“Blown-pack” Spoilage of Beef

by

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Abstract

"Blown-pack" spoilage of beef by psychrotrophic *Clostridium* spp. occurs sporadically and resistance of the endospores to most interventions makes them very difficult to control. Current interventions used to control spoilage and pathogenic bacteria, such as acid washes and heat treatments, do not inactivate the endospores and do not control "blown-pack" spoilage. Research was done to identify bacteria isolated from "blown-pack" spoiled beef. The heat and pressure resistance of psychrotrophic *Clostridium* spp. isolated from "blown-pack" spoiled beef was determined and the impact of antimicrobials on heat and pressure resistance was assessed. Presence of small acid soluble (SASPs) proteins was detected because of their role in heat resistance. Genomes of psychrotrophic *Clostridium* spp. were sequenced to confirm identity and to confirm the presence of genes that encode for SASPs.

Psychrophilic and psychrotrophic *Clostridium* spp. were isolated from the meat and purge of a vacuum packaged beef loin that had evidence of "blown-pack" spoilage. During isolation of bacteria, the meat samples and cultures were kept under anaerobic conditions, and organisms were cultivated on rich media under anaerobic conditions at 7°C. Presumptive identification of anaerobic organisms by species-specific PCR and sequencing of the 16S rDNA confirmed them to be *Clostridium estertheticum* and *Clostridium putrefaciens*. HPLC analysis of volatile compounds in the purge detected the presence of volatiles. The strain of *Cl. estertheticum* isolated was only able to grow at

temperatures below 10°C whereas the strain identified as *Cl. putrefaciens* was able to grow at 7 to 21°C.

When *Clostridium*endospores were heated to 90°C under aerobic conditions, no survivors were detected; however, when endospores were heated under anaerobic conditions they survived heating at 70°C for 8 min. Endospores of a strain of *Cl. estertheticum* were able to survive heating to 90°C for 4 min under anaerobic conditions. Small acid soluble proteins, which are critical for heat resistance of endospores, were detected in endospores of cold-tolerant *Clostridium*.

Combination of high hydrostatic pressure (HHP) at 400 MPa and heat (4, 40 and 70°C) increased inactivation of *Clostridium*endospores; however, additives altered the efficacy of HHP. When endospores were subjected to HHP in meat with the antimicrobial preparation Micocin X™, endospores survived 15 min of pressure at 4°C, whereas without the Micocin X,endospores of *Clostridiumestertheticum* BP09-13 and ATCC 51377 were inactivated at 8 min.Similar results were observed when the supernatant of a strain of *Carnobacterium maltaromaticum* or APT were added to the meat prior to pressure treatment. To determine if the presence of peptidoglycan could account for the increased survival, peptidoglycan from *Bacillus* spp. was added to saline containing endospores and samples were subjected to pressure treatment. In this case, the addition of peptidoglycan increased the inactivation of the endospores; thus peptidoglycan is not responsible for the protective effect observed with the addition of Micocin X™.

The genomes of the 3 isolates from this study were sequenced. This was the first time genomes of *Clostridium* spp. isolated from "blown-pack" spoiled beef there sequenced.Comparison of the genomes of the two strains of psychrophilic *Cl. estertheticum* and the strain of psychrotrophic *Cl. putrefaciens* revealed that the *Cl. estertheticum* had a larger genome than *Cl. putrefaciens*. The strain that was presumptively identified by 16S rDNA sequencing as *Cl. putrefaciens* was closely aligned to *Clostridium algidicarnis* and was thus renamed. Annotation of the genes indicated that nearly half of the genes present were hypothetical for all three genomes analyzed. Analysis of the genomes of the strains of *Cl. estertheticum* revealed 16 single nucleotide polymorphisms in transport proteins. The presence of genes that encode small acid soluble proteins was confirmed in *Cl. estertheticum* and *Cl. algidicarnis*.

Current interventions used in industry are insufficientto prevent the occurrence of "blown-pack" spoilage in vacuum packaged beef,therefore the organisms responsible for "blown-pack" spoilage continue to be a problem for beef producers.Combinations of pressure and antimicrobial preparations did not increase activation, but instead increased survival of the *Clostridium* spp. Despite unsuccessful control of*Clostridium* spp. in meat, genomic information could identify key weaknesses in the life cycle or structure of the endospore to target. Further research and analysis of the genome could reveal the answers required to prevent the sporadic occurrence of "blown-pack" spoilage of vacuum packaged meats.

Preface

Chapter 4 was conducted in collaboration with Dr. Ryan Mercer. Dr. Mercer is acknowledged for his contribution to genome annotation.I was responsible for all other analysis as well as the manuscript composition.

Dedication

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."
Marie Curie

I dedicate this work to my son and any future children. Don't ever give up on your dreams. Even when the road is long and hard and when things don't work out the way you think or want, it is worth it when you get there.Thank you to my loving husband, Richardson Mah, for all the support during my time as a graduate student. For all the tears, frustrations and long nights, you were there and I couldn't have done it without you. Thank you to my mom and dad for laying the foundation and instilling the love for education and learning. It truly is something that nobody can take from me. Thanks dad for showing me that it's never too late to finish.

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List of Abbreviations

ANOVA – analysis of variance

APT – All Purpose Tween

ATCC – American Type Culture Collection

ATP - Adenosine triphosphate

B. – *Brochothrix* or *Bacillus*

BP – "blown-pack"

bp – base pair

C. – *Carnobacterium*

ca. – approximately

Ca^{2+} - Calcium ion

Cl. – *Clostridium*

CFU – colony forming units

dH_2O – distilled water

DPA - Pyridine-2-6-dicarboxylic acid (dipicolinic acid)

En – enriched environmental sample

ERP – enriched purge

Fig – figure

HHP – high hydrostatic pressure

HPLC – high performance liquid chromatography

LAB – lactic acid bacteria

LacP – Lacombe purge culture

MAL - muramic acid-δ-lactam

μL – microliter

MPa – megapascal

n – number

N – normal

NCBI - National Center for Biotechnology Information

ND – not determined

nGR – non germinant receptor

P. – *Pediococcus*

PCA – plate count agar

PYGS – peptone yeast glucose starch

R - ramp

RCM – reinforced clostridial media

RFLP – restriction length polymorphism

RNA - Ribonucleic acid

SASP – small acid soluble protein

SDS-PAGE - Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SNK - Student–Newman–Keuls

SNP – single nucleotide polymorphism

SFP – Shahihi-Ferguson perfringens

Spl - Spore photoproduct lyase

STAA - Streptomycin thallous acetateactidione

spp. – species

TIGR – The Institute of Genomic Research

TSC - Tryptose Sulfite Cycolserine

U – unit

UV – ultraviolet

VRBG – Violet red bile glucose

1. Introduction and Literature Review

1.1 Introduction

Meat spoilage caused by clostridia can be categorized in three different groups: "blown-pack", "surface spoilage" and "bone taint" (1, 2). "Blown-pack" spoilage is characterized by distention of the package and production of off odours within four to six weeks of storage at -1.5 to 2ºC (3, 4). Spoilage during refrigeration is a problem in the meat industry because refrigeration is intended to extend the storage life and prevents growth of mesophilic pathogens on meat. Vacuum packaging is used to prevent the growth of aerobic spoilage organisms; however, these conditions are optimal for the growth of psychrophilic *Clostridium* species. These organisms produce endospores that are extremely resistant to interventions used during the slaughter process. Although the incidence of "blown-pack" spoilage is considered sporadic, the occurrence of "blown-pack" spoilage leads to decreased confidence in producers and therefore leads to loss of lucrative contracts or agreements with various suppliers. What makes "blown-pack" spoilage unique is that it occurs relatively quickly, under anaerobic conditions and at refrigeration temperature.

