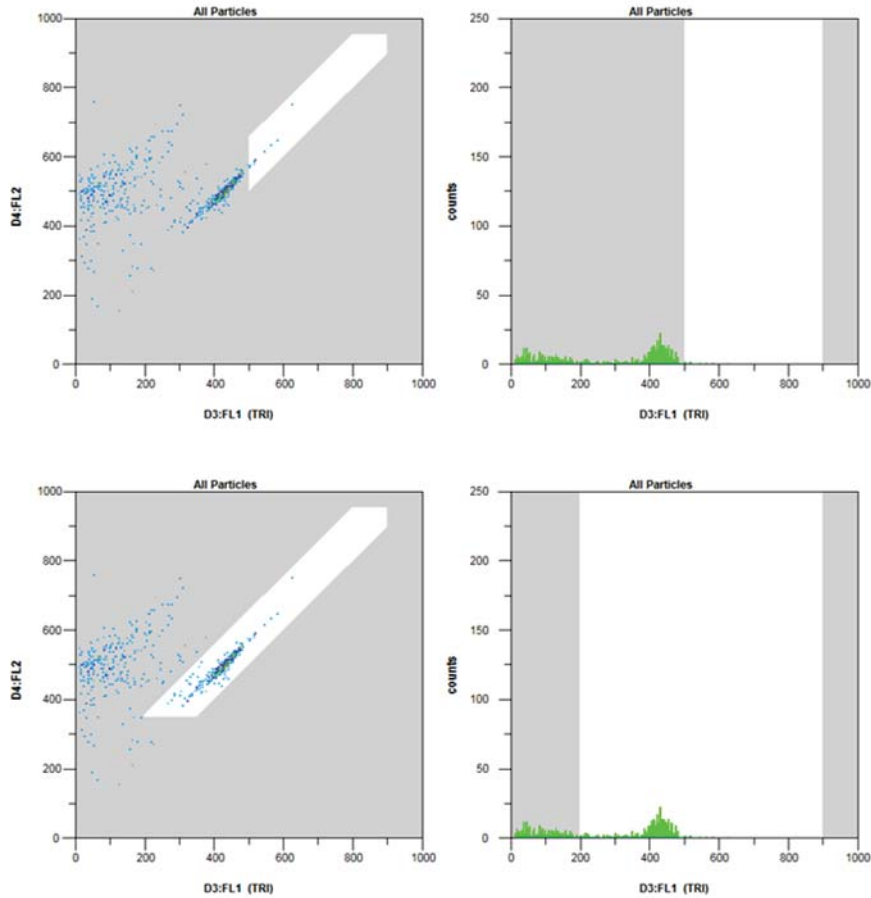
Both spore and vegetative cell samples exhibited a degree of smearing resulting in some overlap of area in which events were recorded for the two samples resulting in a count being recorded when the spore gate was applied to the vegetative sample (**Table 4.2**).

**Table 4.2** Flow cytometry counts using both the *Cl. frigidicarnis* vegetative gate and the *Cl. frigidicarnis* spore gate on a vegetative culture and a clean spore preparation.

| Cell type                | Cell count (per ml)                      |                                     |
|--------------------------|--|-------------------------------------|
|                          | <i>Cl. frigidicarnis</i> vegetative gate | <i>Cl. frigidicarnis</i> spore gate |
| Vegetative (16h culture) | $7.1 \times 10^7$                        | $3.3 \times 10^6$                   |
| Spore                    | $< 10^3^*$                               | $4.1 \times 10^5$                   |

\* Minimum level of detection

To determine if changes in spores occurring at the time of germination could be detected using the BactiFlow flow cytometer a sample of clean spores was exposed to PYGS and analysed at 0, 150 and 180 min. Following 15 min exposure to PYGS the population had shifted horizontally to the right of the graph, indicating increased uptake and binding of SYTO 16. A lack of vertical shift indicates propidium iodide emission, recorded by the FL2 detector, remained constant. The D3:FL1 minimum gate setting was adjusted to 200 to take this into account (**Figure 4.5**). Vegetative cells showed higher levels of emission from both SYTO 16 and propidium iodide (**Figure 4.4 and Figure 4.5**). Changes in counts, in the adjusted vegetative gate and the spore gate, following exposure to PYGS are shown in **Table 4.3**. While the count increased in the vegetative gate there was no corresponding reduction in the spore gate count.



**Figure 4.5** Flow cytometry analysis of *Cl. frigidicarnis* spores following 150 min exposure to PYGS. The population of freshly germinated spores is in a different position on the graph requiring an adjustment of the gate parameters in order to obtain a count.

**Table 4.3** Flow cytometer counts from vegetative and spore gates of a sample of clean *Cl. frigidicarnis* spores exposed to PYGS.

| Sampling time (min) | Cell count (per ml) |                   |
|---------------------|---------------------|-------------------|
|                     | Vegetative gate     | Spore gate        |
| 0                   | $10^3$ *            | $1.5 \times 10^5$ |
| 150                 | $3.6 \times 10^6$   | $2.2 \times 10^5$ |
| 180                 | $3.3 \times 10^6$   | $3.4 \times 10^5$ |

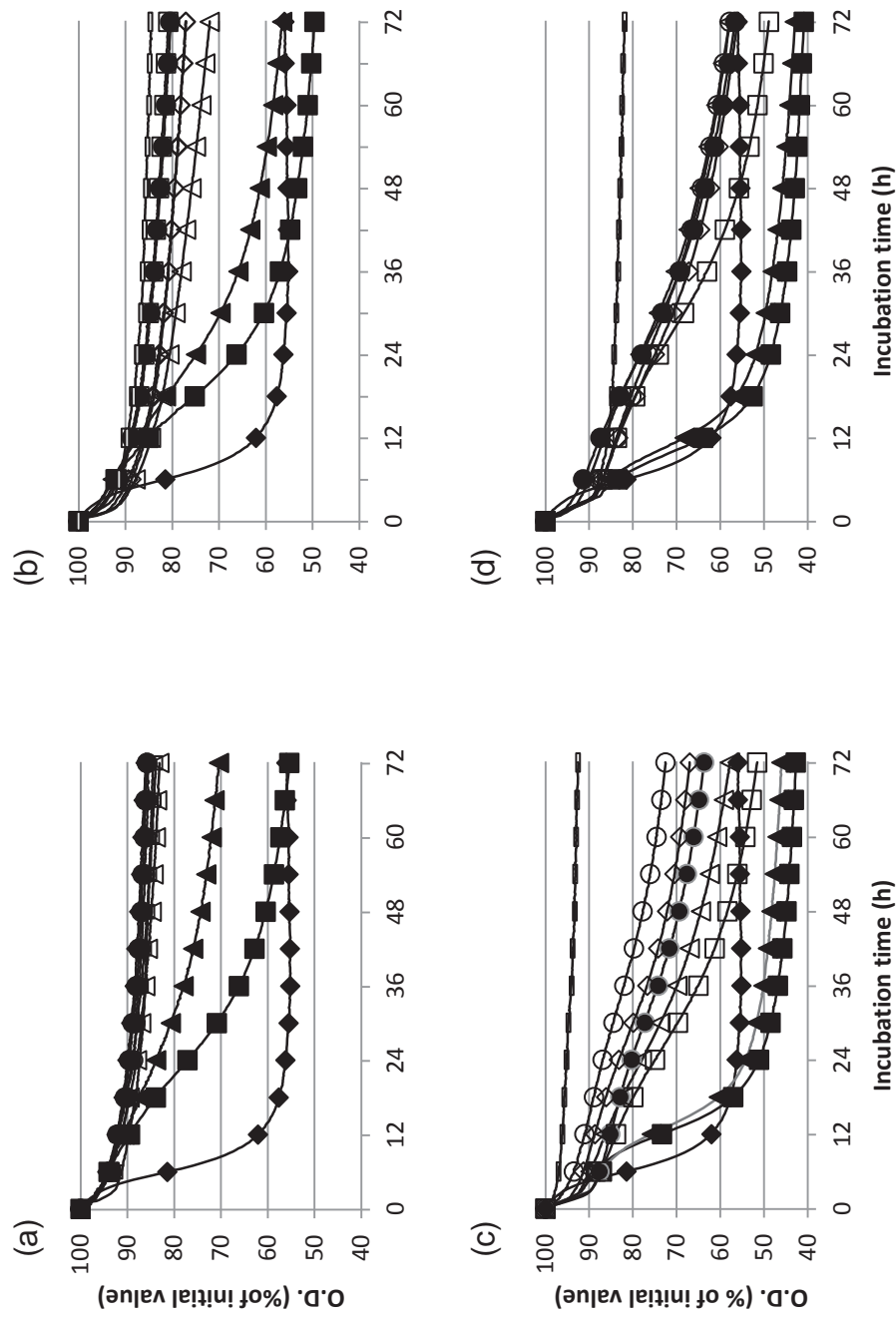
\* At or below the limit of detection.

Having determined that flow cytometry lacked the accuracy necessary to measure germination, all further experiments were carried out using the Bioscreen C method or the microscopy based method.

#### 4.3.5 Germination of *Cl. frigidicarnis* in defined media

In order to determine the specific germinant(s) responsible for inducing germination in *Cl. frigidicarnis*, single potential germinants were tested. The addition of single nutrient germinants [glucose, fructose, sucrose (all at final concentrations of 50 mmol l<sup>-1</sup>); inosine (10 mmol l<sup>-1</sup>); L-valine, L-norvaline, glycine, L-threonine, L-alanine, L-serine and L-cysteine (all at 100 mmol l<sup>-1</sup>)] to sodium phosphate (100 mmol l<sup>-1</sup>) or sodium phosphate (100 mmol l<sup>-1</sup>) plus NaHCO<sub>3</sub> (100 mmol l<sup>-1</sup>) and L-cysteine (50 mmol l<sup>-1</sup>) all failed to induce germination of heat shocked spores (<10 % of the spores became phase dark in 72 h at 10 °C). The addition of lysozyme (625 units ml<sup>-1</sup>), taurocholate (10 mmol l<sup>-1</sup>) or bryostatin (10 mmol l<sup>-1</sup>) to sodium phosphate or sodium phosphate plus NaHCO<sub>3</sub> and L-cysteine also failed to induce germination of heat shocked spores of *Cl. frigidicarnis*. Germination of unheated spores, in sodium phosphate, was induced with L-lactate combined with L-valine or L-norvaline (**Figure 4.6a**). These combinations also induced germination of heat-shocked spores (60 °C for 10 min), as to a lesser extent did L-serine in combination with L-lactate in sodium phosphate (**Figure 4.6b**). The extent and rate of germination were both greater when NaHCO<sub>3</sub> and L-cysteine were also present (**Figure 4.6c, d**). For both unheated and heat-shocked spores, the greatest extent of germination was observed with the combinations of L-lactate / L-valine and L-lactate / L-norvaline, followed by L-lactate / L-alanine. Microscopic examination, following 72 h incubation, confirmed that more than 95 % of spores had germinated. All amino acids tested (L-valine, L-norvaline, glycine, L-threonine, L-alanine, L-serine, and L-cysteine) induced germination of heat-shocked spores in combination with L-lactate in sodium phosphate containing NaHCO<sub>3</sub> and L-cysteine (**Figure 4.6d**). Heat shock (60 °C for 10 min), although not necessary for germination to occur, resulted in faster germination in all L-lactate /amino acid combinations. For example, for unheated spores incubated in L-lactate / L-valine, there was a 16 % fall in OD<sub>600</sub> after 24 h while for heat shocked spores, there was a fall in OD<sub>600</sub> of 25 %. L-Lactate was an

essential germinant and could not be replaced by any of the tested amino acids. The amino acids L-valine, L-norvaline, glycine, L-threonine, L-alanine, L-serine and L-cysteine (all at 50 mmol l<sup>-1</sup>) did not induce germination of heat-shocked spores when tested in pairs or threes in sodium phosphate only, with microscopic examination showing <10 % of the spores became phase dark (data not shown). The components of AGFK and AFR\*, individually or in combination, also failed to induce spore germination of *Cl. frigidicarnis*.

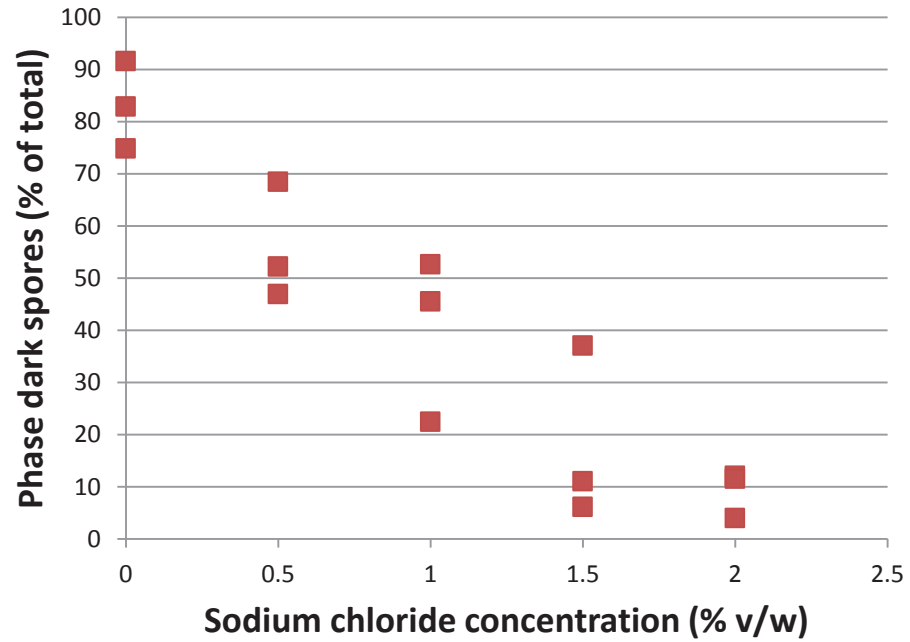


**Figure 4.6** Effect of heat shock and amino acid additions on germination of spores of *Cl. frigidicarnis*. Treatments: unheated spores in sodium phosphate (a), heat shocked spores (60 °C for 10 min) in sodium phosphate (b), unheated spores in sodium phosphate, L-cysteine and NaHCO<sub>3</sub> (c), heat shocked spores in sodium phosphate, L-cysteine and NaHCO<sub>3</sub> (d). Potential germinants: L-valine/L-lactate (▲), L-norvaline/L-lactate (■), L-norvaline/L-lactate (●), glycine/L-lactate (◇), L-threonine/L-lactate (◇), L-alanine/L-lactate (□), L-serine/L-lactate (△), L-cysteine/L-lactate (○), no addition [buffer only] (◻). PYGS (◆). Reproduced from (Adam et al. 2011).

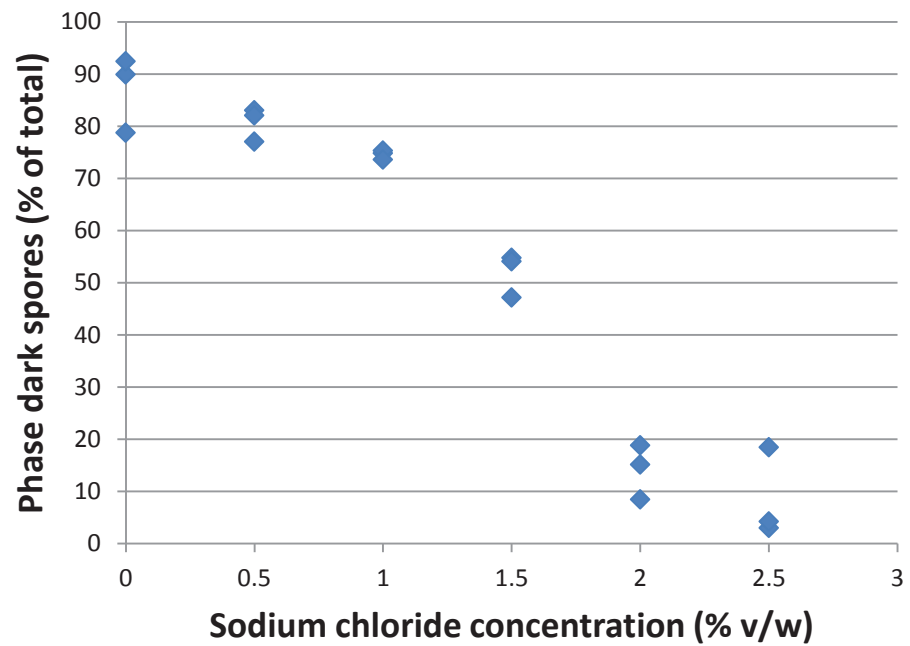
#### 4.3.6 Effect of NaCl concentration, pH and temperature on germination of *Cl. frigidicarnis*

NaCl concentration, pH and temperature all vary along the meat processing and transport chain. To determine how they are likely to affect germination, spores were exposed to germinants over a range of NaCl concentrations (0 – 2.5 % v/w), pH values (4 – 10) and temperatures (0 – 50 °C). Germination decreased with increasing NaCl concentration in both PYGS and dCMM (**Figure 4.7**). It is important to note that unsupplemented dCMM was expected to have a higher initial NaCl concentration than PYGS.

A



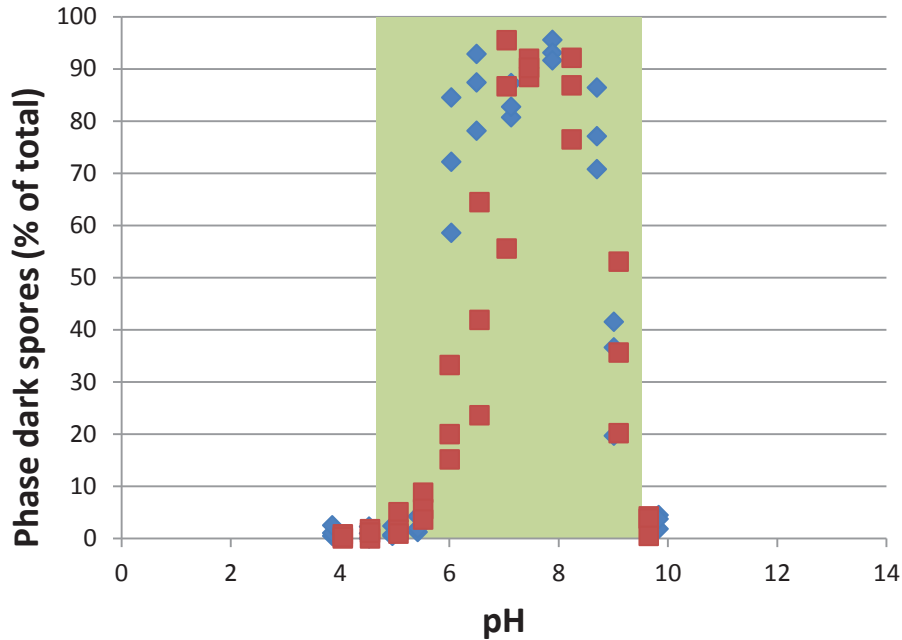
B



**Figure 4.7** Germination of spores of *Cl. frigidicarnis*, incubated for 3 days at 10 °C, in the presence of a range of NaCl concentrations in dCMM (A) and PYGS (B).

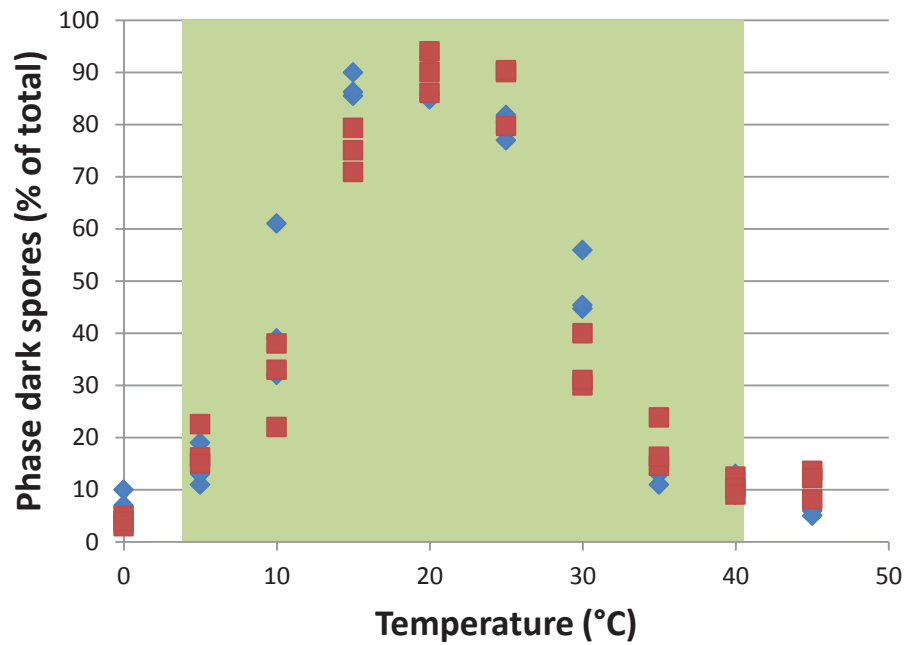
The extent of germination of *Cl. frigidicarnis* spores was determined at 11 different pHs. The range of pHs at which germination is able to occur is similar in dCMM and PYGS and closely matches the range of pHs at which growth can occur (**Figure 4.8**).



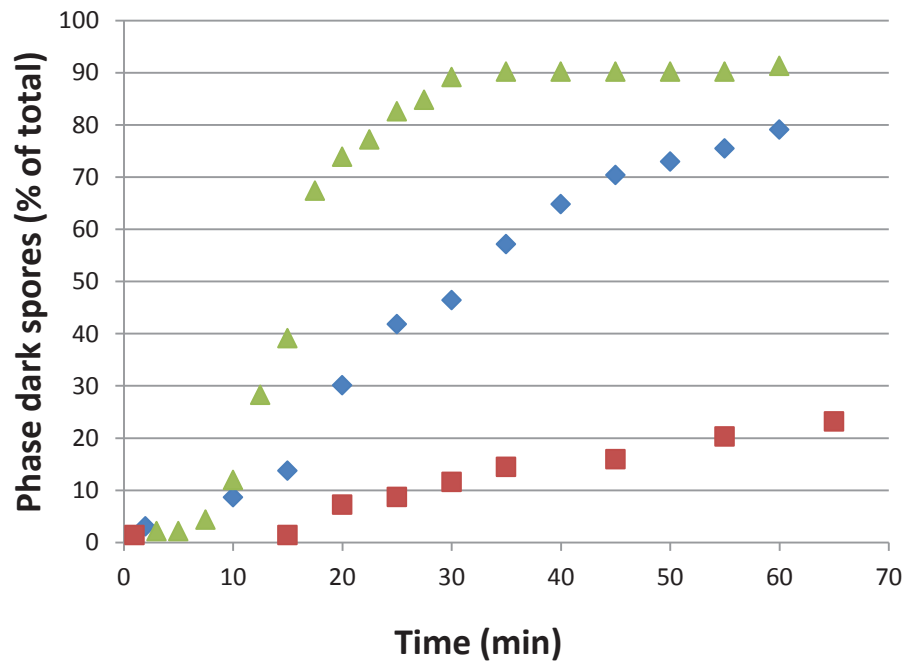


**Figure 4.8** Germination of spores of *Cl. frigidicarnis* incubated for 3 days at 10 °C, in (■) dCMM and (◆) PYGS at a range of pHs. The growth range of vegetative *Cl. frigidicarnis* is shown in green (Broda et al. 1999).

The effect of temperature (0 to 45 °C), on germination, was measured (**Figure 4.9**). Germination approached the lower level of detection (10 %) near the upper and lower temperature limits for growth [3.8 and 40.5 °C (Broda et al. 1999)]. The effect of temperature was similar in PYGS and dCMM. The effect of pre exposure of spores, to the complex germinant dCMM at non lethal temperatures unsuitable for germination, increased the rate at which *Cl. frigidicarnis* spores germinated (**Figure 4.10**). Cooling and heating curves of slides recorded with the Squirrel logger are given in **Appendix 2**.



**Figure 4.9** Germination of spores of *Cl. frigidicarnis* incubated, for 180 min, in (◆) PYGS and (■) dCMM at a range of temperatures. The growth range of vegetative *Cl. frigidicarnis* is shown in green (Broda et al. 1999).



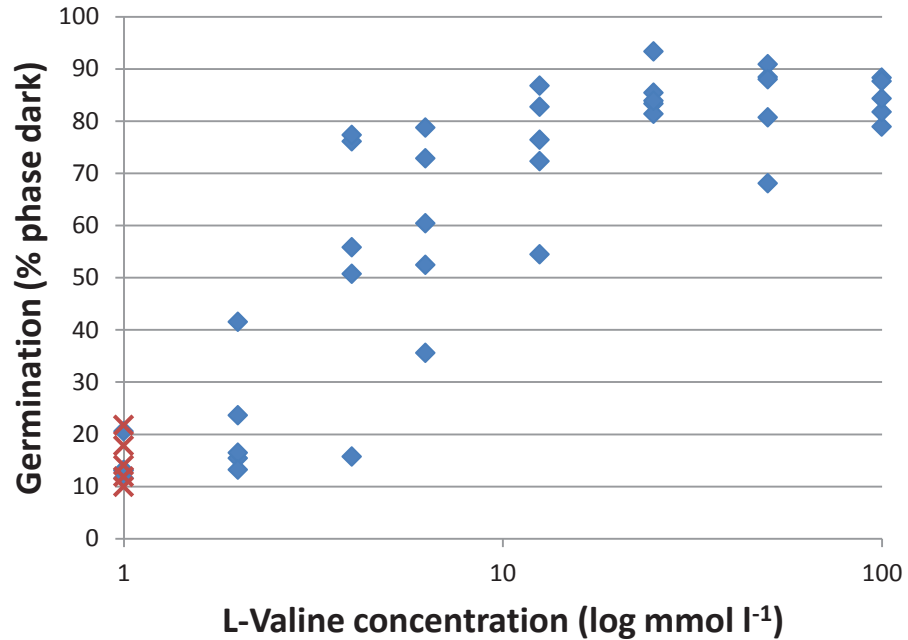
**Figure 4.10** Comparison of germination of *Cl. frigidicarnis* spores, in dCMM, at room temperature (21 °C), following exposure to PYGS at 0 °C for 3 hours (◆), 45 °C for 3 hours (▲) and without exposure (control, ■).

#### 4.3.7 Germination of *Cl. frigidicarnis* in aerobic conditions

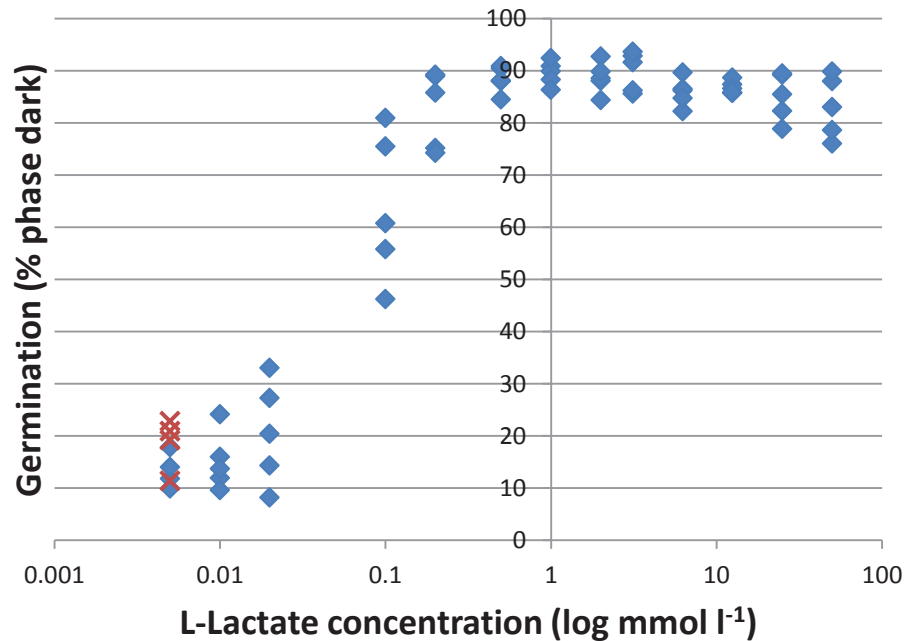
To determine if spores of *Cl. frigidicarnis* require anaerobic conditions to germinate or if, like non-proteolytic *Cl. botulinum* it is able to germinate under aerobic conditions (Plowman and Peck 2002), spores were exposed to known germinants under aerobic conditions. Germination was very poor when heat-shocked spores were incubated aerobically instead of anaerobically. Less than 10 % germination (the minimum level of detection, as judged microscopically) occurred in spores incubated for 72 h at 10 °C in PYGS, dCMM or defined germinant mixtures of sodium phosphate, L-cysteine, NaHCO<sub>3</sub> and L-lactate, in combination with an amino acid (L-valine, L-norvaline, glycine, L-threonine, L-alanine, L-serine, or L-cysteine).

#### 4.3.8 Concentration of L-valine and L-lactate necessary to induce germination in *Cl. frigidicarnis*

The level of L-valine and L-lactate necessary to induce germination in *Cl. frigidicarnis* was determined in order to make a comparison with those present in red meat (**Figure 4.11** and **Figure 4.12**).



**Figure 4.11** The effect of concentration of the co-germinant L-valine, in L-lactate (50 mmol l<sup>-1</sup>) and sodium phosphate buffer (50 mmol l<sup>-1</sup>), on germination of *Cl. frigidicarnis* after 72 h at 10 °C. L-Valine in L-lactate (50 mmol l<sup>-1</sup>) and sodium phosphate buffer (50 mmol l<sup>-1</sup>, ◆), L-lactate (50 mmol l<sup>-1</sup>) and sodium phosphate buffer (50 mmol l<sup>-1</sup>, ×).



**Figure 4.12** The effect of concentration of the co-germinant L-lactate, in L-valine (100 mmol l<sup>-1</sup>) and sodium phosphate buffer (50 mmol l<sup>-1</sup>), on germination of *Cl. frigidicarnis* after 72 h at 10 °C. L-lactate in L-valine (100 mmol l<sup>-1</sup>) and sodium phosphate buffer (50 mmol l<sup>-1</sup> ◆), L-valine (100 mmol l<sup>-1</sup>) and sodium phosphate buffer (50 mmol l<sup>-1</sup>, ×).

