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Characterization of germination of *Clostridium estertheticum* spores

by

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To my parents, who have made me the person I am today

ABSTRACT

The germination of *Clostridium estertheticum,* the primary organism responsible for early blown pack spoilage, was investigated. Presence of *C. estertheticum* in blown packages was confirmed. The effect of pH, temperature and oxygen on germination of spores was established. Germination rate of spores increased as pH approached neutrality, with an optimal germination rate at pH 7.0. Temperatures between -1.5 and 10°C did not affect germination of spores. Spores germinated equally well in aerobic conditions as they did in anaerobic conditions. A fraction of heat activated *C. estertheticum* spores germinated and died when heated in meat juice medium at 80°C for 10 min. Amino acids and lactate were screened for possible germinants of *C. estertheticum*. *C. estertheticum* spores germinate in meat with lactate as the potential germinant. This information can be used to design intervention methods for prevention of blown pack spoilage.

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LIST OF ABBREVIATIONS

AGFK	Asparagine, glucose, fructose and potassium ions
AK	Asparagine and potassium
atLAB	Acid tolerant lactic acid bacteria
BPS	Blown pack spoilage
Ca-DPA	Calcium-dipicolinic acid
CBA	Columbia blood Agar
CFDA	Carboxyfluorescein diacetate
Ct	Cycle threshold
DFD	Dark firm dry
$DiOC_6$	Dihexyioxacarbocyanine
DNA	Deoxyribionucleic acid
DPA	Dipicolinic acid/ 2,6 pyridine dicarboxylic acid
FAO	Food and Agricultural Organization
HACCP	Hazard analysis critical control point
HHP	High hydrostatic pressure
LAB	Lactic acid bacteria
MJM	Meat juice medium
MRS	de man Rogosa and Sharp Agar
NAM	N-acetyl muramic acid
OD	Optical density
PG	Peptidoglycan
PYG	Peptone yeast glucose

PYGSB	Peptone yeast glucose starch broth
qPCR	Quantitative polymerase chain reaction
RCM	Reinforced clostridial medium
RFU	Relative fluorescence units
RNA	Ribonucleic acid
SASP	Small acid soluble proteins
tLAB	Total lactic acid bacteria
TSB	Tryptone soya broth
VP	Vacuum packaged
VRBG	Violet red bile glucose agar

I. Literature Review

1.1 Food spoilage

Food spoilage is the process via which food quality deteriorates to the point that it is no longer acceptable to the consumer. The food becomes undesirable to the consumer due to changes in sensory properties of the food. According to a survey done by the Food and Agricultural Organization (FAO), in 2011, 1.3 billion tons of food is wasted every year (FAO, 2011). The food wasted includes food lost or spoiled during the manufacturing process, at the retail level and in households, which translates to economic losses and a significant impact on the environment. Some spoilage is inevitable; however, some types of microbial spoilage can be avoided or at least delayed. Meat and other animal derived foods are very susceptible to microbial spoilage. Most bacteria are ubiquitous in soil; they can be transferred onto food during the manufacturing process either from equipment, personnel or the animal, in the case of animal derived foods. Once contamination has occurred, in conditions conducive to growth, certain microbes metabolize carbohydrates and/or proteins in the food, thus emitting compounds that produce offensive odours. There is a great need in the industry to control spoilage of food, especially meat. To reduce contamination from pathogenic and spoilage bacteria, meat packing plants today follow a controlled process paying close attention to sanitation and hygiene.

1.2 Decontaminating treatments used in beef processing

Most North American beef processing facilities follow hazard analysis critical control point (HACCP) systems to ensure safety of their products. As per rules laid out by USDA, plants are expected to comply with sanitary requirements, have at least one antimicrobial treatment prior to chilling of carcasses, and mandate daily microbial testing to determine if targets for reduction of pathogens are being met (USDA, 1996). Federally inspected plants in Canada are required to use decontaminating treatments as part of their HACCP program during the carcass dressing process (CFIA, 2005). Traditionally, plants use steam vacuuming to decontaminate areas on the carcass contacted by knives or machines during the skinning process (Koohmaraie et al., 2005). Subsequently, a pre-evisceration wash with hot water and 2-3% lactic acid is done to reduce the load of microbes. After evisceration and splitting, a steam pasteurization step is used. Sometimes another lactic acid wash is done before the carcasses are sent to the cooler. This use of multiple treatments is very effective in reducing the load of bacteria on the carcass. The chilled carcass has only 1 log aerobic bacteria/cm^2 remaining on its surface compared to 8 log cfu/cm^2 on the animal that enters the slaughter floor (Arthur et al., 2004). However, none of the treatments can eliminate bacteria from carcasses and clean carcasses do not result in similarly clean beef trimmings, cuts and ground beef. This indicates that bacteria either survive decontaminating treatments or are introduced onto the carcass after

chilling or during carcass breaking. Endospores transferred from the hide to the skinned carcass during the de-hiding process would survive all the decontaminating treatments, thus remaining on the chilled carcass (Broda et al., 2002; Boerema et al., 2003; Setlow, 2007). The numbers of bacteria found on cuts, and trimmings are much higher than that found on carcasses entering the breaking process, which suggests that there is a transfer of bacteria from personal equipment to the final meat cuts (Yang et al., 2012). Even with several decontaminating treatments, bacteria that survive the treatments or are introduced later on during the breaking process have the potential to spoil meat and therefore decrease its storage life.

1.3 Spoilage microflora on beef

The physical and chemical properties of beef vary based on several environmental conditions. These properties have an impact on spoilage microflora that can grow on beef. Lactate and residual glucose concentrations are inversely related to the ultimate pH of the meat (Immonen and Puolanne, 2000). High pH meat or Dark Firm Dry (DFD) meat is produced as a result of glycogen reduction in the animal before slaughter. Residual glucose levels and lactate levels are lower because of the low initial glycogen levels, therefore resulting in a high ultimate pH. The ultimate pH and glucose concentrations of beef will affect the types of organisms that grow on meat and when spoilage occurs (Gill and Newton, 1978).

The packaging atmosphere also has an effect on beef properties. The color of the meat is based on the oxygen content of the packs. In the absence of oxygen,

meat is most likely to retain the ability to bloom to a fresh, red color (Gill and Mcginnis, 1995). This is because oxidation of the pigment, myoglobin to metmyoglobin is prevented. The absence of residual oxygen ensures that the stable pigment, metmyoglobin is not formed. If present on beef, it gives it a dull, brown color. Packaging also has an impact on the type of spoilage microflora that develops on meat during storage.

Pseudomonads are the predominant spoilage bacteria on aerobically stored raw beef (Gill and Newton, 1978). After the initial levels of glucose are depleted in beef, pseudomonads have an advantage over other spoilage bacteria since they can readily catabolize gluconates and amino acids. Breakdown of these compounds results in the production of amines, ammonia, sulfides and the biogenic amines, putrescine and cadaverine (Doyle, 2007). Spoilage only becomes evident when breakdown of amino acids is initiated once growth ceases, as a result of glucose depletion. In addition, pseudomonads will cause spoilage faster in high pH beef; since glucose content of high pH meat is lower (Gill and Newton, 1978). Growth of aerobic bacteria can be inhibited by changing the environment of packs. High CO_2 concentrations and low O_2 concentrations will inhibit the growth of pseudomonads. High CO_2 concentrations are achieved via modified atmosphere packaging and mainly used for packaging retail ready meats. Another approach is vacuum packaging which is commonly used for fresh beef.

Vacuum packaging of beef will significantly reduce the growth of aerobic bacteria thereby extending the shelf life of meat to around 12 weeks (Egan, 1983). Due to their ability to grow on many substrates including glucose, glucose-6-

phosphate, glycogen and amino acids, lactic acid bacteria (LAB) are the dominant population on anaerobically packaged raw beef stored at low temperatures. Enterobacteriaceae, such as *Shewanella putrifaciens,* are gram negative, facultative anaerobes, and are usually found on high pH vacuum packaged (VP) beef; they produce hydrogen sulfide and organic acids from amino acids and glucose respectively (Doyle, 2007). Spoilage of VP beef can result in acidic odors, slime, and greening on the surface of meat at late times during storage. Gas production from LAB and Enterobacteriaceae in small volumes is a possibility (Chaves et al., 2012). As expected, the lower the storage temperature of meat, the slower the rate of spoilage. Storage of beef at abusive temperatures will lead to rapid spoilage by Enterobacteriaceae (Gill and Mcginnis, 1995).

Psychrotrophic, anaerobic, spore forming *Clostridium* spp. have been found in vacuum packs that were grossly distended (Broda et al., 2003). If spores present on the final primal cut germinate and outgrow into vegetative cells, they can produce copious amounts of gas in the pack. The first case of vacuum pack spoilage by a *Clostridium* spp. was reported in 1989 (Dainty et al., 1989). Thereafter cases of vacuum pack spoilage by psychrotolerant *Clostridium* spp. have been reported in lamb, venison, beef and cooked dog rolls (Broda et al., 1996; Kalinowski and Tompkin, 1999). Spoilage resulting from high volumes of gas production in VP beef is referred to as blown pack spoilage (BPS). A combination of factors promotes BPS including anaerobic conditions inside the vacuum pack, low storage temperature and potentially less competition from other bacteria. The inside of a vacuum pack can present ideal growth conditions for

anaerobic, psychrophilic organisms. A number of organisms have been implicated in causing vacuum pack spoilage. These include *C. estertheticum, Clostridium gasigenes, Clostridium frigidicarnis, Clostridium algidicarnis* and *Clostridium tagluense* (Broda et al., 2002; Broda et al., 1999; Broda et al., 2000; Silva et al., 2011; Cavill et al., 2011). Of these, only *C. estertheticum* and *C. frigidicarnis* have the potential to produce gas in the pack (Yang and Badoni, 2013).

Blown pack spoilage of VP beef is known to be primarily caused by *C. estertheticum* spores present on the surface of the meat (Cavill et al., 2011). Quantitative PCR (qPCR) using specific *C. estertheticum* primers (16 SEF, 16 SER), were used to quantify numbers of *C. estertheticum* present in spoiled vacuum packs of beef; it was revealed that the most common cause of BPS is *C. estertheticum*. VP beef inoculated with *C. estertheticum* spores showed signs of swelling in as early as 35 d (Yang et al., 2014a). Spoilage caused by other organisms involves modest distension of packs at late times during storage at chiller temperatures or after temperature abuse (Broda et al., 2000; Brightwell et al., 2007). *C. estertheticum* is a gram positive, anaerobic, psychrophilic organism that grows optimally at 10°C. This organism is probably introduced onto the carcass from soil particles and faeces on the hide of the animal. When samples of animal faeces were tested for *C. estertheticum*, nearly all of the samples tested were positive for *C. estertheticum* (Broda et al., 2009). *C. estertheticum* spores were also found in a large fraction of the samples collected from commercial abattoirs in Ireland (Moschonas et al., 2010). Samples were collected from equipment, faeces and hide. Growth of *C. estertheticum* on the surface of meat is

limited by glucose availability. However, after glucose is exhausted, the organism does subsequently ferment lactate leading to the production of hydrogen and carbon dioxide gases. In addition, esters such as butyrate, formate, 1-butanol and ethanol are produced (Yang et al., 2009a). Although blown packs occur sporadically in each consignment they are a major concern for the industry because one blown pack can mean that the whole consignment has to be discarded. If the initial numbers of *C. estertheticum* are comparable to those of LAB, they might be able to out-compete LAB, and cause early BPS. Therefore, finding a practical and valid way to inhibit pack spoilage is a priority in the meat industry. Spores can survive harsh treatments subjected to carcasses in the meat plant because of their unique structure. It is critical to understand endospore structure before we can find a solution to preventing BPS of VP meat.

1.4 Endospore structure and resistance

Endospores are dormant structures formed by certain Gram positive bacteria, usually under nutritionally deprived conditions. The dormant state of the spore enables the organism to survive harsh environmental conditions. Thereafter, when nutrients are available, the spore returns to its vegetative state. The structure of endospores, which is considerably different from that of vegetative cells, is such that they are highly resistant to extreme heat, pH, desiccation and chemicals (Setlow, 2007). The outermost layer of the spore is the exosporium (Figure 1). Not every spore has an exosporium; it has been found in *Clostridium botulinum* 78A spores. The formation of the exosporium is initiated prior to

formation of the spore cortex and it might be involved in resistance to inert surfaces and interactions with epithelial cells. (Severson et al., 2009; Stevenson and Vaughn, 1972). Inside the exosporium is the spore coat. The spore coat is important in resistance to exogenous lytic enzymes but has little or no role in the resistance of spores to heat and radiation (Driks, 1999; Nicholson et.al., 2000). In *Bacillus* spp., the spore coat houses enzymes with direct germination roles including cortex lytic enzymes, such as CwIJ, so that they are in direct contact with the cortex during germination (Driks, 2002; Baygan and Setlow, 2002). The second layer of the spore is the outer membrane. Removal of outer membrane from *Bacillus* endospores seemed to have no effect on its resistance to heat, radiation or chemicals (Setlow, 2000). The next layer is the cortex, which is composed of peptidoglycan (PG) very similar to vegetative PG, with a few specific modifications. As opposed to vegetative PG, a large percentage of the N-acetyl muramic acid (NAM) residues in the spore PG have no peptide side chains while another 25% of the NAM residues have only an L-alanine side chain. Due to these modifications, only 3% of the spore NAM residues contain side chains that are crosslinked (Popham, 2002). However, this low level of crosslinking does not alter spore resistance; it is required for substrate recognition by germination specific lytic enzymes (Atrih and Foster, 2002). Inside the cortex is the inner membrane, which acts as a permeability barrier to small molecules that could potentially damage DNA in the spore core. The low permeability of the inner membrane is likely because of immobility of the lipids in the membrane and accounts for resistance of spores to chemicals (Cowan et al., 2003). The final

layer of the spore is the spore core, which has the most important components of the spore including DNA, RNA and metabolic enzymes. Most of the resistant properties of the spore come from components present in the spore core (Setlow, 2006). It is vastly different from the protoplast of a growing cell. In a growing cell, water makes up 75-80% of the wet weight of a protoplast, whereas in the spore core, water is only 27-55% of the core wet weight (Gerhardt and Marquis, 1989). This dehydrated state of the core accounts for resistance of spores to wet heat, which is described as the resistance of the spore when suspended in an aqueous environment. Spores are able to survive at temperatures 40°C higher than most growing cells. In the absence of water, spore proteins are rotationally immobilized, which prevents the aggregation of heat denatured proteins and protects spore enzymes in extreme heat (Leuschner and Lilford, 2000). Another core molecule important for resistance is pyridine 2,6 dicarboxylic acid (DPA). This molecule makes up 5-15% of the dry weight of spores and is usually chelated to Ca^{2+} cations in the spore core. The amount of DPA in the core is well above its solubility; this huge accumulation of DPA helps further reduce core water content (Setlow, 2006). DPA less spores have a higher water content than wild type spores (Paidhungat et al., 2000). In addition, the photochemistry of spore DNA when exposed to UV light is remarkably different from what is observed in DNA from growing cells (Nicholson et.al., 2000). This altered photochemistry of DNA allows spores to be resistant to UV light. The last group of molecules that contribute to spore resistance are the small acid soluble proteins (SASP). The α/β SASP are found exclusively in the spore core where they saturate the DNA,

protect it from dry heat and prevent the spore from being killed by harsh chemicals (Frenkiel-Krispin et al., 2004; Loshon et al., 1999). In addition, DNA repair mechanisms activated during outgrowth protect the spore from dry heat and UV radiation (Leggett et.al., 2012). Overall, spores are resistant to several treatments including radiation, wet heat, dry heat and chemicals. There are only a few chemicals available that can kill spores. These include formaldehyde, nitrous acid and alkylating agents. These chemicals kill the spores by causing DNA damage (Setlow, 2006). To date, there is no chemical that is able to destroy a spore quickly without drastically affecting the surface intended to be disinfected or without causing significant toxicity (Leggett et al., 2012). It is almost impossible to use very harsh treatments for fresh meat without destroying the quality of the food product. Therefore, another reasonable approach to prevent spores from causing spoilage in food has to be designed. Inhibition of spore germination would prevent spores from progressing into their vegetative state, and therefore stop growth of cells.