1.2 "Blown-pack" Spoilage

The defining feature of "blown-pack" spoilage is the distention of the package which is caused by production of copious amounts of carbon dioxide gas by psychrophilic bacteria under refrigeration storage. Production of volatile compounds such as 1-butanol, butyl butyrate, butyl acetate, and butyric and acetic acid contribute to the off odours (1). Different volatile profiles can be obtained from different types of meat (2) and various

spoilage organisms will contribute to different volatile profiles. Spoilage of vacuum packaged meat stored at less than 4°C generally occurs slowly and is caused by lactic acid bacteria(1). "Blown-pack" spoilage is unique because spoilage occurs at refrigeration temperature within three weeks of packaging and involves a large amount of gas production(1).

Psychrophilic *Clostridium* species have been implicated in "blown-pack" spoilage. *Clostridium estertheticum*(5, 6) and *Clostridium estertheticum*subsp. *laramiense*(7, 8) are generally the main causative agents of "blown-pack" spoilage. Not every incident of "blown-pack" spoilage is due to the presence and growth of *C. estertheticum* and *C. laramiense*. Other psychrophilic clostridia have been detected in "blown-pack" spoiled meats and may play a role in the overall spoilage of the meat. At least eight other psychrotolerant *Clostridium* spp. have been isolated and associated with "blown-pack" meat products in New Zealand (1). *Cl. gasigenes* has been implicated in "blown-pack" spoiled lamb (3). The vector for the psychrophilic *Clostridium* spp. is most likely the hide of the animal. *Clostridium* spp. are generally found in soil, animal feed and on plant surfaces (9). Contamination of the surface of the carcass could occur during carcass dressing and evisceration (10, 11). The open carcass is vulnerable to contamination from surfaces of the equipment in the processing facility or the organs of the animals. During processing, organs can be punctured and fecal contamination can occur. *Clostridium estertheticum* has been isolated from the gastrointestinal tract of farm animals (12). Removal of endospores from carcass is difficult because carcasses do not consist of smooth surfaces. There are several interventions that are used in carcass

decontamination. Some interventions that are used to decontaminate the carcasses of spoilage organisms are also effective in removing endospores. High pressure water or steam and chemical decontamination can facilitates some removal of endospores in some cases. Endospores are extremely resistant and can persist in meat. Most interventions are not effective in removing all endospores. In some cases the *Clostridium* endospore may be present on the meat, but no "blown-pack" spoilage occurs (1, 13). The viable cell must grow for gas production to occur.

Post-packaging heat shrink treatments are thought to increase the likelihood of occurrence of "blown-pack" spoilage (14). Heat shrink is used in the meat industry to improve the appearance of the package of meat. Heat applied during a heat shrink process may be enough to stimulate or induce germination endospores (14). It is thought that low optimal growth temperature leads to lowered heat resistance as well as lower temperatures required for endospore activation. The heat shrink process may provide enough heat that activates temperature dependent germination.

The presence of psychrophilic and psychrotrophic *Clostridium* may depend on climate in which the animals are raised and slaughtered. In more temperate climates, psychrotolerant species will dominate over mesophilic species (9). This is due to the adaptation to lower temperatures. To develop targeted interventions to control the outgrowth of "blown-pack" spoilage, it is important to characterize these bacteria.

1.2.1 Structure of Endospores

The multiple layers that make up an endospore are responsible for a portion of the resistance to various environmental stressors. Depending on the species, there are six to seven layers that make up an endospore. Structures that are present in all species include an exosporium, a cortex and an endospore coat. Within these structures there are other layers that are of importance to the protection of the bacteria. The outer membrane, germ cell wall, and inner membrane also serve as barriers to external factors. The exosporium is the outermost layer and is generally found in *Bacillus* spp. (15) and acts as the initial barrier to an external stress.

The layer of proteins under the exosporium forms the endospore coat. The endospore coat contains at least 40 different proteins that are specific to the species of endospore (16). No individual coat protein has been identified as essential to protecting the endospore (17). The endospore coat acts as the first barrier to large compounds. Some small compounds have the ability to pass through the endospore coat. The outer membrane is directly underneath the endospore coat. There is no known protective or barrier function of the outer membrane (18, 19) and it may just be a remnant of the vegetative cell.

The cortex is primarily peptidoglycan and the peptidoglycan is homologous to that of vegetative cells (20). One major difference between cortex peptidoglycan and vegetative cell peptidoglycan is that muramic acid-δ-lactam (MAL) and muramic acid are linked to alanine (21, 22). Without the cortex, the endospore will not stay dormant. The main

function of the cortex is to protect the inner membrane and the endospore core by excluding the water from the endospore. The mechanism by which the cortex excludes water is unknown (23). The germ cell wall is the next layer that makes up the endospore and there is no direct role in the resistance of the endospore. The germ cell wall is also made up of peptidoglycan and will form the cell wall of an outgrowing endospore (23).

The inner membrane is the most impermeable layer of the endospore and is impermeable to most small molecules. The strong permeability barriers of the inner membrane confer resistance to a variety of chemicals. The inner membrane is especially important in protecting the DNA in the endospore from being damaged (18, 24). Lipid molecules found in the inner membrane are immobile during dormancy. However, during germination they become fully mobile (25). As with the peptidoglycan, the lipids that are found in the inner membrane are similar to those found in the membrane of growing and vegetative cells (24). The precise type and position of the lipids does not lead to increased permeability instead, it is the degree of compression of the lipid molecules that dictates the permeability. The lipid membrane is far more compressed and rigid in an endospore compared to a vegetative cell and results in a highly impermeable layer (25).

At the center of the endospore is the core, which contains the enzymes required for catabolic and metabolic functions, DNA, RNA and nucleic acids. The DNA, RNA, and nucleic acids are identical to those found in vegetative cells (26, 27). Although the DNA is identical, the conformation of the DNA is different due to the presence of pyridine-2-6-dicarboxylic acid (dipicolinic acid, DPA). The endospore core is relatively dehydrated

which is thought to confer enzymatic dormancy (without water, enzymes are not active) and resistance to heat and chemicals (17, 28, 29).

1.2.2 Factors Indicating Resistance in Endospores

Resistance to heat, pressure and chemicals are the central characteristics of bacterial endospores. Although the multiple layerstructure of the endospore plays a major role in the resistance of the endospore it is not the only factor in the resistance of endospores to heat and pressure.

DPApresent in the core confers resistance to environmental stress. Approximately 5 to 15% of the dry weight of an endospore is comprised of DPA (30). In the endospore core, DPA will chelate with divalent cations, such as Ca^{2+}. Reduction in the water content in the core of the endospore during sporulation could be due to synthesis and accumulation of DPA. The concentration of DPA found in the endospore core is above its solubility (23).

Small acid soluble proteins (SASPs) are a group of molecules that are thought to play a role in protection of DNA in the endospore core(31). They are the third most abundant molecule in the endospore core(23). These proteins are responsible for saturating and protecting the endospore DNA from UV radiation, heat and chemicals (30, 32). SASPs make up 3 to 6% of the total protein in the endospore (23). They are approximately 60 to 72 residues and they are synthesized in the developing forespore during late sporulation and before the uptake of DPA. During germination, they are rapidly degraded and amino

acids are used for other cellular functions. These molecules are highly conserved and specific to bacteria that produce endospores. There is no known homology to any structural motifs identified in proteins that are present in bacteria that do not produce endospores (23). There are different types of SASPs, including α/β-type and γ-type SASPs. The α/β-type SASPs are the most abundant. The α/β-type SASPs are coded by at least 7 genes and their amino acids sequences are highly conserved in *Clostridium* and *Bacillus* spp.(23). Bacterial DNA is saturated and tightly bound by α/β-type SASPs, which is part of the mechanism that protects DNA from wet and dry heat, UV radiation, desiccation and genotoxic chemicals (23). When α/β-type SASPs bind to the DNA, the proteins force a conformational change from B-DNA to A-DNA (33). A-DNA is much more compact compared to B-DNA and therefore more stable. The compact nature of the DNA does not allow for chemicals to oxidize the DNA. Instead, genotoxic chemicals will preferentially oxidize the methionine residues in the α/β-type SASPs (34). The binding of SASPs will also protect the DNA from depurination. For instance, α/β-type SASPs protect DNA from UV irradiation by forming a thyminyl-thymine adduct (23), referred to as a photoproduct. The adduct is formed instead of pyramidine dimers in A-DNA. These photoproducts are repaired within minutes of spore outgrowth by at least three repair mechanisms. Spore photoproduct lyase (Spl), which is unique to spores, is responsible for some of the repair process. Spore photoproducts are also repaired by sec-A dependent recombination and excision (35, 36).