#### 4.4 Discussion

The main purposes of this study were to determine an accurate practical method for measuring germination in bacterial spores and to identify a germinant system(s) in *Cl. frigidicarnis* a psychrotolerant, red meat spoilage clostridia and the range of environmental conditions, such as temperature, pH and NaCl concentration, at which germination occurs.

Of the six methods of measuring germination, that have been described in the literature, monitoring OD<sub>600</sub>, monitoring of DPA release, microscopy, flow cytometry, genetic alteration to produce bioluminescence and alcohol or heat treatment, three were tested in the laboratory. Genetic alteration to produce bioluminescence was dismissed as the chemical reaction resulting in bioluminescence requires oxygen rendering this system unsuitable for use in strict anaerobes. Alcohol or heat treatment was ruled out due to the impracticality of analysing a large number of samples using this method. Measuring DPA release lacks the necessary level of accuracy and requires large spore samples. Flow cytometry failed to produce accurate counts. A combination of monitoring OD<sub>600</sub> and microscopic counts of phase bright and phase dark spores was found to be the most accurate and practical method for measuring germination in *Cl. frigidicarnis*.

Differences in the uptake of SYTO 16 and propidium iodide by dormant and germinated spores of *B. subtilis* allowed for enumeration of these cells by flow cytometry (Vepachedu and Setlow 2004; Black et al. 2005). The same was not true for cells of *Cl. frigidicarnis*. Based on microscopy dye did not appear to penetrate dormant spores rather it adhered to the outside of the cells. Unlike with *B. subtilis*, analysis of dormant *Cl. frigidicarnis* spore samples failed to produce an accurate cell count. This was seen in a comparison of cell counts done using the flow cytometer in comparison with those done by plate count or with a haemocytometer. *Cl. frigidicarnis* spore samples were analysed before and after exposure to PYGS for 150 min at 25 °C, a period of time sufficient to induce germination in 80-90 % of spores, but not sufficient for cell division to have occurred (**Table 4.3**). Counts representing germinated cells at

150 min were an order of magnitude higher than the original spore counts. In addition, the count in the spore gate did not decrease following exposure to PYGS.

Using a combination of monitoring of OD<sub>600</sub> in a Bioscreen C analyser and microscopy, a germinant system consisting of L-lactate in combination with L-valine or L-norvaline was identified in *Cl. frigidicarnis*. The rate and extent of germination were increased by the presence of the co-germinants, L-cysteine and NaHCO<sub>3</sub>, and in the presence of these co-germinants, other amino acids (L-alanine, and to a lesser extent glycine, L-threonine, L-serine and L-cysteine) were also effective germinants in combination with L-lactate. L-Valine and L-alanine are both nonpolar amino acids and likely to be present on the surface of vacuum-packed red meat, as are L-lactate and the co-germinants NaHCO<sub>3</sub> and L-cysteine. Lactic acid, and lactate build up in red meat post slaughter, and valine makes up 5.7 % of crude protein in beef (Lawrie 1998). The concentration of lactate and valine necessary to induce germination in *Cl. frigidicarnis* in a simple germinant system was determined. How this relates to the concentration of lactate and valine available in fresh meat is difficult to determine as this changes with time in storage, and a complex range of amino acids and chemicals are present on the surface of meat. L-norvaline is a non-proteinogenic amino acid unlikely to be present in red meat, and may owe its effectiveness as a germinant to it being an isomer of L-valine.

Germination of spores of *Cl. frigidicarnis* and non-proteolytic *Cl. botulinum* is induced by a combination of L-lactate and an amino acid, in the case of *Cl. frigidicarnis*, the amino acid showing the strongest response was L-valine, while for non-proteolytic *Cl. botulinum*, L-alanine, L-serine and L-cysteine show the strongest response (Plowman and Peck 2002). L-Lactate can be replaced by an amino acid to induce the germination of spores of non-proteolytic *Cl. botulinum* (Plowman and Peck 2002), but not those of *Cl. frigidicarnis*. There would therefore seem to be subtle differences in germination between these two psychrotolerant clostridia.

In the present study L-lactate failed to stimulate germination on its own, but acted in a synergistic manner with various amino acids. Several germinant systems found to induce germination, in other species of bacillus and clostridia, did not germinate

spores of *Cl. frigidicarnis*. These included the single amino acid L-alanine, the AGFK mixture, inosine and bryostatin, that induced germination in *B. cereus* or *B. subtilis* (Moir and Smith 1990; Gounina-Allouane et al. 2008; Wei et al. 2010); taurocholate that induced germination in *Cl. difficile* (Wilson et al. 1982) and AFR\*, that induced germination in *Cl. sordellii* (Ramirez and Abel-Santos 2010). Lysozyme did not induce germination in either unheated or heated spores of *Cl. frigidicarnis*. Lysozyme-induced germination has been shown to occur in sub-lethally heat-damaged spores of non-proteolytic *Cl. botulinum* (Peck et al. 1992; 1993). However, the heat treatment used here (60 °C for 10 min) is unlikely to have damaged the spores, as heat-shocked spores germinated normally in PYGS or filtered dCMM. A greater heat treatment, may lead to sub-lethal spore injury and spores of *Cl. frigidicarnis* being unable to germinate in the absence of lysozyme [as observed previously with spores of non-proteolytic *Cl. botulinum* (Peck et al. 1992)]. The heat shock applied in the present study (60 °C for 10 min) activated *Cl. frigidicarnis* spores, leading to more complete and faster germination especially in suboptimal conditions. This is a well known phenomenon affecting spores from a number of species including *B. subtilis*, proteolytic *Cl. botulinum* and non-proteolytic *Cl. botulinum* (Plowman and Peck 2002; Alberto et al. 2003; Setlow 2003).

For spores of *Bacillus* species, experimental evidence suggests that the amino acid L-valine binds directly to the GerA germination receptor (Atluri et al. 2006; Yi and Setlow 2010). However, until more sequence data become available for *Cl. frigidicarnis*, it can only be speculated that *Cl. frigidicarnis* has a similar system.

Spores of *Cl. frigidicarnis* do not germinate under aerobic conditions, unlike spores of the psychrotolerant pathogen non-proteolytic *Cl. botulinum* (Plowman and Peck 2002). This may indicate that the germinant receptor protein(s) in *Cl. frigidicarnis* need to be in a particular oxidative state. In the context of producing a method for spoilage reduction, involving triggering germination, the need for anaerobic conditions complicates matters.

NaCl has long been used as a food preservative as high concentrations result in osmotic conditions that are unfavourable to vegetative bacterial cells. As with non-proteolytic *Cl. botulinum*, the concentration of NaCl necessary to reduce germination of *Cl. frigidicarnis* was far in excess of the 0.16 g / 100 g in beef and 0.18 g / 100 g in lamb (Graham et al. 1996; Graham et al. 1997; Stringer and Peck 1997; Eoin 2006).

The pH range for germination of *Cl. frigidicarnis* spores was similar to its growth range. Germination reduced to below the level of detection as the pH dropped from 6.0 to 5.0. The ultimate pH of red meat varies considerably, for example from 5.2 to 7.0 in beef (Young 2001). These results show that pH of fresh meat is an important factor influencing the transition of *Cl. frigidicarnis* from a spore to a spoiler. The influence of pH on meat spoilage has been observed in connection with *Cl. estertheticum*. In a survey of spoiled meat samples from vacuum-packed meat with pH < 6.0 and stored at <3 °C, they almost always contained *Cl. estertheticum*, where the pH was > 6.0 there was an increase in spoilage without the presence of *Cl. estertheticum* (Cavill et al. 2011).

Germination of spores of *Cl. frigidicarnis* occurred at a temperature range similar to the growth range of vegetative cells. Germination was less than the limit of detection, at 0 °C, indicating that the optimum storage temperature, of -1.5 °C, is sufficient to prevent germination. A longer incubation time may have resulted in measurable germination. However, as growth of *Cl. frigidicarnis* does not occur at 0 °C, germination would not result in spoilage. Germination would be possible in temperature abused meat (low levels occurred at 5 °C) and at 10 °C, the temperature at which boning rooms are typically maintained. Care must also be taken to maintain the storage temperature. Spores exposed to germinants at suboptimal temperatures germinated rapidly when returned to more favourable temperatures. A spike in the surface temperature of meat, of 21 °C for 20-60 min, could be sufficient to allow 30 to 80 % of spores previously exposed to germinants to germinate.



#### 4.5 Conclusion

For measuring germination, in *Cl. frigidicarnis*, a combination of monitoring OD<sub>600</sub> and microscopy are recommended, for both accuracy and practicality. L-lactate in combination with L-valine or L-norvaline induces germination *Cl. frigidicarnis*. *Cl. frigidicarnis* only germinates under anaerobic conditions. *Cl. frigidicarnis* is unlikely to spoil properly chilled, good quality (pH 5.5) red meat. Temperature and pH, in vacuum-packs of meat, may play a role in determining if spores of *Cl. frigidicarnis* can germinate, as the minimum temperature for germination is higher than the ideal storage/shipping temperature for fresh meat and the pH range of fresh beef and lamb extends below the minimum pH for germination in *Cl. frigidicarnis*. Temperature spikes must be avoided during processing, chilling and shipping as spores pre exposed to germinants, at temperatures inhibitory to germination, germinate rapidly when returned to favourable temperatures. The amount of NaCl required to reduce germination, sufficiently to affect spoilage, is well in excess of naturally occurring levels of NaCl in fresh meat.

## Chapter 5

### Spore germination in *Cl. estertheticum* and LA1

#### 5.1 Introduction

Having characterised a germinant system for *Cl. frigidicarnis* in the previous chapter, the following work was aimed at identifying germinant systems in other psychrotolerant, red meat spoilage related clostridia. *Cl. estertheticum* is one of the major causes of blown-pack spoilage, of red meat, in the absence of temperature abuse (Helps et al. 1999). It has been detected in blown-packs, originating from Europe, USA and New Zealand, meat processing plants, and farm environments (Dainty et al. 1989; Kalchayanand et al. 1989; Broda et al. 2002; Broda et al. 2009). K21, L1, and V1 are *Cl. estertheticum* like strains that were all locally isolated from a blown-pack, a venison hide sample and a meat processing plant respectively (Bell et al. 2001). LA1 is a local strain isolated from spoiled beef. Germination has not been studied in *Cl. estertheticum* or LA1.

Germinant systems differ within the genus *Clostridium*. For example spores of proteolytic *Cl. botulinum* types A, B and F germinated in response to a three-component system comprising of L-alanine, L-lactate and NaHCO<sub>3</sub> (Peck 2009; Peck et al. 2011), while spores of non-proteolytic *Cl. botulinum* types B, E and F responded to a combination of L-lactate and either L-alanine, L-cysteine, or L-serine (Plowman and Peck 2002) and spores of *Cl. sordellii* are triggered by the presence of AFR\* (Ramirez and Abel-Santos 2010).

A previous study (Clemens et al. 2010) found that gas was produced in 4/5 vacuum-packed lamb steaks and 5/5 vacuum-packed beef steaks inoculated with 10<sup>-1</sup> spores (determined by heat shock followed by plate counting on CBA), incubated at 2 °C for 105 days. Theoretically an inoculum of 10<sup>-1</sup> spores would result in one in ten packs containing a spore. As the number of packs that accumulated gas was inconsistent with this, it was suspected that the proportion of the population of *Cl. estertheticum*

spores able to germinate, grow and cause spoilage, on a red meat substrate, was higher than the proportion able to form colonies on CBA. If only 10 % or less of laboratory produced *Cl. estertheticum* spores can be germinated, *in vitro*, microscopy and observation of changes in OD<sub>600</sub> will be insufficient to measure germination. Differences in populations of spores, newly germinated spores and vegetative cells have been observed using flow cytometry (Laflamme et al. 2005; Mathys et al. 2007). The high throughput nature of flow cytometry may offer a faster and more accurate method for measuring germination in bacterial cells. Germination in *Cl. frigidicarnis* could not be measured accurately. However, this may have been a strain specific problem.

The purpose, of the present study, was to compare the effectiveness of different methods to measure germination, of meat spoilage clostridia, in a number of different matrices and in the presence of different germinants. These methods were then used to expand the range of red meat spoilage related clostridia for which germination could be measured, and where possible to identify their germinant systems in order to compare them with those of *Cl. frigidicarnis* and other clostridia.

## 5.2 Methods

### 5.2.1 Spore production

Spores of LA1 were produced in biphasic dCMM as described in Section 3.5. Spores of *Cl. estertheticum* subsp. *estertheticum*, of *Cl. estertheticum* subsp. *laramiense* and three locally isolated *Cl. estertheticum* like strains were produced in PYGS as described in Section 3.5. Third generation spores were produced from *Cl. estertheticum* subsp. *estertheticum* by three successive spore propagations (spores<sup>3</sup>). All spores were separated from vegetative cells and debris by centrifugation through an Urografin 370 density gradient as described in Section 3.6.

### 5.2.2 Assessment of spore germination

The extent of germination of LA1, *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense*, the three local *Cl. estertheticum* like strains and spores<sup>3</sup> was determined microscopically as described in Section 3.7. All chemicals were obtained from Sigma-Aldrich (Auckland, New Zealand) unless otherwise stated. Germination of LA1 was tested in the presence of the single nutrient germinants [L-alanine, L-cysteine, glycine, L-norvaline, L-serine and L-valine (all at 100 mmol l<sup>-1</sup>); sodium lactate (50 mmol l<sup>-1</sup>) in sodium phosphate buffer (100 mmol l<sup>-1</sup>)], combined germinants [L-alanine, L-cysteine, glycine, L-norvaline, L-serine and L-valine (all at 100 mmol l<sup>-1</sup>) combined with sodium lactate (50 mmol l<sup>-1</sup>) in sodium phosphate buffer (100 mmol l<sup>-1</sup>)], PYGS, dCMM, supernatant from dried media and sodium phosphate buffer (100 mmol l<sup>-1</sup>). Inoculated slides were incubated for 3 days at 10 °C prior to analysis. Germination of *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense*, the three local *Cl. estertheticum* like strains and spores<sup>3</sup> was determined in the presence of sodium phosphate buffer (100 mmol l<sup>-1</sup>), PYGS, dCMM supernatant, fCMM supernatant and drip collected from frozen beef mince. Slides were incubated for 3 weeks at 10 °C prior to analysis.

### 5.2.3 Spore and cell counts using flow cytometry

Dilution series of a clean *Cl. estertheticum* subsp. *estertheticum* spore suspension and an exponentially growing culture of vegetative cells, in PYGS, were made and 600 µl samples of each dilution analysed using a BactiFlow flow cytometer (Chemunex, France), following staining with propidium iodide and SYTO 16 (Invitrogen) as described in Section 3.9. Counts of the suspensions were also made using a haemocytometer (Bright-Line, Hausse Scientific, Horsham, PA, USA).

#### 5.2.4 Meat slurry production

Sterile lamb meat homogenates were prepared using an adaption of the method described by Brightwell et al. (2007). Whole lamb legs were obtained from a local lamb processing plant on the day of slaughter. A surface area of approximately 15 cm × 10 cm, on each lamb leg was sterilised by searing with a superheated metal plate. 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 one part meat to one part sterile 0.85 % NaCl to obtain a lamb meat homogenate. Finally, 10 g aliquots of sterile homogenate were dispensed into sterile plastic Universal bottles for use in gas production studies.

#### 5.2.5 *Cl. estertheticum* subsp. *estertheticum* germination in meat slurry

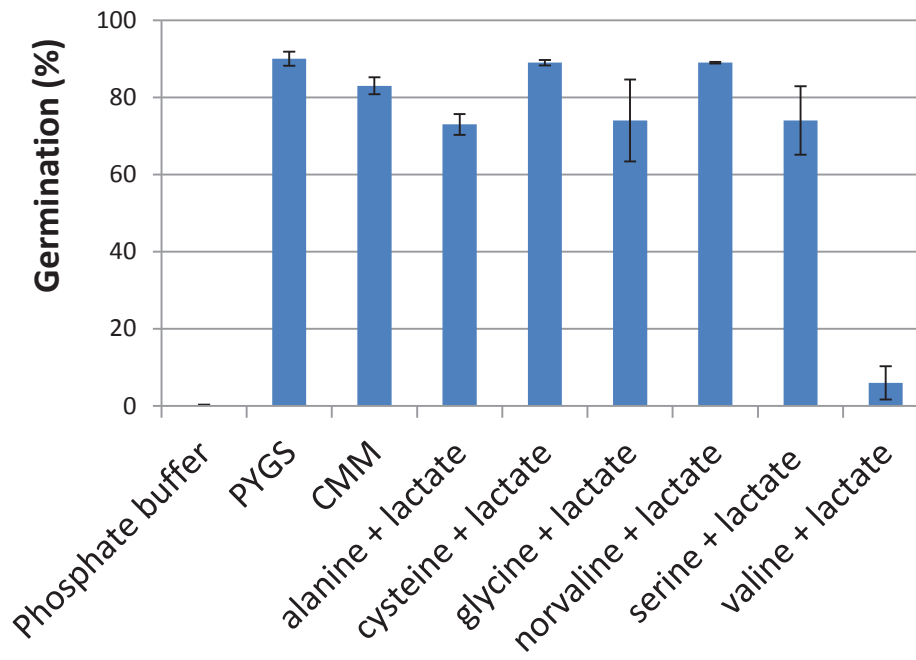
A suspension containing approximately  $10^5$  spores was prepared. From this 10 fold dilutions were produced. Plate counts were carried out on PYGS agar and CBA incubated at 10 °C for 21 days. Each homogenate was inoculated with 100 µl of spore suspension, with five replicates prepared. The inoculated homogenates were stirred, with a sterile inoculating loop, to ensure an even distribution of the 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 10 °C for 35 days. Five uninoculated lamb meat homogenates served as negative controls. The homogenates were removed

from chilled storage, twice a week, 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.

## 5.3 Results

### 5.3.1 Germination of LA1

To determine their germinant system(s), LA1 spores were exposed to a range of possible germinants. Germination of LA1 was initially tested using microscopic methods in sodium phosphate buffer, PYGS and dCMM at 10 °C for 3 days (**Figure 5.1**). Germination in phosphate buffer was less than 1 %. Germination in PYGS was 90.4 %. In all subsequent work sodium phosphate buffer was included as a negative control and PYGS as a positive control. The addition of a single nutrient germinant [L-alanine, L-cysteine, glycine, L-norvaline, L-serine and L-valine (all at 100 mmol l<sup>-1</sup>); or sodium lactate (50 mmol l<sup>-1</sup>)] to sodium phosphate (100 mmol l<sup>-1</sup>) failed to induce germination (<5 % of spore became phase dark). When combined with sodium lactate L-alanine, L-cysteine, glycine, L-norvaline and L-serine each induced 70 to 90 % germination (**Figure 5.1**). The highest level of germination, in this simple germinant system, was 89.0 % in a combination of lactate and L-cysteine or L-norvaline. In contrast a combination of sodium lactate and L-valine resulted in no detectable germination.



**Figure 5.1** Germination of LA1 in peptone, yeast, glucose, starch (PYGS) broth, filtered cooked meat medium from dried pellets (CMM) and a selection of lactate/amino acid combinations, following incubation for 3 days at 10 °C.

### 5.3.2 Germination of *Cl. estertheticum* and *Cl. estertheticum* like strains

To determine if they germinate in complex media, *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense*, several *Cl. estertheticum* like strains and spores<sup>3</sup> were exposed to PYGS, dCMM and fCMM using microscopic methods (**Table 5.1**). In all cases germination was less than 10 % and consistent with germination in phosphate buffer. Germination was not increased by repeated culturing of spores in the laboratory. Finally, to simulate the chemical composition of the surface of freshly slaughtered red meat, germination was tested in drip collected from frozen minced beef. This failed to stimulate more than 10 % germination.



**Table 5.1** Germination as a percentage of phase dark spores of *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense* three local *Cl. estertheticum* like strains and third generations *Cl. estertheticum* subsp. *estertheticum* spores (spores<sup>3</sup>) in phosphate buffer (PB), peptone, yeast, glucose, starch (PYGS) broth, filtered cooked meat medium from dried pellets (dCMM) and fresh meat (fCMM) and drip collected from frozen beef mince as % of spores that were phase dark.

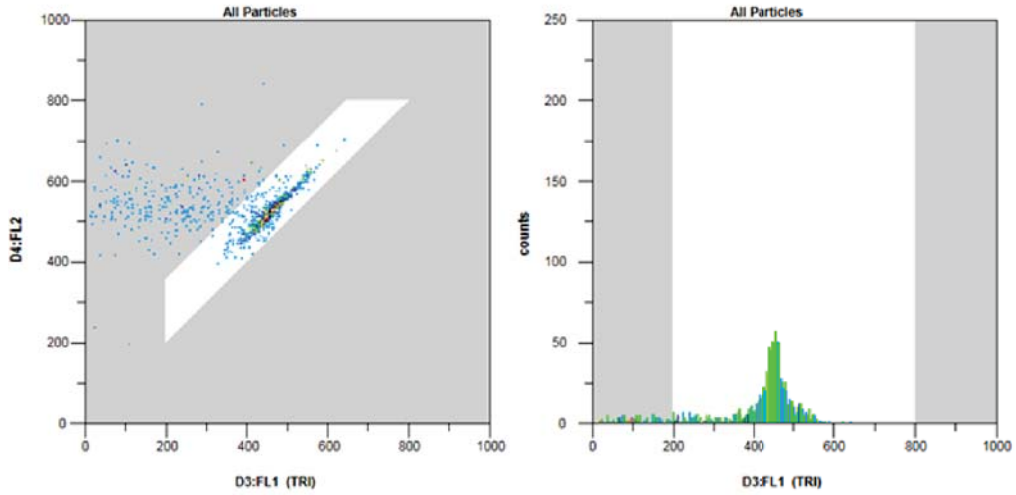
| Strain   | Germination (% phase dark spores) |      |      |      |      |
|--|-----------------------------------|------|------|------|------|
|  | PB                                | PYGS | dCMM | fCMM | Drip |
| <i>Cl. estertheticum</i><br>subsp.<br><i>estertheticum</i> | 7.2                               | 7.2  | 7.1  | 5.9  | 5.8  |
| <i>Cl. estertheticum</i><br>subsp. <i>laramiense</i>       | 0.3                               | 1.8  | 0.9  | 1.5  | 2.1  |
| K21  | 2.3                               | 2.2  | 2.1  | 2.2  | 2.6  |
| V1   | 2.5                               | 3.8  | 4.1  | 4.0  | 3.7  |
| L1   | 1.5                               | 1.8  | 1.6  | 2.1  | 2.1  |
| Spores <sup>3</sup>  | 4.5                               | 4.5  | 4.8  | 2.7  | 1.7  |

### 5.3.3 Analysis of *Cl. estertheticum* subsp. *estertheticum* vegetative cells and spores using a flow cytometer

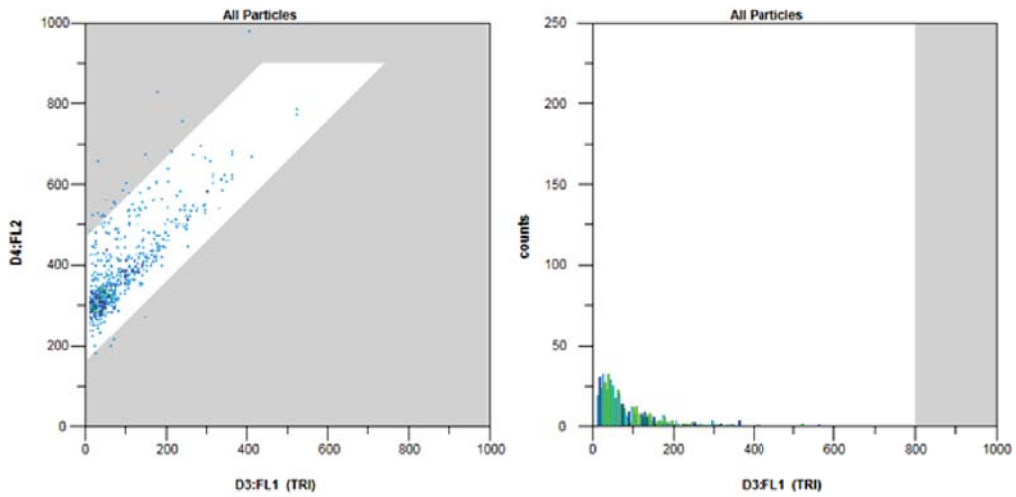
To determine if flow cytometry could be used to measure germination, in spores of *Cl. estertheticum* subsp. *estertheticum*, samples from a vegetative culture and a clean spore suspension were analysed using a flow cytometer. The spores and vegetative cells formed two distinct populations (**Figure 5.2**). Based on a visual assessment of the results the gates for vegetative cells and spores were set as shown in **Appendix 1**. Haemocytometer and flow cytometry counts for vegetative cells were in the same order of magnitude. However, counts for spores were higher by approximately half a log (**Table 5.2**). Both samples exhibited a degree of smearing resulting in some overlap of the area in which events were recorded for the two samples resulting in a count being recorded when the spore gate was applied to the vegetative sample (**Table 5.3**). By reducing the FL2 maximum to 400 only those spores outside the area of overlap

were counted resulting in a final count of  $4.3 \times 10^6$  cells/ml and a count  $1.9 \times 10^5$  cells/ml (30 % of the total count) in the area of the overlap.

A



B



**Figure 5.2** Graphical representation of flow cytometry analysis of vegetative cells (A,  $9.0 \times 10^3$  cells/ml) and spores (B,  $6.3 \times 10^3$  cells/ml) of *Cl. estertheticum* subsp. *estertheticum* stained with propidium iodide and SYTO 16.

**Table 5.2** Comparison of haemocytometer counts and flow cytometry counts of *Cl. estertheticum* subsp. *estertheticum* cells stained with propidium iodide and SYTO 16.

| Cell type  | Cell count (per ml) |                   |
|------------|---------------------|-------------------|
|            | Flow cytometry      | Haemocytometer    |
| Vegetative | $9.0 \times 10^7$   | $1.1 \times 10^8$ |
| Spores     | $6.3 \times 10^6$   | $2.6 \times 10^7$ |

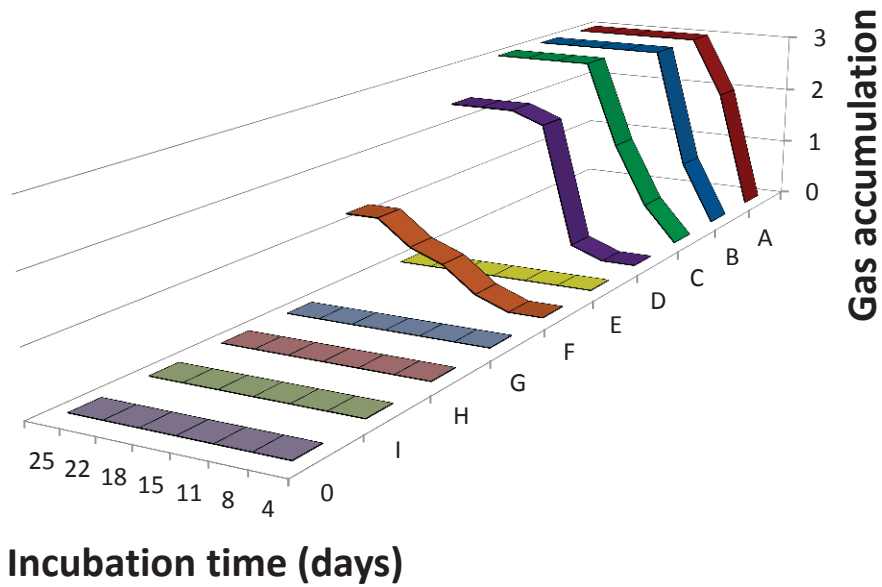
**Table 5.3** Flow cytometry counts using both the *Cl. estertheticum* subsp. *estertheticum* vegetative gate and the *Cl. estertheticum* subsp. *estertheticum* spore gate on a vegetative culture and a clean spore preparation respectively.

| Cell type  | Cell count (per ml)  |   |
|------------|--|---|
|            | <i>Cl. estertheticum</i> subsp. <i>estertheticum</i> vegetative gate | <i>Cl. estertheticum</i> subsp. <i>estertheticum</i> spore gate |
| Vegetative | $9.0 \times 10^7$  | $2.2 \times 10^7$   |
| Spore      | $< 10^{2*}$  | $6.3 \times 10^6$   |

\* Minimum level of detection

#### 5.3.4 Germination of *Cl. estertheticum* subsp. *estertheticum* in a meat slurry model

To determine if *Cl. estertheticum* subsp. *estertheticum* germinates better in a meat model, than in complex media, tubes of meat slurry were inoculated with spores. The initial inoculation suspension contained  $9.7 \times 10^5$  spores  $\text{ml}^{-1}$  (haemocytometer count) and  $5.8 \times 10^3$  CFU  $\text{ml}^{-1}$  and  $5.7 \times 10^3$  CFU  $\text{ml}^{-1}$  on PYGS and CBA respectively. The final inoculation levels ranged from  $9.7 \times 10^4$  spores per tube to 0.01 spores per tube (i.e. 1 spore in 100 tubes). Gas accumulation in the tubes implied growth, and by extension germination of *Cl. estertheticum* subsp. *estertheticum* spores. The first signs of gas production were visible eight days after inoculation, and no new gas production was seen following 22 days of cold storage (**Figure 5.3**). All tubes in which gas was observed reached the maximum level of gas accumulation possible (level 3). The tubes containing the minimum level of inoculum in which gas production was observed contained 1 spore/tube or 0.006 CFU/tube. Of the 5 tubes theoretically inoculated with 1 spore each gas accumulation was observed in 2 out of 5 tubes (**Table 5.4**).



**Figure 5.3** Average level of gas production, by *Cl. estertheticum subsp. estertheticum*, in lamb meat homogenate. 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. A contained  $9.7 \times 10^4$  spores/tube, B contained  $9.7 \times 10^3$  spores/tube and so on. The control tubes, 0, were uninoculated.