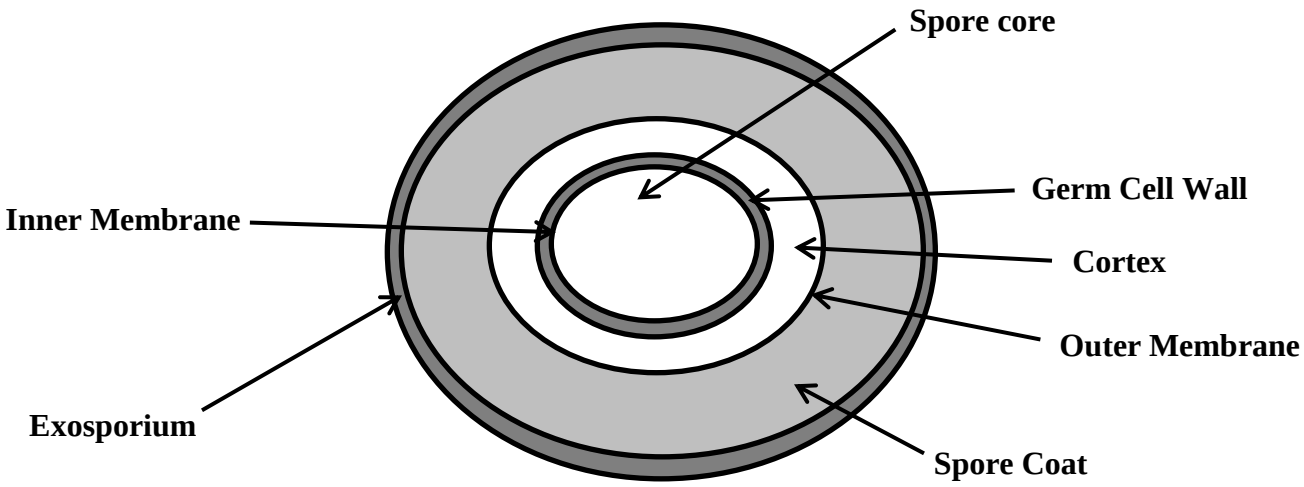


Figure 1. The structure of the endospore. The multiple layers of the endospore provide resistance to heat, radiation, and chemicals (Setlow, 2006; Paredes-Sabja et.al., 2011).

1.5 Spore germination

Dormant spores have the ability to rapidly exit dormancy and transition into vegetative cells upon sensing that the environment is conducive to growth. These vegetative cells can metabolize like any other growing bacterial cells and are no more resistant to harsh conditions like spores are. The process via which dormant spores, in the presence of nutrients, exit dormancy is called spore germination. Spore germination is a crucial event because disease progression and food spoilage caused by spores requires germination. The process of spores becoming vegetative cells can be divided into three steps: activation, germination and outgrowth. Activation is reversible, and most often the result of heat

treatment (Keynan et al., 1964). Not all spores go through an activation step before they germinate. Heat activation has been shown to be required for many *Clostridium* spores (Paredes-Sabja et al., 2008b; Adam et al., 2011). However, preliminary studies on *C. estertheticum* spores indicated that activation temperature or lack of spore activation did not affect the extent of germination Yang, unpublished data). It is likely that activation of spores alters molecular interactions on the surface of spores, thus increasing the ability of germinants to interact with germinant receptors (Keynan et al., 1964). Unlike activation, germination of spores is not reversible; spores that have initiated germination are committed to the process. Once they have germinated, spores have lost most of their resistance properties, although they are physiologically very different from vegetative cells. A germinated spore must outgrow before it is considered to be a dividing vegetative cell. Nutrient germinants include amino acids, ribosides and sugars. Non-nutrient pathways that induce germination of spores have also been reported in the literature. These include initiation by exogenous Ca-DPA, ions, PG fragments and high hydrostatic pressure (Paredes-Sabja et al., 2011). Stage I of nutrient induced germination is initiated by germinants penetrating the outer layers of the spore coat and cortex to interact with germinant receptors present on the inner membrane of spores (Ross and Abel-Santos, 2010; Moir, 2006). This is usually followed by receptor mediated changes to the permeability of the spore's inner membrane, resulting in the movement of Na^+, K^+, H^+ ions and other small molecules through the membrane from the spore core, with water moving into the spore (Swerdlow et al., 1981). The release of cations increases the internal core

pH of the spore, such that metabolism can begin. This step is followed by release of Ca-DPA from the core of the spore. In *Clostridium perfringens*, SpoVA proteins are essential for uptake of DPA into spores but spores lacking SPoVA proteins germinate similarly to wild type spores, thus indicating that Ca-DPA is not crucial for spore stability or for germination as in some *Bacillus* spp. (Paredes-Sabja et al., 2008a). It is also possible, but less likely, that other proteins are activated in the absence of SpoVA for uptake of Ca-DPA into the spore. Stage II of germination involves depolymerisation of the PG cortex by specific hydrolases. In *C. perfringens*, the serine protease, CSpB is activated through an unknown mechanism. It cleaves proSleC converting it to its active form, SleC which hydrolyzes the PG cortex. At this point, protein mobility inside the spore resumes, marking the end of germination and entry into outgrowth (Paredes-Sabja et al., 2011). Non-nutrient spore germination has been well defined in *Bacillus* spp. Exogenous Ca-DPA will activate the cortex hydrolase, CwIJ which leads to full core hydration and completion of spore germination (Yi and Setlow, 2010). Moderate high hydrostatic pressure (HHP) activates germinant receptors and triggers germination in this way. Recently, foodborne isolates of *C. perfringens* were shown to germinate in the presence of KCl and inorganic phosphate by activating germinant receptors (Paredes-Sabja et al., 2009). A eukaryotic-like serine/threonine kinase PrkC on the surface of spores upon binding to PG fragments can activate subsequent events of germination (Shah et al., 2008). PrkC has a PG binding domain that recognizes mesodiaminopimelic acid residues present in PG. PG fragments or muropeptides signify the presence of growing

bacteria in the environment, which might be the reason for triggering germination of spores in response to these molecules. PrkC dependent germination has only been shown in *Bacillus* spp. However, homologues of this protein are present in most other spore-forming organisms. There have been few studies done on germination in psychrophilic, anaerobic Clostridia such as *C. estertheticum*. Determining the optimal conditions required for spore germination of *C. estertheticum* is a much needed first step to be able to inhibit germination, and therefore spoilage caused by these spores. Determination of the optimal pH and temperature required for germination of these spores could be very valuable since these might be the easiest parameters to manipulate when working with meat. Identification of germinants required by *C. estertheticum* might also give us insight into potential ways to inhibit BPS.

1.6 Effect of pH on germination

Previous studies on germination of *Clostridium* spores showed that pH has an effect on germination of spores. Plowman and Peck (2002) demonstrated that germination of a strain of non-proteolytic *Clostridium botulinum* was greatest at pHs ranging from 5.5 to 8.0, where more than 90% of the spores germinated. The extent of germination at pH 5.3 was lower, where only 50% of the spores germinated after 4 h. Only 10% of the spores germinated at pH 9.9 and pH 5.0. It seems as though pH affects the extent of germination since several other investigations by the same authors indicated that three other strains of non-proteolytic *C. botulinum* spores also completed germination within 4 h. There

was minimal loss in optical density between 3 and 4 h. Studies on *Clostridium difficile* showed similar results where spores germinated optimally between pH 6.32 and 7.53. The extent of germination at pH 8.47 was lower than germination at pH 7.53. The extent of germination at pH 5.56 was significantly lower when compared with the other pHs. *C. difficile* spores germinated much faster than *C. botulinum* spores. A 2 log reduction in spore count was detected in 70 min at pHs where spores germinated (Wheeldon et al., 2008). Germination responses to pH in most *Bacillus* spp. are also comparable to what is observed for spores of *Clostridium* (White et al., 1974; Ishida et al., 1976). Studies have shown that pH has an effect on germination of *C. perfringens* spores. When spores were germinated in buffer containing asparagine and potassium ions (AK) the most optimal rate of germination was observed between pH 7.0 and 7.5, with a lower rate detected below pH 7.0 and above pH 7.5(Paredes-Sabja et al., 2008b). However, when germinated in buffer containing alanine or KCl, the germination response with respect to pH was much different. The maximum rate of germination steadily declined as pH increased, with a maximum rate of 3% per min and 1.5% per min in the presence of KCl and alanine, respectively. Non nutrient germination pathways are probably significantly different from nutrient induced germination. Strain differences and varying germination responses to pH with different germinants have been reported in other organisms. It should be noted that in all of the above studies, germination of spores was monitored in a buffer system to which nutrient or non-nutrient germinants were added. As of yet, there are no studies that have monitored germination in a food system.

The effect of pH on growth of *C. estertheticum* and other organisms isolated from blown packs has also been studied. *Clostridium estertheticum* subsp. *estertheticum* and another *C. estertheticum* isolate did not grow at a pH below 5.5 in MJM but grew at the same rate between pH 5.5 and 7.5 (Yang et al., 2009b). *Clostridium gasigenes* and *Clostrdium algidicarnis* isolated in Brazil grew at a pH between 6.2 to 8.2 (Silva et al., 2011).

1.7 Effect of temperature on germination

There have been several reports on the effect of temperature on germination of spores. As opposed to pH, since the optimal growth temperature among different spore forming species differs substantially, the optimal temperature at which they germinate also varies. For example, *C. botulinum* Type E strain Eklund 17B germinates between 2-40°C whereas the type E strain Iwanai and Minnesota can germinate between 5 and 45°C and 2 and 50°C, respectively. The optimal germination temperatures for these strains are also quite different at 35, 30 and 37°C for strains Eklund 17B, Iwanai and Minnesota, respectively (Plowman and Peck, 2002). It is most likely that the enzymes required for germination and growth are not related and therefore, respond differently to temperature. Most organisms have an optimal germination temperature below and above which the rate of germination slows down considerably, which is consistent with most other physiological processes. The effect of temperature on *C. perfringens* spore germination indicates an optimal spore germination of temperature of 41 and 40°C for the two strains. In response to different

germinants AK and 100 mM KCl, the maximum germination rates differed, with strain NB16 germinating at double the rate when AK was present rather than KCl (Paredes-Sabja et al., 2008b). *C. difficile* spores germinate at a higher rate when incubated at higher temperatures (Wheeldon et al., 2008). The extent of germination was not affected by changing the temperature.

The studies mentioned above were all done on mesophilic organisms. Since VP beef is stored at chiller temperatures, the organisms that are responsible for BPS are mainly psychrophilic. To date, there have been no investigations into the effect of temperature on germination in psychrophilic organisms, which could be entirely different. At temperatures below 0 °C freezing effect might change response of germination to temperature. Investigations into the effect of temperature on growth of *C. estertheticum* in meat juice medium (MJM) showed that this organism grows in MJM at temperatures between -2 to 20°C (Yang et al., 2009b). At 20°C, two isolates of *C. estertheticum* stopped growing in peptone yeast glucose starch broth (PYGSB). Other psychrophilic organisms that have been involved in BPS such as *C. algidicarnis* and *C. gasigenes* grow at temperatures between 4 and 37°C (Silva et al., 2011). *C. frigidicarnis* can grow at temperatures between 3.8 and 40.5°C (Broda et al., 1999). When VP beef was inoculated with *C. estertheticum* subsp. *estertheticum* and stored at -1.5°C, time until gas production was longer than when the meat was stored at 1 or 4°C. Meat inoculated with 10^2 cfu/cm^2 *C. estertheticum* stored at -1.5°C showed signs of gas production within 42 d which is the expected arrival time in an overseas market.

With increasing temperature, the time to gas production reduced significantly, at 1 and 4°C, gas production was seen within 23 and 21 d, respectively (Moschonas et al., 2009).

1.8 Effect of oxygen on germination

Obligate anaerobic spore former organisms can only grow in an anaerobic atmosphere. Some of these organisms can tolerate a little oxygen but exposure to too much oxygen will kill most anaerobic bacteria. These organisms die in the presence of oxygen because of a combination of factors. Historically, it was thought that oxygen killed obligate anaerobes because of the absence of enzymes such as superoxide dismutase and catalase, which convert the lethal superoxide ions formed in cells from exposure to oxygen. However, genes for the superoxide dismutase proteins are present in obligate anaerobes like *Clostridium butyricum* (Kim et.al., 2008). There are other factors that could explain the inhibition of growth of anaerobic organisms by oxygen. Oxygen increases the redox potential inside the cell, which inhibits growth. Molecular oxygen oxidizes sulfide to disulfide; sulfide is an important component of cellular enzymes. In the presence of oxygen, electrons are exhausted by organisms in reducing oxygen, which inhibits metabolism. Since a combination of factors affects the growth of obligate anaerobes in the presence of oxygen, some organisms are more tolerant to oxygen than others. Germination of spores from anaerobic organisms in presence of oxygen has been described several times in the literature. Oxygen had no effect on either the extent or the rate of germination of *C. difficile* spores (Wheeldon et al.,

2008). If spores can germinate equally well in aerobic conditions, surfaces in the hospital that have possibly been contaminated with *C. difficile* can be exposed to germinant solution under aerobic conditioins and then killed by milder treatments. Killing of *C. difficile* spores incubated in germinant solution by heating, UV radiation and drying was demonstrated recently (Nerandzic and Donskey, 2010). Many researchers have found that germinated spores are much more sensitive to heat than dormant spores, as one would expect. A decrease of 1.5 log cfu/mL was observed when germinated spores were exposed to 80°C for 5 min (Nerandzic and Donskey, 2013). Numbers of dormant spores did not decrease much after heating. Similar observations were made with non-proteolytic *C. botulinum* where organisms germinated similarly in the presence and absence of oxygen. All the investigations done by Paredes Sabja et.al on *C. perfringens* were done in aerobic conditions (Paredes-Sabja et al., 2008b). It seems likely from the above evidence that spores germinate just as well in aerobic environments as they do in anaerobic environments.