Another major group of SASPs include the γ-type SASPs. These are encoded by a single gene. Their protein sequences are conserved, but not as highly conserved as that of the

α/β-type SASPs. Generally, γ-type SASPs do not associate with the DNA, but they do act as a protein reservoir during spore germination (21, 37).

1.2.3 Endospore Germination

Endospores can germinate after several years of dormancy, if the proper germinants are present. Germinants have been studied since the 1950s and early research identified L-alanine and a variety of other amino acids (*i.e.*,L-aminobutyric acid, L-cysteine, L-valine and L-leucine) as chemicals that induce germination (38). Other common nutrients that can trigger germination include D-sugars and purine nucleosides (21). Bicarbonate anions stimulate germination and lactate is an effective co-germinant in *Clostridium* spp. (39). Nutrient-dependent germination begins with the nutrients binding to germinant receptors in the inner membrane. There is an initial release of monovalent cations (*i.e.*, H^+, K^+) and DPA with divalent cations from the endospore core when the nutrients binds to the receptors on the endospore coat (21, 40). With an increase in free ions in the endospore core, the water content increases. However, the amount of water is not sufficient for protein movement in the core or for endopsore dormancy to end (29). The flooding of water into the endospore coat will trigger a cascade of events that ends with germination of the endospore. Water uptake leads to the hydrolysis of cortex peptidoglycan and expansion of the endospore core(41). Germination is completed when the water content within the endospore core is equal to that of a growing cell(42). The whole germination process occurs without ATP production and membrane synthesis does not occur at this stage(43, 44).

With an increase in the concentration of water, there is an increase in protein mobility(43, 44). This signals the end of dormancy as there is enzyme activity in the endospore core. With enzyme activity, the endospore enters the outgrowth stage. During endospore outgrowth there are several key events that occur including SASP hydrolysis, metabolism of exogenous and endogenous compounds, macromolecular synthesis, and it concludes with DNA replication (21).

Germination can be induced by "non-nutrient" means such as stimulation by muropeptide fragments. Muropeptide fragments originate from a variety of sources and are generally found in the cell walls of vegetative cells. There are different receptors on the endospore coat that act as receptors for these fragments (45). Another method by which germination can be induced is via exogenous calcium DPA (46). Surfactants, such as *n*-dodecylamine, can also be use a germinants. However, surfactant induced germination can result in death of germinated spores (47) as the surfactant will change the permeability of the membrane resulting in cell death. Treatment with disulfide bond-breaking reagents can also permeabilize the endospore coat and trigger germination (48). High hydrostatic pressure (HHP) stimulates germination as well(48).

Early endospore research revealed that endospores could be activated with mild heating (38). Rapid germination occurs when both mild heat and specific germinants are present. By pre-heating, the endospore may enter a temperature-induced glass transition state (49) that may lead to activation; however, this mechanism has not been completely validated.

Germination of psychrophilic endospores can be affected by a variety of different factors. Psychrophilic endospores, such as *Cl. estertheticum*, germinate at a slower rate than other endospores (50). Factors such as pH, temperature, oxygen, and presence of germinants can aid or prevent germination(51, 52).

Optimal pH for germination of *Cl. estertheticum* in meat juice medium is 7.0 (50). Germination of *Cl. estertheticum* is significantly decreased at pH values of 8.0, 6.0 and 5.0 (50). Generally the pH of beef after aging is around 5.7. Only at pH below 5.5 does the growth and germination of *Cl. estertheticum* stop (51). Changing the pH of the meat to prevent spore germination and outgrowth is not an option because it would be difficult to overcome the buffering capacity of meat as well as it would drastically change the quality of the meat(51).

As long as endospores stay dormant, they do not cause problems for food spoilage. However, if a *Cl. estertheticum* endospore germinates and grows, food spoilage will occur. Since viable cells are more susceptible to interventions, exploiting or forcing germination of the endospore may be a method in decontaminating food.

1.3 High Hydrostatic Pressure Processing (HHP)

The ability of high hydrostatic pressure processing (HHP) to inactivate vegetative bacteria near ambient temperatures has made HHP an attractive alternative to thermal processing. HHPis defined as pressure treatments ranging from 100 to 900 MPa (53). Low temperature pasteurization can be achieved in a number of foods using HHP while

retaining quality. Unlike thermal and chemical processes, HHP involves instantaneous, even pressure transmission, as well as uniform heating of the entire food product (54). Generally, pressures higher than 400 MPa are required for significant inactivation of bacteria (54); however, with addition of moderate heat and antimicrobials, lower pressures may be used to have similar effects. The combination of nisin and moderate heating with HHP improved the pressure inactivation of highly heat resistance endospores of *Bacillus sporothermodurans* in skimmed milk (55). However, that is observed with increased pressure, superdormant endospores will exist in the endospore population leading to a tailing effect in inactivation curves (56). In most cases, addition of pressure (*i.e.*, increasing the pressure to 600 MPa) will accelerate bacterial inactivation. Pressures of this nature can be used for liquids and processed meats, but are generally not suitable for raw meat because of colour changes caused by denaturation of proteins. A major benefit to using HHP is that there are few chemical reactions affected by hydrostatic pressure, especially at lower temperatures (54). Although application of 400 to 600 MPa without significant heating can inactivate vegetative cells (57, 58), endospores of the genera *Bacillus* and *Clostridium* can withstand pressures greater than 1000 MPa at ambient temperatures (59). Therefore, addition of heat or natural antimicrobials may be required to inactivate endospores in food products.

1.3.1 Mechanism of Inactivation

The mechanisms of inactivation of bacteria in either a vegetative or endospore state are different. HHP will change the cell membranes, cell walls, proteins and enzyme-mediated functions (53, 60). HHP effectively damages the bacterial membranes of

vegetative cells and therefore affects a myriad of different cellular processes. Damaging the membranes results in a leaky cell. At 100 MPa there is a decrease in lipid bilayer fluidity and some reversible conformational changes in proteins (61) which results in malfunction of membrane bound proteins. Reversible phase transition of the lipid bilayer into the gel phase is observed between 100 and 220 MPa (53). When the lipid bilayer is in the gel phase, transmembrane tunnels are formed because of dissociation and/or conformational changes to the protein subunits. Above 220 MPa, irreversible damage will occur to the proteins due to unfolding. Under some pressure treatment, presence of intracellular fluid compounds has been observed in the cell suspending fluid (62), which indicates leakage of the cell during pressure treatment.

Damage to the cell walls and cell membranes also reduces the potential gradient across the membrane. This results in both autolytic enzyme degradation and decreased ATP synthesis (60, 63). Generally the outer membrane of gram negative bacteria will provide sufficient protection against environmental stressors; however, pressure can permeabilize the outer membrane (64) and make the cells more vulnerable to antimicrobials. There are some who hypothesize that pressure may induce some mechanical stress on microbial cell walls which may lead to inactivation of bacteria (65). Permeabilization of the outer membrane has been elucidated via bud scars, nodes in the cell wall and separation of the cell wall from the membrane (66, 67).