**Table 5.4** Initial inoculation levels of spores of *Cl. estertheticum* subsp. *estertheticum* and the number of meat homogenate tubes containing gas after 25 days of incubation.

| Inoculum    | Spores/tube<br>(haemocytometer<br>count) | CFU/tube<br>(CBA)    | CFU/tube<br>(PYGS)   | Tubes containing<br>gas |
|-------------|--|----------------------|----------------------|-------------------------|
| A           | $9.7 \times 10^4$                        | $5.7 \times 10^2$    | $5.8 \times 10^2$    | 5/5                     |
| B           | $9.7 \times 10^3$                        | $5.7 \times 10^1$    | $5.8 \times 10^1$    | 5/5                     |
| C           | $9.7 \times 10^2$                        | 5.7                  | 5.8                  | 5/5                     |
| D           | $9.7 \times 10^1$                        | $5.7 \times 10^{-1}$ | $5.8 \times 10^{-1}$ | 4/5                     |
| E           | 9.7                                      | $5.7 \times 10^{-2}$ | $5.8 \times 10^{-2}$ | 0/5                     |
| F           | $9.7 \times 10^{-1}$                     | $5.7 \times 10^{-3}$ | $5.8 \times 10^{-3}$ | 2/5                     |
| G           | $9.7 \times 10^{-2}$                     | $5.7 \times 10^{-4}$ | $5.8 \times 10^{-4}$ | 0/5                     |
| H           | $9.7 \times 10^{-3}$                     | $5.7 \times 10^{-5}$ | $5.8 \times 10^{-5}$ | 0/5                     |
| I           | $9.7 \times 10^{-4}$                     | $5.7 \times 10^{-6}$ | $5.8 \times 10^{-6}$ | 0/5                     |
| J (control) | 0  | 0                    | 0                    | 0/5                     |

## 5.4 Discussion

In order to broaden both the techniques available to measure spore germination and the strains of cold tolerant red meat spoilage clostridia for which germination systems have been identified the germination of spores of LA1, *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense* and *Cl. estertheticum* like strains was investigated using microscopy, measurement of OD<sub>600</sub> and flow cytometry. A germinant system, consisting of L-lactate and L-norvaline or cysteine in sodium phosphate buffer, was identified for LA1. It was also determined that the extent of germination in spores *Cl. estertheticum* subsp. *estertheticum* was greater in meat slurry than on CBA or PYGS agar.

LA1 germinates in a similar range of amino acid/L-lactate combinations, in sodium phosphate buffer, to *Cl. frigidicarnis* with the notable exception of the combination of L-valine and L-lactate. In *Cl. frigidicarnis* the highest levels of germination were seen in a combination of L-norvaline/L-lactate followed by a combination of L-valine/L-lactate. While L-valine makes up 5.7 % of the crude protein in beef (Lawrie 1998), L-norvaline is a nonproteinogenic amino acid and unlikely to be present in red meat. L-norvaline is an isomer of L-valine and this close relationship may explain why both these amino acids are germinants for *Cl. frigidicarnis* (Adam et al. 2011). The inability of L-valine to induce germination in LA1 suggests a subtle difference in the germinant receptor(s) with which amino acid/L-lactate germinant systems interact. The germination system described here for LA1 also shares similarities with the L-lactate/amino acid germination system of non-proteolytic *Cl. botulinum* which shows the greatest response in a combination of L-lactate and L-alanine, L-serine or L-cysteine (Plowman and Peck 2002). Sequencing and analysis of sequence data for *Cl. frigidicarnis* and LA1 would potentially clarify the situation.

A comparison between haemocytometer counts and plate counts demonstrated that only approximately 1 % of spores of *Cl. estertheticum* subsp. *estertheticum* produced in the laboratory were able to germinate, divide and form colonies on CBA or PYGS agar.

The occurrence of gas accumulation in tubes containing  $9.7 \times 10^{-1}$  spores (determined by haemocytometer count) suggests that germination and growth of *Cl. estertheticum* subsp. *estertheticum* is better in meat homogenate than on CBA or PYGS agar. It is likely that *Cl. estertheticum* subsp. *estertheticum* is also able to germinate better on vacuum-packed red meat than in CBA or PYGS agar. However, further work is required to confirm this.

The low levels of germination seen for *Cl. estertheticum in vitro* pose a challenge to the accurate measurement of the extent of germination and to determining the triggers of germination. PYGS and CMM are both complex media containing a range of amino acids, sugars, salts, nucleosides and other components. Under anaerobic conditions both support growth and sporulation of *Cl. estertheticum*. The ability of spores manufactured in the laboratory to cause symptoms of spoilage, in meat homogenate containing only 1 spore, indicates that laboratory manufactured spores are equipped with functioning germination apparatus. This indicates that the trigger of germination in *Cl. estertheticum* spores requires a combination of germinants and conditions present in meat homogenate but not in PYGS or CMM. Temperature, pH and atmospheric conditions tested, 1-15 °C, 6.5-7.2, and strictly anaerobic respectively, were all suited to the growth of *Cl. estertheticum*, making them unlikely to have inhibited germination (Collins et al. 1992; Spring et al. 2003). Prior to preparation the major ingredient of CMM, ground beef heart, theoretically contains the same constituent parts as freshly slaughtered meat indicating that the trigger of germination is destroyed or deactivated during preparation. This suggests an enzyme may be involved in triggering germination of *Cl. estertheticum* spores in vacuum-packed red meat. Alternatively the triggers of germination of *Cl. estertheticum* may be produced during the storage of vacuum-packed red meat, for example mucopeptides, which have been shown to trigger germination in *B. subtilis* (Setlow 2008; Shah et al. 2008; Wei et al. 2010). This would not account for germination of spores in aseptically prepared meat slurry.

Three methods for measuring germination in *Cl. estertheticum*, microscopy, changes in OD<sub>600</sub>, and flow cytometry, were investigated. None provided sufficiently accurate



results to detect germination of less than 10 % of a population of spores. An alternative to increasing the accuracy of a system for measuring germination would be to determine the method by which *Cl. estertheticum* germinates in the presence of red meat.

#### 5.4 Conclusion

Germinant systems differ between cold tolerant red meat spoilage clostridia. The germinant system for LA1 discovered during this study (L-lactate and L-norvaline or L-cysteine in sodium phosphate buffer) is similar to those of *Cl. frigidicarnis* and non-proteolytic *Cl. botulinum*, which also germinate in response to a combination of an amino acid and L-lactate. *Cl. frigidicarnis* differs considerably from *Cl. estertheticum* which does not germinate well in complex growth media PYGS and CMM. A simple germinant system has not been identified for *Cl. estertheticum*. However, it was found to germinate more readily in red meat slurry than in complex growth media.



## Chapter 6

### Inactivating germinated spores

#### 6.1 Introduction

Ultimately, this work aims to develop a method of reducing red meat spoilage by cold tolerant clostridia involving a two step process. First the spores would be treated to induce germination then they would be inactivated. Previous chapters have identified triggers of germination in *Cl. frigidicarnis* and LA1 consisting of L-lactate in combination with an amino acid. This chapter focuses on the second step. A defining feature of the genus *Clostridium* is that they are strictly anaerobic, that is they do not grow under aerobic conditions (Stackbrandt and Rainey 1997). Conceivably, the simplest method of inactivating them would be through exposure to air. This chapter aims to determine if exposure to air or a hot water wash (HWW) are practical and sufficient to inactivate or kill germinated spores on fresh red meat. For the purpose of this work, germinated spores are defined as cells that are phase dark but have not fully elongated and are not able to divide.

In meat processing plants, HWW is usually applied to the whole carcass following slaughter; removal of the head, hooves and pelt; and prior to boning out which may be done immediately (hot boning) or following a period of chilling (cold boning). Ideally hot water (80 °C) is applied evenly to the whole carcass. Application time is limited to approximately 10 s to prevent permanent discolouration of the meat (Castillo et al. 1998). HWW has been shown to be effective in reducing *Escherichia coli* 0157:H7 and *Salmonella typhimurium*, but not been specifically investigated for use against spores of cold tolerant clostridia (Castillo et al. 1998). A peroxyacetic acid based wash has been shown to be effective in delaying the onset of spoilage by *Cl. estertheticum* spores (Boerema et al. 2007). However, Europe does not accept chemically treated red meat. HWW and exposure to air would also constitute non-chemical intervention, and treated product would be acceptable in Europe. The tolerance of clostridia to oxygen has been studied in the context of culturing methods. Tally et al. (1975) investigated

the effect of exposure, of five isolates of *Clostridium* sp. on pre-reduced CBA plates, and found that they survived 72 h of exposure to aerobic conditions. If red meat spoilage clostridia exhibit similar tolerance, to aerobic conditions, exposure to air will not be a practical option for their inactivation.

Tyndallisation involves the exposure of cells to nutrients which causes them to enter an actively growing vegetative state. Actively growing vegetative cells have reduced resistance in comparison to spores. The cells are then exposed to heat. The process is repeated three times to ensure the maximum number of spores are killed. The timing and temperature of treatments depends on the target organisms and the product being treated. It is important that fresh meat retain its colour and texture following tyndallisation limiting the possible exposure time.

This study looked at the ability of germinated spores and vegetative cells, of red meat spoilage clostridia, to survive exposure to air *in vitro* and on lamb. It also looked at the effect of hot water wash (HWW) and cold water wash (CWW) treatments in conjunction with tyndallisation and exposure to air on the spoilage of lamb by *Cl. estertheticum*.

## 6.2 Methods

### 6.2.1 Cultures

Vials of PYGS (described in Section 3.4) were inoculated with *Cl. algidicarnis*, *Cl. algidixylanolyticum*, *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense*, *Cl. frigidicarnis*, LA1 and *Cl. gasigenes* (described in Section 3.3) and incubated anaerobically until late growth phase. *Cl. estertheticum* subsp. *estertheticum* and *Cl. estertheticum* subsp. *laramiense* were incubated at 10 °C for 7 to 15 days and *Cl. algidicarnis*, *Cl. algidixylanolyticum*, *Cl. frigidicarnis*, LA1 and *Cl. gasigenes* were incubated at 25 °C for 20 to 36 h. Germinated *Cl. frigidicarnis* spores were prepared as described in Sections 3.5 and 3.6 prior to being exposed to PYGS for 150 min and 25 °C. Based on the results of Chapter 4, exposure to PYGS for 150 min is sufficient time to induce germination in 80 to 90 % of a population of *Cl. frigidicarnis* spores, but insufficient time to allow spores to complete outgrowth. Spores of *Cl. estertheticum* subsp. *estertheticum* were prepared as described in Sections 3.5 and 3.6. All cultures were prepared under strict anaerobic conditions.

### 6.2.2 Air exposure *in vitro*

Prior to exposure to air, cultures were diluted to approximately  $10^6$  CFU ml<sup>-1</sup>, in deoxygenated physiological saline. One ml aliquots were then transferred into vials containing nine ml aerated, dCMM supernatant, prior to incubation at room temperature (21 °C) or 10 °C. During the course of the experiment, sub-samples were taken for enumeration. The time between samplings increased with the duration of the experiment. All cultures and freshly germinated spores of *Cl. frigidicarnis* were exposed to air at 21 °C, *Cl. estertheticum* subsp. *estertheticum* and *Cl. frigidicarnis* were also exposed at 10 °C, and the effect of exposure of *Cl. estertheticum* subsp. *estertheticum* to 21 °C under anaerobic conditions was tested.

### 6.2.3 Ethanol treatment

Samples containing recent germinants were ethanol treated to determine the portion of viable dormant spores using a method adapted from Broda et al. (1998). Deoxygenated absolute ethanol was added to sub-samples of cultures at a final concentration of 50 % and incubated for 60 min at 15 °C prior to enumeration.

### 6.2.4 Enumeration of *in vitro* samples

Sub-samples were enumerated on deoxygenated CBA plates containing 5 % defibrinated sheep's blood. *Cl. estertheticum* subsp. *estertheticum* and *Cl. estertheticum* subsp. *laramiense* plates were incubated anaerobically at 10 °C for 21 days, *Cl. algidicarnis*, *Cl. algidixylanolyticum*, *Cl. frigidicarnis*, LA1 and *Cl. gasigenes* plates were incubated anaerobically at 25 °C for 48 to 72 h.

### 6.2.5 Lamb

Fresh lamb was obtained from a local lamb processing plant either as whole carcasses or as flaps. The whole carcasses were boned out and cut into steaks with a surface area of approximately 100 cm<sup>2</sup>, in a cool room (10 °C). Lamb flaps were used during the preliminary HWW experiment as their surface area consists largely of tissue exposed prior to removal from the carcass under a normal processing regime in New Zealand. The exposure of meat to air and combined treatments to reduce spoilage were carried out on lamb steaks consisting of surfaces that had been both internal and exposed prior to boning out simulating the surface of meat cuts commonly shipped vacuum-packed.

### 6.2.6 Air exposure on meat

Lamb steaks were exposed to aerobic conditions, at various stages in a simulated shipping regime, to determine if oxygen exposure could extend shelf life. Spore preparations of *Cl. estertheticum* subsp. *estertheticum* were diluted to give estimated

final concentrations of 1 and 10 CFU ml<sup>-1</sup>. The inocula were plated onto CBA and incubated anaerobically for 21 days at 10 °C to enumerate. Lamb steaks were randomly assigned to 120 barrier bags (70 µ Cryovac, New Zealand) representing five replicates for each of three inoculums, four exposure times and two temperatures. Forty packs were inoculated with 1 ml each 10 CFU ml<sup>-1</sup> inoculum, forty with 1 ml each 1 CFU ml<sup>-1</sup> inoculum and the remaining packs were left uninoculated (controls). The packs were vacuum-packed using a Securepak 10 Controlled Atmosphere Packaging Machine (Securefresh Pacific, Auckland, New Zealand). The packs were then subject to heat-shrinking treatment by complete immersion in 78 °C water for 2 to 3 s, to simulate best practice. 'Best practice' cooling was simulated by placing meat packs in boxes in a -1.5 °C chiller. After 24 h half the packs from each treatment were transferred to a 2 °C chiller. Five packs from each temperature/inoculum treatment (six treatments) were collected and opened in a 10 °C room and the steaks exposed to air for 15 min. The steaks were turned over and left for a further 15 min before being resealed in fresh barrier bags and returned to the appropriate chillers. The exposure process was repeated, with fresh packs, after 72 h and 6 days.

A second set of 30 vacuum-packs were prepared. Ten packs were inoculated with 1 ml each 10 CFU ml<sup>-1</sup> inoculum, ten with 1 ml each 1 CFU ml<sup>-1</sup> inoculum and the remaining packs were left uninoculated (controls). These were stored at -1.5 °C for 21 days prior to half of the packs, from each inoculum, being exposed to air and repackaged. The packs were then all stored at simulated display temperatures for 10 days.

#### 6.2.7 Hot water wash

To determine if HWW decreased the time until spoilage, by *Cl. estertheticum*, gas accumulation was compared, in lamb steaks that received a high inoculation and were stored under sub optimal refrigeration conditions. *Cl. estertheticum* subsp. *estertheticum* spores were diluted to approximately 10<sup>2</sup> CFU ml<sup>-1</sup>. Plate counts were carried out on CBA incubated anaerobically for 21 days at 10 °C for confirmation. Five lamb flaps were pinned to each of two polystyrene boards. Each flap was inoculated



with 1 ml of  $10^2$  CFU ml<sup>-1</sup> spore suspension. The inoculum was spread over an area 100 cm<sup>2</sup> before sitting for 10 min to allow for attachment.

The first sets of flaps, the control CWW set, were sprayed for 10 s with 24.5 °C water. The second set of flaps (HWW) was sprayed for 10 s with 77.0 °C water. The wash treatments were delivered in a cabinet set up to simulate the decontamination procedures used in the New Zealand industry. Commercially available spray nozzles shown in **Figure 6.1** delivered water at 6 bar at the nozzles. Flaps were placed 0.3 m from the nozzles. The flaps were allowed to dry for 10 min, then individually vacuum-packed in barrier bags before heat shrinking, by immersion in 78 °C water for 2 to 3 s. The samples were initially incubated at 0 °C, with a chiller failure simulated at day 30, after which the samples were stored at 4 °C for a total of 10 weeks.



**Figure 6.1** Commercially available spray wash nozzles attached to a rotating wand.

### 6.2.8 Treating meat to reduce spoilage

HWW post packaging and tyndallisation were investigated as methods of reducing spoilage by spores of *Cl. estertheticum* subsp. *estertheticum*. Lamb steaks were

randomly assigned to 40 barrier bags representing five replicates each for eight treatments. The treatments were: uninoculated control; inoculated control; inoculated and CWW; inoculated and HWW; inoculated, stored at -1.5 °C for 3 days then exposed to aerobic conditions; inoculated, stored at -1.5 °C for 3 days then HWWed; tyndallised and tyndallised, stored at -1.5 °C for 3 days and HWWed. The initial inoculum was approximately 1 CFU ml<sup>-1</sup> and was enumerated on pre-reduced CBA plates. Tyndallisation consisted of heat shrinking, by immersion in 78 °C water for 4 to 5 s, then chilling packs, in iced water, for 10 min, three times. The packs were stored at -1.5 °C for 8 weeks. Exposure to air, HWW and CWW were carried out as described in Section 6.2.6 and 6.2.7.

#### 6.2.9 Monitoring of packs and chillers

Packs were examined twice a week, and assessed for gas accumulation and assigned a score from 0 to 5 as previously described (Clemens et al. 2010). Score 0 was assigned to packs with no gas bubbles in the drip; score 1 was assigned to packs that had gas bubbles in the drip; score 2 was assigned to packs at the 'loss of vacuum' stage; score 3 was assigned to obviously 'blown', puffy packs; score 4 was assigned to fully distended packs without tightly stretched packaging; and score 5 was given to tightly stretched, 'overblown' packs. It should be noted that in commercial practice, packs with a distension score of 1 would not be recognized as being abnormal. The operating temperatures of the storage chillers were monitored using KOOLTRACK data loggers (KoolTrack Inc., Palm Beach Gardens, FL, USA).

#### 6.2.10 Molecular detection of *Cl. estertheticum*

To confirm its presence, PCR detection of *Cl. estertheticum* was carried out on a representative selection of the exudates. Nucleic acid was extracted, from 1 ml exudate samples, using methods described in Section 3.10. Eluted DNA was stored at -20 °C, pending PCR amplification. *Cl. estertheticum* specific PCRs were carried out as described in Section 3.11. Following amplification aliquots of the PCR mix were subjected to electrophoresis as described in Section 3.12.

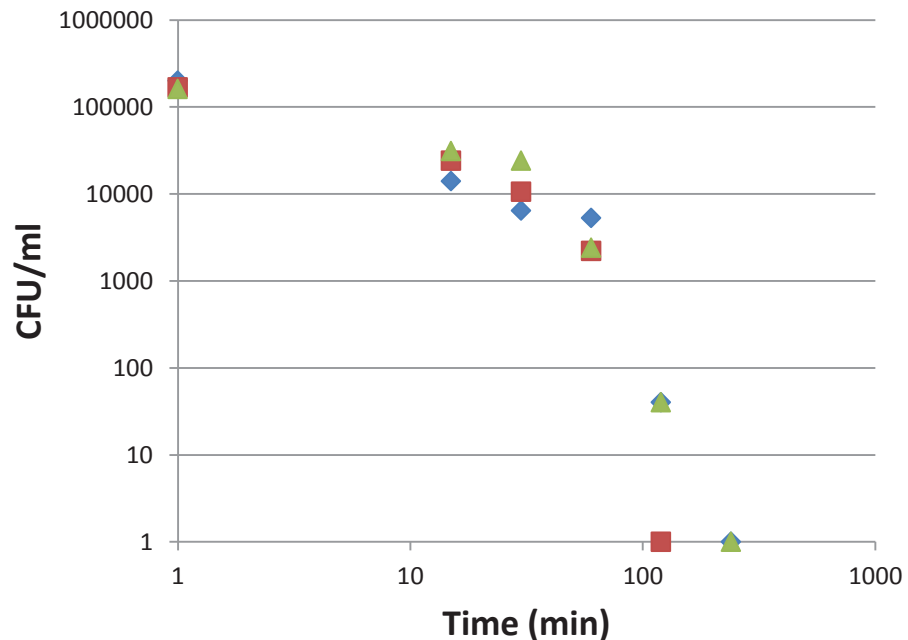
### 6.2.11 Statistical analysis

ANOVAs were carried out on the results of the 'exposure of meat to air' experiment and the 'treating meat to reduce spoilage' experiment using GenStat (2012, GenStat for Windows, Thirteenth Edition, Release 13.2.0.4682, VSN International Ltd., Oxford).

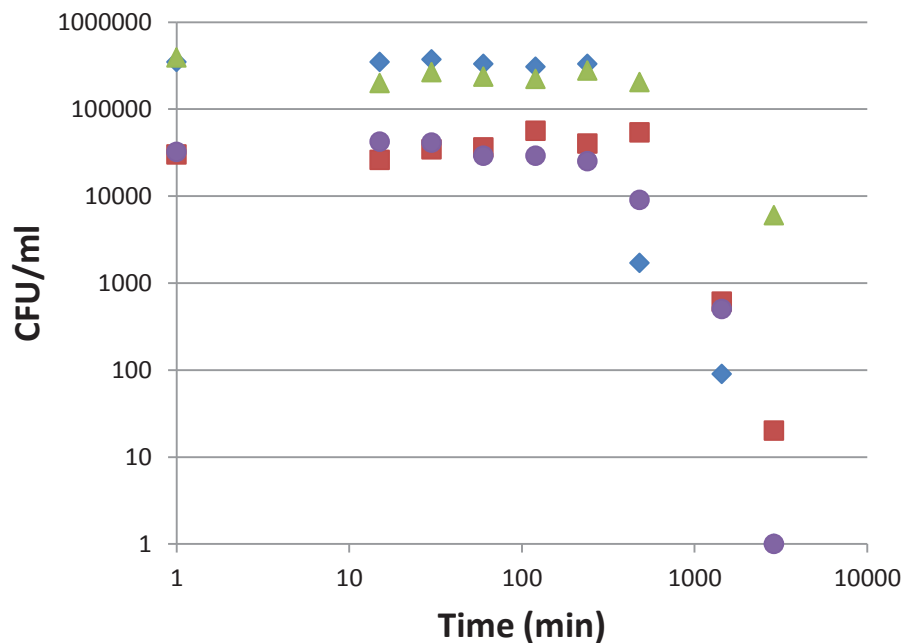
## 6.3 Results

### 6.3.1 Exposure of freshly germinated and vegetative cells of psychrotolerant clostridia *in vitro*

To determine how long vegetative cells, of red meat spoilage clostridia, could survive, when exposed to atmospheric levels of oxygen, late growth phase cultures were added to aerobic dCMM supernatant at 21 °C. Subsamples were plated onto pre-reduced CBA to determine the viable population remaining. *Cl. frigidicarnis*, LA1 and *Cl. gasigenes* numbers were all reduced to below the limit of detection after 240 min exposure to air (Figure 6.2). *Cl. algidixylanolyticum* and *Cl. estertheticum* subsp. *laramiense* were reduced to below the limit of detection after 24 h exposure to air and some *Cl. algidicarnis* and *Cl. estertheticum* subsp. *estertheticum* cells were still viable after 48 h exposure to air at 21 °C (Figure 6.3). As a general rule the lower the preferred growth temperature the longer the strains survived exposure to air.

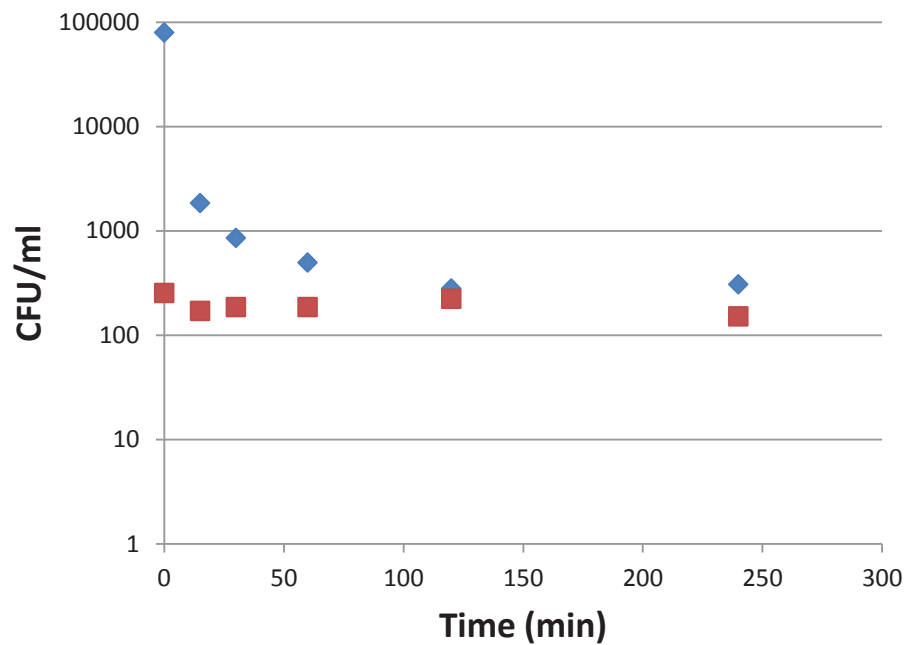


**Figure 6.2** Viable vegetative cells of *Cl. frigidicarnis* (◆), LA1 (■) and *Cl. gasigenes* (▲) exposed to air in dCMM supernatant at room temperature (21 °C), as determined by plate count on CBA. The limit of detection of the test was 10 CFU ml<sup>-1</sup> or 1 log. Values shown are the mean of three replicates.



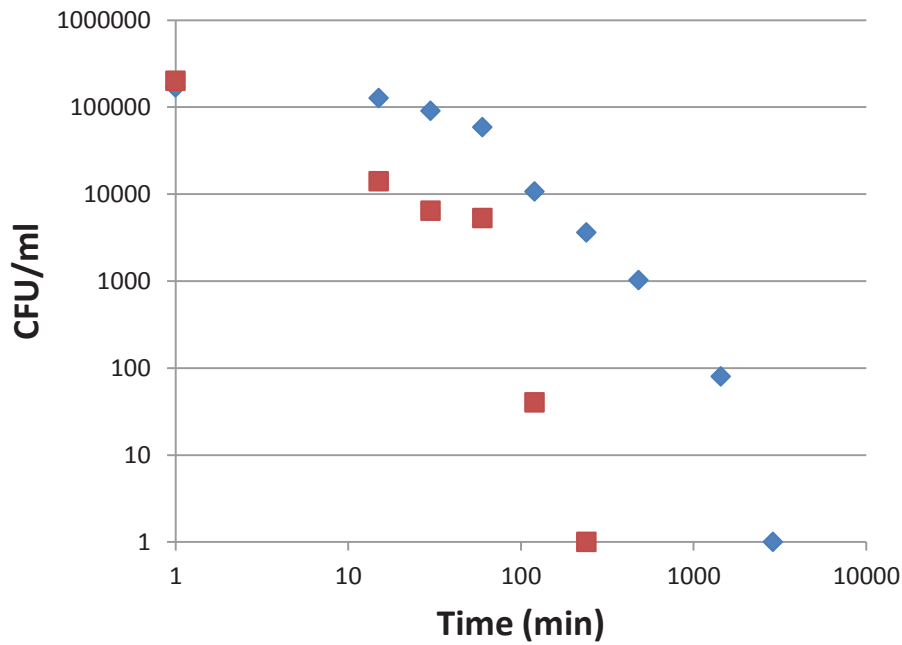
**Figure 6.3** Viable vegetative cells of *Cl. algidixylanolyticum* (◆), *Cl. algidicarnis* (■) *Cl. estertheticum* subsp. *estertheticum* (▲) and *Cl. estertheticum* subsp. *laramiense* (●) exposed to air in dCMM supernatant at room temperature (21 °C), as determined by plate count on CBA. The limit of detection of the test was 10 CFU ml<sup>-1</sup> or 1 log. Values shown are the mean of three replicates.

To determine if the rate of die off in vegetative cells of *Cl. frigidicarnis* mirrored that of freshly germinated spores, a spore preparation was exposed to PYGS, to induce germination, then enumerated before and after ethanol treatment following exposure to air (**Figure 6.4**). The total population (consisting of germinated and dormant spores) was reduced to the level of the portion of the population that survived ethanol treatment (viable dormant spores) after 120 min exposure. The rate of reduction was similar to that of vegetative cells, warranting using vegetative cells to model the response of freshly germinated cells exposed to air.

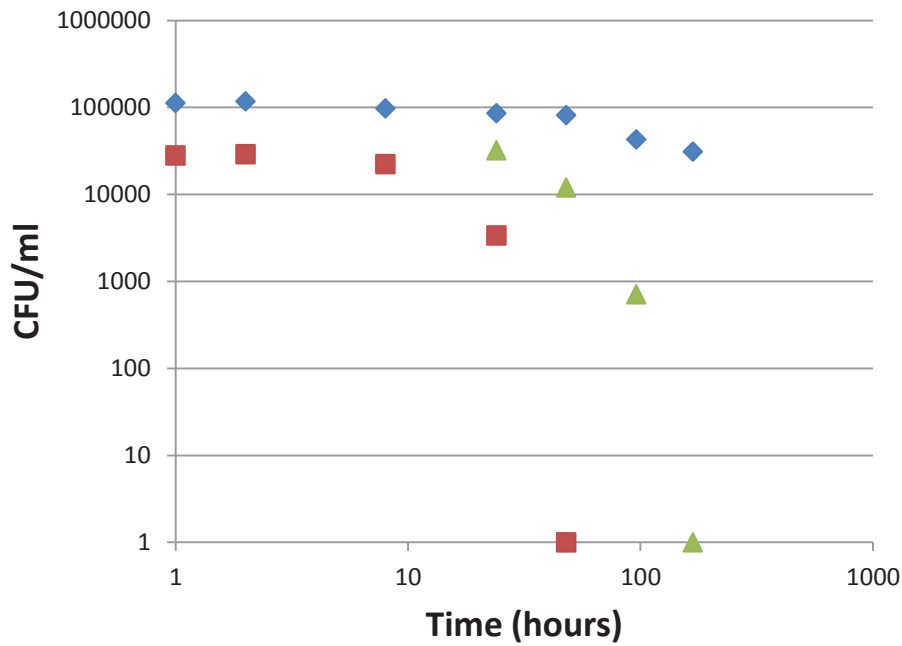


**Figure 6.4** Viable cells of germinated *Cl. frigidicarnis*, as determined by plate count on CBA, following exposure to air in dCMM supernatant. Total population of viable cells based on counts on CBA (◆) and cells resistant to ethanol treatment (dormant spores, ■). Values shown are the mean of three replicates.