1.9 Germinants

Identifying nutrient or non-nutrient germinants required for germination can be very useful when trying to inhibit spore germination or using the 'activate to eradicate' strategy to prevent the growth of spore forming organisms. As mentioned earlier, nutrient germinants include amino acids, sugars, ribosides, purines or bile salts. Most *Bacillus* spp. use L-alanine as a germinant and germination with L-alanine can be blocked via competitive inhibition with D-

alanine in a germinant solution (Paidhungat and Setlow, 2000). Germination with asparagine, glucose, fructose and potassium ions (AGFK) has been defined in *Bacillus* and *C. perfringens* spores (Paredes-Sabja et al., 2008b, Cabrera-Martinez et al., 2003). The use of a co-germinant for germination is common among *Clostridium* spores and has been observed for *Clostridium bifermantans*, *C. difficile* and *C. botulinum* (Waites and Wyatt, 1971; Wheeldon et al., 2011; Plowman and Peck, 2002). *C. botulinum* strains Eklund 17B and Craig 610 germinated optimally in germinant mixtures of L-alanine/L-lactate, L-cysteine/L-lactate, and L-serine/L-lactate. More than 90% of the spores germinated in these mixtures. L-lactate is the most effective co-germinant in combination with amino acids (Plowman and Peck, 2002). Other chemicals tested in combination with amino acids did not increase the extent of germination significantly, except for adenosine and inosine, which were particularly effective with L-alanine. It can be concluded that most germination was significantly enhanced in solutions containing amino acids and lactate. Out of the 15 pairs of chemicals in which over 60% germination was established, 12 were combinations of an amino acid and lactate (Plowman and Peck, 2002). Spores of C. *frigidicarnis*, another non-proteolytic *Clostridium*, germinated optimally when amino acids were combined with lactate. L-valine and L-lacatate in sodium phosphate buffer supplemented with sodium bicarbonate and cysteine was the germinant solution in which maximum spore germination was detected (Adam et al., 2011). The inducing effect of lactate has also been shown in *C. botulinum* type A and B spores, where spores germinated in the presence of L-alanine, L-lactate and sodium bicarbonate

(Alberto et al., 2003). Germination of *C. difficile* spores with sodium taurocholate and glycine is enhanced when histidine is added to the mixture (Wheeldon et al., 2011). Sodium taurocholate and glycine are also both co-germinants (Sorg and Sonenshein, 2008). As mentioned previously, L-lactate seems to be a common co-germinant for several *Clostridium* spores. Further research is needed to understand the process of spore germination. To study the effects of oxygen, temperature and pH on germination, an efficient method of monitoring spore germination needs to be determined. There are multiple techniques that have been described in the literature.

1.10 Monitoring spore germination

1.10.1 Loss in optical density (OD)

One of the most common techniques used to monitor spore germination is to measure the maximum rate of loss of optical density. Spore germination has been characterized by a loss of OD when the initial OD of the solution is > 0.8. A linear correlation has been found between phase darkening and loss in OD by several researchers (Paredes-Sabja et al., 2008b; Plowman and Peck, 2002; Adam et.al., 2011). The maximum loss in OD is equivalent to maximum rate of germination. A loss in OD is seen because of the loss in refractility of spores as they germinate (Hashimoto, 1969). As spores germinate, the higher water content of the spore means that light does not refract as much when passing through, therefore resulting in a lower optical density. There are several advantages to this technique. With a Bioscreen analyzer this method was automated by Plowman

and Peck (2002). Using this analyzer, measurements can be taken at intervals over a period of time automatically. It is very rapid and accurate, and allows for screening of numerous conditions or germinants at one time. The initial concentration of spores required for this technique is very high. If the media from which spores are harvested is very dense, media components will interfere with the OD reading.

1.10.2 Heat shock/Ethanol Shock

When spores germinate they lose their resistance to heat and chemicals. This loss in resistance can be measured by subjecting germinating spores to heat treatments (80°C for 10 min). The reduction in log cfu/mL or spore count will theoretically correlate with germination. This method has been used with *C. difficile* (Wheeldon et al., 2008, Nerandzic and Donskey, 2013). Germinating spores can also be subjected to ethanol (100 % v/v), and plated to obtain maximum recovery of spores. When spore germination was carried out under aerobic conditions, no reduction in log cfu/mL was observed when germinating spores were subjected to ethanol shock. However, under anaerobic conditions, ethanol shock had just as much of an effect as heat shock (Nerandzic and Donkey, 2010). This means that exposure to oxygen inhibits progression of germination into outgrowth since only vegetative cells are sensitive to killing by ethanol shock. Monitoring spore germination via ethanol shock or killing by other chemicals can give insight into the mechanism of spore germination. In this way,

the above techniques can be very valuable. On the other hand, these techniques are also very labor intensive and lengthy.

1.10.3 Spore staining

Spores can be differentially stained from vegetative cells using the modified Wirtz-Conklin stain, which uses malachite green (Hamouda et al., 2002). The principle behind the Wirtz- Conklin stain is that the dye is taken up by the spores and attaches to the peptidoglycan once the spores have been heated extensively to penetrate the outer protective layers of the spore. This dye is washed away from the vegetative cells with water but not from the spores. A counter-stain, safranin, is used to stain vegetative cells red. This relatively simple method can be used to monitor spore germination. It is very similar to using phase darkening or reduction in phase bright spores to monitor germination. In that case, a loss in refractility of spores makes them appear phase-dark when germinated and phase bright as spores, as light cannot penetrate spores. This loss in refractility is related to the increased water content of the germinated spore (Hashimoto, 1969). However, detecting green spores might be easier than detecting phase bright spores. Spore staining is cost-effective, does not require an anaerobic chamber and uses minimal amount of spores. The percentage of remaining spores in the medium can be checked several times during germination to gain a better understanding of germination. This technique is labor intensive since spores have to be distinguished manually in images. However, the Wirtz-

Conklin staining technique has been used successfully to monitor germination in several species (Bischof et al., 2007).

1.10.4 Dipicolinic acid fluorescence

One of the most rapid and sensitive techniques that can be used to monitor spore germination is dipicolinic acid (DPA) fluorescence. Spores release their store of Ca-DPA very early in the germination process. The release of DPA from spores is correlated with germination. The amount of DPA released from spores can be measured using fluorescence techniques. DPA, by itself, does not emit high intensity light when excited but when bound to lanthanide ions such as terbium, fluorescence of the complex can be measured fairly easily. The DPA-Tb complex emits light at a wavelength of 545 nm when excited with UV light of 275-280 nm. DPA released from 10^4 spores/mL has been reported in the literature as the minimum concentration of DPA required for reliable interpretation (Kort et al., 2005). A higher number of spores may be required to monitor change in DPA fluorescence and germination. Measuring the time resolved fluorescence of this complex ensures that background fluorescence is minimized. Since the fluorescence lifetime of this complex is very high, measuring fluorescence a few milliseconds after excitation still gives an intense signal (Ammann et.al., 2011). Several conditions can be tested at one time with this technique. This method is most well suited for screening of germinants such as amino acids, sugars and muropeptides. However, to measure several conditions at one time, a high volume of spores is needed. The availability of an anaerobic

chamber would make it much easier to work with lower volumes of spores and media. If germination has to be monitored over time at different points, access to a low temperature incubator inside the chamber would be necessary. If this is not the case, a large amount of spores would be required. Even though DPA fluorescence is very useful and a reliable methodology, it has many costs associated with it. Another important point to note is that sporulation media and germination media might quench or enhance fluorescence readings of samples. This should be taken into consideration when media is selected. Preliminary studies indicated that components of MJM might have a quenching effect on fluorescence emitted by the DPA-Tb complex (data not shown).

1.10.5 Flow cytometry

Differential staining of spores and germinating spores has been employed by several groups to monitor spore germination. Examples of these dyes include Syto 9 combined with propidium iodide or carboxyfluorescein diacetate (CFDA) combined with Hoecsht 33342. The staining procedures using Syto 9 and propidium iodide assume that since the membranes of germinated spores are more permeable than non-germinated, non-damaged spores, Syto 9 can get through the membranes of germinated spores but not non-germinated spores. Propidium iodide will go through the membranes of non-viable spores or germinated dead spores. Non-germinated viable spores will only exhibit peripheral staining with both of the above dyes. It is not easy to distinguish different spores types based on the epifluorescent images generated by this technique. Flow cytometry in

combination with the above dyes generates 4 different populations of spores that have to be distinguished manually. This might introduce biases into the methodology. Correlation was found between Syto 9 and loss in OD but the germination rate based on Syto 9 was much lower than was seen with plate counts (Cronin and Wilkinson, 2008). Dye based techniques need to be explored more before they can be used reliably. The higher membrane potential of germinated spores versus non-germinated spores has also been used to detect germination. Germinated spores are hyperpolarized and therefore, carbocyanine dyes accumulates on its membranes and eventually gets translocated to the lipid bilayer. An example of such a dye is dihexyioxacarbocyanine iodide ($DiOC_6$). Within 15 min of germination, an increase in signal by $DiOC_6$ was observed in a study done on *Bacillus* spores (Laflamme et al., 2005). However, the authors also noticed a signal at time 0 whereas no signal was observed in the control treated with an ionophore to reduce membrane potential to zero. Dormant spores might have minimal membrane potential. To confirm this theory, more research would be necessary. Other methodologies explored have measured respiratory activity in spores. The principle behind this approach is that as spores germinate, they begin to show signs of respiratory activity (Laflamme et al., 2004). Respiratory activity and the signs of it might differ from organism to organism. The one disadvantage of all of the above flow cytometry techniques is that the effect of aerobic conditions on membrane permeability or membrane potential in anaerobic organisms is not known. Unfortunately, aerobic conditions might inhibit or stop esterase activity in germinated spores; therefore the CDFA/Hoecsht 33342

methodology is not applicable to anaerobic spore forming organisms. Respiratory activity would be abolished once aerobic atmosphere is attained.

1.11 Research objectives

Several sporadic blown packs were observed in VP meats stored at 2°C (Youssef et.al., 2014). The purges of these vacuum packs were monitored for the presence of *C. estertheticum* and other spoilage microflora. *C. estertheticum* was expected to be found on blown packs and sporadically on beef stored at 2°C. PCR analysis from the purge of several swollen packs was done to determine if *C. estertheticum* was present in packs.The second objective of this research was to determine the optimal pH and temperature range in which *C. estertheticum* can germinate in MJM. The effect of oxygen on germination and the effect of heating spores in the presence of germinants was also explored. The final objective was to identify germinants required by *C. estertheticum* spores. The overall purpose of the study was to determine optimal conditions required for germination of *C. estertheticum* spores in VP beef. To achieve this, spore germination was monitored at different pH values, temperatures and atmospheres. In addition, potential spore germinants were evaluated for their ability to stimulate germination of *C. estertheticum* endospores.

II. Materials and Methods

2.1 PCR analysis of purge collected from blown packs

The purge from 3 blown packs of VP beef was collected at different times during storage. The first 2 blown packs were obtained from Plant A and were opened after 37 weeks and 18 weeks of storage. Blown pack 3, was from Plant B and was opened after 16 weeks of storage. Purge from non-blown vacuum packs at analogous storage times was obtained from the same plants. pH of all purges was measured using an Accumet pH probe (Fisher Scientific, Ottawa, ON, Canada). All purge samples were centrifuged at 9,000 *x g* for 10 min. The pellet was re-suspended in 5 mL of 0.1 % peptone water and stored at -80°C. DNA was extracted from both sets of purge samples using the FastPrep Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). All procedures in the kit were followed; elution in 50 µL of elution buffer was done twice. Quantitative PCR(qPCR) analysis of the extracted DNA was done using *C. estertheticum* 16S rRNA gene primers: 16SEF and 16SER (Helps et al., 1999, Cavill et al., 2011) with the QuantiFast SYBR green PCR kit (Qiagen, Fisher-Scientific) and the Stratagene Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA, USA). Primers were obtained from Sigma-Aldrich (Mississauga, ON, Canada). Stock solutions of primers were first prepared at 100 µM. A ten-fold dilution of the stock solution was used as a working solution. Each reaction mixture contained 0.5 µL of a 10 µmol/L solution of each primer, 12.5 µl of SYBR green II master mix, 6.5 µL of sterile milliQH_2O and 5 µL DNA. For the negative control, sterile water was

added to the reaction mixture in place of DNA. The following PCR program was used: 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 10 min and extension at 72°C for 1 min. Cycle threshold (C_t) values were converted to log cell numbers/mL using the equation y= -3.538x +37.765 (Yang et al., 2014a). The standard curve was obtained by extracting DNA from several ten-fold consecutive dilutions of cells of *C. estertheticum* and performing qPCR. C_t values were then plotted against log cell numbers/mL. Each sample was analyzed in triplicate.

2.2. Spoilage microflora of vacuum packaged beef

As part of a previous study, VP boneless steaks obtained from a meat plant in Canada were stored for 0, 30, 50 and 70 d at 2°C. A 1000 cm^2 square surface on 3 steaks were sampled on each of the day(s) by rinsing with 100 mL of 0.1 % peptone water. A 10 mL portion of each rinse fluid was centrifuged to pellet cells, each pellet was then re-suspended in 1 mL glycerol supplemented tryptone soya broth (TSB). All suspensions were frozen and stored at -80°C. A 0.2 mL portion of each sample was used to prepare the first dilution in 0.1% peptone water. One half a mL of the first dilution was used for subsequent serial ten-fold dilutions up to 10^{-7}. The remaining 1.5 mL from the first dilution was used for DNA extraction. Portions (1 mL) of each of the dilutions was pour plated with Violet Red Bile Glucose (VRBG; Oxoid, Missisauga, ON, Canada) agar and incubated at 35°C for 24 h. Pink colonies with a halo on plates bearing 25-250 colonies were counted as Enterobacteriaceae. Portions (0.1 mL) of each of the dilutions were

also plated onto de Man Rogosa and Sharpe (MRS) agar and incubated anaerobically using 2.5 L Anaerogen sachets (Oxoid) at 25°C for 72 h and colonies were counted to enumerate the numbers of acid-tolerant lactic acid bacteria (atLAB). A modified version of this agar excluding sodium acetate was prepared using: 10 g proteose peptone, 10 g beef extract, 5.0 g yeast extract, 20.0 g dextrose, 1.0 g of Tween 80, 2.0 g of ammonium citrate, 0.1 g of magnesium sulphate, 0.05 g of manganese sulfate, 2.0 g of dipotassium phosphate and 15.0 g of agar (Difco, Becton-Dickinson, Sparks, MD, USA; Sigma-Aldrich). pH of the media was adjusted to pH 8.0 using 3N NaOH. Portions (0.1 mL) of dilutions were plated onto modified MRS media to enumerate total numbers of lactic acid bacteria (tLAB). These plates were incubated anaerobically using 2.5 L Anaerogen sachets at 25°C for 72 h. DNA extraction using the Fastprep kit followed by qPCR, as before, was done to quantify *C. estertheticum* present in all samples.