Bacterial endospores do not respond to pressure treatment in the same manner as a vegetative cell. Endospores are extremely resistant and generally require a combination

of pressure and temperature (68). Endospores of *Cl. botulinum* only experienced a one log decrease when 800 MPa was applied, but combined with heat (80°C) there was a 7 log decrease (68). The DNA of an endospore is very well protected by a number of layers but how pressure affects all of these layers is not completely understood. Changes to the structure of an endospore are not as clear as those that happen in the cell membrane of vegetative cells. The endospore coat, being the first layer of defense, should be the first affected by high pressure. However, relatively little is known about how pressure and how the combination of heat and pressure affects the endospore coat and its proteins (60). There has been some success in the use of fluorescent dyes to understand what happens with the inner membrane of the endospore (69) during pressurization.

At mild pressures (100 to 200 MPa), non-nutrient receptors (nGR) are activated in *Bacillus subtilis* and *Bacillus cereus*(51, 52, 70, 71). However, endospores without major nGRs do not germinate with mild pressure treatments (52, 72). Germination will occur at pressures between 100 and 200 MPa, but at higher pressures, the impact of pressure on the nGR is not as pronounced (52, 73). Inactivation of endospores at higher pressures may not be dependent on nGRs.

1.3.2 HHP and Meat

High pressure processing has been successfully used in a variety of products but has limited use for fresh meat due to changes in colour, texture and water holding capacity. High pressure processing has been utilized effectively to cause textural changes by exploiting changes in endogenous enzymes. Pressure can also cause defects in colour.

One of the biggest effects that high pressure processes has on muscle quality is colour. The colour of red meat increases in lightness (L* value) above 250 MPa and redness (a* values) decreases at pressure treatments between 400 and 500 MPa (74-76). The decrease in redness results in a cooked, grey colour that is undesirable to consumers(74-76). Myoglobin, the major pigment of meat, undergoes conformational changes at 250 to 500 MPa, and grey metmyoglobin will increase when the meat is treated at 400 to 500 MPa (74). Meat colour is dependent on the valence of the iron in myoglobin (77). HHP causes irreversible denaturation of myoglobin (78) which leads to unwanted colour changes. At higher temperatures, in addition to HHP, the denaturation of the myoglobin is exacerbated. Meat discolouration due to HHP is described as a whitening effect resulting from myoglobin denaturation and/or haem displacement (74). One of the major limitations to using HHP in meat products is the oxidation of the inrom from ferrous to ferric state at pressures above 400 MPa (74). To mitigate colour changes in meat, processors can vacuum package the meat, add an oxygen scavenger and NaCl to protect meat from discolouration up to 500 MPa (79).

Overall the application of HHP to fresh meat products is limited by discolouration. However, it still remains a useful tool to control the presence of pathogenic bacteria. Little work has been done with HHP in fresh meat at lower temperatures. Most of the research focuses on processed meat and ambient or elevated temperatures. Addition of antimicrobials could also be explored as an additional treatment to HHP to control endospores in meat.

1.4 Bacteriocins

The movement towards "natural" food products has led to the investigation of different type of minimal processes that preserve food and extend its shelf life. Bacteriocins, produced by bacteria, have been studied as possible biopreservatives in foods. Bacteriocins are antimicrobial peptides with a narrow spectra of antibacterial activity against closely related species (80, 81). Very little bacteriocin is required to have a strong antimicrobial effect(80, 81). Different bacteriocins will have different spectrums of activity, and may have different mechanisms of actions. The size, weight, and genetic origin also differ. Most of the current research is focused on bacteriocins produced by lactic acid bacteria (LAB); however, other bacteria also have the ability to produce bacteriocins. Gram negative bacteria produce their own bacteriocins, which are called colicins or microcins(80, 81).

Bacteriocins produced by LAB have little to no effect on Gram negative organism as the cell wall in Gram negative bacteria offers an added protective barrier to bacteriocins(82). Similarly, endospores are generally not killed by bacteriocins, but some bacteriocins prevent the outgrowth of some endospores (82). Sensitization to bacteriocins can be achieved by combining bacteriocins with sublethal temperatures (heating or freezing), chelating agents orHHP(83).

The class I bacteriocins are small peptides (19 to 38 amino acid residues) and contain a β-lanthionine ring (84, 85). Lantibiotics are produced ribosomally and undergo post-translational modification before they are released as active peptides. Nisin is the most studied and well known of all lantibiotics. Lantibiotics are broad spectrum and prevent the outgrowth of endospores. However, one of the major limitations of class I bacteriocins is that they are inactivated in raw meats because of the presence of glutathione(86).

Class II bacteriocins are of small thermostable peptides (<10 kDa) and have been divided into three subclasses (84). Subclass IIa are classified as antilisteral-pediocin bacteriocins. These bacteriocins have 37 to 48 amino acids residues with an N-terminal portion with β-pleated sheet conformation and a C-terminus that contains one or two α-helices (87). Subclass IIb is composed of two-component bacteriocins. These bacteriocins require the combined activity of the two peptides (85). Subclass IIc bacteriocins are distinctive because of their cyclical structure. Several circular bacteriocins have been described (84). Carnocyclin A from *Carnobacterium maltaromaticum* UAL307 is an example of class IIc bacteriocin (88). The circular bacteriocins are large and posttranslationally modified (88).

1.5.1 Mode of Action of Bacteriocins

The mode of action of bacteriocins varies among the bacteriocins of the different classes. Generally, the action of the bacteriocin is exerted on the cell membrane. The lantibiotics have two actions on the cell membrane. There is interference with the cell wall synthesis and promotion of the pore formation in the cell wall(84, 89). This results in changes to

the permeability of the cell. Changes to the permeability results in a leaky cell (84, 89) and loss of essential compounds, such as ions, amino acids and ATP, through the pores (90). The formation of pores eventually leads to the cell's death. Bacteriocins of class II also promote the formation of pores. These bacteriocins target the microorganisms by its C-terminus. By forming pores, there is dissipation of the proton motive force (91, 92). If high pressure can induce germination or disrupt the endospore outer membrane, addition of bacteriocin may allow for the disruption of the inner membrane.

1.5.2 Bacteriocins as Biopreservatives

Bacteriocins can be added to food products as either a pure or partially pure compound or as a bacteriocin-producing culture. When using purified bacteriocins, control of the amount added is an asset. However, purifying bacteriocins is labour and cost intensive. Bacteriocin-producing cultures can be added to the food product with the bacteriocin produced *in situ* during the storage of the products. The concern with using bacteriocin-producing culture is that the strain chosen must produce an adequate amount of bacteriocin *in situ*. The strain must also not produce excess amounts of unwanted metabolites to avoid premature spoilage (82). Metabolites, such as organic acids and peroxides can cause sensory changes to the food. Another consideration is that bacteriocins are generally encoded on plasmids. Plasmids are not always stable and therefore may be lost during storage or processing (82). Other considerations is that some bacteriocins are only produced at specific temperatures (93) and therefore storage temperatures will play a role in which organism is chosen. Currently, nisin and pediocin PA-1 are bacteriocins licensed as food preservatives (85). Despite all of the

disadvantages, there is still a considerable amount of research on the use of bacteriocin-producing LAB as biopreservatives in food.

1.5.3 Bacteriocins of *Carnobacterium maltaromaticum*

Micocin X™ is semi-purified mixture of bacteriocins produced by *Carnobacteriummaltaromaticum* UAL307. The bacteriocins produced by *C. maltaromaticum* belong to class II. The mechanism of action of these bacteriocins is the formation of pores which causes leakage of internal low molecular weight substances and dissipates the membrane potential (84, 89). Bacteriocin production by *C. maltaromaticum* can be affected by extrinsic factors such as: salt concentration, temperatures, presence of acetate and pH (93). In some cases, presence of bacteriocin-sensitive organisms will also induce bacteriocin production by some species of *Carnobacterium*(94).