Spores encounter decreasing temperatures as the meat that they are attached to moves along the processing chain. To determine if temperature changes the rate at which die off occurs vegetative cells of *Cl. frigidicarnis* were exposed to air at 10 °C, the typical temperature of boning rooms (**Figure 6.5**). Reducing the temperature increased the survival time of vegetative cells. *Cl. estertheticum* subsp. *estertheticum* vegetative cells were also exposed to air at 10 °C, resulting in very little reduction in the number of viable cells (**Figure 6.6**). *Cl. estertheticum* subsp. *estertheticum* is unable to grow at temperatures above 15 °C. To determine if die off, at 21 °C, when exposed to air was a result of the effect of temperature, vegetative *Cl. estertheticum* subsp. *estertheticum* cells were incubated at 21 °C anaerobically. The time until non-viability was doubled from two to four days. *Cl. estertheticum* subsp. *estertheticum* was also exposed to air at 10 °C. Under these conditions it was able to survive for 7 days, the extent of the experiment, with only a slight reduction in the viable cell count.



**Figure 6.5** Viable vegetative cells of *Cl. frigidicarnis*, as determined by plate count on CBA, exposed to air, in dCMM supernatant, at 10 °C (◆) and room temperature, 21 °C (■). The limit of detection of the test was 10 CFU ml<sup>-1</sup> or 1 log. Values shown are the mean of three replicates.



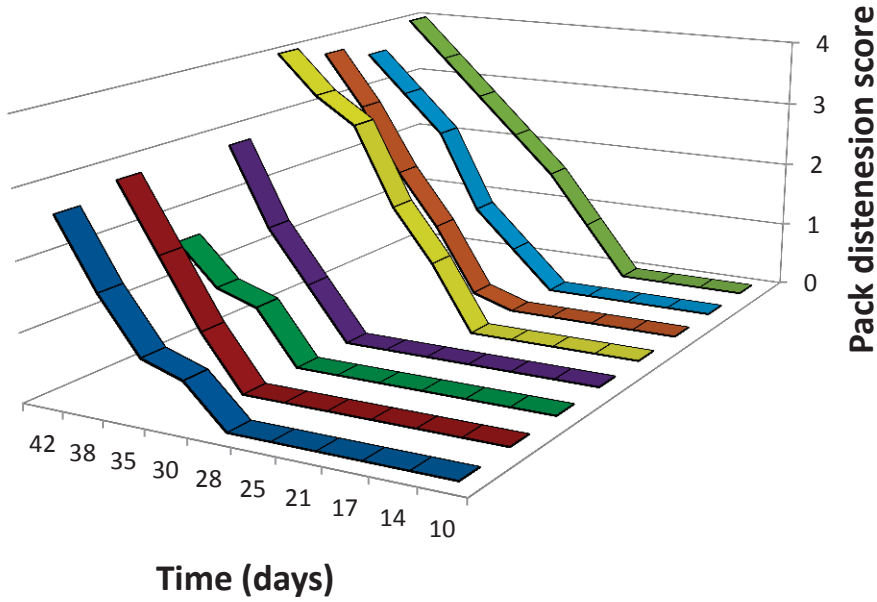
**Figure 6.6** Viable vegetative cells of *Cl. estertheticum* subsp. *estertheticum* exposed to air in dCMM supernatant at 21 °C (■), exposed to air in dCMM supernatant at 10 °C (◆) and under anaerobic conditions in dCMM supernatant at 21 °C (▲), as determined by plate count on CBA. The limit of detection of the test was 10 CFU ml<sup>-1</sup> or 1 log. Values shown are the mean of three replicates.

### 6.3.2 Exposure of *Cl. estertheticum* subsp. *estertheticum* inoculated vacuum-packed red meat to air

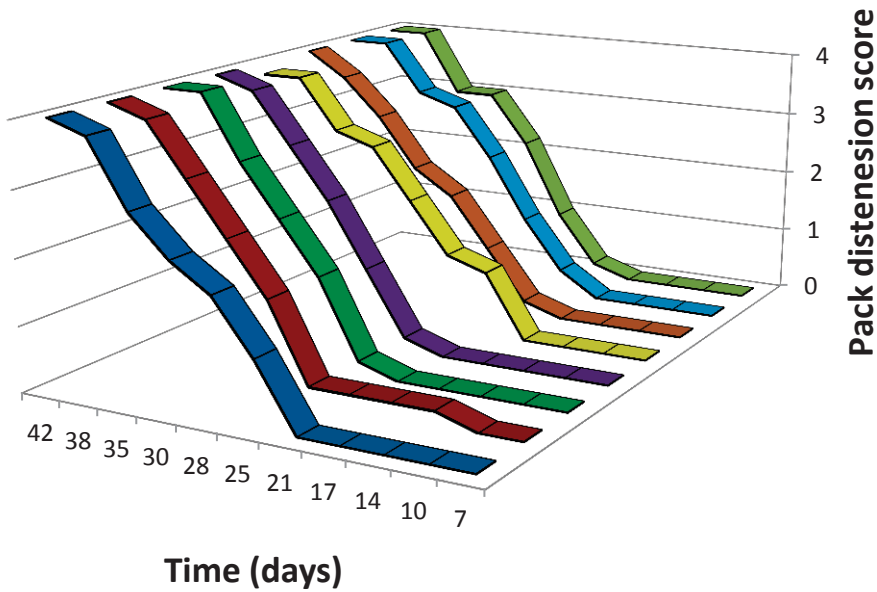
To determine if exposure to air, shortly after packaging, could reduce or delay spoilage, in lamb inoculated with *Cl. estertheticum* subsp. *estertheticum* spores, gas production was monitored in packs opened at 24 h, 3 days, 6 days, and control packs, that had not been opened, stored at -1.5 and 2 °C (**Figure 6.7**). The packs were initially inoculated with 3.7 or 37 CFU ml<sup>-1</sup>, as determined by plate count on CBA. The average level of gas accumulation in the uninoculated control packs remained below level 1 (gas bubbles in the drip) for the duration of the experiment (42 days).



A



B



**Figure 6.7** Average level of gas production, by *Cl. estertheticum* subsp. *estertheticum*, in vacuum-packed lamb stored at A:  $-1.5\text{ }^{\circ}\text{C}$  and B:  $2\text{ }^{\circ}\text{C}$ . 0: packs with no gas bubbles in the drip; 1: packs that had gas bubbles in the drip; 2: packs at the ‘loss of vacuum’ stage; 3: obviously ‘blown’, puffy packs; 4: fully distended packs without tightly stretched packaging; and 5: tightly stretched, ‘overblown’ packs. Unopened/ $3.7\text{ CFU ml}^{-1}$  (■), opened at day 1/ $3.7\text{ CFU ml}^{-1}$  (■), opened at day 3/ $3.7\text{ CFU ml}^{-1}$  (■), opened at day 6/ $3.7\text{ CFU ml}^{-1}$  (■), unopened/ $37\text{ CFU ml}^{-1}$  (■), opened at day 1/ $37\text{ CFU ml}^{-1}$  (■), opened at day 3/ $37\text{ CFU ml}^{-1}$  (■), opened at day 6/ $37\text{ CFU ml}^{-1}$  (■).

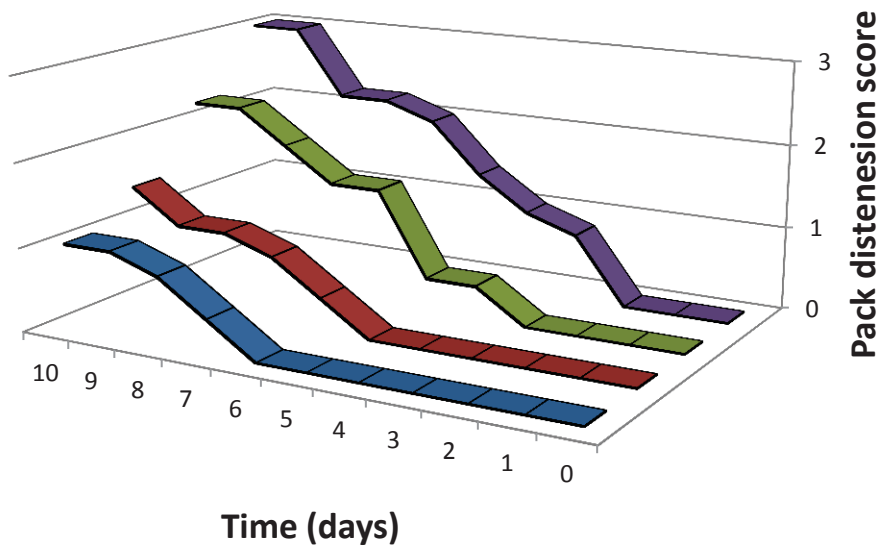
The time until packs reached a gas accumulation level 2 (loss of vacuum) was analysed in an ANOVA. The level of inoculum and the storage temperature both had a statistically significant effect on the time to reach level 2 gas accumulation (F value < 0.001). Higher inoculum and higher storage temperature both shortened the time until level 2 gas accumulation was reached (**Table 6.1**). There was a statistically significant interaction between initial level of inoculum and storage temperature (F value < 0.001). The time at which packs were opened had much less effect (F value = 0.022) although there may have been an interaction between storage temperature and opening time (F value = 0.006), with a greater spread in time until loss of vacuum at -1.5 than 2 °C (**Figure 6.7**).

**Table 6.1** Mean time until vacuum-packed lamb steaks inoculated with spores of *Cl. estertheticum* subsp. *estertheticum* reached level 2 gas accumulation (loss of vacuum) when opened and repackaged at 0, 1, 3 and 6 days after initial packaging. \* had not reached loss of vacuum stage following 42 days of storage.

|                                       | Time (days) until loss of vacuum (mean of 5 replicates) |   |      |      |      |      |
|---------------------------------------|---|---|------|------|------|------|
|                                       | No inoculum   |   | 3.7  |      | 37   |      |
| Inoculum<br>(CFU/pack)                |   |   |      |      |      |      |
| Temperature<br>(°C)                   | -1.5  | 2 | -1.5 | 2    | -1.5 | 2    |
| Pre opening<br>storage time<br>(days) |   |   |      |      |      |      |
| 0                                     | *   | * | 40.4 | 29.4 | 30.0 | 27.8 |
| 1                                     | *   | * | 38.8 | 29.8 | 31.8 | 28.6 |
| 3                                     | *   | * | 43.2 | 29.8 | 34.8 | 27.2 |
| 6                                     | *   | * | 39.6 | 30.4 | 34.2 | 28.6 |

To determine if repackaging, following shipping and prior to retail display, results in delayed spoilage by *Cl. estertheticum* subsp. *estertheticum*, inoculated vacuum-packs

were subjected to simulated shipping and display conditions (**Figure 6.8**). A log of temperature during display is shown in **Appendix 3**. Results were analysed in an ANOVA and showed that both the level of initial inoculum and opening packs prior to placing them at display temperatures had a statistically significant effect on the mean time until level 2 gas accumulation was reached (F values <0.001 and 0.001 respectively, **Table 6.2**).



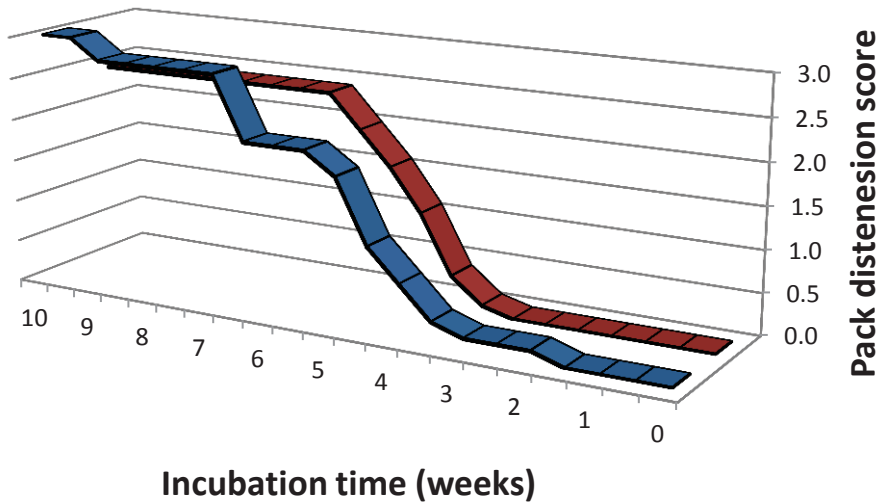
**Figure 6.8** Average level of gas production, by *Cl. estertheticum* subsp. *estertheticum*, in vacuum-packed lamb stored at -1.5 °C for 21 days, to simulate shipping, then opened and repackaged prior to being held at simulated display temperature for 10 days. 0: packs with no gas bubbles in the drip; 1: packs that had gas bubbles in the drip; 2: packs at the 'loss of vacuum' stage; 3: obviously 'blown', puffy packs; 4: fully distended packs without tightly stretched packaging; and 5: tightly stretched, 'overblown' packs. Unopened, 37 CFU ml<sup>-1</sup> inoculum (■); opened, 37 CFU ml<sup>-1</sup> inoculum (■); unopened, 3.7 CFU ml<sup>-1</sup> inoculum, (■) opened, 3.7 CFU ml<sup>-1</sup> inoculum (■). Values shown are the mean of five replicates.

**Table 6.2** Mean time until vacuum-packed lamb steaks inoculated with spores of *Cl. estertheticum* subsp. *estertheticum* reached level 2 gas accumulation (loss of vacuum) following simulated shipping at -1.5 °C for 21 days, repackaging and retail display. \* had not reached loss of vacuum stage following 10 days of display.

| Time (days) from display until loss of vacuum (mean of 5 replicates) |     |     |
|--|-----|-----|
| Pack opened prior to display   |     |     |
| Inoculum (CFU ml <sup>-1</sup> )                                     | N   | Y   |
| 0  | *   | *   |
| 3.7  | 6.8 | 7.8 |
| 37   | 3.2 | 5.2 |

### 6.3.3 Effect of hot water washing on spoilage by *Cl. estertheticum* subsp. *estertheticum*

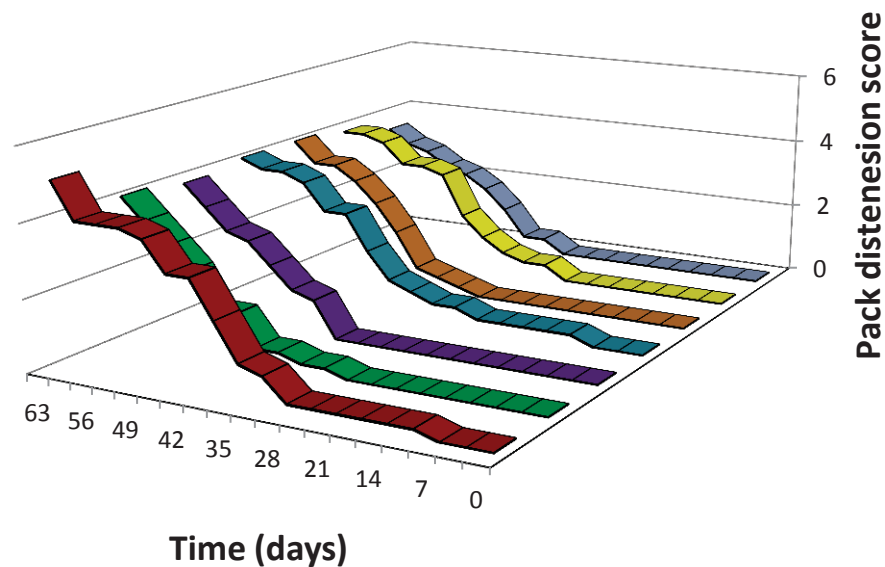
The final inoculum contained  $1.6 \times 10^2$  CFU/pack. The first signs of gas production were visible in a single vacuum-pack in the second week of incubation (**Figure 6.9**). Both cold and HWWed packs reached an average blownness score of 2 (the stage at which the meat would be considered spoiled) during the fifth week of incubation. Gas production plateaued at week 7. After 10 weeks of incubation all packs were obviously spoiled, despite none becoming fully distended. A log of storage temperature is shown in **Appendix 4**.



**Figure 6.9** level of gas production, by *Cl. estertheticum* subsp. *estertheticum*, in vacuum-packed lamb. HWW (■), CWW (■), 0: packs with no gas bubbles in the drip; 1: packs that had gas bubbles in the drip; 2: packs at the 'loss of vacuum' stage; 3: obviously 'blown', puffy packs; 4: fully distended packs without tightly stretched packaging; and 5: tightly stretched, 'overblown' packs. Values shown are the mean of five replicates.

#### 6.3.4 Effect of post packaging hot water washing on spoilage by *Cl. estertheticum* subsp. *estertheticum*

To determine if HWW could be used to reduce spoilage by *Cl. estertheticum* subsp. *estertheticum*, lamb steaks were inoculated with  $2.7 \text{ CFU ml}^{-1}$  of *Cl. estertheticum* subsp. *estertheticum* spores, as determined by plate count on CBA and subjected to a range of treatments including exposure to air, HWW, CWW and tyndallisation prior to chilled storage (**Figure 6.10**). To determine if the time until spoilage (level 2 gas accumulation) was affected an ANOVA was carried out on the results of the on lamb experiment using GenStat. Analysis is summarised in **Table 6.3** shown in full in **Appendix 5**.



**Figure 6.10** Level of gas production, by *Cl. estertheticum* subsp. *estertheticum*, in vacuum-packed lamb stored at -1.5 °C. 0: packs with no gas bubbles in the drip; 1: packs that had gas bubbles in the drip; 2: packs at the 'loss of vacuum' stage; 3: obviously 'blown', puffy packs; 4: fully distended packs without tightly stretched packaging; and 5: tightly stretched, 'overblown' packs. Inoculated control (■), CWW (■), HWW (■), exposed to air at 3 days (■), HWW at 3 days (■), tyndallised (■), tyndallised and HWW at 3 days (■). Values shown are the mean of five replicates.

The average level of gas accumulation for the un-inoculated packs remained below 1 for the duration of the experiment. The time until the inoculated packs reached the loss of vacuum stage varied from 38.3 days, for the samples exposed to air after 3 days of chilled storage and 52.6 days, for the samples that underwent a CWW treatment prior to packaging. Time until loss of vacuum, or shelf life, was improved as a result of a HWW or a CWW by 12.6 and 12.2 days respectively. Exposure to air after 3 days of chilled storage, in a vacuum-pack, did not result in any change in shelf life, compared to the un-inoculated control. A HWW after 3 days of chilled storage, in a vacuum-pack, improved shelf life but not as much as a CWW or HWW prior to packaging. There was no statistically significant improvement in shelf life as a result of tyndallisation compared with the inoculated control packs. Tyndallisation followed by HWW after 3 days of storage resulted in an 8.4 days improvement in shelf life over the inoculated control.

**Table 6.3** Mean time until pack distension reached or exceeded a score of two (loss of vacuum). A vacuum pack with a score of two would be deemed spoiled in a commercial situation. Analysis of variance gave a P value of < 0.001 and the least significant differences of means (5%) was 6.803. The uninoculated packs did not reach or exceed a gas accumulation score of two during the incubation period (8 weeks).

| Treatment              | Inoculated | CWW  | HWW  | Exposed to O <sub>2</sub> | HWW at 3d | Tyndallised | Tyndallised/HWW at 3d |
|------------------------|------------|------|------|---------------------------|-----------|-------------|-----------------------|
| Incubation time (days) | 39         | 51.6 | 51.2 | 38.3                      | 47.4      | 43.2        | 47.4                  |

At the end of storage, drip was collected from one of the replicates from each treatment. PCR tests, for *Cl. estertheticum*, using drip from inoculated samples produced single bands of the expected length (790 base pairs [bp]) and tests using drip from un-inoculated control pack produced no band. Lack of gas accumulation of more than level 1 and negative PCR results for the un-inoculated control samples indicate *Cl. estertheticum* was not present or only present in very small numbers in the natural flora of the meat.

## 6.4 Discussion

The purpose of this study was to investigate options for part two of a two-step process involving inducing germination, of clostridial spores, then treating them to inactivate cells and reduce spoilage. Exposing cells to aerobic conditions is potentially a simple and cost effective method of inactivating cells. The conditions under which germinated spores and vegetative cells, of red meat spoilage clostridia, are inactivated or killed on exposure to anaerobic conditions *in vitro* and on lamb were determined. The effect of HWW and CWW treatments, in conjunction with tyndallisation and exposure to air, on time until spoilage of lamb, by *Cl. estertheticum* spores, was also determined.

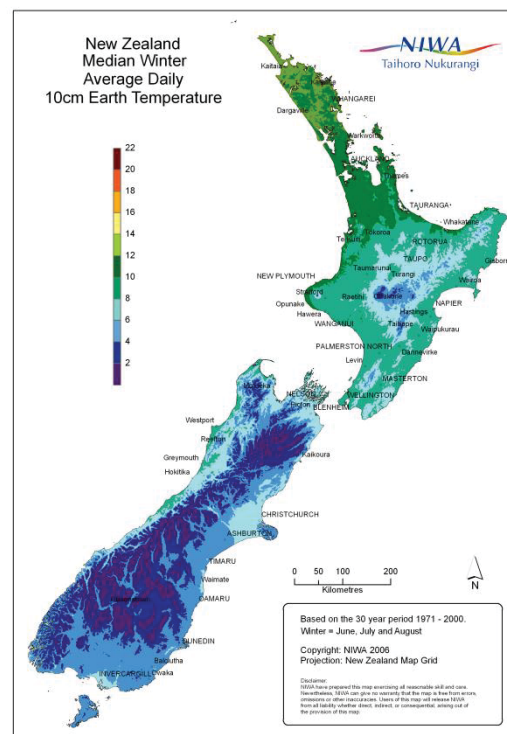
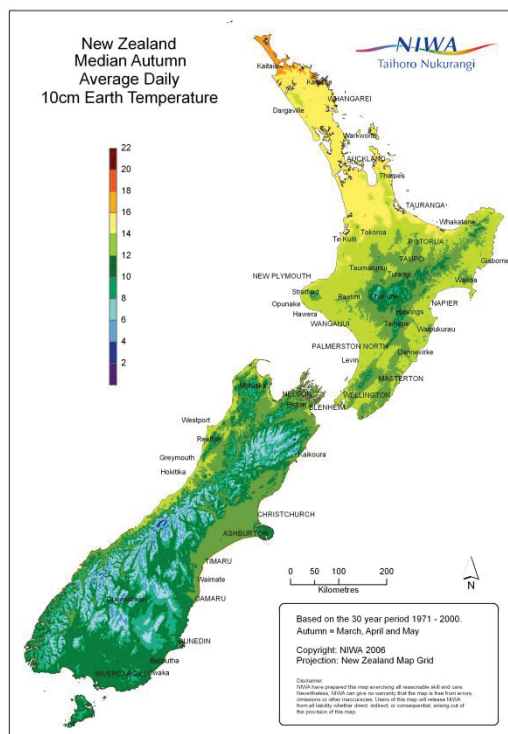
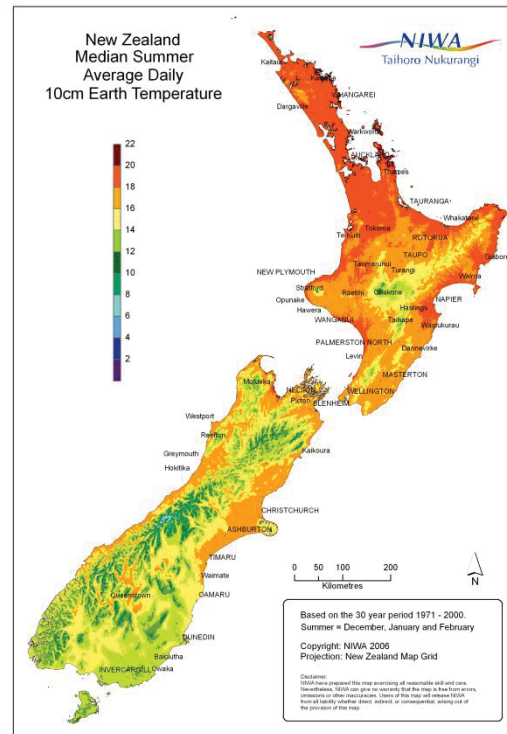
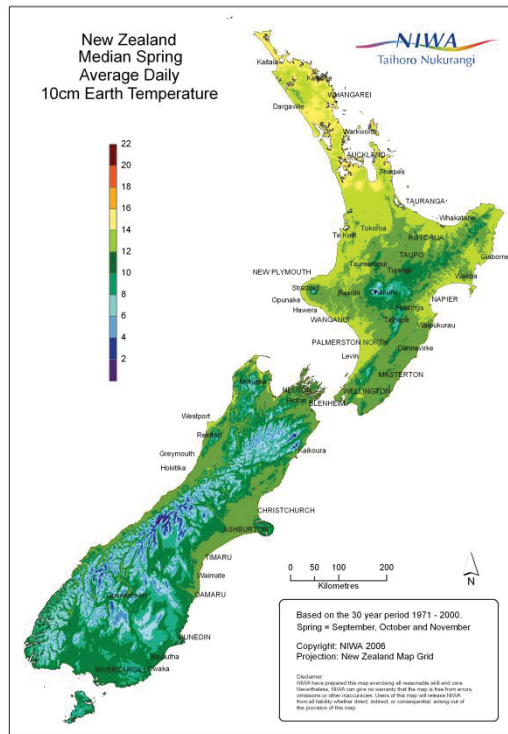
Working with germinated spores is difficult as the time until germination of individual spores varies (Stringer et al. 2009; 2011). The resistance properties of spores are lost rapidly at the time of germination, rendering their survival similar to those of vegetative cells (Foster and Johnstone 1990). Resistance of *Cl. frigidicarnis* vegetative cells was sufficiently similar to that of germinated spores to justify their use as an analogue. The time taken to reduce each of the strains to below the level of detection varied. It may be possible to reduce the number of germinated spores of *Cl. frigidicarnis*, LA1 and *Cl. gasigenes* on red meat by exposure to aerobic conditions as they were all inactivated in 2 to 3 h, under ideal conditions (aerobic media and exponentially growing cells). Longer exposure times become increasingly impractical in a meat processing plant situation. Dehydration would begin to have an impact on both the quality of the meat as well weight, on which payment is calculated.

Temperature had an effect on the length of time *Cl. frigidicarnis* and *Cl. estertheticum* subsp. *estertheticum* survived in aerobic conditions. In both cases reducing the temperature from 21 °C (room temperature) to 10 °C (the temperature boning rooms are typically maintained at) increased the survival time. Unfortunately increasing the temperature at which meat is exposed to air would be impractical, as the benefits with regard to reducing spoilage by psychrotolerant clostridia would be outweighed by the increased risk of growth of pathogens and spoilage by mesophiles. Vegetative *Cl. estertheticum* subsp. *estertheticum* survived seven days of exposure to aerobic



conditions at 10 °C with little effect. This calls into question the assumption that the majority of spoilage is caused by contamination with spores, rather than vegetative cells. Clostridial spores can survive in soil for years with little reduction in viable numbers, less is known about vegetative cells, especially from psychrotolerant strains (Girardin et al. 2005). One of the few published studies reported that vegetative cells of pathogenic *Cl. septicum* survived for 30 days in a range of soils (Garcia and McKay 1969). **Figure 6.11** shows the mean soil temperature for spring, summer, autumn and winter in New Zealand. Even taking into account extremes and variation in microclimates there is potential for soil temperature, in the South Island, to remain suitable for the survival, of vegetative *Cl. estertheticum* subsp. *Estertheticum*, for significant periods of time.

A comparison between HWW and CWW confirmed that exposing spores of *Cl. estertheticum* subsp. *estertheticum* to the short burst of heat that occurs during HWW did not shorten the time until spoilage under worst case conditions (high inoculum, sub optimal refrigeration) or optimal conditions (low inoculum, optimal refrigeration temperature). This differs from the effect of heat shrinking following vacuum-packing which has been shown to shorten the time until spoilage (Bell et al. 2001). HWW and CWW of lamb, inoculated with *Cl. estertheticum* subsp. *estertheticum*, increased the time until spoilage equally in comparison with inoculated control packs. Prior studies have shown HWW is effective in reducing aerobic plate count (APC), coliform, *E. coli*, and vegetative *Cl. sporogenes* counts (Barkate et al. 1993; Dorsa et al. 1997; Gill et al. 1999; Gill and Bryant 2000). The anaerobic clostridia investigated here would not be detected by APC and the effects on spores have not been previously published. From this result we can conclude that the mechanism for improved shelf life, in packs containing *Cl. estertheticum* subsp. *estertheticum*, is a reduction in spore numbers resulting from the mechanical effect of the water rather than the temperature of the water. The reduction may be as a result of removal of spores from the meat. As spores are able to form strong attachments to surfaces rapidly water pressure is likely to play an important role (Bower et al. 1996). This agrees with a study that found no difference in reduction of *E. coli* O157:H7, from contaminated faeces, on beef sprayed with water between 30 and 70 °C (Cutter et al. 1997).



**Figure 6.11** Mean soil temperature for spring, summer, autumn and winter, at a depth of 10cm, in New Zealand (<https://secure.niwa.co.nz/climate-explorer/home.do>, March 2012).

In an attempt to induce germination of *Cl. estertheticum* subsp. *estertheticum* spores prior to inactivation, inoculated steaks were stored chilled and vacuum-packed for one to six days prior to treatment. The shelf life of samples exposed to air was not improved. This is consistent with *in vitro* tests. Inducing germination prior to the application of a HWW treatment would theoretically increase inactivation as spores are highly resistant to heat (Driks 2002). Some improvement in shelf life was seen in samples that underwent a HWW treatment after 3 days in vacuum-packs. However, improvement was not as great as when samples were washed with hot or cold water prior to packaging. Three day old chilled meat has lost flexibility. The stiffness of the meat increases the crevices in the meat decreasing the effectiveness of washing. Low levels of germination may also have played a part in reducing the effectiveness of HWW in reducing spoilage at 3 days. Tyndallisation did not decrease the time until spoilage and the time until spoilage in a sample that had undergone tyndallisation and a HWW at 3 days was slightly less than samples HWWed or CWWed prior to packing.

## 6.5 Conclusion

A CWW or HWW prior to packaging is recommended to delay the spoilage of meat contaminated with *Cl. estertheticum* spores. The ability of vegetative cells of psychrotolerant red meat spoilage clostridia to survive exposure to air varies considerably, from hours to days. Vegetative cells of *Cl. estertheticum* subsp. *estertheticum*, a major cause of blown-pack spoilage, are able to survive more than a week of exposure to air at 10 °C, the typical temperature of boning rooms. This rules out the use of exposure to air as a practical means of inactivating germinated spores. This also introduces the possibility that *Cl. estertheticum* subsp. *estertheticum* is able to persist in aerobic soil, in a vegetative state, for long periods of time, and therefore may play a larger part in contamination of red meat leading to spoilage than originally thought.