2.3 Preparation of spore suspension

Clostridium estertheticum subsp. *estertheticum* ATCC 51377 was used in this study. The organism was maintained and cultured in pre-reduced Reinforced Clostridial Medium (RCM; Oxoid). This media was dispensed into 15 mL Hungate tubes in 7 mL portions and made anaerobic by flushing with an oxygen free gas mixture of 10% H_2, 10% CO_2 and 80% N_2 (Linde Canada Inc., Nisku, AB, Canada). Cultures were incubated at 10°C. In approximately 3-4 days, when cultures were in the log phase (OD=0.60), 75 mL of anaerobic pre-reduced RCM in 160 mL serum bottles were inoculated with 1 mL of the culture. After

incubation at 10°C for 5-6 weeks, wet-mounts of cultures were prepared. Oil immersions of wet mounts were examined under a phase contrast microscope (Olympus, Tokyo, Japan) at 1000X magnification and spores were harvested when > 50% of the bacteria were phase bright. Cultures were centrifuged at 7,500 *x g* for 20 min, washed, and re-suspended in sterile 0.1% peptone water. This was repeated 4 times (X 4). Spore suspensions were heated at 75°C for 20 min to kill vegetative cells, centrifuged at 7,500 *x g* for 20 min, washed and re-suspended in sterile 0.1% peptone water (X 3). Multiple dilutions were plated after heat activation at 80°C for 10 min to obtain an accurate spore count. Portions (0.1 mL) from all dilutions were plated onto Columbia blood agar (CBA) plates (Oxoid) supplemented with 5% defibrillated sterile sheep blood. The plates were incubated at 10°C for 3 weeks in anaerobic jars with 3.5 L Anaerogen pouches (Oxoid). Colonies on plates with 25-250 colonies were counted to enumerate *C. estertheticum* spores. Spore suspensions were distributed into sterile eppendorf tubes in 1.5 mL portions and were stored at -80°C. For all fluorescence assays cultures were maintained and sporulated in pre-reduced peptone, yeast, glucose (PYG) prepared according to the procedure described by Lund et.al. (1990) using Difco materials. To minimize interference from media components in assays, starch was not added to the media. Thereafter, media was dispensed into 15 mL Hungate tubes in 7 mL portions, media was made anaerobic by flushing with an oxygen free gas mixture of, 10% H_2, 10% CO_2, and 80% N_2. Spores were harvested and enumerated using the procedure described above.

2.4 Meat juice medium

Meat juice medium (MJM) was prepared by pummelling 100 g of ground beef in 100 mL of distilled water in a stomacher bag fitted with a filter, for 2.5 min, using a stomacher operated at high speed. The stomacher fluid from each of the bags was drained into a flask (Yang et al., 2009b).The fluid was heated to 80°C and held for 3 min, and immediately cooled to < 30°C. The cooled fluid was filtered to remove large particles using multiple layers of cheese cloth. The pH of the filtrate was recorded or was adjusted when testing the effect of pH to 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 using 2N HCl and 3N NaOH. The media was filtered using a 0.22 µm membrane filter (Nalgene, Fisher Scientific) to give clear, sterile filtrate. Meat juice medium (7 mL) was dispensed into sterile Hungate tubes, and flushed with 10% H_2, 10% CO_2 and 80% N_2 anoxic gas mixture for anaerobic conditions. The media was stored at 4°C. Sodium thioglycolate (Sigma-Aldrich) was prepared at a concentration of 10% weight/volume in milliQ H_2O. The solution was filter sterilized using 10 mL syringes and syringe filters (0.2 µm pore size; Fisher Scientific). The sterile solution was dispensed into a 160 mL serum bottle, sealed and flushed with 10% H_2, 10% CO_2 and 80% N_2. It was stored at room temperature. Immediately before inoculation, 100 µl of the 10% anaerobic sodium thioglycolate was added to each tube of MJM. Temperatures averaged within 0.5°C of the specified temperature for all incubation temperatures. pH of MJM for all temperatures was confirmed to be at pH 5.90.

2.5 Inoculation of MJM

Aliquots of the spore suspension were thawed, centrifuged at 9,000 *x g* for 3 min and re-suspended in an equal volume of 0.1% peptone water. They were heat-activated at 80°C for 10 min. Sterile 1 mL syringes attached onto needles with a gauge of 23G X 1 inch (Becton-Dickinson) were used for all inoculations. They were rinsed with anaerobic MJM several times before being used to inoculate each tube of MJM with 1 mL of 10^6 cfu/mL *C. estertheticum* spores. Inoculated MJM was incubated for 72 h at 10°C. The different incubation temperatures used were at -1.5, 2.0, 6.0 or 10°C for 72 h. At 0, 1, 4, 8, 24, 30, 48 and 72 h, 100 µl aliquots from each of the cultures was withdrawn. From these aliquots 10 µl was used to differentially stain spores. Each experiment was repeated 3 times.

2.6 Wirtz-Conklin Stain

A modified differential spore stain referred to as the Wirtz-Conklin stain was used to distinguish spores from vegetative cells. As described by Hamouda et.al (2002), 10 µl from each aliquot was heat fixed onto glass slides (X 2). The slides were flooded with 5% aqueous malachite green (Sigma-Aldrich) and heated on a flame intermittently for 5 min while making sure that dye on the slide did not boil or dry. The malachite was washed off with distilled water after cooling the slides. The slides were then flooded with the safranin counter stain (Sigma-Aldrich). After 30 s, the slides were washed with distilled water. Slides were air-dried for approximately 30 min. An oil immersion of the each of the slides was

examined under an Olympus light microscope at 1000X magnification. Up to 4 images were obtained from each stain for a total of 8 images for each aliquot. The area of each rectangular image was consistent at 5.62 cm^2. All spores on images were counted manually using Image J software (National Institute of Health, Bethesda, Maryland, USA). Spores were distinguished manually by color, size and shape. Green spheres were considered to be non- germinated spores, pink spheres were germinated spores and pink rods were *C. estertheticum* vegetative cells. Only green spheres were counted.

2.7 Effect of heat on germinating spores

Anaerobic MJM with sodium thioglycolate was inoculated with 1 mL of 10^5 cfu/mL *C. estertheticum* spores in Hungate tubes as described previously. Aliquots (1 mL) were withdrawn from each tube (X 3). The first tube was heated at 80°C for 10 min, immediately after which two consecutive ten-fold dilutions were made in 0.1% sterile peptone water. From the second tube a 10^{-1} and 10^{-2} dilution were made and heated at 80°C for 10 min. The third tube was centrifuged at 9,000 *x g* for 3 min. After discarding the supernatant, the pellet was re-suspended in 1 mL of 0.1 % peptone water. Two consecutive ten-fold dilutions were made and all tubes were heated at 80°C for 10 min. Portions (0.1 mL) of each dilution and aliquot were plated on CBA plates supplemented with 5% defibrillated sheep blood (Oxoid, Mississauga, ON, Canada) and incubated anaerobically at 10°C for 3 weeks. Colonies on plates with 25-250 colonies were counted to enumerate the number of *C. estertheticum* spores. As described, spores

were either diluted from MJM in peptone water and then heated, or were centrifuged, re-suspended in peptone water, diluted and heated. No differences in log counts were found between the spore counts obtained from dilutions, in both of these treatments. These two methods were carried out to eliminate clumping as a factor for different counts. For simplicity purposes, these two methods were considered duplicates of the same treatment and are represented as heated in peptone water. Each experiment was replicated 3 times.

2.8 Preparation and inoculation of amino acid solutions

Leucine, lysine, histidine, arginine, and aspartic acid stock solutions were prepared at 100 times (100 X) the final concentration. Tyrosine, glutamine and glutamic acid stock solutions were 10 X the final concentrations because of their lower solubility. All amino acids used were obtained from Sigma-Aldrich and were dissolved in milliQ H_2O. All solutions were filter-sterilized using Nalgene bottles with a 0.2 µm membrane filter. 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris) was dissolved in milliQ H_2O, the pH was adjusted to 7.5. Thereafter, it was sterilized by autoclaving at 120°C for 20 min. Leucine, lysine, histidine, arginine, and aspartic acid were diluted 100 X in 20 mL of 20 mM Tris-HCl. Glutamine, glutamic acid and tyrosine were diluted 10 X in 20 mL of Tris-HCl buffer. The pH of all solutions was adjusted to 7.50 using 2 N HCl and 3 N NaOH before they were filter sterilized using 10 mL syringes and 0.2 µm syringe filters into clean, sterile, centrifuge tubes. The final amino acid concentrations were: 0.78 mmol/L L- leucine, 0.47 mmol/L L-lysine 0.21 mmol/L L-histidine, 0.18

mmol/L L-arginine, 0.07 mmol/L L-aspartic acid, 0.89 mmol/L, L-glutamic acid, 2.33 mmol/L L-glutamine and 0.19 mmol/L L-tyrosine. All other amino acids including asparagine, serine, glycine, threonine, alanine, tryptophan, valine, phenylalanine and isoleucine were combined to make one amino acid solution at a concentration of 70 X final concentration as done before. The final concentration of amino acids in this solution was : 0.22 mmol/L L-asparagine, 0.66 mmol/L serine, 0.88 mmol/L L-glycine, 0.51 mmol/L L-threonine, 2.89 mmol/L L-alanine, 0.08 mmol/L L-tryptophan, 0.30 mmol/L L-valine, 0.36 mmol/L L-phenylalanine and 0.41 mmol/L L-isoleucine. This aliquot was included for screening as one of the germinant solutions. Sodium lactate (4.50 M) was prepared and filter sterilized using a syringe filter. Lactate (200 µL) was added to 20 mL of each amino acid solution. The pH was adjusted to 7.50, and solutions were filter sterilized as described previously. Lactate was added to each of the amino acid solutions to test the effect of lactate in combination with amino acids on germination of spores. A 45 mM solution of pH 7.50 sodium lactate solution in buffer was used as a control. Meat juice medium of pH 7.50 and Tris buffer of pH 7.50 were inoculated with spores and used as a positive control and negative control for germination of spores, respectively. The pH was adjusted as described earlier. Each germinant solution (50 µl) was dispensed into eppendorf tubes (X 2). All tubes (closed) were left in the COY anaerobic chamber (Mandel Scientific, Guelph, ON, Canada) for > 2 h. Following this, solutions were inoculated in the anaerobic chamber with 150 µL of heat activated spores (10^6 spores/mL). One set of solutions was taken out of the chamber immediately and centrifuged at 10,600

x g for 4 min. Supernatants were stored at -20°C until used for the fluorescence assay. The second set of solutions was transferred to anaerobic jars with 3.5L Anaerogen sachets (Oxoid) and incubated at 10°C for 24 h, after which the solutions were centrifuged at 10,600 *x g* for 4 min. Supernatants were stored at -20°C for determination of DPA using the Terbium-DPA (Tb-DPA) fluorescence assay. Each experiment was replicated 3 times.

2.9 DPA fluorescence Assay

Terbium chloride ($TbCl_3$) was dissolved in milli Q H_2O to make a 2 µM $TbCl_3$ stock solution (Sigma-Aldrich). Filter sterilization was done using a Nalgene bottle with 0.2 µm membrane filter. This solution was diluted 10 X in pH 7.50, 20 mM Tris-HCl buffer. DPA stock solutions were prepared by dissolving DPA in 500 mL of milliQ H_2O in a volumetric flask to get a concentration of 1 mM. Final working solutions of DPA standards used were 0.5, 0.75, 1, 1.25, 2.5, 3, 4.5 and 5 µM.

For the Tb-DPA fluorescence assay, 150 µL of 200 nM $TbCl_3$ and 50 µl of supernatant (from samples) were dispensed into a 96 well plate (Nunc, Fisher-Scientific). Time resolved fluorescence was measured with excitation at 280 nm, emission at 545 nm, with a delay time of 50 µs and an interval of 1200 µs Ammann et.al., 2011). A gain of 1279 was used for all measurements. All fluorescence measurements were done using the PolarStar Omega Microplate Reader (Mandel Scientific).

Standard curves for DPA were generated by measuring the fluorescence of increasing concentrations of DPA at the same settings. The total DPA content of

spores was determined by autoclaving 150 µl of 10^6 *C. estertheticum* spores/mL in 50 µl of 0.1% peptone water at 121°C for 20 min. DPA released for each sample was calculated by subtracting RFU values obtained at 24 h from initial RFU values. The change in RFU for buffer was zeroed to obtain final change in RFU for all samples. Percent DPA released was determined using the Total DPA values obtained previously. Each sample was analyzed in triplicate.

2.10 Statistical analyses

All statistical analyses were done using Statistical Analysis System (SAS; Cary, North Carolina, USA). To determine if differences in numbers of *C. estertheticum* cells between swollen packs and non-swollen packs were significant, a one-tailed t-test was employed. A Tukey test was used to determine if differences between counts of atLAB and tLAB on vacuum packaged beef were significant. The same test was employed for the effect of storage time on counts of Enterobacteriaceace and LAB. The effect of time, pH and temperature on germination at different conditions was tested using ANOVA (Lowest Significant Determinant test). For the effect of oxygen on germination, a one tailed t-test was performed. A one tailed t-test was also used for the effect of heat on viability of germinating spores. Percent total DPA released was compared between MJM and all other conditions, lactate and all other conditions using ANOVA (Lowest Significant Determinant test).

III. Results

3.1 Analysis of purge from Blown pack and Non blown pack samples

Blown pack 1 and 2 were from Plant A and had been stored for 37 weeks and 18 weeks, respectively, before they were opened and the purge was analyzed. The first blown pack showed gross distention, surface of meat was slimy, cheesy sour odors were detected upon opening the pack. The second blown pack was moderately distended. Green discoloration and a slimy surface were observed but no pungent odors were detected. Sample 3 was obtained from Plant B and had been for stored for 16 weeks. Blown packs 1 and 3 had more than 5 log cells of *C. estertheticum* present in the purge (Table 1). Non-blown packs from the same sets (samples 4-6) had 2-5 log units lower numbers of *C. estertheticum*. The average log number of cells/mL of *C. estertheticum* in blown packs is higher than the average log number of cells/mL *C. estertheticum* in non-blown packs. Blown packs 1 and 3 had a slightly lower than normal surface pH at 5.3. Non blown packs for both samples had a pH of 5.4 and 5.9, respectively. The blown pack from sample 2 had a relatively higher surface pH than blown packs from other sets and the internal pH was also higher than normal. The non-blown pack sample from the same batch had a relatively normal surface pH of 5.6 and purge pH of 5.2 (Table 1).