C.maltaromaticum UAL307 produces carnobacteriocin BM1, piscicolin 126 and carnocyclin A. Carnobacteriocin BM1 and piscicolin 126 are class IIa bacteriocins (93, 95, 96). Carnocyclin A was the first circular bacteriocin to be isolated from a *Carnobacterium* spp. (88). Carnocyclin A, like other circular bacteriocins, has a high isoelectric point and a high concentration of hydrophobic residues. It also has potent bioactivity, stability to variation in temperature and pH, and is resistant to proteolysis (97, 98).

When adding bacteriocin-producing cultures to food, considerations have to be made about changes to the sensory properties. Metabolites that may be produced can cause off flavours and odours. Some *C. maltaromaticum* strains are considered moderate spoilage organisms. For example, when *C. maltaromaticum* is in the presence of *Br. thermosphacta*, a synergistic spoilage effect will occur in modified atmosphere packaged shrimp (99, 100). *C. maltaromaticum* is homofermentative and will utilize glucose to produce lactic acid (101). Generally, carbohydrate metabolism, with the exception of fermented milk products, does not affect sensory properties of food as much as the breakdown of proteins, lipids and fats. Other organisms that are present in naturally contaminated meat products generally have a greater effect on sensory and spoilage. The use of Micocin X™ (a cell free supernatant containing bacteriocins) does not result in the breakdown of food constituents initially.

1.5.4 Bacteriocins and *Clostridium* spp.

Addition of bacteriocin-producing cultures, especially LAB, has been studied in different food products including: yogurt, cheese, sausages and other vacuum packaged meat products. The bulk of the research has examined the effect of bacteriocins on *L. monocytogenes* in ready-to-eat meat products(102). There has been little investigation on the effect of bacteriocins on the outgrowth of clostridial endospores. *Clostridium botulinum* and nisin-producing strains have been co-incubated *in vivo*. At 10ºC, *Cl. botulinum* was not detected after 10 days of co-incubation with a protective culture (*Lactococcus lactis* and *Pediococcus pentosaceus;*(102). Temperature played a role in the ability of *L. lactis and P. pentosaceus* to be effective protective cultures. At 15ºC, *Cl.*

botulinum was not inhibited by the protective cultures (102). See Table 1 for other examples of co-inoculation of bacteriocin-producing cultures and *Clostridium* spp. in food.

Table 1.1 Application of bacteriocin-producing culture in non-fermented refrigerated foods

Bacteriocin producing cultures	**Target organism**	**Food**	**Reference**
Enterococcus faecium	*Cl. botulinum*	Sous vide fish	(103)
Lactococcus lactis	*Cl. botulinum*	Chicken a la king	(104)
Lactobacillus plantarum ATCC 8014	*Cl. botulinum* type A, B, and E	Refrigerated can pea soup	(105)
Lactobacillus plantarum	*Cl. botulinum*	Cured meat	(106)
Pediococcus acidilactici	*Cl. botulinum*	Chicken salad	(107)
Carnobacteriummaltaromaticum UAL307	*Cl. putrefaciens, Cl. frigidicarnis*	Raw beef steaks	(108)

Adapted from (102, 109)

Although there is little information on the effects of bacteriocin-producing cultures and its effect on *Clostridium* spp., this does not mean bacteriocin-producing cultures do not prevent the growth or outgrowth of *Clostridium* spp. In combination with other interventions, such as HHP, bacteriocins may be a viable option in preventing the outgrowth of clostridial endospores. Little or no work has been done to examine the effect of class II and circular bacteriocins on *Clostridium* spp. in meat products (especially vacuum packaged fresh beef). Some work has been done with psychrophilic *Clostridium* spp., fresh meat and antimicrobial addition with some success (101). However, addition of HHP in combination with an antimicrobial would be novel.

1.5.5 Bacteriocins and High Hydrostatic Pressure (HHP)

Combining bacteriocins and high hydrostatic pressure (HHP) may be a viable option to control spoilage in fresh beef. The thought is that addition of HHP will increase sensitivity of the bacteria to antimicrobial treatments. Generally, experiments involving bacteriocins and HHP have been completed in a liquid medium. Most of the research has focused on ready-to-eat meats and non-spore forming bacteria. There has been some success with combining bacteriocins and HHP in ready-to-eat meats. The combination of nisin and 400 MPa for 10 min at 17°C was effective in inactivating *Escherichia coli* and some *Salmonella* spp. in fermented sausages (110). Some work has been completed in raw chicken with some success as well. High pressure processing at 350 MPa for 15 min at 2°C in chicken with nisin was effective in decreasing mesophilic and psychrotrophic bacterial populations (111) in raw mechanically deboned chicken. Psychrotrophs were more sensitive to the presence of nisin compared to the mesophilic bacteria.

Endospores are generally resistant to HHP treatment. However, when pressure is added, germination can be induced and the endospores will be more susceptible to chemicals or even biopreservatives. When the endospores are at the outgrowth stage, the endospores are susceptible to a variety of different treatments. *Cl. sporogenes*, *Cl. perfringens*, *Cl. tertium*, and *Cl. estertheticum* subsp. Laramie were controlled using pedocin AcH and nisin A in combination with 345 MPa for 5 min at 60°C in roast beef (112). There has been no work completed in endospores, refrigeration temperatures, high pressure and bacteriocins in raw meat.

1.6 Research Objectives

"Blown-pack" spoilage continues to be a problem for the fresh meat industry in Alberta and novel interventions are needed to control the outgrowth of these endospores. HHP and bacteriocins, especially in combination, can be viable options to controlling the outgrowth and presence of endospores in food. Understanding the effectiveness of interactions of HHP and bacteriocins and how it affects psychrophilic endospores has not been elucidated.

This research tested the following hypotheses:

1. Psychrotrophic or psychrophilic *Clostridium* spp. can be isolated from blown pack spoiled meat and these strains do not produce small acid soluble proteins;
2. The temperature and pressure resistance of psychrotrophic or psychrophilic *Clostridium* spp. will decrease in the presence of bacteriocins.

To test these hypotheses, the specific objectives of this research are to:

1. isolate, identify, and characterize psychrophilic endospores from beef that have undergone "blown-pack" spoilage;
2. determine the presence of small acid soluble proteins in endospores of psychrophilic clostridia isolated from "blown-pack" spoiled meat;
3. determine the resistance to heat and pressure of psychrophilic endospores in saline;

4. understand the interaction of pressure and bacteriocins on the survival of psychrophilic clostridial endospores in raw meat.

1.7 Research Objectives

(1) Broda DM, DeLacy KM, Bell RG, Penney N. Association of psychrotrophic *Clostridium* spp., with deep tissue spoilage of chilled vacuum-packed lamb. Int J Food Microbiol. 1996 Apr;29(2-3):371-8.

(2) Broda DM, DeLacy KM, Bell RG, Braggins TJ, Cook RL. Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Int J Food Microbiol. 1996 Apr;29(2-3):335-52.

(3) Broda DM, Saul DJ, Lawson PA, Bell RG, Musgrave DR. *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum-packed meat. Int J Syst Evol Microbiol. 2000 Jan;50:107-18.

(4) Broda DM, Saul DJ, Bell RG, Musgrave DR. *Clostridium algidixylanolyticum* sp. nov., a psychrotolerant, xylan degrading, spore-forming bacterium. Int J Syst Evol Microbiol. 2000 Mar;50:623-31.

(5) Collins MD, Rodrigues UM, Dainty RM, Edwards RA, Roberts TA. Taxonomic studies on a psychrophilic *Clostridium* from vacuum-packed beef: description of *Clostridium estertheticum* sp. FEMS Microbiol Lett. 1992 Sep;96:235-40.