## Chapter 7

### Germinant receptor proteins and their sequences

#### 7.1 Introduction

Germination is generally triggered by the interaction of a germinant or group of germinants with the spore's germinant receptors (GRs) (Setlow 2003). Germinants are small molecules, often nutrients required by the cell for growth and division. They are highly specific to given strains but can vary between species and strains. After penetrating the outer layers of the spore coat and cortex, germinants interact with GRs, which are present in the inner membrane (Moir 2006). This interaction triggers a cascade of degradative germination events (Johnstone 1994). Spores of most strains contain multiple proteins from the GR family with different specificities for different germinants (Paredes-Sabja et al. 2011). GRs have been best studied in *B. subtilis*. The GRs of *B. subtilis* are GerA which responds to L-alanine and GerB and GerK which collaboratively respond to AGFK (Paidhungat and Setlow 2000; Cabrera-Martinex et al. 2003). GR proteins are classified as belonging to the GerA family. Sequence coding for GerA family proteins has been identified in the genomes of a wide range of strains of spore formers studied. In many cases the germinants responsible for triggering specific GRs identified through sequencing remain unknown. Of the spore formers studied *Cl. difficile* and *Cl. bartlettii* were the only species with no identifiable GR genes (Paredes-Sabja et al. 2011). The GRs of *B. subtilis* are composed of three protein subunits termed A, B and C, encoded by homologous tricistronic *gerA* family operons; this is common but not universal in other species (Moir et al. 1994; Paredes-Sabja et al. 2011). Precisely how and where germinants bind to germinant receptors remains unclear although suggestions have been made for binding sites in *B. megaterium* QM B1551 (Christie et al. 2010; Paredes-Sabja et al. 2011).

Genetic analysis identified putative GR genes in several clostridial strains including *Cl. botulinum*, *Cl. perfringens* and *Cl. sporogenes* (Paredes-Sabja et al. 2008b; Peck 2009; Peck et al. 2011). The *gerA* and *gerK* genes in *Cl. perfringens* Type A have been partially

characterized using mutants (Paredes-Sabja et al. 2008b). The receptor encoded by *gerA* receptor plays an auxiliary role in germination in the presence of KCl and *gerK* is involved in germination in response to L-asparagine. *Cl. sporogenes* and proteolytic *Cl. botulinum* both germinate in response to a combination of L-alanine, L-lactate and NaHCO<sub>3</sub> and have putative *gerA* operons. However, a link has not been made between the two (Broussolle et al. 2002). Non-proteolytic *Cl. botulinum* germinates in response to an amino acid (e.g. L-alanine, L-cysteine, L-serine) in combination with L-lactate (Plowman and Peck 2002). The genomes of non-proteolytic *Cl. botulinum* strains Eklund 17B (Type B) and Alaska E43 (Type E) each contain a single tricistronic germinant receptor operon (Peck et al. 2011). Again it is not known if the response is linked to the GR operon.

The aim of this chapter was to screen psychrotolerant red meat spoilage clostridia for the presence GR genes. Where GR genes are identified and sequenced it may be possible to predict which germinants are likely to trigger germination based on their similarity to GR genes from species with known germinants.

## 7.2 Methods

### 7.2.1 Screening for germinant receptor genes using PCR

To determine if genes for specific germinant receptors were present in the genomes of cold tolerant meat spoilage clostridia several PCRs were performed. Primers used are shown in **Table 7.1**. Primers specific to *gerB* type sequence were designed based on alignments of *B. cereus* m1293, *Bacillus mycoides* DSM 2048, *Bacillus pseudomycooides* DSM 12442 and *Bacillus thuringiensis*. Primers specific to the *gerK* type sequence were designed based on alignments of *B. subtilis* (*gerK*), *Cl. perfringens* str. 13 and *Cl. acetobutylicum* (*gerKA*) (**Appendix 6**). A fourth set of primers was designed based on a fragment of sequence from *Cl. estertheticum* subsp. *estertheticum* and *Cl. sporogenes* spore germination protein (*gerAA* and *gerAB*) sequence (EGER5F and EGER1R). DNA sequences were obtained from the The National Center for Biotechnology Information website (NCBI, <http://www.ncbi.nlm.nih.gov>). Alignments were performed using Toffee software (<http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi>). All primers were obtained from Invitrogen. PCR conditions were optimised by performing gradient PCRs on whole genomic DNA extracted from *Cl. sporogenes* (*gerA*), *B. cereus* (*gerB*) and *Cl. perfringens* Type B and C for (*gerK*) using the High Pure PCR Template Preparation Kit as described in Section 3.10. Gradient PCRs were performed in a Techne TC-512 thermocycler (Total Lab Systems, Auckland, New Zealand) at 3 x MgCl concentrations (1.0 mmol l<sup>-1</sup>, 1.5 mmol l<sup>-1</sup> and 2.0 mmol l<sup>-1</sup>) and 12 annealing temperatures ranging between 40 and 55 °C. All other conditions were consistent with those used in the 16S rRNA PCR described in Section 3.11. Following optimisation, PCRs for *gerA*, *gerB* and *gerK* were performed on genomic DNA extracted from *Cl. algidicarnis*, *Cl. algidixylanolyticum*, *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense*, *Cl. frigidicarnis*, *Cl. gasigenes* and LA1.



**Table 7.1** Germinant receptor specific PCR primers. AAF2 and AAR2 were modified versions of AAF1 and AAR1 described by Broussolle et al. (2002). GERBF, GERBR, GERKF, GERKR, EGER5 and EGER1 were designed specifically for this project.

| Target gene          | Name  | Forward/<br>Reverse | Primer (5' to 3')                  |
|----------------------|-------|---------------------|------------------------------------|
| <i>gerA</i>          | AAF2  | F                   | ACW GAA AGA CCD GAT GTW GC         |
|                      | AAR2  | R                   | CTT AAT CCW SCY TCT CTT AW         |
| <i>gerB</i>          | GERB  | F                   | AAA YCG TTG AAT GAA AGG AAC        |
|                      | GERB  | R                   | ATT TTA CCT ATT TTK ACR CAT TCA CC |
| <i>gerK</i>          | GERK  | F                   | AGY WGA TGG WAC WCC TWT TGT TC     |
|                      | GERK  | R                   | YTA WWG CAA CAT ATA WKC CTG GT     |
| <i>Cl.</i>           | EGER5 | F                   | AGR GGM CCT AAR GAA GCY TT         |
| <i>estertheticum</i> | EGER1 | R                   | AGG GCA ATT CCT ACC CTT GAC TGA    |

Gel electrophoresis was carried out on PCR products as described in Section 3.12. A number of bands were cleaned, as described in Section 3.13, and sent to the Massey Genome Service for sequencing (Palmerston North, New Zealand). The resulting sequences were trimmed at each end and analysed by nucleotide BLAST (basic logical alignment search tool, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>, 2012). Results from forward and reverse primers were combined prior to nucleotide BLAST analysis where possible.

### 7.2.2 Screening for germinant receptor genes by Southern Blotting.

Southern blots were performed to further screen for germinant receptor genes in psychrotolerant meat spoilage clostridia. Whole genomic DNA was digested prior to blotting using enzymes from Invitrogen. Probe production and blotting were carried out as described in Section 3.14. **Table 7.2** lists the combination of probe and digestion used for the blots. Fragment length was calculated based on the DNA Molecular Weight Marker VI (Roche) using Image Lab™ software (BioRad). Expected fragment

length was calculated by performing virtual digests, of the sequences used to design the primers, using <http://www.restrictionmapper.org> (2012).

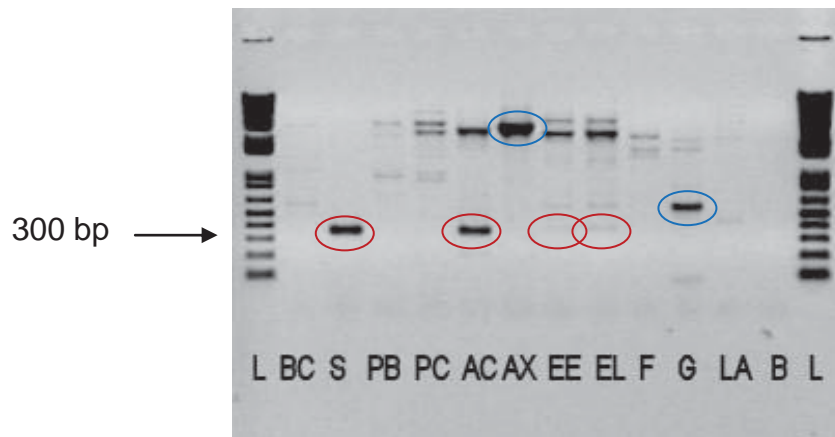
**Table 7.2** Digestion treatments and probes used in Southern blotting. Digestions were carried out in 25  $\mu\text{l}$  volumes containing the enzyme, 100 ng of DNA and 2.5  $\mu\text{l}$  of 10 x REact<sup>®</sup> 2 buffer giving a final concentration of 50 mmol  $\text{l}^{-1}$  Tris-HCl (pH 8.0), 10 mmol  $\text{l}^{-1}$   $\text{MgCl}_2$ , 50 mmol  $\text{l}^{-1}$  NaCl. The mixes were incubated for one h, an additional volume of enzyme was added and the incubation continued overnight.

| Probe  | Digestion enzyme | Volume of enzyme ( $\mu\text{l}$ ) | Temperature of digest ( $^{\circ}\text{C}$ ) | Volume of second addition of enzyme ( $\mu\text{l}$ ) |
|--|------------------|------------------------------------|--|---|
| Primers  |                  |                                    |  |   |
| <i>Cl. sporogenes</i>                                | AAF2 / AAR2      | 1 $\mu\text{l}$                    | 37   | 0.5 $\mu\text{l}$                                     |
| <i>Cl. estertheticum</i> subsp. <i>estertheticum</i> | AAF2 / AAR2      | 1 $\mu\text{l}$                    | 37   | 0.5 $\mu\text{l}$                                     |
| <i>B. cereus</i>                                     | GERBF / GERBR    | 2.5 $\mu\text{l}$                  | 65   | 1 $\mu\text{l}$                                       |
| <i>Cl. perfringens</i> Type B                        | GERKF / GERKR    | 2.5 $\mu\text{l}$                  | 37   | 2 $\mu\text{l}$                                       |

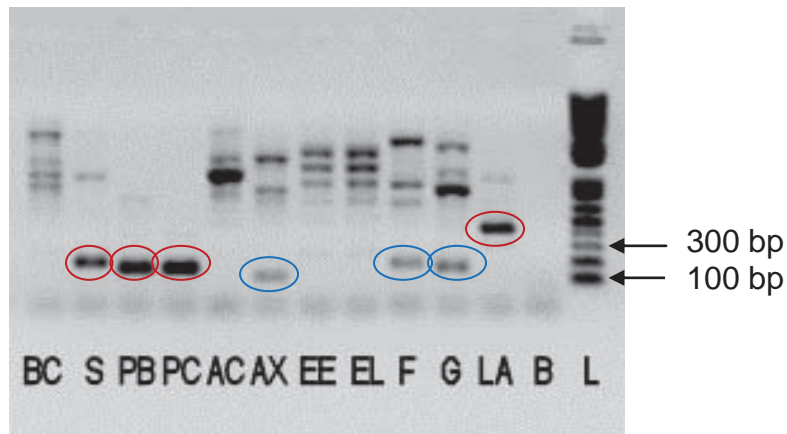
### 7.3 Results

#### 7.3.1 Screening for germinant receptor genes using PCR

The *gerA* and *gerK* PCRs produced bands as shown in **Figures 7.1** and **7.2**. The *gerB* PCR only produced a band in the *B. cereus* control lane.



**Figure 7.1** PCR products from amplification of whole genome DNA using *gerA* primers. The expected band size was 360 bp. *B. cereus* (BC), *Cl. sporogenes* (S), *Cl. perfringens* Type B (PB), *Cl. perfringens* Type C (PC), *Cl. algidicarnis* (AC), *Cl. algidixylanolyticum* (AX), *Cl. estertheticum* subsp. *estertheticum* (EE), *Cl. estertheticum* subsp. *laramiense* (EL), *Cl. frigidicarnis* (F), *Cl. gasigenes* (G), LA1 (LA), blank (B), 1kb<sup>+</sup> ladder (L). Circled bands were sequenced. Those circled in red produced good sequence, those circled in blue failed to return usable sequence.



**Figure 7.2** PCR products from amplification of whole genome DNA using *gerK* primers. The expected band size was 170 bp. *B. cereus* (BC), *Cl. sporogenes* (S), *Cl. perfringens* Type B (PB), *Cl. perfringens* Type C (PC), *Cl. algidicarnis* (AC), *Cl. algidixylanolyticum* (AX), *Cl. estertheticum* subsp. *estertheticum* (EE), *Cl. estertheticum* subsp. *laramiense* (EL), *Cl. frigidicarnis* (F), *Cl. gasigenes* (G), LA1 (LA), blank (B), 1kb<sup>+</sup> ladder (L). Circled bands were sequenced. Those circled in red produced good sequence, those circled in blue failed to return usable sequence.

The *gerB* / *B. cereus* band and the bands, labelled in **Figures 7.1** and **7.2**, were sequenced, and the resulting sequence analysed using a nucleotide BLAST. Bands chosen were close to the expected size. Additionally, three strong bands of varying sizes were sequenced. Nearest matches are shown in **Table 7.3**, and sequences are in **Appendix 7**. Sequences from *Cl. estertheticum* subsp. *estertheticum* and *Cl. estertheticum* subsp. *laramiense* showed a high degree of homology to each other, as did sequences from *Cl. perfringens* Type B and *Cl. perfringens* Type C. The *Cl. estertheticum* subsp. *estertheticum* AAF2 / AAR2 sequence and the *Cl. estertheticum* subsp. *estertheticum* EGER5F / EGER1R bands do not align with each other.

**Table 7.3** PCR product sequenced and nearest match identified by nucleotide BLAST.

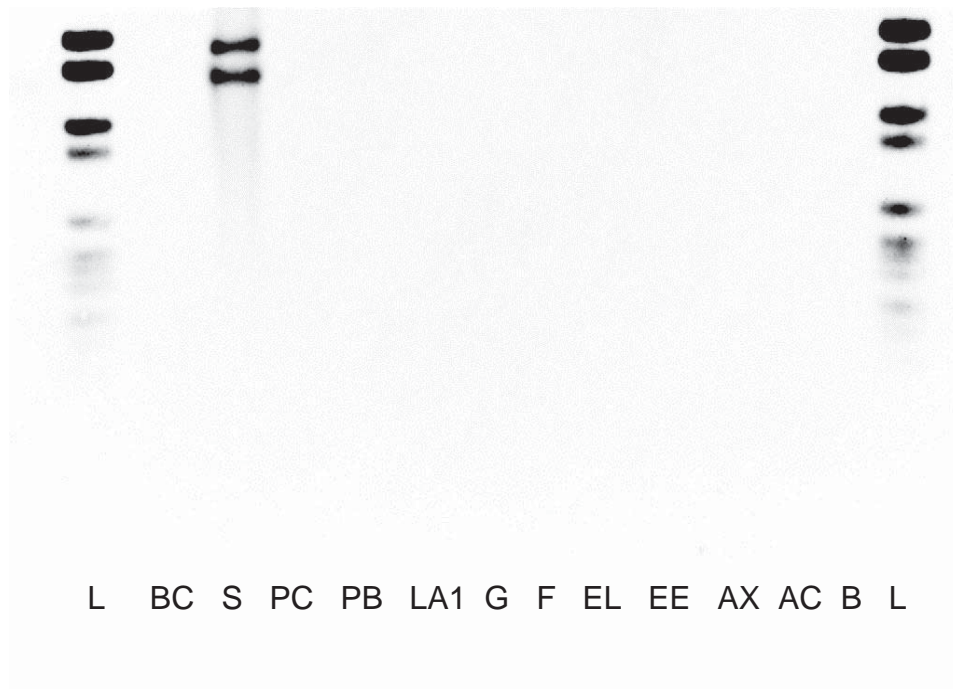
| Template   | Sequencing    |                 |   | BLAST | Similarity (%) |
|--|---------------|-----------------|---|-------|----------------|
|  | Primers       | Primer(s)       | Closest match   |       |                |
| <i>B. cereus</i>                                     | GERBF / GERBR | GERBF           | <i>B. cereus</i> NC740, spore germination protein, GerB family          | 99    |                |
| <i>Cl. sporogenes</i>                                | AAF2 / AAR2   | AAF2 and AAR2   | <i>Cl. sporogenes</i> spore germination protein (GerAA and GerAB)       | 99    |                |
| <i>Cl. algidicarnis</i>                              | AAF2 / AAR2   | AAR2            | <i>Cl. botulinum</i> BKT015925, spore germination protein KA            | 78    |                |
| <i>Cl. estertheticum</i> subsp. <i>estertheticum</i> | AAF2 / AAR2   | AAR2            | <i>Cl. sporogenes</i> spore germination protein (GerAA and GerAB)       | 71    |                |
| <i>Cl. estertheticum</i> subsp. <i>laramiense</i>    | AAF2 / AAR2   | AAR2            | <i>Cl. sporogenes</i> spore germination protein (GerAA and GerAB)       | 71    |                |
| <i>Cl. sporogenes</i>                                | GERKF / GERKR | GERKF and GERKR | <i>Cl. botulinum</i> A3 str. Loch Maree, hydro-lyase                    | 94    |                |
| <i>Cl. perfringens</i> Type B                        | GERKF / GERKR | GERKR           | <i>Cl. perfringens</i> SM101 spore germination protein, GerABKA family  | 96    |                |
| <i>Cl. perfringens</i> Type C                        | GERKF / GERKR | GERKF and GERKR | <i>Cl. perfringens</i> SM101, spore germination protein, GerABKA family | 99    |                |

|  |                 |        |  |    |
|--|-----------------|--------|--|----|
| LA1  | GERKF / GERKR   | GERKR  | <i>Cl. difficile</i> 630, putative cell surface protein                  | 77 |
| <i>Cl. estertheticum</i> subsp. <i>estertheticum</i> | EGER5F / EGER1R | EGER1R | <i>Cl. botulinum</i> Type 5A H04402 065, spore germination protein GerKA | 98 |

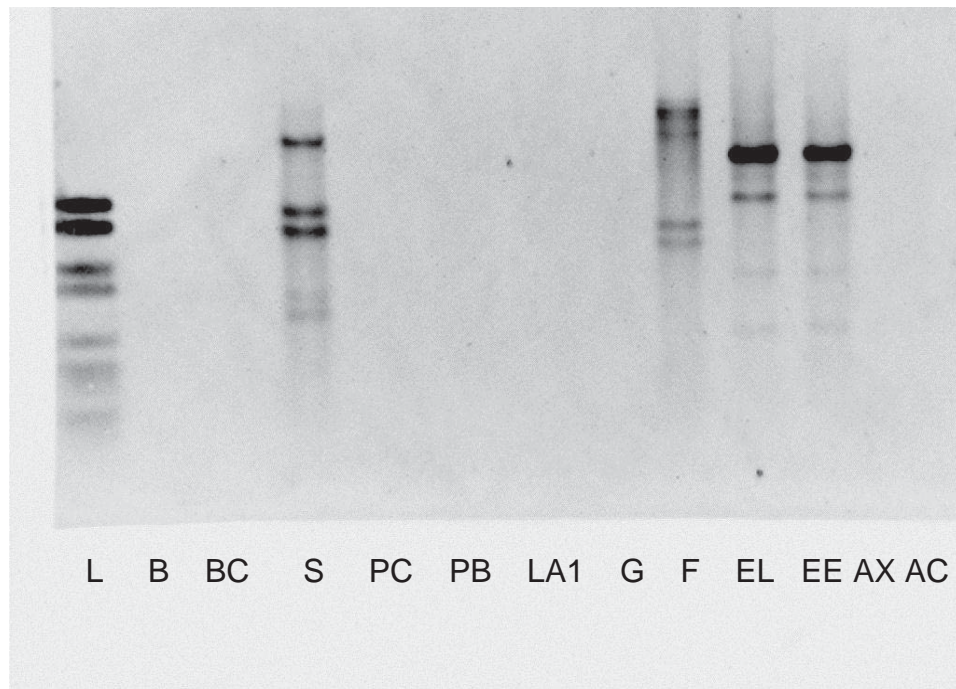
### 7.3.2 Screening for germinant receptor genes by Southern blotting.

PCR may not produce a band even if a gene is present in the bacterium's genome as a result of small changes in the primer region. Germinant receptor genes appear to lack a conserved region increasing the chances of this happening. To further screen for the presence of GR genes four Southern blots were carried out. Two of the blots were produced using probes produced using the AAF2 / AAR2 primers. The *Cl. sporogenes* based probe produced two bands in the *Cl. sporogenes* lane, at 1695 (predicted) and 2100 bp (**Figure 7.3**). The *Cl. estertheticum* subsp. *estertheticum* based probe produced bands in the *Cl. sporogenes*, *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense* and *Cl. frigidicarnis* lanes (**Figure 7.4**). Two of the bands correspond with those produced using the *Cl. sporogenes* based probe; the sizes of the other fragments are given in **Table 7.4**. No predictions could be made about size of the bands as sequence data for *Cl. estertheticum* subsp. *estertheticum* was not available. The *B. cereus* probe produced using GERBF / GERBR primers produced a single band approximately 1160 bp, the predicted size, in the *B. cereus* lane (**Figure 7.5**). The *Cl. perfringens* Type B probe produced using GERKF / GERKR primers produced two bands at 861 bp (predicted) and 519 bp (**Figure 7.6**).





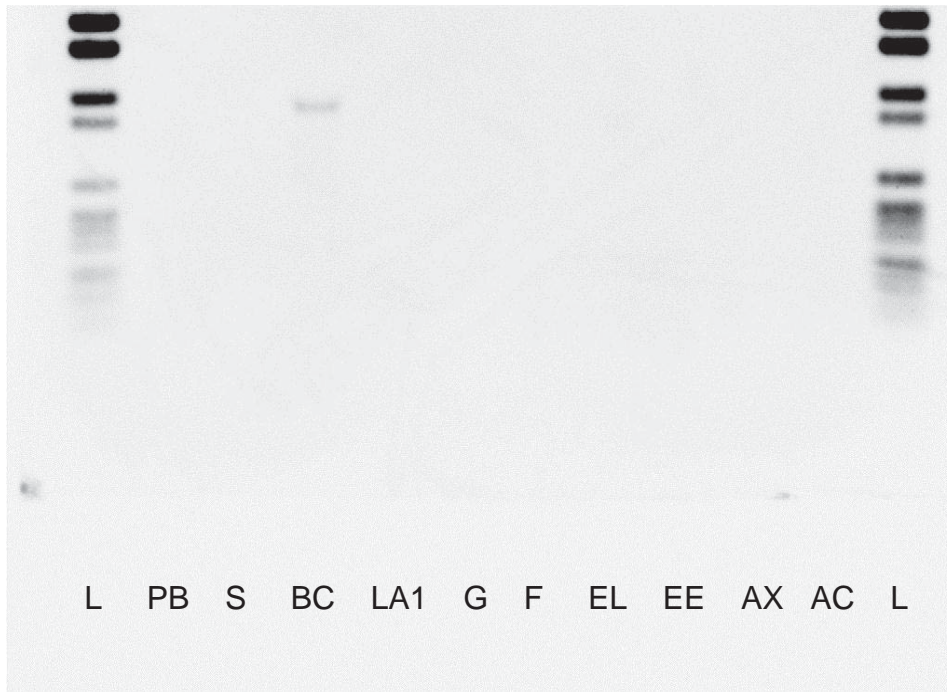
**Figure 7.3** Southern blot of psychrotolerant meat spoilage clostridia using *Cl. sporogenes* probe produced using AAF2 / AAR2 primers. *B. cereus* (BC), *Cl. sporogenes* (S), *Cl. perfringens* Type B (PB), *Cl. algidicarnis* (AC), *Cl. algidixylanolyticum* (AX), *Cl. estertheticum* subsp. *estertheticum* (EE), *Cl. estertheticum* subsp. *laramiense* (EL), *Cl. frigidicarnis* (F), *Cl. gasigenes* (G), LA1 (LA) and DNA Molecular Weight Marker VI (L).



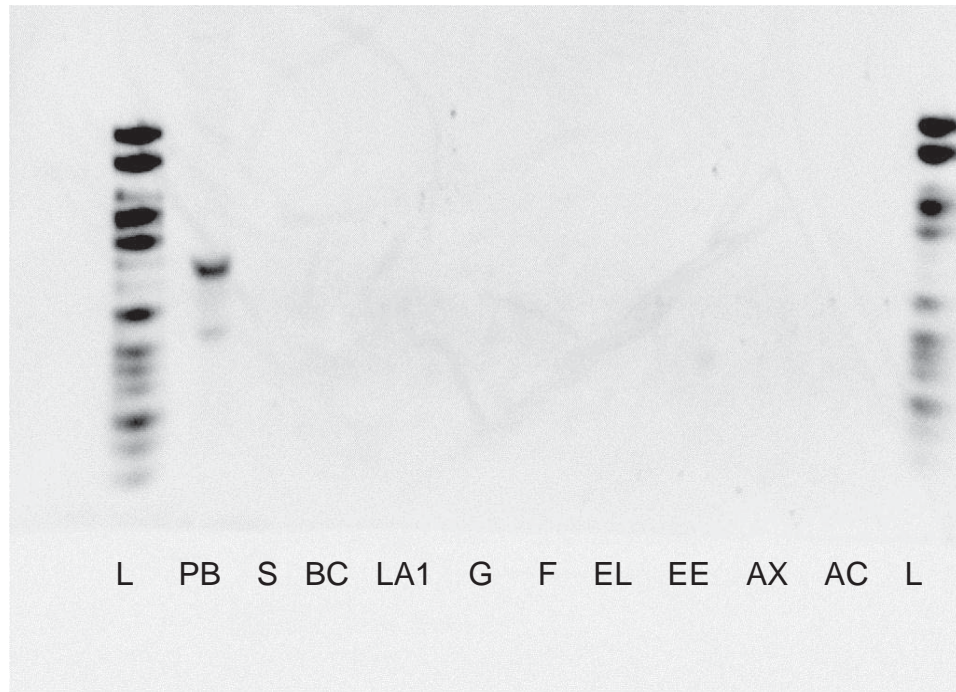
**Figure 7.4** Southern blot of psychrotolerant meat spoilage clostridia using *Cl. estertheticum* subsp. *estertheticum* probe produced using AAF2 / AAR2 primers. *B. cereus* (BC), *Cl. sporogenes* (S), *Cl. perfringens* Type B (PB), *Cl. algidicarnis* (AC), *Cl. algidixylanolyticum* (AX), *Cl. estertheticum* subsp. *estertheticum* (EE), *Cl. estertheticum* subsp. *laramiense* (EL), *Cl. frigidicarnis* (F), *Cl. gasigenes* (G), LA1 (LA), blank (B) and DNA Molecular Weight Marker VI (L).

**Table 7.4** Sizes of bands from Southern blot pictured in **Figure 7.4**.

| Size of bands (bp)    |                          |  |  |
|-----------------------|--------------------------|--|--|
| <i>Cl. sporogenes</i> | <i>Cl. frigidicarnis</i> | <i>Cl. estertheticum</i><br>subsp. <i>laramiense</i> | <i>Cl. estertheticum</i><br>subsp.<br><i>estertheticum</i> |
| 3350                  | 3909                     | 3126   | 3145   |
| 2020                  | 3555                     | 2306   | 2343   |
| 1695                  | 3201                     |  |  |
|                       | 1800                     |  |  |



**Figure 7.5** Southern blot of psychrotolerant meat spoilage clostridia using *B. cereus* probe produced using GERBF / GERBR primers. *B. cereus* (BC), *Cl. sporogenes* (S), *Cl. perfringens* Type B (PB), *Cl. algidicarnis* (AC), *Cl. algidixylanolyticum* (AX), *Cl. estertheticum* subsp. *estertheticum* (EE), *Cl. estertheticum* subsp. *laramiense* (EL), *Cl. frigidicarnis* (F), *Cl. gasigenes* (G), LA1 (LA) and DNA Molecular Weight Marker VI (L).



**Figure 7.6** Southern blot of psychrotolerant meat spoilage clostridia using *Cl. perfringens* Type B probe produced using GERKF / GERKB primers. *B. cereus* (BC), *Cl. sporogenes* (S), *Cl. perfringens* Type B (PB), *Cl. algidicarnis* (AC), *Cl. algidixylanolyticum* (AX), *Cl. estertheticum* subsp. *estertheticum* (EE), *Cl. estertheticum* subsp. *laramiense* (EL), *Cl. frigidicarnis* (F), *Cl. gasigenes* (G), LA1 (LA) and DNA Molecular Weight Marker VI (L).

#### 7.4 Discussion

The aim of the work described in this chapter was to screen the psychrotolerant red meat spoilage clostridia for known GR genes. Due to the lack of a known conserved binding site in the GR genes this was done both with PCR and Southern blotting. Fragments of genes displaying similarity to GR genes were sequenced from *Cl. algidicarnis*, *Cl. estertheticum* subsp. *estertheticum* and *Cl. estertheticum* subsp. *laramiense*. Further to this Southern blotting indicates the possible presence of multiple GR genes in the genome of *Cl. frigidicarnis*.

Following the design of the primers used in this chapter a paper clarifying the naming and relatedness of GR genes was published (Ross and Abel-Santos 2010). Cases of homologous GR genes having different designations as well as dissimilar GR genes having similar or the same designations have been identified (Ross and Abel-Santos 2010). In *B. subtilis* *gerA* was so designated after a study using phenotypic mutants linked it to germination with the single germinant L-alanine (Yasuda and Tochikubo 1997; Ross and Abel-Santos 2010).