Table 1 Estimated log numbers of *C. esterthethcium* found in swollen and non-swollen packs of vacuum packaged beef obtained from Canadian meat plants

Sample Number	Degree of pack swelling	Storage Time (weeks)	Log numbers/ mL	Purge pH	Surface pH	Internal pH
1	+++	37	5.4	5.1	5.3	5.3
2	++	18	1.8	5.7	6.4	6.1
3	+++	16	5.4	5.3	5.3	5.5
4	-	37	2.2	5.7	5.4	5.7
5	-	18	n.d.	5.2	5.6	5.9
6	-	16	3.1	5.8	5.9	5.5

n.d. below detection limit.
++ pack had a slackened look
+++ pack was tightly distended

3.2 Spoilage microflora on vacuum packaged beef

Sporadic BPS was observed in VP beef stored at 2°C. To investigate if *C. estertheticum* could be detected in these packs, major spoilage organisms were enumerated from the purge of VP beef stored at 2°C. At 0 d, the estimated log cfu/cm^2 for all types of gas producers was less than 1.5 log cfu/cm^2 (Table 2). Numbers of Enterobacteriacae and LAB were significantly higher at 30, 50 and 70 d. Most of the vacuum packs had little or no *C. estertheticum*. Two purge samples, 1 pack from 50 d and 1 pack from 70 d had 0.5 log cfu/cm^2 and 3 log cfu/cm^2 *C. estertheticum* respectively (Table 2). There were no significant differences found between numbers of atLAB and tLAB on the meat for any of the samples at any storage time (Table 2).

Table 2 Log numbers of Entertobacteriaceae, total aerobes, tLAB and atLAB and *C. estertheticum* in vacuum packaged beef stored at 2°C for 0, 30, 50 and 70 d

Days of Storage	Enterobacteriaceae (log cfu/cm^2)	Total Aerobes[3] (log cfu/cm^2)	tLAB (log cfu/cm^2)	at LAB (log cfu/cm^2)	*C. estertheticum* (log numbers/cm^2)
0[1]	< 2.0	2.5	1.30[a]	1.2[a]	-
30	4.3[b]	6.6	7.0[b]	6.9[b]	-
50	4.5[b]	7.3	7.9[b]	7.9[b]	0.5[2]
70	5.4[b]	7.7	8.3[b]	8.2[b]	3.0[2]

[1]Log values for 0 d are estimates
[2]At 50 and 70 d, *C. estertheticum* was found in only 1 of the 3 packs.
[3] Data obtained from a concurrent study (Youssef et.al., 2014)

3.3 Effect of pH on germination of *C. estertheticum* spores

Spores appeared to start germinating within 8 h of incubation for most conditions tested. 'Pink' spores were observed in many of the images (Figure 2). In addition, when decrease in spore count is plotted over time, we see that spores do not germinate at a constant rate throughout the 72 h.

The percentage of remaining spores decreased over the course of 72 h in MJM at all pHs inoculated with *C. estertheticum* spores. The standard error for three independent replicates ranged from 0% to 26.2 %. In MJM at pH 5.00, the percentage of remaining spores at 72 h was not significantly ($p > 0.05$) different from 0 h (Figure 3). A similar observation was made for MJM at pH 5.50. At 72 h, more than 60% of the spores had not germinated. It is also important to

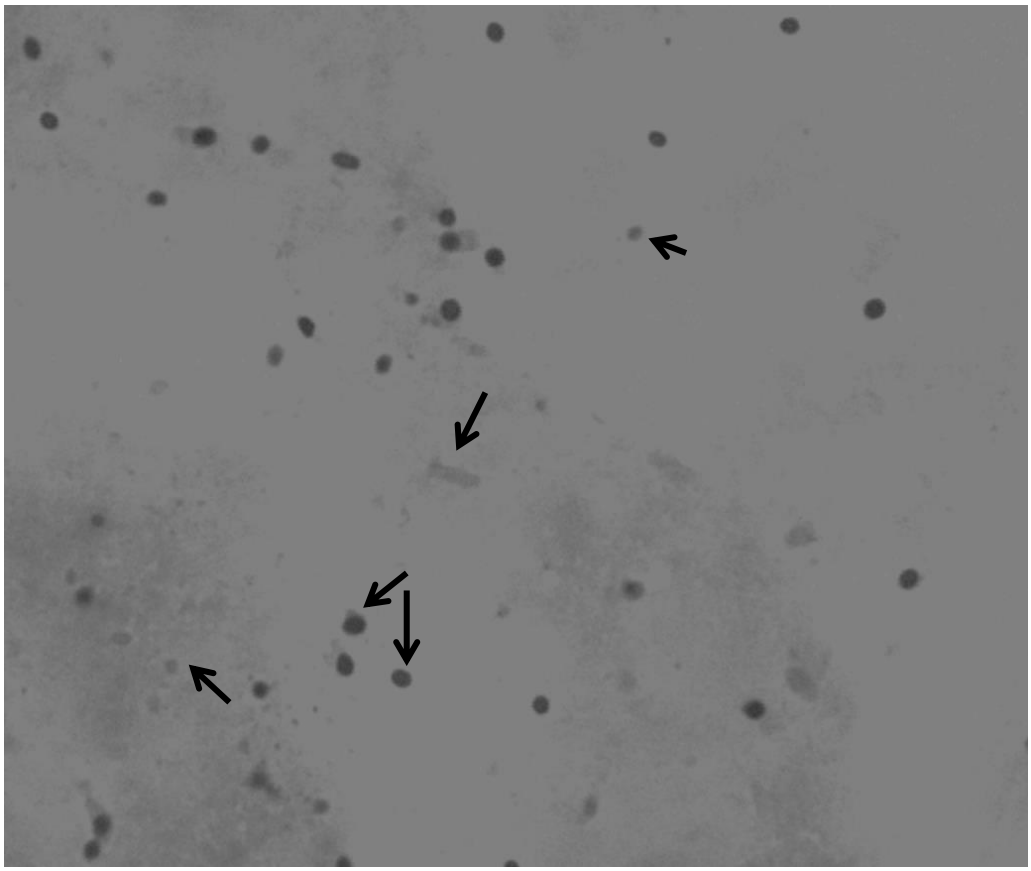
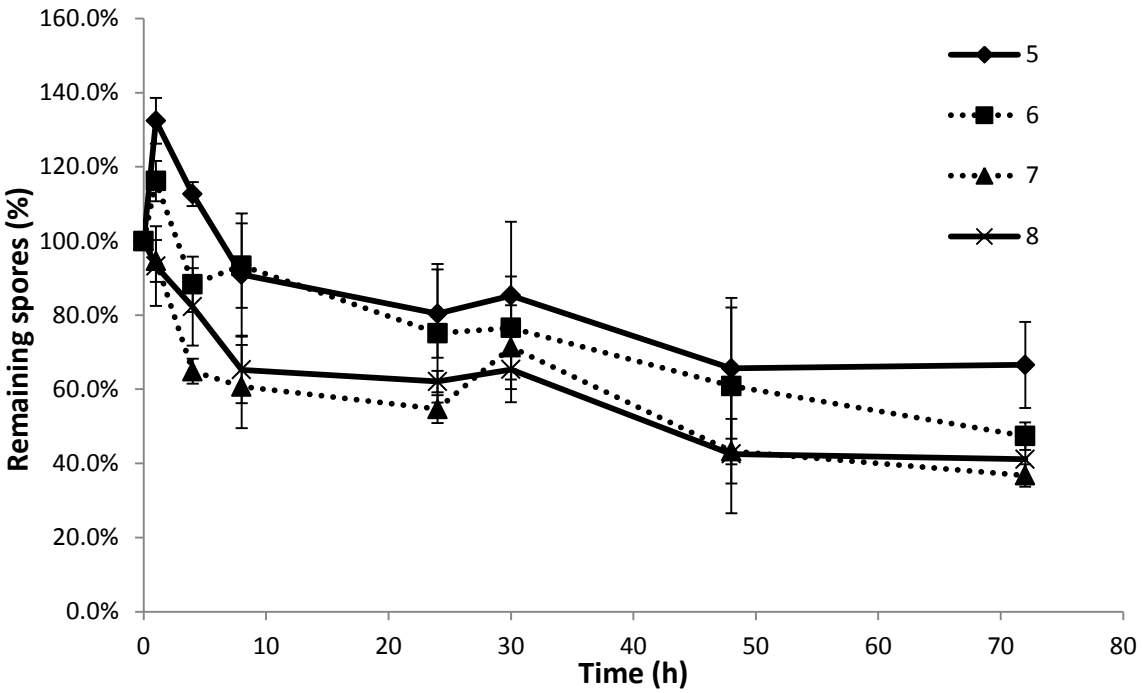


Figure 2. Wirtz-Conklin stain of *C. estertheitcum* spores. The arrows pointing to the grey circle indicate pink spores. The grey rod is an outgrown, vegetative cell. The black circles are stained *C. estertheticum* spores.

note that for both pHs, the percentage of remaining spores stabilized at 48 h and did not decrease any further. In MJM of pH 6.00 and 6.50 there was a significant decrease in spore count by 48 h. The percentage of remaining spores decreased most rapidly at pH 7.00. The percentage of remaining spores was significantly lower at 4 h compared to 0 h. This percentage further decreased from 60% at 4 h to below 40% at 72 h (Figure 3). Similar to pH 7.00, the number of remaining spores at 8 h for pH 7.50 was significantly ($p \leq 0.05$) different from remaining spores at 0 h and this count did decrease significantly from 8 to 72 h.



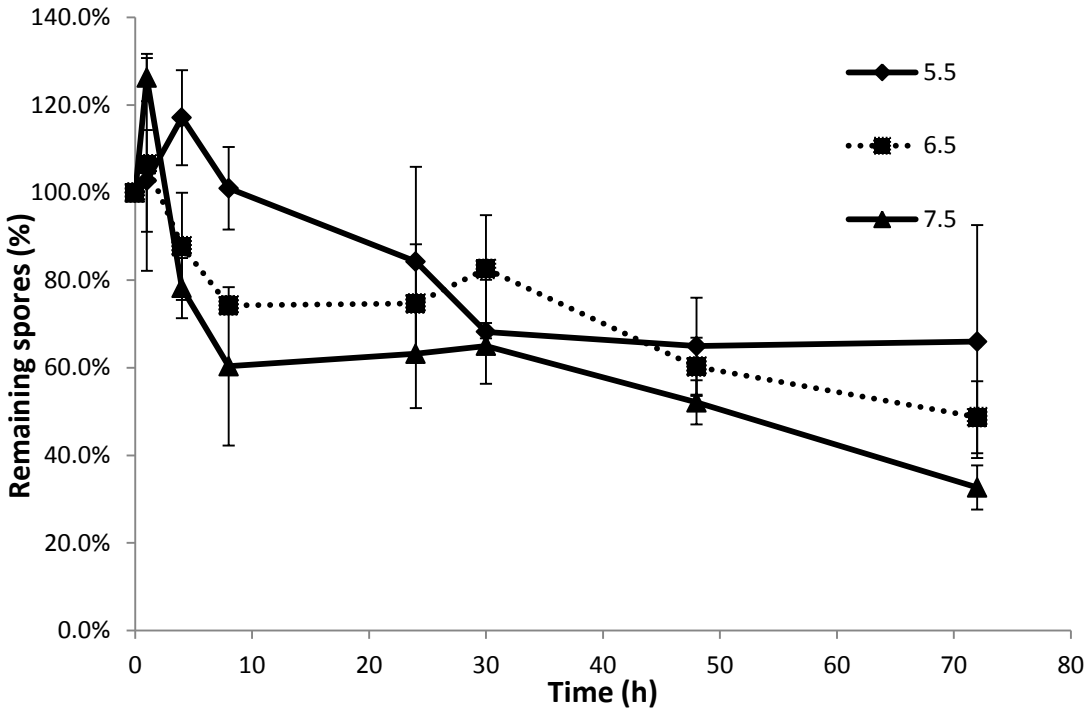


Figure 3. Effect of pH on the germination of spores of *C. estertheticum* when the spores in meat juice medium of pH 5.0, 6.0, 7.0 and 8.0 (A) and meat

juice medium of pH 5.5, 6.5 and 7.5 (B) Incubation was done for 72 h at 10°C. Standard error bars represent three independent replicates.

Less than 40% of the spores were remaining at the end of 72 h incubation (Table 3). Finally, in MJM of pH 8.00, spores germinated slower than at pH 7.00 and pH 7.50. At 8 h, the percentage of remaining spores was significantly ($p \leq 0.05$) less than at 0 h. The percentage of spores decreased to a little more than 40% in 72 h. At 4 h, the percentage of spores differed significantly among the various pHs. The number of remaining spores at pH 7.00 was significantly ($p \leq 0.05$) lower than the number of remaining spores at pH 5.50 (Figure 3).

As pH approaches neutrality, the rate of spore germination increases with the most optimal rate of germination at pH 7.00. Above pH 7.00, the rate either remained stable or decreased. The decrease in percentage of remaining spores for pH 5.00 and 5.50 was not significant ($p > 0.05$) whereas the decrease in number of remaining for all other pH was significant. At pH 7.00 and 7.50, the percentage of remaining spores further decreased after the first significant drop between 0 h and 8 h. This was not observed for any of the other pH values, where a significant decrease in the percentage of spores was either never observed, or was detected only once.

3.4 Effect of temperature on germination of *C. estertheticum* spores

To determine the effect of temperature on germination of spores, MJM inoculated with *C. estertheticum* spores was incubated at different temperatures. At -1.5 and 2°C, the number of remaining spores did not decrease significantly (p

> 0.05) in 72 h (Figure 4). At 6 and 10°C, the extent of spore germination was significant. At 6 and 10°C, a significant drop was observed in non-germinated spores by 30 and 24 h, respectively. For both temperatures, a steady drop in remaining spores was noticed up until 30 to 48 h, after which percentages remained stable. However, for all times tested, the percentage of remaining spores among temperatures did not differ. The number of remaining spores in inoculated MJM incubated at 6 and 10°C seemed to steadily decrease for up to 30 h after which the rate of decrease slowed considerably. The variation among the percentage of remaining spores was high.