(6) Cato EP, George WL, Finegold SM. Genus *Clostridium* Prazmowski, 1880. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editors. Bergey's Manual of Systematic Bacteriology. Baltimore: The Williams & Wilkins Co.; 1986. p. 1141-200.

(7) Kalchayanand N, Ray B, Field RA, Johnson MC. Spoilage of vacuum-packaged refrigerated beef by *Clostridium*. J Food Prot. 1989 Jan;52:424-6.

(8) Lawson P, Dainty RH, Kristiansen N, Berg J, Collins MD. Characterization of a psychrotrophic *Clostridium* causing spoilage in vacuum-packed cooked pork: description of *Clostridium algidicarnis* sp. nov. Lett Appl Microbiol. 1994 Feb:153-7.

(9) Lund BM. Anaerobes in relation to foods of plant origin. In: Barnes EM, Mead GC, editors. Anaerobic Bacteria in Habitats Other Than Man. 13th ed. Oxford: Blackwell Scientific; 1986. p. 351-72.

(10) Broda DM, Musgrave DR, Bell RG. Use of restriction fragment length polymorphism analysis to differentiate strains of psychrophilic and psychrotrophic clostridia associated with 'blown pack' spoilage of vacuum-packed meats. J Appl Microbiol. 2000 Jan;88:107-18.

(11) Boerema JA, Broda DM, Bell RG. PCR detection of psychrotolerant clostridia associated with deep tissue spoilage of vacuum-packed chilled meats. Lett Appl Microbiol. 2002 Oct;35:446-50.

(12) Broda DM, Bell RG, Boerema JA, Musgrave DR. The abattoir source of culturable psychrophilic *Clostridium* spp. causing 'blown pack' spoilage of vacuum-packed chilled venison. J Appl Microbiol. 2002 Oct;93(5):817-24.

(13) Broda DM, Boerema JA, Bell RG. PCR detection of psychrophilic *Clostridium* spp. causing 'blown pack' spoilage of vacuum-packed chilled meats. J Appl Microbiol. 2003 Feb;94(3):515-22.

(14) Bell RG, Moorhead SM, Broda DM. Influence of heat shrink treatments on the onset of clostridial "blown pack" spoilage of vacuum packed chilled meat. Food Res Int. 2001 Jul;34(4):271-5.

(15) Setlow P. Summer meeting 2013-when the sleepers wake: the germination of spores of *Bacillus* species. J Appl Microbiol. 2013 Dec 2013;115(6):1251-68.

(16) Kim H, Hahn M, Grabowski P, McPherson DC, Otte MM, Wang R, et al. The *Bacillus subtilis* spore coat protein interaction network. Mol Microbiol. 2006 Jan;59(2):487-502.

(17) Leggett MJ, McDonnell G, Denyer SP, Setlow P, Maillard J-. Bacterial spore structures and their protective role in biocide resistance. J Appl Microbiol. 2012 Sep:113(3):485-98.

(18) Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. MicrobiolMol Biol Rev. 2000 Sep;64(3):548-72.

(19) Setlow B, McGinnis KA, Ragkousi K, Setlow P. Effects of major spore-specific DNA binding proteins on *Bacillus subtilis* sporulation and spore properties. J Bacteriol. 2000 Dec;182(24):6906-12.

(20) Popham DL. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. Cell Mol Life Sci. 2002 Mar;59(3):426-33.

(21) Setlow P. I will survive: DNA protection in bacterial spores. Trends Microbiol. 2007 Apr;15(4):172-80.

(22) Sunde EP, Setlow P, Hederstedt L, Halle B. The physical state of water in bacterial spores. Proc Natl Acad Sci U S A. 2009 Nov;106(46):19334-9.

(23) Setlow P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol. 2006 Sep;101(3):514-25.

(24) Cortezzo DE, Setlow P. Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. J Appl Microbiol. 2005 Dec;98(3):606-17.

(25) Cowan AE, Olivastro EM, Koppel DE, Loshon CA, Setlow B, Setlow P. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. Proc Natl Acad Sci U S A. 2004 May;101(20):7733-8.

(26) Setlow P. Germination and outgrowth. In: Hurst A, Gould GW, editors. The Bacterial Spore. Vol. II ed. London: Academic Press; 1983. p. 211-54.

(27) Setlow P. Mechanisms which Contribute to the long-term survival of spores of *Bacillus*species. J Appl Bacteriol. 1994 Jun;76:S49-60.

(28) Paidhungat M, Setlow B, Driks A, Setlow P. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. J Bacteriol. 2000 Oct;182(19):5505-12.

(29)Cowan AE, Koppel DE, Setlow B, Setlow P. A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: Implications for spore dormancy. Proc Natl Acad Sci U S A. 2003 Apr;100(7):4209-14.

(30) Gerhardt P, Marquis RE. Spore thermoresistance mechanisms. In: Smith I, Slepecky RA, Setlow P, editors. Regulation of Prokaryotic Development. 1st ed. Washington, DC: American Society for Microbiology; 1989. p. 43-63.

(31) Huang S, Chen D, Pelczar PL, Vepachedu VR, Setlow P, Li Y. Levels of Ca(2+)-dipicolinic acid in individual *Bacillus* spores determined using microfluidic raman tweezers. J Bacteriol. 2007 Jul;189(13):4681-7.

(32) Setlow P. Small, Acid-Soluble Spore Proteins of *Bacillus* Species - Structure, Synthesis, Genetics, Function, and Degradation. Annu Rev Microbiol. 1988 Oct;42:319-38.

(33) Lee KS, Bumbaca D, Kosman J, Setlow P, Jedrzejas MJ. Structure of a protein-DNA complex essential for DNA protection in spores of *Bacillus* species. Proc Natl Acad Sci U S A. 2008 Feb;105(8):2806-11.

(34) Paredes-Sabja D, Raju D, Torres JA, Sarker MR. Role of small, acid-soluble spore proteins in the resistance of *Clostridium perfringens* spores to chemicals. Int J Food Microbiol. 2008 Mar;122(3):333-5.

(35) Setlow B, Setlow P. Thymine-containing dimers as well as spore photoproducts are found in ultraviolet-irradiated *Bacillus subtilis* spores that lack small acid-soluble proteins. Proc Natl Acad Sci U S A. 1987 Jan;84(2):421-3.

(36) Nicholson WL, Setlow B, Setlow P. Ultraviolet-irradiation of DNA complexed with alpha-beta-type small, acid-soluble proteins from spores of *Bacillus* or *Clostridium* species makes spore photoproduct but not thymine dimers. Proc Natl Acad Sci U S A. 1991 Oct;88(19):8288-92.

(37) Raju D, Waters M, Setlow P, Sarker MR. Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. BMC Microbiol. 2006 Jun;6:1-13.

(38) Hills GM. Chemical Factors in the Germination of Spore-Bearing Aerobes - the Effects of Amino-Acids on the Germination of *Bacillus anthracis*, with some Observations on the Relation of Optical Form to Biological Activity. Biochem J. 1949 Dec;45(3):363-70.

(39) Gould GW. History of science - spores - Lewis B Perry Memorial Lecture 2005. J Appl Microbiol. 2006 Sep;101(3):507-13.

(40) Setlow P. I Will Survive - Protecting and Repairing Spore DNA. J Bacteriol. 1992 May;174(9):2737-41.

(41) Setlow P. Spore germination. Curr Opin Microbiol. 2003 Dec;6(6):550-6.

(42) Moir A. How do spores germinate? J Appl Microbiol. 2006 Sep;101(3):526-30.

(43) Paredes-Sabja D, Sarker MR. Germination response of spores of the pathogenic bacterium *Clostridium perfringens* and *Clostridium difficile* to cultured human epithelial cells. Anaerobe. 2011 Apr;17(2):78-84.