The initial aim was to design primers based around the *gerB* and *gerK* genes in *B. subtilis* as this approach had been successful for identifying GR genes, in putative *gerA* operons, in proteolytic *Cl. botulinum* Type B and *Cl. sporogenes* (Broussolle et al. 2002). Grouping of neighbour forming trees based on protein sequences of GR genes indicates *Clostridium* GR proteins designated GerB and GerK have little similarity to their counterparts in *B. subtilis* (Ross and Abel-Santos 2010). The sequences used to design *gerB* primers for this study were all from *Bacillus* species so it is unsurprising that both PCR and Southern blots were negative for the psychrotolerant meat spoilage clostridia. Sequences used to design the *gerA* and *gerK* came from multiple clades suggested by Ross and Abel-Santos (2010), both *Bacillus* and *Clostridium* based. The *Cl. algidicarnis* sequence and *Cl. estertheticum* subsp. *estertheticum* sequences identified during this study all appear to be part of the suggested C3 clade also containing GerAA from *Cl. botulinum*.

The putative GR gene fragment sequenced from *Cl. estertheticum* subsp. *estertheticum* and *Cl. estertheticum* subsp. *laramiense* was most closely related to the *Cl. sporogenes* *gerA* operon. *Cl. sporogenes* germinates in response to L-alanine, L-lactate and NaHCO<sub>3</sub>. However, a link has not been made between the germinants and the *gerA* operon (Broussolle et al. 2002). A second fragment was sequenced from *Cl. estertheticum* subsp. *estertheticum*. This most closely resembles proteolytic *Cl. botulinum* Type 5A H04402 065, spore germination protein *gerKA* (Carter et al. 2011). Other strains of proteolytic *Cl. botulinum* germinate in response to L-alanine, L-lactate and NaHCO<sub>3</sub> (Broussolle et al. 2002). It is not known if the germination response is the same in *Cl. botulinum* Type 5A H04402 065.

Southern blotting results identified sequence in *Cl. frigidicarnis* similar to the presumptive GR sequence from *Cl. estertheticum* subsp. *estertheticum* (AAF2 and AAR2). The same probe produced bands in the *Cl. sporogenes* lane but not the *Cl. algidicarnis* lane despite a PCR producing a band with homology to a GR gene. PCR and Southern blotting is limited as genes identified may or may not be transcribed. Appropriate designation of clostridial GR genes is limited by lack of studies linking genes and proteins with responses to specific germinants. Multiple bands were seen in several of the Southern blotting results. This may be due to incomplete digestion or multiple GR genes being present which is consistent with other spore formers (Moir 2006).

## 7.5 Conclusions

Gene fragments similar to known GR genes were sequenced from *Cl. algidicarnis*, *Cl. estertheticum* subsp. *estertheticum* and *Cl. estertheticum* subsp. *laramiense*. PCR results indicate that *Cl. estertheticum* subsp. *estertheticum* possesses at least two distinct GR genes. Insufficient data, linking the most closely related GR genes to germination in the presence of specific germinants, is available to draw any conclusions about the germination pathway(s) in *Cl. estertheticum*. Southern blotting results support the possible existence of multiple GR genes in *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense* and *Cl. frigidicarnis*.

## Chapter 8

### Discussion

#### 8.1 *Final discussion*

Several strains of psychrotolerant clostridia have been identified as causing spoilage of vacuum-packed, fresh, red meat (beef, lamb and venison). These bacteria are able to cause spoilage at low temperatures and under anaerobic conditions that restrict growth of other spoilage organisms. The spoilage caused is obvious and highly unpleasant resulting in loss of customer confidence as well as financial penalties. Spoilage results from meat becoming contaminated with bacteria, from hides and faeces of animals, during slaughter and processing. Clostridia are strict anaerobes so it was believed that the majority of cells surviving transfer from soil to meat are highly resistant spores. For spores to cause spoilage they must first germinate outgrow and resume a vegetative state. The first aim of this work was to determine the mechanisms of germination of spores of psychrotolerant clostridia. Knowing the triggers for spore germination is an important part of understanding the process of spoilage and determining how to reduce it.

Literature on methods for measuring germination of bacterial spores was reviewed. Several of the methods were tested for their effectiveness for measuring germination of spores of psychrotolerant clostridia. A combination of microscopy and measuring decrease in OD<sub>600</sub> were found to be effective and practical. Using these methods germination in *Cl. estertheticum*, *Cl. frigidicarnis* and LA1 were measured (Chapters 4 and 5). A germinant system consisting of a combination of L-lactate and L-valine or L-norvaline was identified as inducing germination in *Cl. frigidicarnis*. Lactate and valine are both present in fresh meat (Lawrie 1998). Further to this, the conditions under which *Cl. frigidicarnis* germinates were investigated. The salt content of fresh red meat is too low to impact on germination of *Cl. frigidicarnis*. Germination was reduced to below the level of detection at 0 °C indicating that germination and subsequent spoilage by *Cl. frigidicarnis* can be prevented by maintaining meat at the optimal



chilled temperature of -1.5 °C. The pH of meat also plays an important role in spoilage with the lowest pH value at which germination will occur falling within the pH range for fresh red meat. Unlike non-proteolytic *Cl. botulinum*, *Cl. frigidicarnis* only germinates under anaerobic conditions (Plowman and Peck 2002). Sub-lethal heat shock increased the speed of germination especially under sub-optimal conditions. This study highlighted the importance of avoiding temperature spikes during storage of meat as pre-exposure to germinants at temperatures low enough to prevent germination increased the speed of germination.

Locally isolated strain, LA1, germinates in response to L-lactate and L-norvaline or L-cysteine. Nutrient germinant systems consisting of lactate in combination with an amino acid have been identified in other clostridia. Non-proteolytic *Cl. botulinum* germinates in response to L-lactate in combination with L-alanine, L-cysteine or L-serine (Plowman and Peck 2002). Unlike *Cl. frigidicarnis*, LA1 germinated under aerobic conditions. Subtle differences in germinant responses are common. Unlike non-proteolytic *Cl. botulinum*, proteolytic *Cl. botulinum* germinates in response to hydrophobic L-amino acids without L-lactate. Subtle changes to the germinants can also have a big impact on their ability to induce germination, for example alterations to the amine group or the carboxylate group of glycine significantly decreases or stops germination of *Cl. difficile* (Howerton et al. 2011).

No germinant system(s) were identified for *Cl. estertheticum* subsp. *estertheticum*, subsp. *laramiense* or three locally isolated *Cl. estertheticum* like strains. Germination of the *Cl. estertheticum* and *Cl. estertheticum* like strains was very poor in media and beef drip compared to germination in a lamb slurry based meat model. Poor germination in complex media and beef drip, which contains a wide range of amino acids and other potential germinants, suggests that some non germinant condition may play an important role.

Chapter 6 focused on methods for killing or inactivating recently germinated spores. Several methods were investigated, using *in vitro* methods and artificially inoculated lamb steaks, including exposure to air, hot and cold water spray washing and

tyndallisation. As clostridia are strict anaerobes it was thought that exposing germinated spores to air would be an easy and cost effective method of killing and inactivating them. Die off of vegetative cells, on exposure to air, was sufficiently similar to that of germinated spores to warrant using them as models. Reduction of populations of vegetative cells of all seven strains of psychrotolerant clostridia on exposure to aerobic dCMM varied from 2 h to 3 days at 21 °C. Dropping the temperature to 10 °C extended the survival time of *Cl. estertheticum* subsp. *estertheticum* to more than 1 week. Experiments with artificially inoculated lamb steaks were in keeping with the *in vitro* results. No improvement was seen in shelf life of meat packs inoculated with *Cl. estertheticum* subsp. *estertheticum* and exposed to air following 3 days of chilled storage compared with inoculated control packs.

Of the methods tested HWW and CWW, at the time of packaging, resulted in the largest extension of shelf life, of 12 to 13 days, of artificially inoculated lamb steaks. As both the effect of HWW and CWW was the same it is thought that the effect is due to mechanical removal of spores rather than heat. This agrees with a study that found the reduction in numbers of *E. coli* O157:H7, from contaminated faeces, on beef was constant when sprayed with water at 30 and 70 °C (Cutter et al. 1997). Heat shrinking vacuum-packed meat has been shown to shorten the time until spoilage of beef striploin inoculated with spores of *Cl. estertheticum* presumably as a result of heat activating the spores (Bell et al. 2001). In the present study the heat from HWW did not shorten the time until spoilage.

The ability of vegetative psychrotolerant clostridia, especially *Cl. estertheticum* subsp. *estertheticum*, to survive exposure to air for extended periods of time calls into question the belief that spoilage is largely the result of contamination of meat with spores. The survival time of both *Cl. frigidicarnis* and *Cl. estertheticum* subsp. *estertheticum* increased when the temperature was lowered from 21 °C to 10 °C. Soil temperature varies with season, latitude, altitude and exposure to the elements. Conditions in soil parts of new of New Zealand, especially the cooler South Island are likely to be suitable for extended survival of vegetative cells.

Given the difficulties in studying germination of *Cl. estertheticum* spores a molecular approach was applied (Chapter 7). PCR and Southern blotting were employed to screen psychrotolerant clostridia for GR genes. Gene fragments, similar to known GR genes, were sequenced from *Cl. algidicarnis*, *Cl. estertheticum* subsp. *estertheticum* and *Cl. estertheticum* subsp. *laramiense*. PCR results indicate that *Cl. estertheticum* subsp. *estertheticum* possesses at least two distinct GR genes. Insufficient data, linking the most closely related GR genes to germination in the presence of specific germinants, is available to draw any conclusions about the germination pathway(s) in *Cl. estertheticum*. Southern blotting results support the possible existence of multiple GR genes in *Cl. estertheticum* subsp. *estertheticum*, *Cl. estertheticum* subsp. *laramiense* and *Cl. frigidicarnis*.

The work covered by this thesis is part of a larger project aimed at developing a two part method for reducing red meat spoilage by psychrotolerant clostridia. Part one would involve treating the meat to induce any spores on the surface to germinate. Part two would involve treating the germinated spores to kill or inactivate them. Progress has been made with regards to both parts. Germinant systems have been identified in *Cl. frigidicarnis* and LA1 and exposure to air has been ruled out as a means of killing or inactivating germinated spores. Supplemental to this the survival abilities of vegetative cells on exposure to air were determined to be greater than thought and HWW and CWW were found to extend the shelf life of meat inoculated with *Cl. estertheticum* subsp. *estertheticum* spores.

Two part methods for inactivating bacterial spores have been successfully tested in cooked meat products and on hospital laboratory bench tops. Akhtar et al. (2009) used a three part strategy involving, a primary heat treatment of 80 °C for 10 min, to activate spores; cooling of the product to 55 °C in about 20 min and incubation at this temperature for about next 15 min to induce spore germination; followed by pressure-assisted thermal processing (586 MPa at 73 °C for 10 min) to inactivate spores of *Cl. perfringens* in ground chicken. Nerandzic and Donskey (2010) found that spraying hospital laboratory bench tops inoculated with *Cl. difficile* spores with a germinant

solution, containing amino acids, minerals and taurocholic acid reduced the time needed to kill spores using ultraviolet C sterilization.

A two part method for eliminating spores of pathogens, such as *Cl. botulinum* has been ruled out as spore populations contain superdormant spores (Wei et al. 2010). Superdormant spores are a subpopulation of spores that do not germinate in response to conditions that trigger germination in the rest of the population. Due to the seriousness of botulism it is important to eliminate all *Cl. botulinum* spores from food. There is no food safety issue associated with psychrotolerant meat spoilage clostridia and reducing the number of spores in a meat pack extends the time until spoilage (Clemens et al. 2010). While eliminating clostridial spoilage is preferable extending the time until spoilage would be advantageous to the industry.

## 8.2 Recommendations for future work

A number of questions regarding germination of spores of psychrotolerant, red meat clostridia and of scientific and industry importance have been raised during the course of this work.

Germination of *Cl. estertheticum* was too low to be accurately measured in any of the media tested including beef drip. This prevented a specific germinant system(s) from being identified. Germination in a lamb slurry based meat model system was considerably higher suggesting the presence of specific germinants was not the limiting factor for germination. A study of other factors affecting bacterial spore germination such as redox potential, pH and aging may prove fruitful.

Reduced germination was not the only barrier to identifying germinant systems. Several of the strains of interest failed to produce sufficient numbers of spores *in vitro* to carry out germination studies. Whole genome sequencing and screening for germinant receptor and eukaryotic-like Ser/Thr membrane kinase genes would provide useful insight into the potential germinant systems in psychrotolerant clostridia.

An unexpected find was the ability of *Cl. estertheticum* subsp. *estertheticum* to survive in excess of 7 days of exposure, to air, in dCMM, at 10 °C. This raises questions around initial contamination of meat. There is a need to determine the ratio of spoilage caused by vegetative cells as opposed to spores. The ability of vegetative cells to survive in soil, on the hides or in the guts of cattle on their way to meat processing plants and once transferred to meat is unknown.

This study also noted the poor performance of media used to recover spores of psychrotolerant clostridia. The development of media with greater specificity, especially with regards to *Cl. estertheticum* would be helpful for both research and commercial purposes. A *Cl. estertheticum* specific media with good recovery rates would allow meat to be screened post boning as well as during and after storage answering questions around initial contamination and CFU necessary for gas production.

### 8.3 Final conclusion

The main objective of this work was to determine the triggers of germination in psychrophilic and psychrotrophic clostridia associated with spoilage of New Zealand red meat. Initially methods for measuring germination were surveyed and a combination of microscopy and measuring decrease in OD were found to be effective and practical. A germinant system for *Cl. frigidicarnis*, consisting of L-lactate in combination with L-valine or L-norvaline was identified and characterised. Achieving and maintaining chilled storage at -1.5 °C and the pH of the meat were identified as being important in preventing spoilage by *Cl. frigidicarnis*. A germinant system for LA1 consisting of L-lactate and L-norvaline or L-cysteine was identified. Lactate, valine and cysteine are present on the surface of red meat. Conditions necessary for germination varied between psychrotolerant red meat spoilage clostridia. *Cl. frigidicarnis* required anaerobic conditions to germinate whereas LA1 germinated under aerobic conditions. Germination in *Cl. estertheticum*, the most widely reported cause of blown-pack spoilage, was extremely poor in media compared with meat slurry or fresh meat preventing the identification of a specific germinant system(s). Poor germination amongst *Cl. estertheticum*, and several locally isolated *Cl. estertheticum* like strains, in the presence of drip from beef heart, indicates the failure to germinate may not have resulted from the absence of a specific nutrient(s). Two distinct nonchemical interventions, HWW and CWW, were found to reduce spoilage of vacuum-packed, chilled, lamb inoculated with spores of *Cl. estertheticum*. Where problems with blown-packs spoilage caused by *Cl. estertheticum* have been identified, meat processing plants could consider the use of HWW or CWW in conjunction with the use of best practices, such as preventing roll back while making the opening cut. Survival of vegetative cells of psychrotolerant clostridia was found to be much better than expected, especially in *Cl. estertheticum* subsp. *estertheticum*, indicating that vegetative cells may play a larger role in initial contamination than previously assumed.



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## Appendix 1

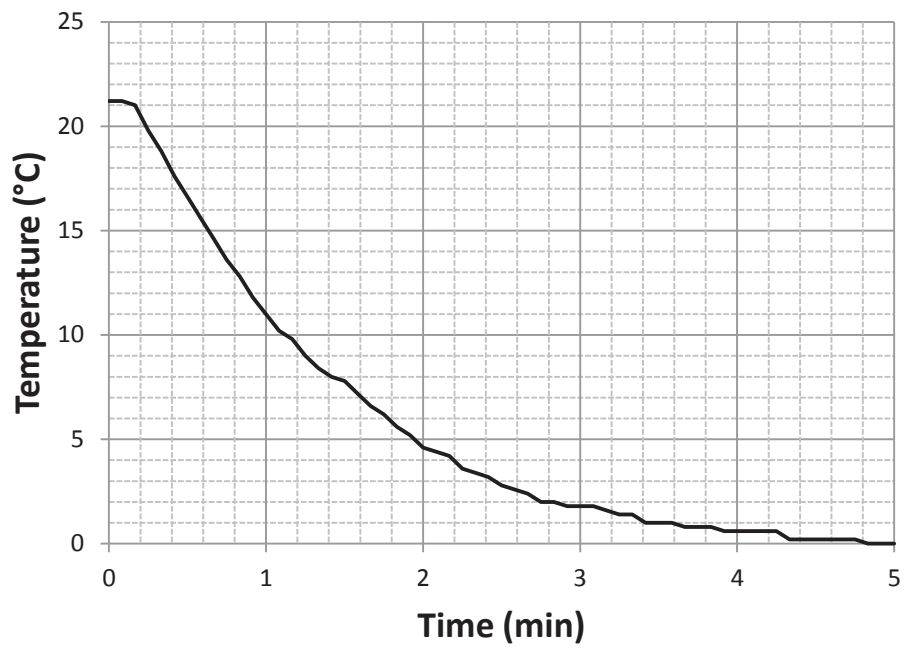
Settings for gates on BactiFlow flow cytometer for analysis of spores and vegetative cells of *Cl. frigidicarnis*.

| Cell type  | D3:FL1  |         | D4:FL2  |         | FL2/FL1 |         |
|------------|---------|---------|---------|---------|---------|---------|
|            | minimum | maximum | minimum | maximum | minimum | maximum |
| Vegetative | 500     | 900     | 350     | 950     | 1.0     | 3.0     |
| Spore      | 0.5     | 800     | 0.5     | 900     | 5.0     | 30      |

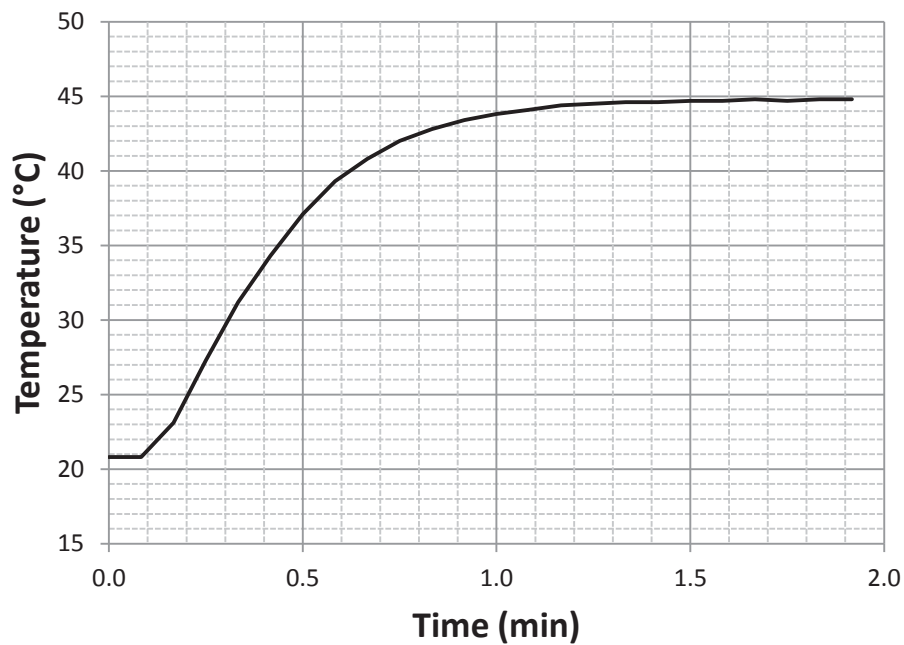
Settings for gates on flow cytometer for analysis of spores and vegetative cells of *Cl. estertheticum* subsp. *estertheticum*.

| Cell type  | D3:FL1  |         | D4:FL2  |         | FL2/FL1 |         |
|------------|---------|---------|---------|---------|---------|---------|
|            | minimum | maximum | minimum | maximum | minimum | maximum |
| Vegetative | 200     | 800     | 0.5     | 800     | 1       | 3       |
| Spore      | 0.5     | 800     | 0.5     | 900     | 3       | 25      |

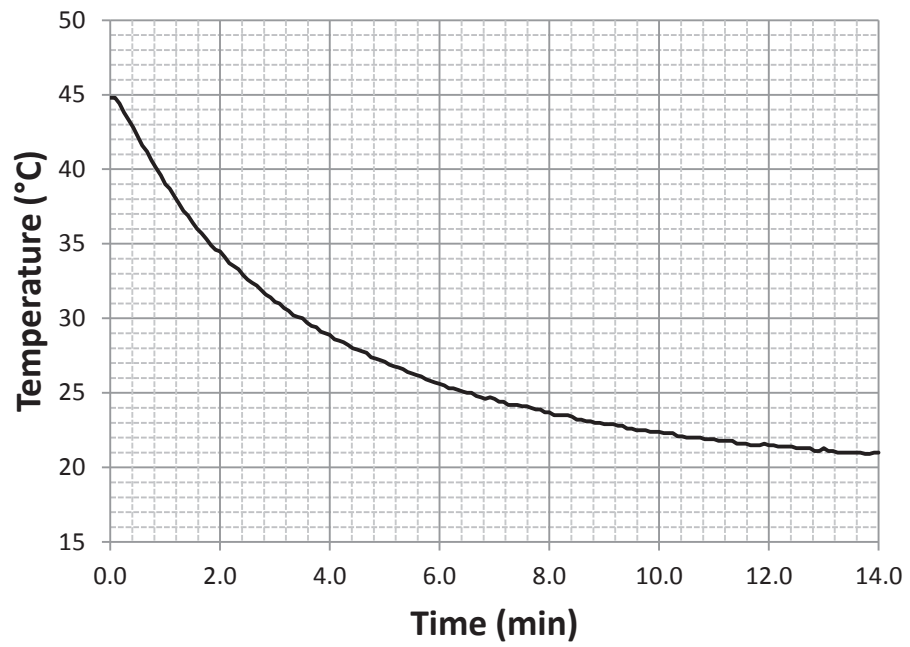
## Appendix 2



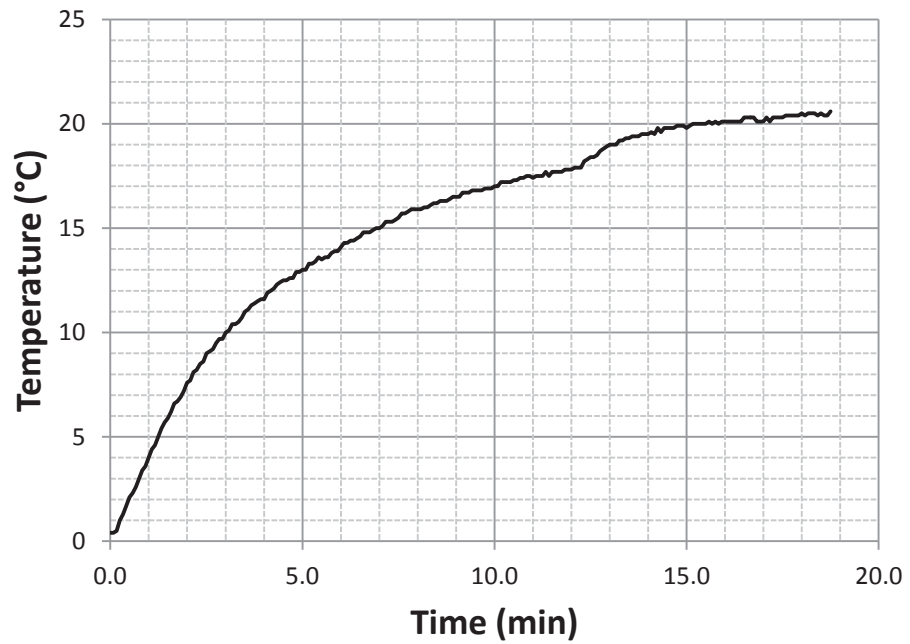
Cooling curve of a glass microscope slide from 21 to 0 °C in water bath (Section 4.3.6).



Heating curve of a glass microscope slide from 0 to 45 °C in water bath (Section 4.3.6).



Cooling curve of a glass microscope slide from 45 to 21 °C in air (Section 4.3.6).

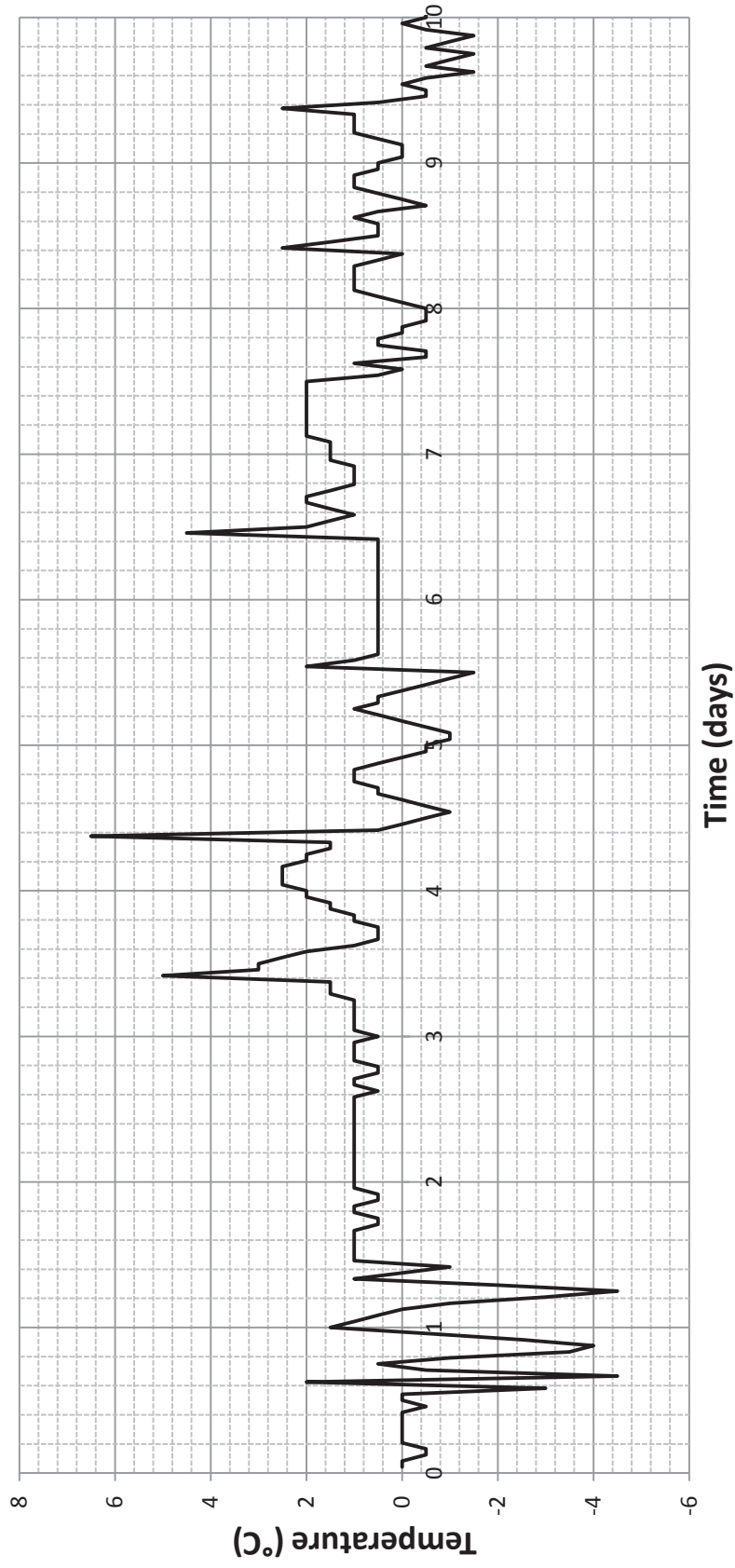


Heating curve of a glass microscope slide from 0 to 21 °C in air (Section 4.3.6).



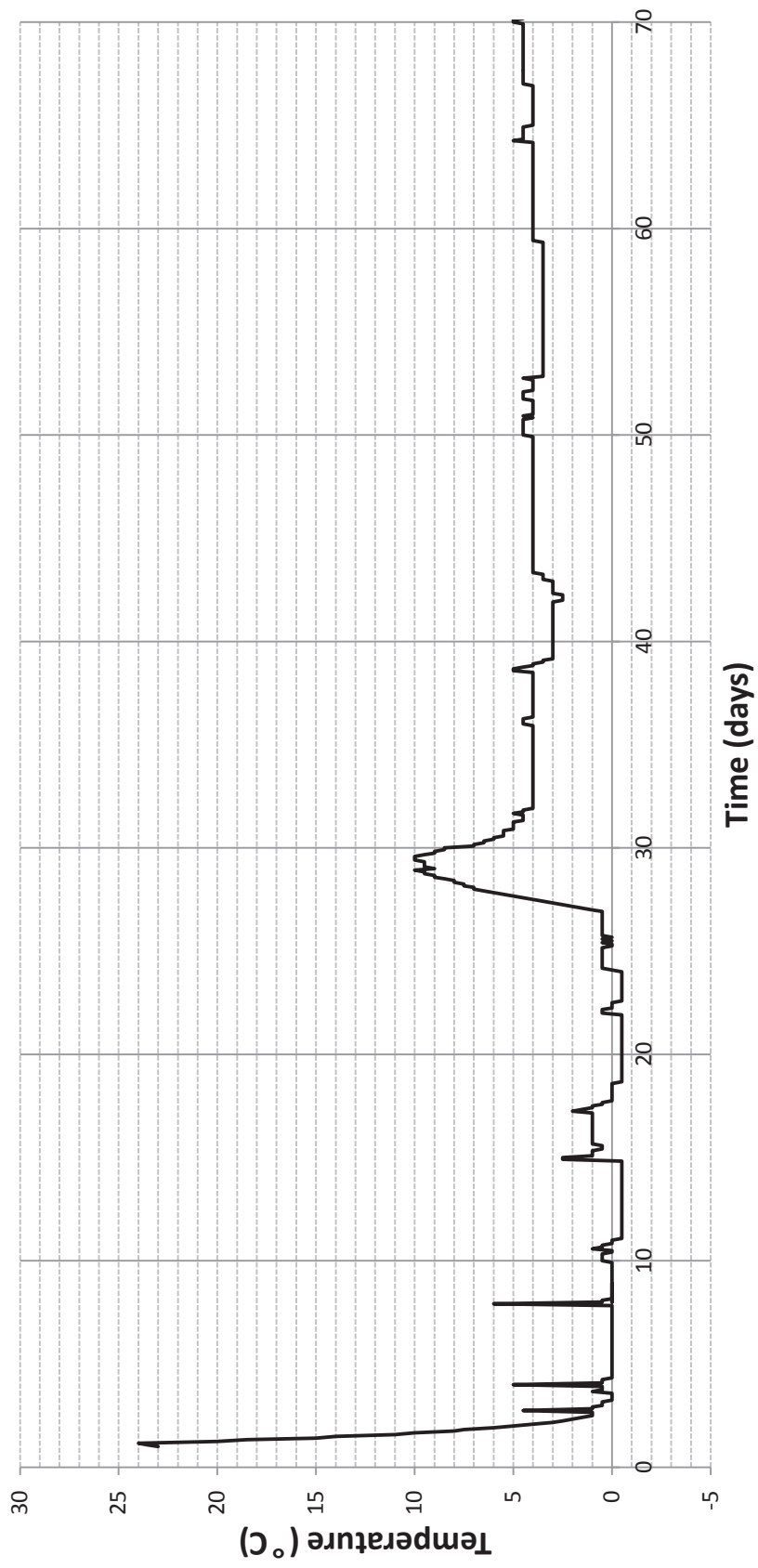
## Appendix 3

Temperature log from display portion of experiment exposing lamb to oxygen following chilled storage at  $-1.5^{\circ}\text{C}$  or  $2^{\circ}\text{C}$  (Section 6.3.2).