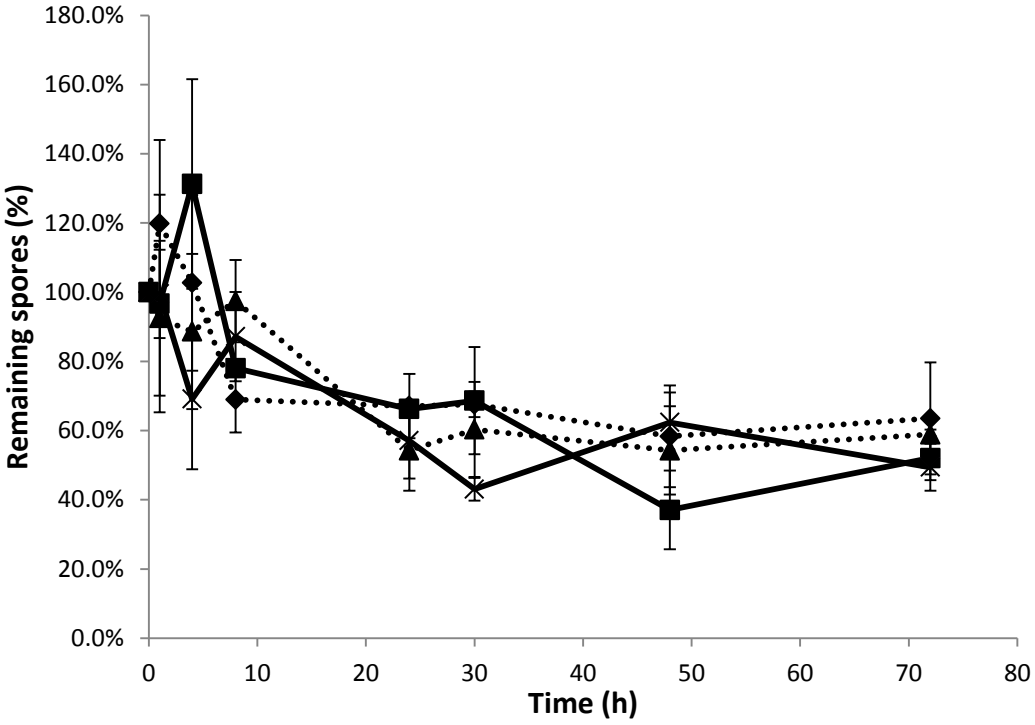


Figure 4. Percentage of remaining spores of *C. estertheticum* when the spores were incubated in meat juice medium at -1.5(♦), 2(■), 6(▲) and 10°C (X) for 72 h. Standard error bars represent three independent replicates.

3.5 Effect of oxygen on germination of *C. estertheticum* spores

The decrease in percentage of spores in aerobic and anaerobic MJM varies; however, this difference was not significant ($p > 0.05$) at any of the times (Figure 5). Variation among spore counts at different times is again high. Consistent with the observations in Figure 3, the spore count in anaerobic MJM decreased significantly ($p \leq 0.05$) by 30 h. Between 0 and 48 h incubation, the difference in the percentage of remaining spores for anaerobic and aerobic media was minimal. The extent of germination of *C. estertheticum* spores in an anaerobic MJM was much higher at 66% compared to an aerobic environment, where only 37% of the spores germinated over 72 h. As seen previously, the rate of decrease was higher between 0 and 8 h, after which the rate of germination decreased.

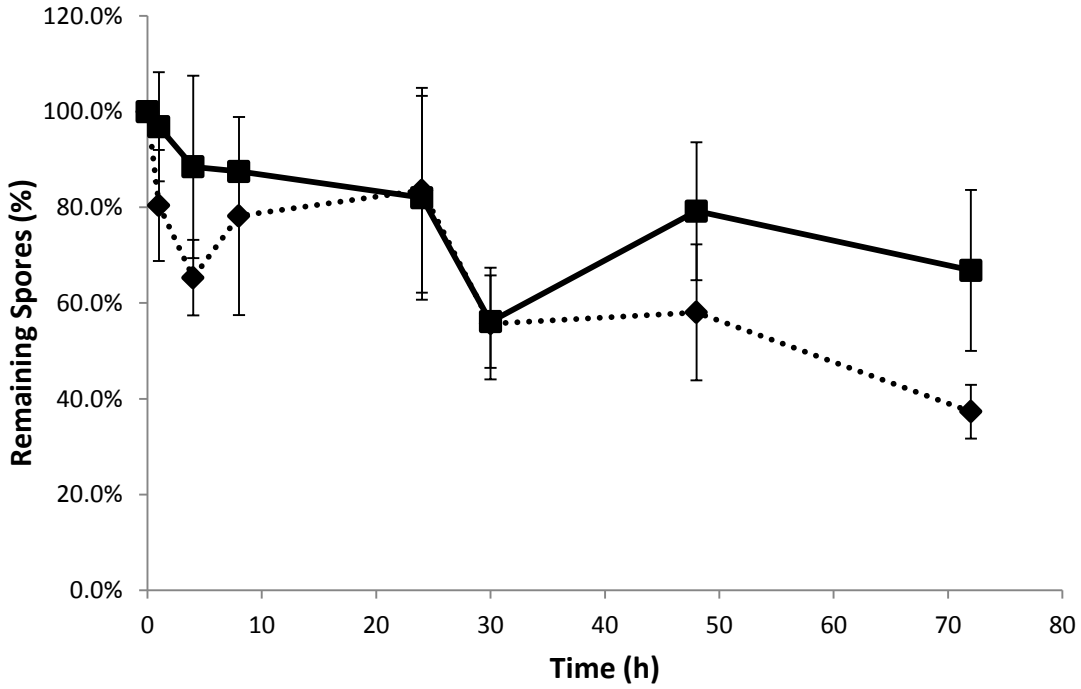


Figure 5. The effect of oxygen on germination of *C. estertheticum* spores in meat juice medium. Anaerobic (♦) and Aerobic (■) MJM were incubated at 10°C and the percentage of remaining spores was monitored for 72 h. Standard error bars represent three independent replicates.

3.6 Effect of heat on *C. estertheticum* spores

The spore count enumerated on CBA plates after heating *C. estertheticum* spores in MJM at 80°C for 10 min varied significantly ($p \leq 0.05$). from the spore count obtained on plates after heating in 0.1% peptone water at 80°C for 10 min (Figure 6). The difference in spore concentration between aliquots subjected to the two different treatments was about 0.7 log cfu/mL. This difference was significant as determined by a one tailed t-test ($p \leq 0.05$). Since the dilutions of MJM aliquots were made in peptone water, these dilutions were assumed to be peptone water aliquots when comparing treatments.

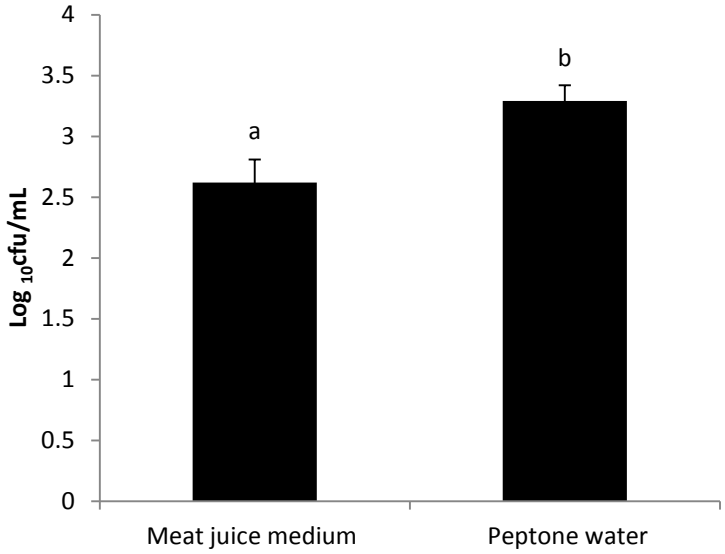


Figure 6. Log values obtained from heating spores (80°C for 10 min) in meat juice medium and in 0.1 % peptone water. Error bars are the standard errors for values from three independent replicates. Bars with different letters are significantly different ($p \leq 0.05$).

3.7 Germinants of *C. estertheticum* spores

To screen amino acids for their potential as germinants, three mixtures of amino acids were used. When using the Wirtz-Conklin stain to monitor germination in three germinant solutions: one containing asparagine, serine, glycine, threonine, alanine, tryptophan, valine, phenylalanine and isoleucine, a second solution containing, glutamic acid, glutamine and tyrosine, the third one containing leucine, lysine, histidine, arginine and aspartic acid, a decrease in percentage of remaining spores was observed in the second and third solution and not in the first one (Table 3). The amino acids in the second and third solution were then tested individually for their role in the germination of *C. estertheticum* spores. Dipicolinic acid fluorescence was used to monitor germination. The standard curve generated for increasing concentrations of the Tb-DPA complex against fluorescence showed a linear relationship (Figure 7). Total DPA released was between 50,000 - 60,000 RFU for 10^6 *C. estertheticum* spores. Spores released very minimal amount of DPA when incubated with individual amino acids. Less than 1% of total DPA was released in most germinant solutions with just individual amino acids (Figure 8). In germinant solutions with leucine and aspartic acid, the average % DPA released was about 1.5%. DPA from spores incubated in germinant solution containing

Table 3. Percentage remaining spores in various germinant solutions inoculated with *C. estertheticum* spores. Solutions were incubated anaerobically at 10°C for 144 h. n=1.

Time (h)	Glu, Gln, Tyr	Leu, Lys, His, Arg, Asp	Asp, Ser, Gly, Thr, Ala, Tyr, Val, Phe, Iso	MJM	Buffer
0	100%	100%	100%	100%	100%
24	91%	127%	100%	56%	101.%
48	71%	82%	105%	51%	136%
144	70%	39%	132%	27%	-

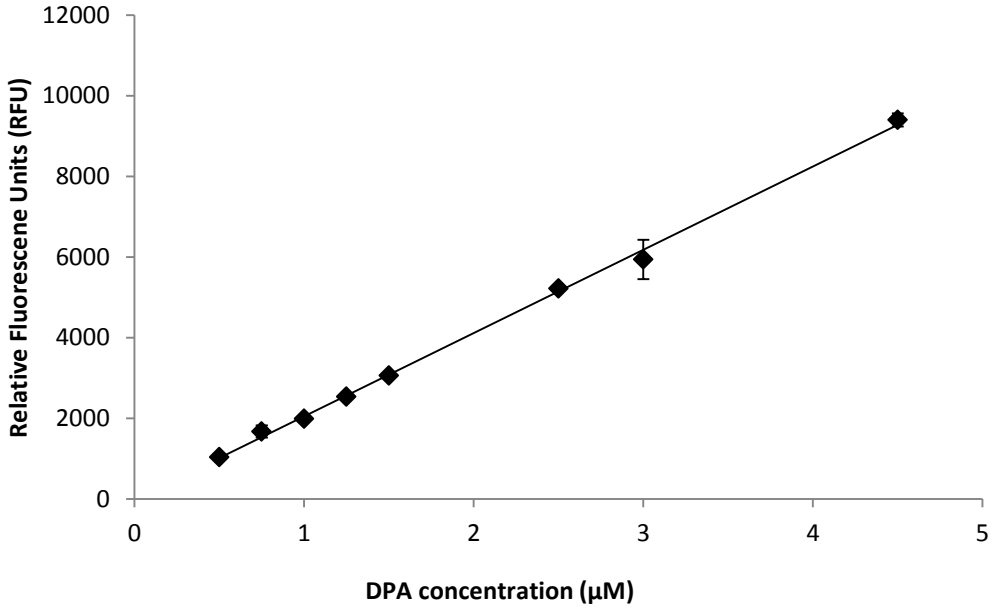


Figure 7. Standard curve for relative fluorescence of the Tb-DPA complex. The time resolved fluorescence of increasing concentrations of pyridine 2,6 dicarboxylic acid (DPA) in combination with terbium was measured as described in Materials and Methods. Standard error bars show values from three independent replicates.

'other amino acids' was also very low. 'Other amino acids' included asparagine, serine, glycine, threonine, alanine, tryptophan, valine, phenylalanine and

isoleucine combined into one germinant solution. This is consistent with results presented in Table 3.

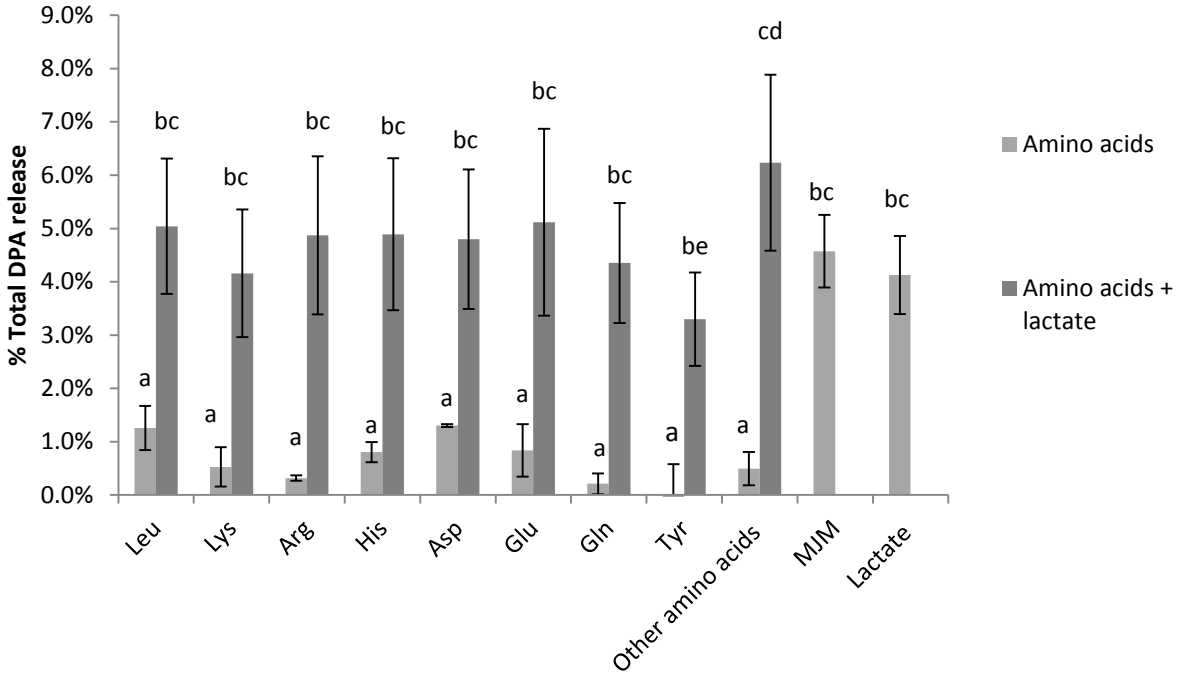


Figure 8. Percentage of total DPA released from *C. estertheticum* spores incubated in various amino acid solutions and amino acid + lactate solutions after incubation at 10°C for 24 h. All solutions had a pH of 7.50. Error bars are the standard errors for values from three independent replicates.