(44) Paredes-Sabja D, Setlow P, Sarker MR. Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and proteins involved. Trends Microbiol. 2011 Feb;19(2):85-94.

(45) Shah IM, Laaberki M, Popham DL, Dworkin J. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell. 2008 Oct;135(3):486-96.

(46) Riemann H, Ordal ZJ. Germination of bacterial endospores with calcium and dipicolinic acid. Science. 1961 May;133(346):1703.

(47) Rode LJ, Foster JW. The action of surfactants on bacterial spores. Arch Mikrobiol. 1960 Mar;36(1):67-94.

(48) Clouston JG, Wills PA. Initiation of germination and inactivation of *Bacillus pumilus* spores by hydrostatic pressure. J Bacteriol. 1969 Feb;97(2):684.

(49) Ablett S, Darke AH, Lillford PJ, Martin DR. Glass formation and dormancy in bacterial spores. Int J Food Sci Tech. 1999 Feb;34(1):59-69.

(50) Rajagopal S. Characterization of germination of *Clostridium estertheticum* spores [dissertation]. Edmonton, Alberta: University of Alberta; 2014.

(51) Black E, Koziol-Dube K, Guan D, Wei H, Setlow B, Cortezzo D, et al. Factors influencing germination of *Bacillus subtilis* spores via activation of nutrient receptors by high pressure. Appl Environ Microbiol. 2005 Oct;71(10):5879-87.

(52) Reineke K, Doehner I, Schlumbach K, Baier D, Mathys A, Knorr D. The different pathways of spore germination and inactivation in dependence of pressure and temperature. Innov Food Sci Emerg Technol. 2012 Jan;13:31-41.

(53) Campus M. High pressure processing of meat, meat products and seafood. Food Eng Rev. 2010 Dec;2(4):256-73.

(54) Toepfl S, Mathys A, Heinz V, Knorr D. Review: Potential of high hydrostatic pressure and pulsed electric fields for energy efficient and environmentally friendly food processing. Food Rev Int. 2006 Oct to Dec;22(4):405-23.

(55) Aouadhi C, Rouissi Z, Mejri S, Maaroufi A. Inactivation of *Bacillus sporothermodurans* spores by nisin and temperature studied by design of experiments in water and milk. Food Microbiol. 2014 Apr;38:270-5.

(56) Hofstetter S, Gebhardt D, Ho L, Gänzle M, McMullen LM. Effects of nisin and reutericyclin on resistance of endospores of *Clostridium* spp. to heat and high pressure. Food Microbiol. 2013 May;34(1):46-51.

(57) Islam M, Inoue A, Igura N, Shimoda M, Hayakawa I. Inactivation of *Bacillus* spores by the combination of moderate heat and low hydrostatic pressure in ketchup and potage. Int J Food Microbiol. 2006 Mar;107(2):124-30.

(58) Vercammen A, Vanoirbeek KGA, Lemmens L, Lurquin I, Hendrickx MEG, Michiels CW. High pressure pasteurization of apple pieces in syrup: Microbiological shelf-life and quality evolution during refrigerated storage. Innov Food Sci Emerg Technol. 2012 Oct;16:259-66.

(59) San Martin M, Barbosa-Canovas G, Swanson B. Food processing by high hydrostatic pressure. Crit Rev Food Sci Nutr. 2002 Jun;42(6):627-45.

(60) Torres JA, Velazquez G. Commercial opportunities and research challenges in the high pressure processing of foods. J Food Eng. 2005 Mar;67(1-2):95-112.

(61) Kato M, Hayashi R, Tsuda T, Taniguchi K. High pressure-induced changes of biological membrane - Study on the membrane-bound Na+/K+-ATPase as a model system. Eur J Bioch. 2002 Jan;269(1):110-8.

(62) Shimada S, Andou M, Naito N, Yamada N, Osumi M, Hayashi R. Effects of hydrostatic-pressure on the ultrastructure and leakage of internal substances in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol. 1993 Oct;40(1):123-31.

(63) Kato M, Hayashi R. Effects of high pressure on lipids and biomembranes for understanding high-pressure-induced biological phenomena. Biosci Biotechnol Biochem. 1999 Aug;63(8):1321-8.

(64) Gänzle M, Vogel R. On-line fluorescence determination of pressure mediated outer membrane damage in *Escherichia coli*. Syst Appl Microbiol. 2001 Dec;24(4):477-85.

(65) Ludwig H, van Almsick G, Schreck C. The effect of hydrostatic pressure on the survival of microorganisms. In: Taniguchi, Yoshihiro Stanley, Harry E. Ludwig,Horst, editor. Biological and Medical Physics Series. 175 Fifth Avenue, New York, NY, 10010-7858, USA Heidelberger Platz 3, D-14197, Berlin, Germany: Springer-Verlag New York Inc., 175 Fifth Avenue, New York, NY, 10010-7858, USA Heidelberger Platz 3, D-14197, Berlin, Germany; 2002. p. 239-56.

(66) Park SW, Sohn KH, Shin JH, Lee HJ. High hydrostatic pressure inactivation of *Lactobacillus viridescens* and its effects on ultrastructure of cells. Int J Food SciTech. 2001 Oct 2001;36(7):775-81.

(67) Ritz M, Tholozan JL, Federighi M, Pilet MF. Morphological and physiological characterization of *Listeria monocytogenes* subjected to high hydrostatic pressure. Appl Environ Microbiol. 2001 May;67(5):2240-7.

(68) Lenz CA, Vogel RF. Effect of sporulation medium and its divalent cation content on the heat and high pressure resistance of *Clostridium botulinum* type E spores. Food Microbiol. 2014 Dec;44:156-67.

(69) Hofstetter S, Denter C, Winter R, McMullen LM, Gänzle MG. Use of the fluorescent probe LAURDAN to label and measure inner membrane fluidity of endospores of *Clostridium* spp. J Microbiol Methods. 2012 Oct;91(1):93-100.

(70) Wuytack EY, Boven S, Michiels CW. Comparative study of pressure-induced germination of Bacillus subtilis spores at low and high pressures. Appl Environ Microbiol. 1998 Sep;64(9):3220-4.

(71) Wuytack EY, Soons J, Poschet F, Michiels CW. Comparative study of pressure- and nutrient-induced germination of Bacillus subtilis spores. Appl Environ Microbiol. 2000 Jan;66(1):257-61.

(72) Paidhungat M, Setlow B, Daniels WB, Hoover D, Papafragkou E, Setlow P. Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. Appl Environ Microbiol. 2002 Jun;68(6):3172-5.

(73) Reineke K, Mathys A, Heinz V, Knorr D. Mechanisms of endospore inactivation under high pressure. Trends Microbiol. 2013 Jun;21(6):296-304.

(74) Carlez A, Veciananogues T, Cheftel JC. Changes in color and myoglobin of minced beef meat due to high-pressure processing. Food Sci Technol-LEB. 1995 Mar;28(5):528-38.

(75) Jung S, Ghoul M, de Lamballerie-Anton M. Influence of high pressure on the color and microbial quality of beef meat. Lebensm Wiss Technol. 2003 May;36(6):625-31.

(76) Tintchev F, Bindrich U, Toepfl S, Strijowski U, Heinz V, Knorr D. High hydrostatic pressure/temperature modeling of frankfurter batters. Meat Sci. 2013 Jul;94(3):376-87.

(77) Mancini RA, Hunt MC. Current research in meat color. Meat Sci. 2005 Sep;71(1):100-21.

(78) Defaye AB, Ledward DA, Macdougall DB, Tester RF. Renaturation of metmyoglobin subjected to high isostatic pressure. Food Chem. 1995 Dec;52(1):19-22.