## Appendix 4

Temperature log, from storage trail comparing HWW and CWW treated meat, under poor storage conditions with a simulated failure (Section 6.6.3).



## Appendix 5

Statistical analysis of results of results shown in **Figure 6.10.** and **Table 6.3.**

### Analysis of variance

Variate: day at which blowness level equals or exceeds 2

| Source of variation | d.f. | (m.v.) | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------|--------|---------|--------|------|-------|
| ntrt                | 6    |        | 884.97  | 147.49 | 5.37 | <.001 |
| Residual            | 27   | (1)    | 741.95  | 27.48  |      |       |
| Total               | 33   | (1)    | 1573.76 |        |      |       |

*Message: the following units have large residuals.*

|            |                     |      |
|------------|---------------------|------|
| *units* 20 | -10.25 approx. s.e. | 4.60 |
| *units* 35 | 15.60 approx. s.e.  | 4.60 |

### Tables of means

Variate: day at which blowness level equals or exceeds 2

Grand mean 45.44

|                   |            |             |       |       |
|-------------------|------------|-------------|-------|-------|
| ntrt Uninoculated | inoculated | CWW         | HWW   | O2    |
|                   | 39.00      | 51.60       | 51.20 | 38.25 |
| ntrt 3d HWW       | Tynd       | Tynd 3d HWW |       |       |
| 47.40             | 43.20      | 47.40       |       |       |

### Standard errors of differences of means

|        |       |
|--------|-------|
| Table  | ntrt  |
| rep.   | 5     |
| d.f.   | 27    |
| s.e.d. | 3.315 |

(Not adjusted for missing values)

### Least significant differences of means (5% level)

|        |       |
|--------|-------|
| Table  | ntrt  |
| rep.   | 5     |
| d.f.   | 27    |
| l.s.d. | 6.803 |

(Not adjusted for missing values)



```

B_thuringiensis TLTMLKIIKVPFIQRFEYIIIFVWFLIILPNLCLTIWSSCQTMKRSFRIS
B_mycoides TLTMLKIIKVPFIQRFEYIIIFVWFLIILPNLCLTIWSSCRISKYSFHIP
B_pseudomycoides TLTMLKIIKVPFIQRFEYIIIFVWFLIILPNLCVTIWSSCQSMKRSFHIP
*****:*****:*****: * **:.

B_cereus FKFTLPFFIFIVFTASLFFKNRESINTLNTVLSQAGLYIVYAYIPILFLF
B_thuringiensis FKFTLPFFIFIVFTASLFFKNRESINTLNTVLSQAGLYIVYAYIPILFV
B_mycoides FNITLPLFIATIFVSSLFFTNRESINTLNTVLSQAGLYIVYAYIPILFLF
B_pseudomycoides FKFTLPFFIFVYIASLFIINREHVNTLNTLLSRAGLYIVYVYIPLLFLW
*::***:** : :***: *** :*****:**:*****.***:**::

B_cereus HSVRWHFKNRSKSSPDTP
B_thuringiensis HSLRWHFKNRSKSSPDTP
B_mycoides HSLRWRFKNQSKSSPDTP
B_pseudomycoides HSIRWRLKNKQNKTS---P
**:**:***:..*: *

```

## 6.2

Alignment of *B. cereus* m1293, *B. mycoides* DSM 2048, *B. pseudomycoides* DSM 12442 and *B. thuringiensis* GR amino acid sequences used to design GERBK/GERBR primers  
Section 7.2.1.

```

B_pseudomycoides ctatg-----gtgaagttttattttgtttatttttcaatcgccagc
B_mycoides ctatg-----gtgaagttttattttgtttatttttcaatcgccagc
B_thuringiensis ttatgggtgtgtcgggtgaagattttttgaccgatttttgaaatgccatc
B_cereus ttacgggtgtgtcgggtgaagattttttgaccgatttttgaaatgccatc
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

B_pseudomycoides gaatggaatgccatagaaaaaggagaggatataaacgtatacgtatac
B_mycoides gaatggaatgccatagaaaaaggagaggatataaacgtatacgtatac
B_thuringiensis gtagtgaatggaatacaataaaatcgggatataagcgtatacaatatat
B_cereus gaactgaatggaatagaaataaaattgggatataagcgtatacaatgtat
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

B_pseudomycoides agaccagcccgggataagagcgtgttcaatgtgttaacatgttctctatt
B_mycoides agaccagcccgggataagagcgtgttcaatgtgttaacatgttctctatt
B_thuringiensis aagccagcttgagataggactgtgttaatgtgttgatactttcacggtt
B_cereus aaaccagcttgagatagactgtatttaatgtgttgatactttcacggtt
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

B_pseudomycoides tataataaataaagacgcgatatacacaacgaaaatgaaaaaggcaatg
B_mycoides tataataaataaagacgcgatatacacaacgaaaatgaaaaaggcaatg
B_thuringiensis tttaaagaataaagaagctgtaaaaacgatgaaaatgaaaaacggtaatg
B_cereus tttaaaaataaagaagctgtaaaaacgatgaaaataaaaaacggtaatg
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

B_pseudomycoides taaacttaaacggaatatggaacgatcgtttcatggattggcaagaagac
B_mycoides taaacttaaacggaatatggaacgatcgtttcatggattggcaagaagac
B_thuringiensis taaatttaaatgaaatacgaatatgaacgcttcatcgtttggcatgatgac
B_cereus taaatttaaatgaaatatgaaatgaacgcttcatcgtttggcatgatgac
**** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

B_pseudomycoides caaattgttacgcaaagattaggtaaaatgatgagaaccaacaaaaat

```

```

B_mycoides      caaattggttacgcaaagattaggtaaaatgatgagaaaccaaacaaaaat
B_thuringiensis caaatagttaacaaagggttggcaaaataatcagaaaccaaacaaaaat
B_cereus        caaatggttaacaaagggttggcaaaataattaaaaaccaaacaaaaat
*****
          →
B_pseudomycoides aatgatgtactcaaatcgttgaatgaaaggaac ttttaataattttaagca
B_mycoides      aatgatgtactcaaatcgttgaatgaaaggaac ttttaataattttaagca
B_thuringiensis aataatatattcaaacggttgaatgaaaggaac ttttaattatttttaaca
B_cereus        aataatatattcaaacggttgaatgaaaggaac ttttaattatttttaaca
*** ** *
B_pseudomycoides ttgттаaggттgcccaagттgtacgctgcaaaagtccttcactaaagtac
B_mycoides      ttgттаaggттgcccaagттgtacgctgcaaaagtccttcactaaagtac
B_thuringiensis tcgттаaggттaggccaaatagtatgatttaattgtccttcactataatac
B_cereus        tcgттаaggттaggccaaatagtatgatttaattgtccttcactataatac
* ***** ** * ***** * ** * * * ***** * **
B_pseudomycoides atgaaagaaatgagtgctaagataacgtaaataatggttgttaagcaat
B_mycoides      atgaaagaaatgagtgctaagataacgtaaataatggttgttaagcaat
B_thuringiensis ataaaagatacaatagctaaaactacataaatcaaagtgctaaaaaaaat
B_cereus        ataaaagatacaatagctaaaactacataaatcaaagtgctaaaaaaaat
** ***** * * ***** * ** ***** * ** * *** ***
B_pseudomycoides tccaccgtgtgcccatcttttagagattttcctttttcaatgagtgggт
B_mycoides      tccaccgtgtgcccatcttttagagattttcctttttcaatgagtgggт
B_thuringiensis tcctgcatgagcccatcttttaagacttacctttttcaataaatggat
B_cereus        tcctgcatgagcccatcttttaagacttacctttttcaataaatggat
*** * ** ***** ***** ** * ***** * ** *
B_pseudomycoides atacaaatagtaatgcttcaaataccgagaaattctaataatagaatctttt
B_mycoides      atacaaatagtaatgcttcaaataccgagaaattctaataatagaatctttt
B_thuringiensis agaagataaggattgtttcaaagccgagaaactctaataatgagattgttt
B_cereus        agaagataaggattgtttcaaagccgagaaactctaataatgagattgcttt
* * * ** * ** ***** ***** ***** ** * ***
          ←
B_pseudomycoides gctgattgaaaaatttgtaaa ggtgaatgcgtcaaaataggtaaaat att
B_mycoides      gctgattgaaaaatttgtaaa ggtgaatgcgtcaaaataggtaaaat att
B_thuringiensis gcagatataagcatgtcaaaa ggtgaatgtgtaaaaaataggtaaaat att
B_cereus        gcagatataagcatgtcaaaa ggtgaatgtgtaaaaaataggtaaaat att
** *** ** ** * ***** ** ***** *****
B_pseudomycoides acggaaatgagcatatttcattgggaaaattaaaaaaagattacaaaaa
B_mycoides      acggaaatgagcatatttcattgggaaaattaaaaaaagattacaaaaa
B_thuringiensis acggaaatgagcgtatttcattggatagactaaaaagaataacaacgaaaa
B_cereus        acggaaatgagcgtatttcattggataaattaaaaagaataacaacgaaaa
***** ***** * * ***** ** * ** *****
B_pseudomycoides aaggaatgacgatcccccaaaagcagataaccggttacagagcgaaatcca
B_mycoides      aaggaatgacgatcccccaaaagcagataaccggttacagagcgaaatcca
B_thuringiensis taggaagtacaatacccccaaaacatatccctgtcacagaacggaagcca
B_cereus        taggaagaacgactcccccaaaacatatcccggttacagagcgaaatcca
***** ** * ***** ** ** ** ***** ** ** **
B_pseudomycoides ccttttataacataaaaaacaatgaataaaaagagtacattcaacttcca
B_mycoides      ccttttataacataaaaaacaatgaataaaaagagtacattcaacttcca
B_thuringiensis ccttttataatgtagtaagtgactaataaaaacataagggtaagtttcca
B_cereus        ccctttataatataagcaagtcactaataaaaacataaagggttagtttcca
** ***** ** ** * ***** * * * ** *****
B_pseudomycoides cgatttaattgtgggaaatacccaaacttgaatgacttcaatatatgттt

```

```

B_mycoides      cgatttaattgtgggaaatacccaaacttgaatgacttcaatatatgttt
B_thuringiensis taacttaatggtaggggaagatccaaacttgtatcacttccatatatgtac
B_cereus        taatttaatggtagggaaaatccaaacttgtatcacttccatatatgtac
                * ***** ** ** * ***** ** ***** *****

B_pseudomycoides gcagaatggtaaggcaaaataaaagaaaataagcagtaaagcatagagaa
B_mycoides      gcagaatggtaaggcaaaataaaagaaaataagcagtaaagcatagagaa
B_thuringiensis gaagaacagtaaggcaaaataataagcaataaagagtgaaaatgacagaa
B_cereus        gaagaacagtaaggcaaaataataagcaataaagagtgaaaatcacggaa
                * **** ***** * ***** ** ** * ***

B_pseudomycoides aagaaagatccgatccactttccaaagcatggtgtatgtatattgattaa
B_mycoides      aagaaagatccgatccactttccaaagcatggtgtatgtatattgattaa
B_thuringiensis aatatagagccaaaccattttccaaaacttgtgtatgaatgctaattaa
B_cereus        aatatagaaccaatccattttccaaaagttgtgtatgaatgctaattaa
                ** * *** ** * *** ***** ***** ** * *****

B_pseudomycoides gtcgttatctttctctagcatcgctaataatacaaaaataaaacaatgtgag
B_mycoides      gtcgttatctttctctagcatcgctaataatacaaaaataaaacaatgtgag
B_thuringiensis atctccatctttttctaactttttaacatacaaaatagcacaatatggg
B_cereus        atctccgtctttttctaactttttaacatacaaaacagcacaatatggg
                ** ***** ** ** * ***** * ***** ** *

B_pseudomycoides ttgcaaatcccataaggataagggaaatccaagcgtcatatccagcgctt
B_mycoides      ttgcaaatcccataaggataagggaaatccaagcgtcatatccagcgctt
B_thuringiensis ttgcaatccctgcaataataagagaaatccatgcatcataaccagcgctt
B_cereus        ttgcaatccccgcaataataagagaaatccagcgtcatataaccagcgctt
                ***** ** ** ***** ***** ** ***** **

B_pseudomycoides ttaatcaggatacgttgaaatcctaaaattcctactccgatttgaagaga
B_mycoides      ttaatcaggatacgttgaaatccta-----
B_thuringiensis tttaaaattacacgttgatacccaagaactcctattccaatttgaagtga
B_cereus        tttaaaattacacgttgatacccaagaactcctattccaatttgaagcga
                ** * * * ***** * ** *

B_pseudomycoides atgaactaaaagaaagcgaagtaaggagatactgttagttctttgctgc
B_mycoides      -----a-----
B_thuringiensis atgtaataatataaaggcaaagtatggagagactgtctttgatttattca
B_cereus        atgtaataatataaaggcaaagtatggagagactgtctttgctttattca
                *

B_pseudomycoides gggatttcat
B_mycoides      -----aat
B_thuringiensis ttgtattcat
B_cereus        ttgtattcat
                **

```

### 6.3

Alignment of *B. subtilis* (*gerK*), *Cl. perfringens* SM101 and *Cl. acetobutylicum* ATCC 824 (*gerKA*) protein sequences used to design GERKF/GERKR primers Section 7.2.1.

```

B_subtilis      MCMRLFskRKNNTDSKDKQNTDERNQEQQEKERPVLISPSLAKNIAETK
Cl_acetobutylicum MFNKIFNKEEASKNNA-----KTVSTSLKTNKHTIE
Cl_perfringens  MEKQL-----DFLK
                *  ::                               :

```

```

B_subtilis      KEVGSSSDVIIREIKIGEQDHVHLAVIYISGLVDNNTIHESLIDPLVQDE
Cl_acetobutylicum TLFTNCSDLTIREFKIANNRNFSAMIVYINNLIQSDVIESSVIKKLIEED
Cl_perfringens  DKLKDSFDVKYREVDTALG---PATIVFMDVLCSTQFISEYIVKPLTLVK
                . . . *: **.. .          :... * ... * . :.. * .

B_subtilis      SIQNTHA-IQEILRKTAPLGGVKAEKSWDKLFSLEMLGNALIFADGHDEA
Cl_acetobutylicum YNNHPLNIFEYTKSIFAVNDKNVYKSMDDVILSILKGSIALFLDGESKA
Cl_perfringens  DGVKDEND---IMTKVIDINITNWSKDKNDTLLHVLSGDVVVIIFDKFDKV
                : . . . . : * . : : : * . : : * . : .

B_subtilis      LICSTQGGEQRSIQEPSTQVSFRGPRQGFTEESLQTNISMIRRYIKNPNLW
Cl_acetobutylicum IISIGKPPERAITTEPTVETVIRGPREGFTESLTTNVALLRKKIRSTDFK
Cl_perfringens  IYCETKGYTRRGVGPITEAVIKGPREGFNEAFVDNVTLRRRIKHNHLK
                : . . . . * . : . : : * * : * : * : * : * : . :

B_subtilis      VGMKEKGSVTNTDIALMYIQGICDEKVLKEVKQRLEKIDIDSILESGYIE
Cl_acetobutylicum VEELTLGENSKTTVAITYLANVANPKIVEEIRRRIKGITIDSIIIGMNYIK
Cl_perfringens  FEPLYVGEDTQTVVVCISYIKNKAPKELIDEIREKIKNLDYKFIILDTNYIE
                . : * . : * : . : . : : * : : : : . * : . * :

                →
B_subtilis      QLIEDETFFTTFFPTMYHTERPDVVAGNLLLEGRFAIIVDGTPFVLIAPALFV
Cl_acetobutylicum EYIQDEAFSAFPTVFSTERPDVVAGKIFEGKIAILADGTPIVCTAPAIFF
Cl_perfringens  AKLRED-RTLFDTVGYTEKADEVAAKLLEGRVAIIVDGTPFVLTVPFFFI
                : : : : : * * : * * : * * : * * : * * : * * : * * : * * :

                ←
B_subtilis      QFFQSVEDYYSRFDIATSIRILRVLVFFISLVAFAVYVAATTFHQEMIPT
Cl_acetobutylicum EFLETNEDFFINFIPATINRWIRYTSLLISILLPGLYVAITTFHQELIQT
Cl_perfringens  ENFQTPDDYYLNRIFTSFTRILRWIAFFIAMFLPGIYVALVTHHFSVLPS
                : : : : * : : . : : * * : * : * : * : * : * : * : :

B_subtilis      QLLVVIAAQREIVPFPAPVVEALTMEVAFKILREAGVRLPRVVGSAVSIVG
Cl_acetobutylicum PLLITFIQTHASVPYPTSTETFFLLVAYQIMIEAGIRMPRAVGQSISIIG
Cl_perfringens  LFFVRLAVARAGVPFTTVAEVIIMMLLFQIIKEAGRLRPQPIGTSMSLVS
                : . : : : * * : . * . : : : : * : * * : * : * : . :

B_subtilis      ALVIGQAAVQAGIVSPAMVIIVALTAIASFATPAFAMAI SARLIRFIFII
Cl_acetobutylicum ALIIGQSAVEAGLISTPVIIVVAITYIASFAVPVNEMHNALTLPRFIFMV
Cl_perfringens  GLILGEAAVGAGIASRITIVVVALSAVCYFLIPKLYG--AVSIWSIGIVI
                . * : * : * * : * : * : * : : . * * : : : : :

B_subtilis      ASAVMGFYGLIILGIIMMFVHLCSLRSFGVPYMSPLAPFSSQGVKDALFRV
Cl_acetobutylicum LGTTLGLFGLTCGYLVLSLKLISIRSFVGPYATPIAPTSKENLLDIVIRK
Cl_perfringens  LAALFGIPGVILISVVLSSHHLAHLRSCEYQYLFPLGTLRSRYKFKDIIIRG
                . : : * : * : : : * : * * * * : . * : * :

B_subtilis      PWWADEKRPEHSVSKEDKVRQGDQRPEPAASRGMVN---KDL---EEGDQ
Cl_acetobutylicum PLWAKLKRSPKVSDKNSTK-----RQTRGHLNLAIKDIRKAIDKIK
Cl_perfringens  -----RLEEISKDIV---GKDGKNEP-----KE
                * . : * . . :

B_subtilis      NGT
Cl_acetobutylicum KSH
Cl_perfringens  SYS
                .

```

## 6.4



Alignment of *B. subtilis* (*gerK*), *Cl. perfringens* SM101 and *Cl. acetobutylicum* ATCC

824 (*gerKA*) amino acid sequences used to design GERKF/GERKR primers Section 7.2.1.

```

B_subtilis      cttcgaaaaacagctccgctgggcggggtga--aagcggaaaagagctg-
Cl_acetobutylicum --acaaaatcaatTTTTGCTGTTaatgataaaaaatgtatataaaagcat-
Cl_perfringens  -tatgactaaagttatagata-taaacataacaaattggtca-aaggata
                *          * *          * * *          * **

B_subtilis      -ggacaag-ctt--TTTTCCGagTTaatgcttggcaatgcgctcattttt
Cl_acetobutylicum -ggatgatgtttatattgtccat---tttaaaaggaagtatagcattattc
Cl_perfringens  aaaacgatacatta--cttcat-gtattatcaggggatgtagtaataatt
                * *          * *          * ** *          * *

B_subtilis      gctgatggccatgatgaagctttg-atttg-cagcactcaagggggag-a
Cl_acetobutylicum ctagatggtgaaagtaaagcaatt-attat-aagtattggaaaaccccct
Cl_perfringens  tttgataagtttgataaggaatTTATTGCGaaacaaagggTTataca-a
                ***          * * *          * ***          * *

B_subtilis      g-cagcgcctccatccaagagccgagcaccagg-tgtcattccgcgggcc
Cl_acetobutylicum gaaagagct--ataacagaaccaactgTTgaaactgtaa-taagaggTCC
Cl_perfringens  g-aagaggt--gTTggattccaataacggaagcagtaa-taaagggacc
                * ** * *          * ** *          * ** * *          ** **

B_subtilis      gcgccaagggTTTACagagTctctgcaaaccaatatttccatgatccggc
Cl_acetobutylicum tagagagggcttCactgaatctTTaaccacaaatgtagccTTTTaagaa
Cl_perfringens  tagagaaggTTTTaatgaggctTTTgTTgataatgTTacTTattaagaa
                * * ** * *          * ** * *          *** *          * * *

B_subtilis      gatacattaaaaatccgaatTTatgggTTgaaaaatgg-aaaaaggttc
Cl_acetobutylicum aaaaaattagaagcactgattTTaaagtggaagaacttac-tttaggtga
Cl_perfringens  gaaggataaaaaatcataatcTaaattcgag-cTTTatattgtagggga
                * ** * **          ** *          * *          *          ***

B_subtilis      tgttacgaatacggatattcgtctcatgtacattcaaggaatat--gtga
Cl_acetobutylicum gaattcaaagacaactgtagctattaccta--tttagctaattgtagctaa
Cl_perfringens  agatactcaaactgtagtatgtatatctta--tataaaaaataaagctcc
                * * * **          * * *          ** * *          ***          *

B_subtilis      tgaaaaggtgctgaaggaagtgaaacagcgcctggaaaaaatagatattg
Cl_acetobutylicum ccctaaaatagtagaggagattagaagacgaataggaaaaataacgatag
Cl_perfringens  aaaggaattaattgatgaatcagagaaaaataaaaaatttagattata
                * * *          * **          * * *          * ** **

B_subtilis      acagtattttggaatctggatatattgagc---agctgattgaagatgaa
Cl_acetobutylicum actccataattggtatgaattacataaagg---aatacacaggatgaa
Cl_perfringens  agtttatattagataactaattatattgaggctaaacttagagaagataga
                * ** * *          ** ** **          *          * * ** *

B_subtilis      accttacgacgtttccgactatgtatcatacagaacgcctgatgttgt
Cl_acetobutylicum gctTTTTctgcctttccaactgtgttctcaacagaaaggcctgatgttgt
Cl_perfringens  acttta-----tttgatactgTTgataacagaaaaagcagatgaggT
                * **          *** ** *          ** ** **          * ** **

B_subtilis      agcgggtaatctgTTagaaggaagattcgcgattatTGTGGACGGAACAC
Cl_acetobutylicum tgccggaagattTTTTgaaggaaaaatagctatactAGCTGATGGTACAC
Cl_perfringens  agcagcaaaactattagaaggaagggTTgcaataatAGTAGATGGAACTC
                ** *          * ** ** **          * ** *          * * ** ** *
    
```

```

B_subtilis      cgtttggtgcttattgccccagccttatttgtccagttttttcagtctgtt
Cl_acetobutylicum ctattgtatgcactgctcctgcaatattttttgaatttcttgagacaaat
Cl_perfringens  cttttgttctttacagtaccttttcttttttatagaaaattttcaaacacca
*   ****   * * **   * *** * * * * * * *

B_subtilis      gaggattattattcgcgttttgacattgcgacaagcataagaattcttcg
Cl_acetobutylicum gaagattttttataaattttattcctgcaaccattaatcgctggattag
Cl_perfringens  gatgattattatttaaataggtattttactagttttactagaatttttaag
**  ****  ** *   *   * * *   * *   * *

B_subtilis      tgttttagtctttttcatttcgcttggtggcaccagctgtatacgtggcag
Cl_acetobutylicum atatacatcgttacttatatccatacttttaccaggcttatatgttgcta
Cl_perfringens  atggatagcattttttattgctatgtttttaccaggaatataatgttgcat
*   ** * ** * * * * *****  **** ** **

←

B_subtilis      cca cgacgtttcatcaagaaatgataccgactc-agctgttagt--ggtc
Cl_acetobutylicum taa ccactttttcatcaagaattaatacaaacgccattactcataactttt
Cl_perfringens  tag ttactcatcatttttcggttttgctagttttttgtttttaggta
* *   ****   * * * * * * * *

B_subtilis      attgcgggccagcgggaaattgtcccgtttccggctggtgtggaggcgct
Cl_acetobutylicum attcaaactca---cgcatctgtgccttaccctacttctacagagacctt
Cl_perfringens  gcagttgctag---agctggagttcctttttacaatagtagcagaggtgat
*   *   * ** ** * *   *** *

B_subtilis      gacgatggaggttgcttttaagattctaaggggaagcgggagtcaggctgc
Cl_acetobutylicum tttccttttagttgcatatcagataatgatagaggctggtattagaatgc
Cl_perfringens  aataatgatgttattattccaaataataaaagaagcaggtctaagattac
*   *   * * ** * * ** ** ** * ** *

B_subtilis      cccgggttgctcggctcggcagtgcaatcgtagggcgcgctcgtaatcggc
Cl_acetobutylicum cacgggctgtagggcaatccataagcatcattggagcactaattataggg
Cl_perfringens  ctcagcctataggaacatctatgagtttagtatcgggacttatttttagga
* * * * * ** * *   * *   * ** * **

B_subtilis      caggctgccgtacaggtcgttatcgtctctccggcaatgggtgattatcgt
Cl_acetobutylicum caatcagccgtagaagctggtttaataagtaaccccgtaattattgtcgt
Cl_perfringens  gaggctgctgtgggagctggaattg---c---ttcaagaactactattgt
* * ** **  **** *   *   * * * * **

B_subtilis      ggcactcaccgcgattgcaagcttcgcaacacc-----ggcgtttgc
Cl_acetobutylicum tgcaattacttatattgcctcttttgctgttcc-----agtcaatga
Cl_perfringens  agtagttgctttaagtgccg--tttgttatttcttgattcctaagcttta
* * * * * ** ** *   *   *

B_subtilis      aatggcgatttcagccccggcttattc--ggtttatctttattattgcctc
Cl_acetobutylicum aatgcaca--acgccttaactttgccaagatttatattcatggttttagg
Cl_perfringens  tgggtgcagtatcgatttgagtagatag--gaatagtaatttttagctgcctt
*   *   * * * * * * *

B_subtilis      agctggtatgggtttttacgggctgattttagggatcattatgatgt--t
Cl_acetobutylicum aactactctaggtctatttggtttaacctgc--ggttatcttgttctatc
Cl_perfringens  att-----tg-gaattcctggtgtgattttaataagtggttgattat--t
*   *   * * * ** * * *   * * * *

B_subtilis      tgtccacttatgcagctcttcgttcattc--ggcgttccatatatgtcgc
Cl_acetobutylicum attaaaattaatttcaatccgctctttt--ggagttccctacgcaacacc
Cl_perfringens  atctcactta-g-----cacatttgagaagttgcaaatat--caa-
*   ***   * ** * *** * * *

B_subtilis      gcttgacactttttcttcacaaggcgtaaaggacgcgctttttccgggttc

```

```

Cl_acetobutylicum  tatagctcctacttctaaagaaaaccttcttgacattggttattaggaac
Cl_perfringens    -----tacttatt-----tccattaggaact
                   * * *                               * **

B_subtilis        cgtggtgg--gccgatgaaaaaaggccggaatcggtcag--caaggaaga
Cl_acetobutylicum ctttgtgg--gcaaagctaaagcgttctccaaggtttctgataagaac-
Cl_perfringens    ttaagtagatacaaatttaagatat-----tattataagagg-
                   ** * * * * ***                               * **

B_subtilis        taaggtgcggcaggggaaagatcaacgccagagccggcgccttcacgag
Cl_acetobutylicum -----tcaaccaaagacagactagaggccatctt-aaccttgccataaa
Cl_perfringens    -----aagattagaagaaatatcaaaagatatagt-ag----g---t-aa
                   * ** * * * * *                               *

B_subtilis        gaatggtgaacaaagatttggagaaggggatcagaatggtacgtaa
Cl_acetobutylicum agatattaga-aaggctattgataaaattaa--aaaat-ctcactaa
Cl_perfringens    ggatggtaaa-aatgaacctaagaaagt-----t-atagctag
                   ** * * ** * * * * * * * * * * * *