The addition of lactate to each of the amino acid solutions increased % DPA release significantly. DPA released from spores incubated in a solution of lactate was not different from spores incubated in MJM ($p > 0.05$). There was no significant difference among the % DPA released from spores incubated in lactate and spores incubated in any of the amino acid solutions with lactate. DPA released from spores incubated in amino acid and lactate and just in lactate were

similar to the amount of DPA released from spores incubated in MJM. The addition of lactate to a group of amino acids increased DPA release more than when lactate was added to individual amino acids. This difference was only significant when compared to DPA release observed in the tyrosine + lactate solution. Another important point to note is that DPA release was higher in some amino acid + lactate solutions when compared to MJM, even though the concentrations of amino acids and lactate used were very similar to the concentration of these amino acids in MJM.

IV. Discussion and Conclusions

In meat processing facilities, spoilage of meat products is controlled by using standardized sanitation practices and following HACCP requirements. Decontaminating treatments used during the carcass dressing process help decrease the bacterial load on the carcass (Gill, 2009). Altering the storage atmosphere of packaged beef can extend the shelf life of chilled beef (Gill and Newton,1978). However, due to their unique structure, spores can survive many harsh chemicals (Setlow, 2007). If present on VP beef, psychrophilic spores have the potential to germinate and grow in the pack when stored at refrigeration temperatures. This can lead to distension of vacuum packs thereby shortening the storage life of the product. Practices in meat processing facilities today are not sufficient to prevent BPS; therefore, it is crucial to understand the conditions required by organisms to cause BPS. A better understanding of the organisms involved in BPS might enable us to inhibit spoilage, thus maintaining the storage life of VP beef.

Vacuum pack spoilage is primarily caused by psychrophilic spore forming *Clostridium* spp., specifically by *C. estertheticum* (Cavill et al., 2011). *C. estertheticum* can also cause early BPS (Broda et al., 2003). Most anaerobic *Clostridium* spp., including *C. estertheticum* utilize glucose with the production of butyric and acetic acids and CO_2 (Hallenbeck and Ghosh, 2012; Yang et al., 2009a). After glucose is exhausted in MJM, the organism utilizes lactate to produce butanol, ethanol, formate, CO_2 and H_2 (Yang et al., 2009a). Presence of

C. estertheticum in blown packs was confirmed when analysis of blown packs and non-blown packs indicated that the presence of *C. estertheticum* was significantly higher in blown packs compared to non-blown packs. No discoloration or sulfurous odors were reported for the blown packs from which *C. estertheticum* was obtained. The pH value of most exudates from blown packs was low, potentially due to the production of lactate. Blown pack 2 had a high internal pH of 6.1, which is much higher than the normal pH of meat at around 5.5-5.7. In addition, the meat had green discoloration which is usually not caused by *C. estertheticum*. Enterobacteriaceae are commonly found on high pH meat, and after 4 months of storage, can produce gas in the vacuum pack (Gill, 2004; Brightwell et al., 2007; Chaves et al., 2012). When vacuum packaged lamb was inoculated with *Hafnia, Enterobacter, Serratia, Rehnella and Ewingella* spp. at 10^5 cfu/cm^2 and stored at 4°C, BPS was observed (Brightwell et al., 2007). LAB can also potentially cause BPS. Gas production was also detected in packs inoculated with LAB and stored at 1°C (Chaves et al., 2012). Therefore, distention in this pack was probably caused by Enterobacteriaceae or LAB. It is unlikely that this pack was distended as a result of gas production by *C. frigidicarnis*, which can produce gas in VP meat at later times during storage, however often produces offensive off odors which were not detected (Broda et.al., 1999). Some non-blown packs had low numbers of *C. estertheticum*. This suggests that the initial load of spores in some packs was not high enough to cause BPS. In addition, sporadic detection of *C. estertheticum* in packs stored at 2°C for 50 and 70d indicates contamination of vacuum packages with spores (Table 2).

Blown pack spoilage would likely occur in these packs if they had been stored for longer times. *C. estertheticum* were detected at 3 log numbers/cm^2 in one pack that had been stored for 70 d. Approximately 5 log numbers/mL is enough to cause pack distention (Table 1). Sporadic occurrence of BPS is probably due to the variable contamination of product with spores of *C. estertheticum* (Boerema et al., 2003). Spores can come from the soil attached to the hide or from equipment (Moschonas et al., 2011a). It can be concluded that *C. estertheticum* is present in blown pack spoiled beef. Gross distension of packs from growth of *C. estertheticum* was observed at 16 weeks of storage. It is important to point out that swelling of packs can happen as early as 5 weeks into storage (Yang et al., 2014a). Other psychrotrophic *Clostridium* spp. known to be associated with vacuum pack spoilage either produce gas in low quantities or at late times during storage (Yang et al., 2014b). To cause early BPS, the organism must be able to rapidly utilize lactate on meat after growth of organism ceases. The only psychrophilic *Clostridium* spp. capable of utilizing lactate in substantial amounts are *C. estertheticum*, *C. frigoris, C. aligidixyalanolyticum* and *C. frigidicarnis* (Yang and Badoni, 2013). *C. frigidicarnis* does not cause BPS at early times because it mostly produces CO_2. *C. frigoris* and *C. estertheticum* were the only organisms found to be capable of causing early BPS (Yang et al., 2014b). Cavill et.al. (2011) concluded that *C. estertheticum* was the most common cause of vacuum pack spoilage. The spoiled vacuum packs tested for *Clostridium* spp. other than *C. estertheticum* yielded very little or no PCR product. The rarity of finding other psychrophilic *Clostridium* spp. suggests that *C. estertheticum* is the

main spore forming organism causing BPS (Cavill et al., 2011). Here, we focused on studying germination of *C. estertheticum* spores. With knowledge of germination, ways to inhibit germination could be designed to prevent BPS.

Enumeration of spoilage bacteria on VP beef stored at 2^{o}C for 70 d revealed that maximum numbers of all gas producers was achieved by 30 d. Most often, spoilage flora on VP meat is dominated by *Carnobacterium* spp. at beginning times during storage (Yang et.al., 2009b). With time, pH of vacuum packs of beef decreases with the accumulation of lactate and other organic acids. Carnobacteria are inhibited at lower pH and due to this, atLAB such as *Leuconostoc meserentoides* form part of the major spoilage flora at later times during storage. However, this was not the case with the VP beef used in this study. Numbers of atLAB and tLAB are not different, suggesting that most LAB present on this particular set of VP beef were acid tolerant. To better understand the distribution of spoilage bacteria in these vacuum packs, PCR analysis or sequencing of isolates would have to be carried out. *Carnobacterium maltaromaticum,* a common organism found on VP beef has a similar growth rate to *C. estertheticum.* The absence of *Ca. maltaromaticum* on VP beef would increase glucose availability for *C. estertheticum* during earlier times in storage. This might allow numbers of *C. estertheticum* to increase substantially and therefore increase the chances of BPS. Further research would have to be done to determine if lower levels of acid intolerant bacteria on VP beef such as *Carnobacterium* spp. are increasing the chances of BPS. When higher numbers of *L. mesenteroides* were inoculated into VP beef with *C. estertheticum*, the

number of days until packaged swelled increased (Yang et al., 2011). This was also observed with other LAB. There seemed to be an association between large populations of *L. sakei* in VP meat and delay in gas production (Jones et al., 2009). The numbers of LAB on VP beef are consistent with the numbers of total aerobes found on VP beef during storage at 2°C (Youssef et.al., 2014). This is expected since the microflora of VP beef at chiller temperatures is dominated by LAB (Doulgeraki et al., 2012).

A large fraction of the spores in MJM seem to complete germination within 24 h. No major changes in percentage of remaining spores were observed after this time. This is the first report in the literature of a germination timeline for spores of *C. estertheticum*. Spore populations of non proteolytic *C. botulinum* completed germination within 4 h (Plowman and Peck, 2002). *C. frigidicarnis*, another psychrotrophic spore forming organism found on spoiled vacuum packs, also germinated within 24 h (Adam et al., 2011).

As the pH of MJM approached neutrality, the overall rate of germination of *C. estertheticum* spores increased with the highest rate observed at pH 7.00. At 4 and 8 h, significant differences in percentage remaining spores were seen between pH 5.50 and pH 7.00. This variability in rate of germination between 4 and 8 h incubation was not detected after 8 h. This suggests that pH affected the rate and not the extent of germination. Some spore-forming organisms such as *C. botulinum* can germinate outside of this pH range, but most can germinate at pH values between 5.5 and 7.0 (Paredes-Sabja et al., 2008b; White et al., 1974; Ishida et al., 1976; Plowman and Peck, 2002; Ramirez and Abel-Santos, 2010).

This implies that pH affects an event in germination that is universal among all spore forming bacteria. The most obvious event to be evaluated is DPA release. However, it has been shown in some *C. perfringens* species that DPA release is not critical for latter spore germination events (Paredes-Sabja et al., 2008a). Further research into mechanisms of spore germination is needed to better understand effects of pH on germination.

Studies on germination of single spores rather than a population of spores suggest that there is a large variation in germination times of individual spores. Non-proteolytic spores of *C. botulinum* have a mean germination time of 2.56 h; however, some spores take as long 12 h to germinate in the same environmental conditions (Stringer et al., 2005). This heterogeneity has been reported in several *Clostridium* and *Bacillus* spp. (Alberto et al., 2003; Wang et al., 2011; Pandey et al., 2013; Elliott and Schaffner, 2001). The high variation among replicates in percentage remaining *C. estertheticum* spores observed early in the incubation time was consistent with this phenomenon. With such a heterogeneous population, it is highly likely that different spore populations at the same pH would have a proportion of spores that germinated faster or slower. For most conditions tested, except pH 6.00, the rate of decrease of remaining spores slowed at some point after 4 or 8h suggesting that spores in the same population were germinating at different rates (Figure 3, 4 and 5). Some fractions of spores germinated much faster than other populations of spores. Another important point to note was that it is this initial rate that differed among pH values. This initial maximum rate was highest at pH 7.00 (Figure 3). Furthermore, germination

events and outgrowth events are not related to each other. Spores that germinate do not necessarily outgrow. In a population of *Bacillus* spores, some spores went from phase bright to phase dark but did not grow out (Pandey et al., 2013). Some spores rapidly transformed into phase dark spores and then divided to form a micro colony. A third group of spores remained phase bright for the entire duration. The authors also observed that initiation of germination (before spores start becoming phase dark) rather than germination time varied the most among spores. Other studies on germination of individual spores did not report time required for initiation of germination. Phase dark spores likely represent an intermediate stage between germination and outgrowth. These different stages have also been shown using the Wirtz-Conklin stain (Hamouda et al., 2002). When incubated in a solution that promoted germination but not outgrowth, Hamouda et.al. observed that *Bacillus* spores stained as pink spheres without completely transitioning into bacilli. In this study, pink spheres were observed in the images (Figure 2); however, these were not quantified. Heterogeneity in spore germination has been linked to the number of functional germinant receptors in individual spores; this number presumably varies significantly in spore populations (Zhang et al., 2010). Levels of activated germinant receptors in superdormant spores was found to be lower than in dormant spores suggesting that spores with less receptors germinate slower, thereby making them superdormant (Ghosh et al., 2012). Overexpression of germinant receptors on spores has been shown to increase the germination rate of spores (Cabrera-Martinez et al., 2003). Recently, it was reported that acidic environments increase

the time of germination of individual *Bacillus cereus* spores and the addition of sorbic acid caused spore germination to be more heterogeneous than in non-acid treated spores (den Besten et al., 2012). It is possible that spores inoculated in lower pH MJM took longer to initiate germination or to complete the germination process.

In addition to heterogeneity of spore populations, variability in counts of spores is expected when manually counting large numbers of spores. Ambiguity in determining color of spores might account for differences. Using cleaner spores would have helped with this problem. However, obtaining clean spores with psychrophilic clostridia is extremely difficult. Changing sporulation media is one way to do it. Using PYG media to sporulate might have resulted in cleaner spores; however, using PYG media would mean a lower yield of spores and longer sporulation times. Despite its downsides, malachite green staining proved to be a very useful, valuable technique in monitoring germination of *C. estertheticum* spores. A clear relationship between pH and spore germination was observed.

To determine which events in germination are affected by pH, earlier events in spore germination (such as DPA release) would have to be measured. If pH affects DPA release in a similar way, then pH probably affects earlier events in spore germination rather than latter events. Another important conclusion is that spore germination outside the pH range for growth is possible. *C. estertheticum* does not grow at pH 5.0; however, minimal germination was seen at this pH. Germination outside the growth range has been shown in other species as

well; this is expected since germination and outgrowth events do not seem to be related to each other (Pandey et al., 2013).

Since *C. estertheticum* spores germinate at a slow rate at a lower pH, a possible strategy to prevent BPS on meat would be to inhibit germination of spores present on VP meat. Incorporation of acid into the packaging might alter the pH of the meat enough to slow germination. This would have to be investigated before making any conclusions. The effect of pH on growth of *C. estertheticum* indicated that generation time was greater at pH 5.5 than at pH 7. Between pH 5.8 and pH 6.8, the generation time is similar (Yang et al., 2009b). Therefore, at a pH below 5.5, where growth of *C. estertheticum* stops and germination is very minimal, BPS can be prevented (Yang et al., 2014a). However, it should be noted that altering the pH of fresh meat is not easy; fresh meat has a very good buffering capacity. Spore germination and outgrowth of *C. perfringens* on cooked ground turkey roast was inhibited quite significantly through addition of buffered vinegar and lemon juice (Valenzuela-Martinez et al., 2010). Acetate has a pKa around 4.5 and at the normal pH of meat (5.5-5.7) there are enough undissociated ions to change the pH of meat and possibly inhibit germination and/or outgrowth.

Temperatures between -1.5 and 10°C did not affect rate of germination in *C. estertheticum* spores inoculated in MJM. The percentage of spores remaining was not significantly different at any of the time points tested. Even though there were no significant differences in rates of germination at different temperatures, spores numbers did go down significantly by 30 h at 6 and 10°C, whereas, spore

counts at 0 and 72 h were not significantly different for solutions incubated at -1.5 and 2°C. Spore germination in each of the populations was heterogeneous. The variation in percentage of spores remaining at different times was much higher at lower temperatures compared to higher temperatures. This was also observed in *C. botulinum* type B strain Eklund 17B spores, where a much broader distribution of germination times was observed when spores were germinated at low temperatures rather than high temperatures (Stringer et al., 2009). Similar trends were noted for *C. botulinum* 56 A spores (Chea, 2000). The high variation makes it much harder to identify differences among temperatures; nonetheless, it can be concluded that for the temperatures tested, *C. estertheticum* spore germination is not affected by temperature. Previous literature indicates that temperature does have an effect on spore germination in most species. For most spores, there is an optimal range of temperatures at which spores germinate rapidly. Outside of this range spore germination is much slower (Plowman and Peck, 2002; Paredes-Sabja et al., 2008b). This was not found with *C. estertheticum* probably because only temperatures between -1.5 and 10°C were tested. If temperatures above 10°C were included, similar results would be expected. Temperatures above 10°C were not investigated because vacuum packs of meat cannot be stored at temperatures above 10°C; this would lead to earlier spoilage from the growth of other organisms. Vacuum packs inoculated with *C. estertheticum* ssp. *estertheticum* stored at cooler temperatures do take longer to distend (Moschonas et al., 2009, Clemens et al., 2010). The growth rate of *C. estertheticum* increases constantly between -1.5 and 10°C in MJM; therefore, this delay in BPS might be because of

slower growth rates at lower temperatures (Yang et.al., 2009b). Since we have demonstrated here that the rate of spore germination of *C. estertheticum* is not affected by temperature, the delay in BPS observed by others is perhaps due to an effect of temperature on growth of cells or outgrowth of spores, rather than on germination. The mechanisms involved in outgrowth are completely unrelated to germination and could be affected by temperature. Lowering the incubation temperature did have a proportionally greater effect on outgrowth and doubling time than on germination time of non-proteolytic *C. botulinum* spores (Stringer et al., 2009).

One would expect that *C. estertheticum* spores would not germinate in aerobic conditions since they cannot grow in oxygen containing atmospheres. However, according to the literature and results obtained in this study, there was no significant difference in spore count at different times in aerobic MJM inoculated with *C. estertheticum* spores and anaerobic MJM inoculated with *C. estertheticum* spores (Wheeldon et al., 2008; Plowman and Peck, 2002). The rate of germination might be slower in aerobic MJM as there were no significant differences between spore counts at 0 and 72 h in aerobic MJM whereas in anaerobic MJM, spore counts were down significantly by 30 h. Between 24 and 72 h the difference between spore counts in aerobic and anaerobic conditions also gets greater. This implies that the rate of germination in aerobic conditions slows down between 24 and 72 h in comparison with anaerobic conditions where spore counts reduce steadily. If spores can germinate aerobically and lose their resistance properties, they can be germinated and then killed on meat. Spores

inoculated in aerobic media probably lose all of their resistance properties upon germination. The initial decrease in spores is observed within 4 h, implying that spores lose their resistance properties within 4 h of incubation in MJM. No difference between germination under aerobic and anaerobic conditions was found for spores of *C. difficile, C. perfringens* or non proteolytic *C. botulinum* (Wheeldon et al., 2008; Plowman and Peck, 2002; Paredes-Sabja et al., 2008b). Furthermore, there was a significant difference in viable spore count after spores were subjected to heat treatment in MJM and water. The difference in viable spore count was close to 1 log cfu/mL, which suggests that a large fraction of the spores become sensitive when heated in MJM within 10 min. The spores themselves are not sensitive to heat because if they were, similar counts would have been obtained from spores heated in 0.1% peptone water. MJM is rich in germinants and it seems likely that a fraction of the spores initiated germination in MJM and were sensitized to heat treatment. For *C. difficile* spores, it was recently reported that when spores in germinant solution were heated, the log reduction was significantly greater when compared to spores that were heated in water (Nerandzic and Donskey, 2010). Germinated *C. difficile* spores were sensitive to heat and UV-C radiation but not to ethanol. This was shown with 4 different strains of *C. difficile*. The log reduction values ranged from 0.9 log cfu/mL to 1.5 log cfu/mL, which was close to what was found in this study. Another study by the authors confirmed this phenomenon in *C. difficile* (Nerandzic and Donskey, 2013). They also reported that heat activation of spores increased log reduction of spores heated in germination medium to 2.5 log cfu/mL, which is much higher

than what was found in the current study with heat-activated spores of *C. estertheticum*. Preliminary work done with spores of *C. estertheticum* indicated that heat shock of spores did not have an effect on germination (Yang, unpublished data). It can be concluded from the current investigation that a fraction of *C. estertheticum* spores initiate germination in a few minutes, and this initiation of germination makes spores sensitive to heat. This is contradictory to reports that have suggested that heat shrinking of vacuum packs reduces the time to spoilage by *C. estertheticum* (Bell et al., 2001; Moschonas et al., 2011b). A hot water wash done on primal cuts before vacuum packaging did not decrease time to spoilage as compared to a cold water wash (Adam et al., 2013). If heating for 4-5 seconds increases germination rate, a decrease in time to spoilage should have been observed. On the contrary, time to spoilage was increased because washing with water mechanically removed spores from the surface of meat. It is unclear how long initiation of germination takes in spores of *C. estertheticum*, though, for a fraction of the spores this time is less than 10 min, when spores are heat activated. If *C. estertheticum* spores can initiate germination aerobically within 10 min, many new avenues for inhibition of BPS are opened up. Tyndalization of meat could be one way of killing germinated spores if spores initiate germination quickly. It is important to understand that tyndalization of fresh meat could lower the quality of meat as heat applied to fresh meat for prolonged times will denature muscle proteins. Since spores initiate germination aerobically, this could be done before vacuum packaging, thereby decreasing the initial load of spores and increasing the time until BPS occurs.

Using the DPA-terbium fluorescence assay, germinants used by *C. estertheticum* spores were identified. Germinants of *C. estertheticum* have never been previously reported in the literature. Germination studies on *C. frigidicarnis*, another psychrotrophic organism revealed that spores germinated most optimally in amino acid and lactate mixtures (Adam et al., 2011). The same phenomenon was observed in non proteolytic *C. botulinum* spores, where spores did not germinate with individual amino acids and germination was significantly enhanced when lactate was added to the amino acids (Plowman and Peck, 2002). Similar observations were made for *C. estertheticum* spores. However, one major difference was that *C. estertheticum* spores also germinated in the presence of lactate. This was not observed with *C. botulinum* spores, where the authors reported minimal germination (<10%) when spores were incubated in L-lactate. Germination with just L-lactate was not tested in *C. frigidicarnis* spores (Adam et.al., 2011). However, L-lactate was reported to be an essential germinant and no pairs of amino acids induced germination of spores like L-lactate/amino acid combinations did. Amino acids were not a requirement for *C. estertheticum* spore germination; germination with L-lactate alone resulted in similar amounts of DPA release (Figure 8). Meat is an abundant source of lactate and it is well established that *C. estertheticum* spores germinate in meat (Yang et al., 2011; Yang et al., 2014a). Therefore, germination of *C. estertheticum* spores using lactate as the major germinant seems logical. It is highly probable that *C. estertheticum* spores germinate with other germinants or germinant mixtures that were not tested in this study. Preliminary studies found that spores germinated in mixtures of L-leucine,

L-lysine, L-histidine, L- arginine and L-aspartic acid and a second mixture of L-tyrosine, L- glutamine and L-glutamic acid (Table 3). Further screening involving pairs of amino acids might reveal additional nutrient co-germinants. Co-germinants have been reported in many *C. difficile* strains. Histidine acts as a co-germinant when germination is carried out in bile salts and glycine and bile salts and glycine themselves have been described as co-germinants (Wheeldon et al., 2011, Sorg and Sonenshein, 2008).

Comparison with other studies indicates that % Total DPA released during germination of spores reported in this study is much lower than what has been reported in the literature. There are several different ways of measuring DPA concentration in a particular sample. One study measured DPA remaining by determining the optical density of the supernatant at 270 nm (Paredes-Sabja et al., 2008b). Other studies use colorimetric assays to measure DPA concentrations (Vepachedu and Setlow, 2007). Measuring total DPA content of spores accurately requires approximately 10^4 spores/mL making it far more sensitive than measuring optical density or using a colorimetric assay (Yang and Ponce, 2009; Kort et al., 2005). The Tb-DPA assay has been used extensively to identify germinant receptors, and to determine the role of proteins involved in germination (Butzin et al., 2012). A higher concentration of spores would be required for the colorimetric and optical density assays, which was not available in the current study. Nevertheless, even with the differences among techniques, % Total DPA release during *C. estertheticum* spore germination seems to be too low. When wild type *C. perfringens* spores were germinated with potassium ions, more than

80% of the total DPA was released within 60 min. Similar observations were made when spores were germinated with dodecylamine (Paredes-Sabja et al., 2008b) Both dodecylamine and potassium ions are non-nutrient germinants. Since non-nutrient germination of spores follows different mechanisms, DPA released might not be comparable to DPA released when using nutrient germinants (Paredes-Sabja et al., 2011). When the same spores were germinated in 100 mM L-asparagine, % DPA released was almost half at close to 45% in 60 min. In addition, monitoring percent loss in OD when incubating in decreasing concentrations of L-asparagine, it was found when using 10 mM L-asparagine as opposed to 100 mM L-asparagine, % germination was significantly lower (Paredes-Sabja et al., 2008b). Other studies measuring DPA release have also reported low % total DPA release, when using lower concentrations of germinants. In *Bacillus subtilis* spores, only 21% of the total DPA was released in 6 h when spores were incubated in 1.5 mM of each component of AGFK. Forty one percent of the total DPA was released when spores were incubated in 0.3 mM L-alanine (Yi and Setlow, 2010). The concentrations of amino acids used in this study were much lower; as they were based on the concentrations of components in MJM. It is possible maximum DPA release or germination is not achieved in MJM. Previous results indicate that 50% of spores germinate in MJM in 24h (Figure 3). It is conceivable then that concentration of germinants in MJM is not sufficient for all spores to germinate. Since the overall objective was to identify germinants used by *C. estertheticum* spores to germinate on meat, it was logical to use concentrations of amino acids similar to MJM. It should be noted that in this

study, % DPA released during germination was reported. Release of DPA upon heat inactivation of spores has been shown previously in *Clostridium* spores (Hofstetter et al., 2013). It is possible that this is the case for *C. estertheticum* spores, where germination is initiated very quickly. This could have decreased % Total DPA released values. However, to avoid background fluorescence from media components, it was more accurate to measure change in fluorescence in 24 h rather than reporting fluorescence at 24 h. There might be a number of explanations for the low % total DPA release observed for *C. estertheticum* spores. Further research into germination of *C. estertheticum* is obviously necessary. Since the percent total DPA released from spores in germinant solutions was comparable to DPA released in the positive control, MJM, the results are valid and can be used to draw conclusions. It can be concluded that in meat, lactate is one of the main germinants used by *C. estertheticum* spores.

To determine if inhibition of germination can lead to delay in BPS, additional research is necessary. There are several strategies that can be explored to prevent BPS. Lowering the pH of meat cuts before vacuum packaging would inhibit germination and growth of *C. estertheticum* on meat. An acetic acid spray or a lactic acid spray might be able to lower the surface pH of meat to below 5.5, thereby, inhibiting growth and germination. The pack of meat with a pH of 5.6 was swollen at 35 days of storage when inoculated with 100 spores/mL of *C. estertheticu*m (Yang et al., 2014a). The buffering capacity of fresh meat is very high; this strategy of inhibition would only work if meat surface pH can be altered. If pH of meat is not low enough to inhibit growth, *C. estertheticum* will

grow faster on low pH meat because glucose concentration of low pH meat is much higher than high pH meat (Immonen and Puolanne, 2000). Another avenue worth exploring to inhibit BPS is to initiate germination and then kill germinated spores. Since more than 40% of spores germinate in MJM within 24 h and lactate is the major germinant of spores, adding lactate salt to the meat surface, vacuum packaging and storing at chiller temperatures for > 24 h, opening up vacuum pack, tyndalizing the meat and/or putting it through a hot water wash before re- packing it would possibly delay BPS. Adding lactate salt would hopefully increase the rate and extent of germination of spores. Tyndalizing the meat after initiation of germination will, in theory, kill germinated spores. Tyndalizing and hot water washes on cuts of meat before packaging has been tried recently (Adam et.al., 2103). A cold water wash worked as well as a hot water wash in delaying gas production and initiation of germination before hot water washes did not significantly change days until spoilage. Increasing time of the wash from 4-5s to at least 30s -1 min would increase the efficacy of the heat treatment. It is critical to point out that extensive heat treatment of muscle tissue will denature protein and lower the quality of meat. Germination of spores and subsequent inactivation using high hydrostatic pressure (HHP) and heat has been used to inactivate *C. perfringens* on meat (Akhtar et al., 2009). Cleaning of equipment in the meat plant with a germinant solution containing amino acids and lactate, with subsequent decontamination might help get rid of spores in the plant. Since many spores are transferred from the hide such a decontaminating treatment on the hide may also prove beneficial. This has been tried with *Bacillus* spores on food

processing equipment. Around 3 log units of the spores germinated in the germinant mixture used and were subsequently killed using standard decontamination protocols (Hornstra et al., 2007; Abee et al., 2011). Another viable approach is inoculation with acid tolerant LAB such as *L. mesenteroides* or *L. sakei* before vacuum packaging. This has been shown to work previously (Yang et al., 2011; Jones et al., 2009). Increasing the initial loads of LAB would increase the chances of spoilage from LAB; however, spoilage from LAB usually does not result in any foul odors so effects of this should be minimal. Addition of bacteriocins or bacteriocins producing organisms that inhibit Gram positive bacteria would perhaps inhibit the outgrowth of germinated spores on meat (Ferrocino et al., 2013; Ho, 2009). All of these approaches would have to be tested before they could be applied in a processing facility. A combination of inhibition strategies might have to be used to control blown pack spoilage. These designs have to be tested out before any conclusions can be made. Inhibition of germination in media might not lead to inhibition on meat.

In conclusion, this study has provided some valuable information regarding germination of *C. estertheticum* spores. This knowledge could be very useful when trying to prevent early BPS. pH had a significant effect on germination of *C. estertheticum* spores in MJM. Percent spores remaining at earlier times during incubation differed substantially among the various pH values of MJM. The effect of temperature on germination is not significant. There was no difference in germination rates when spores were germinated at -1.5, 2, 6 and 10°C. Surprisingly, spore germination in aerobic and anaerobic environments did

not differ. Heat shock at 80°C killed germinating spores within 10 min. It has previously been suggested that heat shrinking of vacuum packs initiates spore germination and possibly induces BPS (Bell et al., 2001). Current data were not consistent with this hypothesis, which suggest that heating spores in MJM might actually kill a fraction of spores that germinate immediately and delay BPS. The findings of this study raise doubts about the effect of heat shock on BPS. Lactate was one of the main germinants of *C. estertheticum* spores in meat. Spores germinated in amino acid/lactate germinant solutions. There were no differences among the pairs except for 'other amino acids + lactate' germinant solution and 'tyrosine + lactate' solution. The reason for this variation is unknown. Further studies are necessary to understand germination of *C. estertheticum* spores, and to inhibit BPS.

V. References

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