```

## 6.5

Alignment of *Cl. sporogenes* spore germination protein (*gerAA* and *gerAB*), *Cl. botulinum* F str. 230613, *Cl. botulinum* A3 str. Loch Maree, and *Cl. kluveri* NBRC 12016 protein sequences used to design EGER5 primer Section 7.2.1.

```

Cl.sporogenes      MENKDLNALQDKINSIKTLVGEKNNLI IKDFIVCKKSNI EAALLYINGLA
Cl_botulinumF      MKNKNLNSLQDKINSIKTLVGEKNDLI I KEFIICKTSNME AALIYINGLV
Cl_botulinumA3     MKNKDLNSLQDKINSIKTLVGEKNDLI I KELIICKTSNME AALIYINGLV
Cl_kluveri         ME-----LDNKINSILESCGNSQQVIVRRFSIGKDTPLDAALIYKDGLV
*:                 *:***** *:*****: : : * : :*****:***.

Cl.sporogenes      NSETIDRDILKPLMFYIEEDLSSTNNLDEILCKKYISLSNTSLEQDINKA
Cl_botulinumF      NNQTIDRDILKPLMFNIEEDLSTVHNLEEYLCCKYISLSNTSLEENINNV
Cl_botulinumA3     NNQTIDRDILKPLMFNIEEDLSVVDHLEEYLCCKYISLSNTYLEEDINNV
Cl_kluveri         NQDIINRDILNPLMHKVNEDLSKAQ SINQEICNKYISMSKIVIQT DINMA
*.: *:*:*:*:*:* .:***** .:*****: :*:*****:*: : : ** .

Cl.sporogenes      VFHLKRGKSVLIVKGGTKFIILD TAGGEYRDISDPINETSIRASREGFVE
Cl_botulinumF      VFHLKRGKTILIVPEKSKFIILD TTGGSYRNISDPVNETSVRSSREGFIE
Cl_botulinumA3     VFHLKRGKTILII PGKSKFIILD TTGGSYRNISDPVNETSVRSSREGFIE
Cl_kluveri         IKDIRRGKTAVLVHKEDTFIVAD TQGTKHRNISEPMDEIGIRGSKEGFIE
: .:***: : : .** * * .:***:*:* * :*:*:*:*:*:

Cl.sporogenes      KLETNVSILRRRIKDN NLVQENLVIGRRNQT DVALMYIDNIADKNIVEDI
Cl_botulinumF      KLETNISLLRRRIRDN NLVEEKLVI GRRSQT DVVLMYIDNIADKNIVADI
Cl_botulinumA3     NLETNISLLRRRIRDN NLVEEKLVI GRRSQT DVLLMYIDNIADKNIVEDI
Cl_kluveri         DIEINLSLIKGLKDKNLI IEPFIIGRRSQKDVALIYIKDIADKDLIEDT
.:* *:*:*:*:*: :*:*:*:*: * :*:*:*:*.* ** *:*:*:*:*:*: *

Cl.sporogenes      KSRLSLIDVDVFGVTGMLEQFIEYSPYSIFPQAFFTERPDVVEANLFEGK
Cl_botulinumF      KSRLSLIDVDGFGVTGMLEQFIEYNPYSIFPQGYFTERPDIVEANLLEGR
Cl_botulinumA3     KSRLSLIDVDGFGVTGMLEQFIEYNPYSIFPQGYFTERPDIVEANLLEGR
Cl_kluveri         RNKLLSIDVDFTFSAGMLEQYIENHPYSVFPQAHASQRPDVVQQNLSEGR
.::* * * * * * .:*****:* * * * * * .:*****:* * * * * :

```

```

Cl.sporogenes      IAIIAEGAPFVMTAPAVFFFEFFQAIEDYTQRTIISSFIRLLRYFAVFIVT
Cl_botulinumF     IAIADGTPFVVTVPTVFFFEFFEAVEDYTQRTVISSFNRLRYIATFIVI
Cl_botulinumA3    IAIADGTPFVVTVPAVFFFEFFEAVEDYTQRTVISSFNRLRYVATFIVI
Cl_kluyveri       IAILMDGTPFALTIPALFMEFFQAVEDYSQRTLLSNFNRLRVFGTFIVI
                  ***:  :*:**.* *:::***:***:***:***:*. * *:* ** ...**

Cl.sporogenes      TLPAYITLIKFNPELIPFKFIGAIIESRKGIALTPFMSILSMNIIIDFL
Cl_botulinumF     TLPAYITLIKFNPELIPFKFIGAIIESRKGIALTPFMSILSMNIIIDFL
Cl_botulinumA3    TLPAYITLIKFNPELIPFKFIGAIIESRKGIALTPFMSILSMNIIIDFL
Cl_kluyveri       LLPSIYLDLIRFNSELIPKFIPIVQSRGIALPPLLEILSMYVVIELL
                  **::*: **:*.****:*** .*:**:****.*::.*** **::**

Cl.sporogenes      REGGLRPFKIAQTLVSVGGIIIGDAAIRAKIVSSTLLVVGSTVATFL
Cl_botulinumF     REGGLRPFKIGQTLVSVGGIIIGDAALKAKIVSSTLLVVGSTVATFL
Cl_botulinumA3    REGGLRPFKIGQTLVSVGGIIIGDAALKAKIVSSTLLVVGISTVATFL
Cl_kluyveri       REGGLRPTKIGQTLVSVGGFIIIGSAALEAKLVSPATLVVVG VATIGTFI
                  ***** **.******:***.**:**:***:*:***:***:*.**.*:

Cl.sporogenes      IPNYEMASISIRLINYPMLFLGNFLGMFGITIGWFFLITYLCTLKSYGVPY
Cl_botulinumF     IPNYEMASISIRLITYPMLFLGNFLGMFGITIGWFFLIAYLCTLESYGVPY
Cl_botulinumA3    IPNYEMASISIRLIAYPMLFLGNFLGMFGITIGWFFLIAYLCTLESYGVPY
Cl_kluyveri       IPNYDMSSSIRLIGFPFLFLTNFLGAVGLIAGWYFLFIHLLSLDSFGVPY
                  ****:*: ***** :*:*** **** .*: **:***:* * :*.**:*

Cl.sporogenes      FKIYK-SDLKDILVRMPVYKMKKRPEVIPNSNPTRQSNFRDLFRRKKN-
Cl_botulinumF     FKIYK-SDLKDFVRSVPYKMNKRPKIIPNSNPTRQSNFRDLFRRRENDE
Cl_botulinumA3    FKIYK-SDLKDFVRSVPYKMNKRPKTIIPNSNPTRQSNFRDLFRRKKNDE
Cl_kluyveri       MPLDKYGDFKDTFIRVPLWKMNRPREGIPNNPKRQTNFRNKLWRNKN--
                  : : * .*:** :* *:::***:***: ***.***:****: : *::*

Cl.sporogenes      E
Cl_botulinumF     Q
Cl_botulinumA3    Q
Cl_kluyveri       E
                  :

```

## 6.6

Alignment of *Cl. sporogenes* spore germination protein (*gerAA* and *gerAB*), *Cl. botulinum* F str. 230613, *Cl. botulinum* A3 str. Loch Maree, and *Cl. kluyveri* NBRC 12016 amino acid sequences used to design EGER5 primer Section 7.2.1.

```

Cl.sporogenes      taattatTTTTatgtatcatatTTTcctataaaggggtaagggtgTTTatgga
Cl_botulinumF     -----a-----tgaa
Cl_botulinumA3    -----a-----tgaa
Cl_kluyveri       -----a-----tgga
                  *                               ** *

Cl.sporogenes      aaataaagatttaaTgcacttcaagataaaattaattc--tattaaaac
Cl_botulinumF     aaataagaatctaaattcacttcaagataaaattaactc--tattaaaac
Cl_botulinumA3    aaataaagacctaaattcacttcaagataaaattaactc--tattaaaac
Cl_kluyveri       a--ttaga---ta-----ataagattaattctatattagaa-
                  * * *      **          **** * ** * ** * **

Cl.sporogenes      ttagtTggagaaaa--aaataatttaataataaaaagattttatagtagt

```

Cl\_botulinumF tttagttggagagaa-aaatgatttaataataaaaagagtttataatagc  
Cl\_botulinumA3 tttagttggagagaa-aaatgatttaataataaaaagagcttataatagc  
Cl\_kluyveri -tcatgtggaacagtcacaagttattgttagaagattttctattggaa  
\* \* \*\*\*\* \* \* \*\* \* \*\*\* \* \*\* \*\*\*\*\* \*\* \* \*

Cl\_sporogenes aaaaaatccaatatagaagctgcattattatatataaatggattagctaa  
Cl\_botulinumF aaaacttccaatatggaagcagctttaatatacataaatggattagctaa  
Cl\_botulinumA3 aagacctccaatatggaggcagctttaatatacataaatggattagctaa  
Cl\_kluyveri aagat-actcccctagatgctgccttaatatataaagatggtttagtaaa  
\*\* \* \* \*\*

Cl\_sporogenes tagtgaaactatagatagagatattcttaagcctttaatgttttatatag  
Cl\_botulinumF taaccagactatagatagagatattcttaaacctttaatgtttaatag  
Cl\_botulinumA3 taaccaaactatagatagagatattcttaaacctttaatgtttaatag  
Cl\_kluyveri tcaagatattataaacagagatatattaatcccctaagtcataaagtta  
\* \* \* \*\*\*\* \* \*\*\*\*\* \* \*\* \*\* \*\*\*\*\* \* \* \*

Cl\_sporogenes aggaagatttaagttctacaaa-taatttagatgaaattctttgtaaaaa  
Cl\_botulinumF aagaggatttaagcactgtcca-taacttagaggaatatctttgtaaaaa  
Cl\_botulinumA3 aagaggatttaagcgttgtcca-tgacttagaggaatatctttgtaaaaa  
Cl\_kluyveri atgaagatttaagtaagcacagtcatta-atcaagaaatttgtaacaa  
\* \*\* \*\*\*\*\* \* \* \* \*\*\* \* \*\* \*\*\*\*\* \*\*

Cl\_sporogenes atatatatccccttagcaacacctctttagaacaagatattaataaggctg  
Cl\_botulinumF gtatatctctcttagcaatacctctttagaagagaatattaataatgttg  
Cl\_botulinumA3 atatatatctcttagtaatacctatttagaagaggatattaacaatgttg  
Cl\_kluyveri atatatctctatgtccaaaattgttattcaactgatataaatatggcta  
\*\*\*\*\* \*\* \* \*\* \* \* \* \*\* \*\*\*\*\* \*\* \* \* \*

Cl\_sporogenes tttttcatttgaaaagaggtaaatctgtactaattgttaaggggtgtaca  
Cl\_botulinumF tttttcatttataaaaagaggaaaacaatcctcattgtaccagaaaaatca  
Cl\_botulinumA3 ttttccatttataaagagagggaaaacaatcctgattataccgggaaaatca  
Cl\_kluyveri taaaagatataagaagaggtaaaacagctgttcttgtagat-aaggagga  
\* \*\* \* \* \*\*\*\* \*\* \* \* \*\* \* \*\* \*

Cl\_sporogenes -aaatttataattctagatacagctgggtggagaatatagagatatttcag  
Cl\_botulinumF -aaatttataattctagataactactgggtggaagttacagaaacatttcag  
Cl\_botulinumA3 -aaatttataattctagataactactgggtggaagttacagaaacatttcag  
Cl\_kluyveri tactttcattgttgctgatacacaaggaacaaaacatagaatatatctg  
\* \*\* \*\* \*\* \*\* \*\* \*\* \*\*\*\*\* \*\* \* \* \*\* \* \*\* \*\* \*

→

Cl\_sporogenes atcctataaacgaaacttctata agagcttctagagaaggcttcgtagaa  
Cl\_botulinumF accctgttaatgaaacttctgta agatcttctagagaaggctttatagaa  
Cl\_botulinumA3 accctgtcaatgaaacttctgta aggtcttctagagaaggctttatagaa  
Cl\_kluyveri aacctatggatgaaatcggaata agaggttcaaaagaaggctttatagag  
\* \*\* \* \* \*\*\*\* \*\*\*\*\* \*\* \* \*\*\*\*\* \*\* \*\*\*\*\*

Cl\_sporogenes aaattagaaactaatgtaagcatattaagaaggagaataaaggataataa  
Cl\_botulinumF aaattagaaaccaatataagcctattaagaagacgaataagggataataa  
Cl\_botulinumA3 aatttagaaaccaatataagcctattaagaagacgaataagggataataa  
Cl\_kluyveri gatattgaaataaatttaagtttaataaaaaagggtttaaagacaaaa  
\* \* \*\*\*\* \*\* \*\* \*\* \*\* \*\* \*\* \*\* \*\* \*\* \* \*\* \*\* \*\* \*\* \*\* \*\*

Cl\_sporogenes tttagtacaagaaaatttagtaattggaagaagaatcaaacagatgtgg  
Cl\_botulinumF tttagtagaagaaaatttagttattgggagaagaagtcaaacggatgtag  
Cl\_botulinumA3 tttagtagaagaaaatttagttattggaagaagaagtcaaacggatgtat  
Cl\_kluyveri ttttaattatagaacctttcatcataggaagacgatcccaaaaggatgtgg  
\*\*\*\* \* \*\*\*\* \*\* \* \*\* \*\* \*\* \*\* \*\* \*\*\*\*\* \*\*\*\*\*

Cl\_sporogenes ccttaatgtatattgacaatatagctgataaaaacatagttgaagatata  
Cl\_botulinumF tgttaatgtacattgataatatagcagataaaaatatagttgctgatata

Cl\_botulinumA3 tgtaaagtacattgataatatagcagataaaaatatagttgaagatata  
Cl\_kluyveri ccttaatatataaaaggatattgcagataaagacttaattgaagataca  
\*\*\*\*\* \*\* \* \* \*\*\*\* \*\* \*\*\*\*\* \* \*\* \*\* \* \*\* \*

Cl\_sporogenes aaatcaagattatctttaatagatgtagatgtatgttaggtactggat  
Cl\_botulinumF aaatcacgattaagcttaatagatgtagacggctttgtaggtactggat  
Cl\_botulinumA3 aaatcacgattaagcttaatagatgtagatggctttgtaggactggat  
Cl\_kluyveri agaaataagctattatcaatagatgtggatacttttacttctgcaggaat  
\* \* \*\* \* \*\*\*\* \*\* \*\*\*\*\* \*\* \*\*\* \* \*\* \*\*

Cl\_sporogenes gttagaacaatttatagaatatagcccctacagtatattccccaggctt  
Cl\_botulinumF gctagagcaatttatagaatataacccttatagtatatttccacagggtt  
Cl\_botulinumA3 gctagaacaatttatagaatataacccttatagtatatttccacagggtt  
Cl\_kluyveri gctggaacagtatattgaaaatcatccatattccggttttccctcaagctc  
\* \* \*\* \* \* \*\* \* \*\* \* \*\* \* \*\* \* \*\* \* \*\* \* \*\* \* \*\* \*

Cl\_sporogenes tttttacagaaagaccagatgtagtagaagcaaattttttgagggcaaa  
Cl\_botulinumF attttacagaaagaccagatatagtagaagccaatctacttgaaggtaga  
Cl\_botulinumA3 attttacagaaagaccagatatagtagaagccaatctacttgaaggtaga  
Cl\_kluyveri atgcgtctcaaaggccagatggtgtgcagcaaaaatctttcagaaggtaga  
\* \* \* \*\*\*\* \*\*\*\*\* \* \*\* \* \*\*\*\* \* \*\* \*\* \*

Cl\_sporogenes attgctatcattgctgaaggagctccctttgtaatgacagcaccagcagt  
Cl\_botulinumF attgctattattgctgatggaaccctttcgtagtaacagtcccaacagt  
Cl\_botulinumA3 attgctattattgctgatggaaccctttcgtagtaacagtcccagcagt  
Cl\_kluyveri attgctattttaatggatggaactccttttgcattaactattcccgttt  
\*\*\*\*\* \* \*\* \*\* \* \*\* \* \*\* \* \*\* \* \*\* \* \*\* \*

Cl\_sporogenes attttttgagttttccaggcaattgaagattatacccaaagaactatta  
Cl\_botulinumF atttttgaaatttttgaagcagttgaagattataactcaaaggacagtaa  
Cl\_botulinumA3 atttttgaaatttttgaagcagttgaagattataactcaaaggacagtaa  
Cl\_kluyveri atttatggaatttttccaggctgttgaagattactctcaaagaactctt  
\*\*\*\* \* \*\* \*\*\*\*\* \* \*\* \*\*\*\*\* \* \*\*\*\* \*

Cl\_sporogenes tatcttcctttataagatta-ttaagatattttgcggtttttatagttac  
Cl\_botulinumF tatcttccttttaatagaata-ttaagatatattgcaactttttatagttat  
Cl\_botulinumA3 tatcttccttttaatagacta-ttaagatatggtgcaactttttatagttat  
Cl\_kluyveri taagtaatttttaatagaattctccgggtatt-tggtaccttcatagttat  
\*\* \* \*\*\*\* \*\* \* \* \* \*\* \* \*\* \*\* \*\*\*\*\*

Cl\_sporogenes gacactacctgctatttacataactctaataaagtttaatccagaattaa  
Cl\_botulinumF aacattaccagctgtctatataactttaataaaatttaaccagaattaa  
Cl\_botulinumA3 aaccttaccggctgtttacataactttaataaaatttaaccagaattaa  
Cl\_kluyveri tttattgccttctatataattagacttaattaggttcaattcagagctta  
\* \*\* \*\* \* \*\* \*\* \*\*\*\*\* \* \*\* \*\* \* \*\* \*

Cl\_sporogenes ttccctttaagttttataggagccattattgaatcaagaaaaggaatagca  
Cl\_botulinumF ttccctttaagttcataggcgccattattgaatcacgaaaaggaatagct  
Cl\_botulinumA3 ttccctttaagttcataggcgccatcattgaatcacgaaaaggaatagct  
Cl\_kluyveri ttccaattaaatttattatacctatagttcaatctagaagagcatagca  
\*\*\*\* \*\*\*\*\* \*\* \* \* \*\* \* \*\* \*\*\*\*\* \*\* \*\* \*\*\*\*\*

Cl\_sporogenes ttaactccttttatgtctatattatctatgaatataatcatagatttttt  
Cl\_botulinumF ttaactccttttatgtctatattgtctatgaatataaattatagacttctt  
Cl\_botulinumA3 ttaactccttttatgtctatattgtctatgaatataaattatagacttctt  
Cl\_kluyveri ctctcctccttttagaaatttatccatgtatggtgtgtagaactttt  
\* \*\*\*\*\* \*\* \* \*\* \*\* \* \*\* \* \*\* \* \*\* \* \*\* \*

Cl\_sporogenes aagagaggggtggattaagactaccctttaagatagctcagactctcagtg  
Cl\_botulinumF aagagaaggtggtttaaggctaccttttaagataggtcaaacccttaagtg  
Cl\_botulinumA3 aagagaaggtggtttaaggctaccttttaaaataggtcaaacccttaagtg

```

Cl_kluyveri      aagagaaggaggctgagacttcctacaaaaatcggtcagacattaagtg
***** ** * * * ** * * * * * ** * * * * * * * * * *

Cl_sporogenes   tgggtgggtggtattataataggtgatgcagccataagggcaaaaatagtt
Cl_botulinumF   tggttgggtggtattataattggcggatgcagcattaaaagcaaaaatagtt
Cl_botulinumA3  tggttgggtggtattataattggcggatgcagcattaaaagcaaaaatagtt
Cl_kluyveri      ttgtaggaggtttttattataggtagtgtgcacttgaagccaattagtt
* ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** * ** ** ** ** ** ** ** ** **

Cl_sporogenes   agttctaccactctacttgtagtaggtgtttctacagtggctacatTTTT
Cl_botulinumF   agttccactactttacttgtggtaggggtttctacagttagctactTTTT
Cl_botulinumA3  agttctactaccttacttgtagtaggaattttctacagttagctactTTTT
Cl_kluyveri      agtcctgcaacttttagtagtagtaggagttgctaccataggaactTTTT
*** * * ** * ** * ** * ** * ** * ** * ** * ** * **

Cl_sporogenes   gatacctaactatgagatggctatTTTctataagattaataaaactatccca
Cl_botulinumF   aatacctaactatgaaatgtctatTTTctataagattaataaacatacccta
Cl_botulinumA3  aatccctaactacgaaatgtctatTTTctataagattaatagcttatcccta
Cl_kluyveri      tataccgaactatgatatgtcaagttcaataagattgataggatttcctt
* * ** ** ** ** ** ** ** ** * * ** * ** * ** * ** * **

Cl_sporogenes   tgttatttttaggtaattttttgggtatgTTTggtattacaataggatgg
Cl_botulinumF   tgctatTTTctaggaaattttttgggtatgTTTggtattacaataggctgg
Cl_botulinumA3  tgctTTTcttaggaaatttttttaggcatgTTTggtattacaataggctgg
Cl_kluyveri      tctTTTcctaacaaactTTTtaggtgctgTTTggtattaattgcaggatgg
* * ** ** * ** * ** * ** * ** * ** * ** * ** * **

Cl_sporogenes   ttttttttaataacctatTTTatgcactTTTaaagagctatggagtaccTTa
Cl_botulinumF   ttttttttaatagcttattTTatgtactTTTtagaaagctatggagtaccTTa
Cl_botulinumA3  ttttttttaatagcttattTTatgtactTTTtagaaagctatggagtcccTTa
Cl_kluyveri      ttttttttatttatccacctcctTTTctTTtagatagctTTTggcgTTccata
* ** ** ** * * * * ** * ** * ** * ** * ** * ** * **

Cl_sporogenes   tttta----aaatatataaaagtgatttaaaagatatattagttagaatg
Cl_botulinumF   tttta----aaatatataagagcgatttaaggatatatttgtaagaagc
Cl_botulinumA3  tttta----aaatatataaaagtgacttaaggatatatttgtaagaagt
Cl_kluyveri      tatgcctctggataaatat-ggtgattttaaagatacctttattagggta
* * * ** ** * ** * ** * ** * ** * ** * ** * **

Cl_sporogenes   cctgTTTataaaatgaaaaaacgcctgaggttattcccaattcaaatcc
Cl_botulinumF   cctgTTTataaaatgaataaacgtccaaaaattatccctaattcaaatcc
Cl_botulinumA3  cctgTTTataaaatgaataagcgtccaaaaactatccctaattcaaatcc
Cl_kluyveri      ccctatggaaaatgaataatagaccagagggatttccaaacaataacc
** * * ** ** ** ** ** ** * ** * ** * ** * ** * ** * **

Cl_sporogenes   aactagacaaagtaatttttagagacttgtttaggagaaaaaagaatgatg
Cl_botulinumF   aactagacaaagtaatttttagagacttgtttaggag-----
Cl_botulinumA3  aactagacaaagtaatttttagagatttgtttaggag-----
Cl_kluyveri      aaaaagacaaaccaactttagaacaaactgtggag-----
** * ** ** * ** * ** * ** * ** * ** * **

(Cl_sporogenes truncated)

```

## Appendix 7

Sequences of bands from germinant receptor PCRs from Section 7.3.1. All sequences shown 5' to 3'.

*B. cereus* template amplified using GERBF and GERBR primers and sequenced using the GERBF primer:

CCAAATAGTATGATTTAATTGTCCTTCACTATAATACATAAAAAGATACAATAGCTAAAACACTACA  
TAAATCAAAGTGCTAAAAAAAATTCTGCATGAGCCCATCTTTTAAAGACTTACCTTTTTCAAT  
AAATGGATAGAAGATAAGGATTGTTTCAAAGCCGAGAACTCTAATGCAGATTGCTTTGCAGA  
TATAAGCATGTCAAAGGTGAATGCGTAAAAATAGG

*Cl. sporogenes* template amplified and sequenced using AAF2 and AAR2 primers.

CATTGCTGAGGAGCTCCCTTTGTAATGACAGCACCAGCAGTATTTTTGAGTTTTCCAGGCAA  
TTGAAGATTATACCCAAAGAACTATTATATCTTCCTTTATAAGATTATTAAGATATTTTTGCGGTT  
TTTATAGTTACGACACTACCTGCTATTTACATAACTCTAATAAAGTTTAATCCAGAATTAATTCC  
CTTTAAGTTTATAGGAGCCATTATTGAATCAAGAAAAGGAATAGCATTAACTCCTT

*Cl. algidicarnis* template amplified using AAF2 and AAR2 primers and sequenced using the AAR2 primer.

GCAATGGTACACCTTGCTTTGCTGCGTATATACTTAGTGCGAGTTTAGTCGGTATAAGCTCTTT  
ATGAAATGCAATAAATGCAACATAAACCCCTGGTGTAGCTACAGTAATAATGAATGCTACTAC  
CCTTAAAATCCTATTTATAGATGCATAAATAAATTATCATAGTAATCTTCATTTATTTGAAAAT  
GTTCTATAAATAAATAAGGTAAGGTTATTGCCATAGGTGATCCGTACATAATATAGCTATTCT  
ACCTTGCAATAATTTAGATGCTGC

*Cl. estertheticum* subsp. *estertheticum* template amplified using AAF2 and AAR2 primers and sequenced using the AAR2 primer.

AATGGAGTTAGGGCAATTCCTACCCTTGACTGAACAATGGGAGTTACAAATTTTATTGGTATA  
AGCTCTACATTAACTTTATAAGTGTTAAATAAATAGAAGGTAGGGTAATAACAATAATTGCC  
GCCAAAATCCTTAGAAGTCTTACAAAAGAGGATACAACCTGTTCTTTTCAGTATAATCCTCCACAG



CCTGAAAAAATTGCATGAATATTGCTGGATATACAAGCACCATTGGCGTTCCTTCTATAATAAG  
AACTATTCTCCCTTCTAGCATATTTCCAATAGCTACATCCGGTCTT

*Cl. estertheticum* subsp. *laramiense* template amplified using AAF2 and AAR2 primers and sequenced using the AAR2 primer.

TAATCTCTAAAATGGAGTTAGGGCAATTCCTACCCTTGACTGAACAATGGGAGTTACAAA  
TTTTATTGGTATAAGCTCTACATTAACCTTTATAAGTGTTAAATAAATAGAAGGTAGGGTAATA  
ACAATAATTGCCGCCAAAATCCTTAGAAGTCTTACAAAAGAGGATACAACCTGTTCTTTTACAGTAT  
AATCCTCCACAGCCTGAAAAAATTGCATGAATATTGCTGGATATACAAGCACCATTGGCGTTC  
TTCTATAATAAGA ACTATTCTCCCTTCTAGCATATTTCCAATAGCTAC

*Cl. sporogenes* template amplified and sequenced using GERKF and GERKR primers.

ACACCTATTGTTCCAAAATACACTGCTTTATTTTTACTATAGATTCTATAACCTCTTTACACCTT  
TTCCCTTTACCTATCATACCTTTTAATCCTAAATCAATAGTTTTGGCGTATATGTATCCATCCTA  
TAACTTG TAGTTGGACCTGCCGCTCCTATTTCCATACCAGGCTTATATGTTG

*Cl. perfringens* Type B template amplified using GERKF and GERKR primers and sequenced using the GERKR primer.

GCTATCCMTCTTAAATTCTAGTAAAAGTAAATACCTATTTAAATAATAATCATCTGGTG  
TTTGAAAATTTTCTATAAAAAAGAAAGGTAAGTGTAAAGAACA AWAGGTGT

*Cl. perfringens* Type C template amplified and sequenced using GERKF and GERKR primers.

CACCTTTGTTCTTACAGTACCTTTCTTTTTATAGAAAATTTTCAAACACCAGATGATTATTATTT  
AAATAGGTATTTTACTAGTCTTACTAGAATTTTAAGATGGATAGCATTTTTTATTGCTATGTTTT  
TACCAGG

LA1 template amplified using GERKF and GERKR primers and sequenced using the GERKR primer.

AAGTGAAGTAGWAAGAAAWTATAATATAGATGGTGTTCAATTTTGATGATTATTTCTACAGAG  
ATGGTATGAATGATGATGAAACATACAAAAAATATGGAAATGGACAAGATAAAGGTGACTGG  
AGAAGAGAGAATGTTAATACTTTATTAAGAGAAGTAAAGTCATCTATAAAAAGTATAAAATCA

AATGTAGATTTTGGAGTAAGCCCAAGCGGTATATGGAGAAATAAATCAAGTGATCCAAGTGG  
GTCTGACACGAGGGGGCAAGAAAGTTATTCAGATTTATTTGCAGATAGTAGAGCTTGGATAA  
AACAAAGGACTTGTTGATTATATAGTACCTCAAGTTTATTGAACAA

*Cl. estertheticum* subsp. *estertheticum* template amplified using EGER5F and EGER1R  
sequenced using the EGER1R

ATTTATCTGGCAGTAATTCCACATTAATTTTACAAGAGTTAAATATATAGAGGTAAACTTAA  
TACTAAAACACTACAGCTATTACTCTAAAAAATCTTATAACTGTGGATAACATAAATTTTTGGTTAT  
AATCTTCTACAGTTTGAAAAAATTCTATAAAAATAGCTGGTACAGTTATCCCTTGCTGAGATCC  
TTCCATtAATATGCCTATTTTCCCCTCCATGATATTAAGTCCATTACATCTGGTCTTTCCGTAGT  
AAAAGACTGTGGAAATACAGTATAGGAATTATTCTCTATATATTCCTCTATATATCCTGCGTTTT  
TTATATGATCTACTTTTATTAATTTACTCTTTCCCTTATCTTATTTACTAATTTTTCATCTGCTAT  
ATCCTCTATATATATAATTGCTACTTCTGTTTGTGATCTTTCTCCAACATAAACCTTTCAATTCT  
TAAATTTTATCTTTTATTCTTCTATTAATTACAGATAAATTACTTTCTATACTTTCTGTAAAGGC  
TT

## Appendix 8

Journal articles listed under publications and DR 16 statements of contributions



**MASSEY UNIVERSITY**  
**GRADUATE RESEARCH SCHOOL**

**STATEMENT OF CONTRIBUTION  
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

**Name of Candidate:** Katharine Helen Adam

**Name/Title of Principal Supervisor:** Steve Flint

**Name of Published Research Output and full reference:**

Adam, K.H., Flint, S.H. and Brightwell, G. (2010). Psychrophilic and psychrotrophic clostridia: sporulation and germination processes and their role in the spoilage of chilled, vacuum-packaged beef, lamb and venison. *International Journal of Food Science & Technology*. 45:1539-1544.

**In which Chapter is the Published Work:** Appendix 8

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:  
and / or
- Describe the contribution that the candidate has made to the Published Work:  
Primary responsibility for the writing of the article in Appendix 8

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**STATEMENT OF CONTRIBUTION  
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

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**Name of Candidate:** Katharine Helen Adam

**Name/Title of Principal Supervisor:** Steve Flint

**Name of Published Research Output and full reference:**

Adam, K.H., Brunt, J., Brightwell, G., Flint, S.H. and Peck, M.W. (2011). Spore germination of the psychrotolerant, red meat spoiler, *Clostridium frigidicarnis*. *Letters in Applied Microbiology*. 53:92-97

**In which Chapter is the Published Work:** Chapter 4, Appendix 8

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:  
and / or
- Describe the contribution that the candidate has made to the Published Work:

All the work in chapter 4, the practical work and primary responsibility for the writing of the article in Appendix 8

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**STATEMENT OF CONTRIBUTION  
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**Name of Candidate:** Katharine Helen Adam

**Name/Title of Principal Supervisor:** Steve Flint

**Name of Published Research Output and full reference:**

Adam, K.H., Flint, S.H. and Brightwell, G. (2012). Mechanisms and reduction of spoilage of chilled vacuum-packed lamb by psychrotolerant clostridia. Submitted to Meat Science.

**In which Chapter is the Published Work:** Chapter 6 and Appendix 8

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:  
and / or
- Describe the contribution that the candidate has made to the Published Work:  
All of the work included in Chapter 6. The practical work in Appendix 8 and primary responsibility for write up.

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