(79) Buckow R, Sikes A, Tume R. Effect of high pressure on physicochemical properties of meat. Crit Rev Food Sci Nutr. 2013 Jan;53(7):770-86.

(80) Tagg JR, Wannamaker LW. Streptococcin-A-Ff22 - Nisin-like antibiotic substance produced by a Group-A-*Streptococcus*. Antimicrob Agents Chemother. 1978 Jul;14(1):31-9.

(81) Klaenhammer TR. Genetics of bacteriocins produced by lactic-acid bacteria. FEMS Microbiol Rev. 1993 Sep;12(1-3):39-86.

(82) Schillinger U, Geisen R, Holzapfel WH. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. Trends Food Sci Technol. 1996 May;7(5):158-64.

(83) Lucke FK. Utilization of microbes to process and preserve meat. Meat Sci. 2000 Oct;56(2):105-15.

(84) Drider D, Fimland G, Hechard Y, McMullen LM, Prevost H. The continuing story of class IIa bacteriocins. Microbiol and Mol Biol Rev. 2006 Jun;70(2):564.

(85) Balciunas EM, Castillo Martinez FA, Todorov SD, Gombossy de Melo Franco,Bernadette Dora, Converti A, de Souza Oliveira RP. Novel biotechnological applications of bacteriocins: A review. Food Control. 2013 Jul;32(1):134-42.

(86) Rose NL, Palcic MM, Sporns P, McMullen LM. Nisin: A novel substrate for glutathione S-transferase isolated from fresh beef. J Food Sci. 2002 Aug;67(6):2288-93.

(87) Fimland G, Johnsen L, Dalhus B, Nissen-Meyer J. Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. J Pept Sci. 2005 Nov;11(11):688-96.

(88) Martin-Visscher LA, van Belkum MJ, Garneau-Tsodikova S, Whittal RM, Zheng J, McMullen LM, et al. Isolation and characterization of carnocyclin A, a novel circular bacteriocin produced by *Carnobacterium maltaromaticum* UAL307. Appl Environ Microbiol. 2008 Aug;74(15):4756-63.

(89) Suzuki M, Yamamoto T, Kawai Y, Inoue N, Yamazaki K. Mode of action of piscicocin CS526 produced by *Carnobacterium piscicola* CS526. J Appl Microbiol. 2005 Feb;98(5):1146-51.

(90) Breukink E, Wiedemann I, van Kraaij C, Kuipers OP, Sahl HG, de Kruijff B. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science. 1999 Dec;286(5448):2361-4.

(91) Hechard Y, Sahl HG. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie. 2002 May-Jun;84(5-6):545-57.

(92) Kaiser AL, Montville TJ. Purification of the bacteriocin bavaricin MN and characterization of its mode of action against *Listeria monocytogenes* Scott A cells and lipid vesicles. Appl Environ Microbiol. 1996 Dec;62(12):4529-35.

(93) Gursky LJ, Martin NI, Derksen DJ, van Belkum MJ, Kaur K, Vederas JC, et al. Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. Arch Microbiol. 2006 Oct;186(4):317-25.

(94) Sip A, Grajek W, Boyaval P. Enhancement of bacteriocin production by *Carnobacterium divergens* AS7 in the presence of a bacteriocin-sensitive strain *Carnobacterium piscicola* . Int J Food Microbiol. 1998 Jun;42(1-2):63-9.

(95) Jack RW, Wan J, Gordon J, Harmark K, Davidson BE, Hillier AJ, et al. Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Appl Environ Microbiol. 1996 Aug;62(8):2897-903.

(96) Quadri LEN, Sailer M, Roy KL, Vederas JC, Stiles ME. Chemical and Genetic-Characterization of Bacteriocins Produced by *Carnobacterium piscicola* Lv17b. J Biol Chem. 1994 Apr;269(16):12204-11.

(97) Kawai Y, Kemperman R, Kok J, Saito T. The circular bacteriocins gassericin A and circularin A. Curr Protein Peptide Sci. 2004 Oct;5(5):393-8.

(98) Ma B, Zhang KZ, Hendrie C, Liang CZ, Li M, Doherty-Kirby A, et al. PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. Rapid Commun Mass Spectrom. 2003 Sep;17(20):2337-42.

(99) Mejlholm O, Boknaes N, Dalgaard P. Shelf life and safety aspects of chilled cooked and peeled shrimps (*Pandalus borealis*) in modified atmosphere packaging. J Appl Microbiol. 2005 Mar;99(1):66-76.

(100) Laursen BG, Leisner JJ, Dalgaard P. *Carnobacterium* species: Effect of metabolic activity and interaction with *Brochothrix thermosphacta* on sensory characteristics of modified atmosphere packed shrimp. J Agric Food Chem. 2006 May;54(10):3604-11.

(101) Leisner JJ, Laursen BG, Prevost H, Drider D, Dalgaard P. *Carnobacterium*: positive and negative effects in the environment and in foods. FEMS Microbiol Rev. 2007 Sep;31(5):592-613.

(102) Rodgers S, Kailasapathy K, Cox J, Peiris P. Co-incubation of *Clostridium botulinum* with protective cultures. Food Res Int. 2004 Mar;37(7):659-66.

(103) Smith LD. Inhibition of *Clostridium botulinum* by Strains of *Clostridium perfringens* Isolated from Soil. Appl Microbiol. 1975 Aug;30(2):319-23.

(104) Saleh MA, Ordal ZJ. Studies on growth and toxin production of *Clostridium botulinum* in a precooked frozen food. Food research. 1955 Aug;20:340-50.

(105) Skinner GE, Solomon HM, Fingerhut GA. Prevention of *Clostridium botulinum* type A, proteolytic B and E toxin formation in refrigerated pea soup by *Lactobacillus plantarum* ATCC 8014. J Food Sci. 1999 Jul-Aug;64(4):724-7.

(106) Tanaka N, Traisman E, Lee MH, Cassens RG, Foster EM. Inhibition of *botulinum* toxin formation in bacon by acid development. J Food Prot. 1980 Jun;43(6):450-7.

(107) Hutton MT, Chehak PA, Hanlin JH. Inhibition of *botulinum* toxin production by *Pediococcus acidilactici* in temperature abused refrigerated foods. J Food Saf. 1991 Apr;11(4):255-67.

(108) Ho L. Characterization of "blown-pack" spoiled beef [dissertation]. Edmonton, Alberta: University of Alberta; 2009.

(109) Rodgers S. Preserving non-fermented refrigerated foods with microbial cultures - a review. Trends Food Sci Technol. 2001 Aug;12(8):276-84.

(110) Garriga M, Aymerich MT, Costa S, Monfort JM, Hugas M. Bactericidal synergism through bacteriocins and high pressure in a meat model system during storage. Food Microbiol. 2002 Oct;19:509-18.

(111) Yuste J, Mor-Mur M, Capellas M, Guamis B, Pla R. Microbiological quality of mechanically recovered poultry meat treated with high hydrostatic pressure and nisin. Food Microbiol. 1998 Aug;15(4):407-14.

(112) Kalchayanand N, Dunne CP, Sikes A, Ray B. Inactivation of bacterial spores by combined action of hydrostatic pressure and bacteriocins in roast beef. J Food Saf. 2003 Nov;23(4):219-31.

2. Isolation, Identification, Characterization of "Blown-pack" SpoilageOrganisms

2.1 Introduction

"Blown-pack" spoiled meat is characterized by spoilage due to gas production via bacterial growth. In most cases, there has been a failure in the supply chain and the packages have been exposed to some sort of temperature abuse. The increased temperatures allow for the proliferation of gas producing organisms from the *Enterobacteriaceae* family with *Serratita liquefaciens*, *Enterobacter aerogenes* and *Hafnia alvei* being the main culprits (1,2). However, "blown-pack" spoilage can occur at refrigeration temperatures. Generally, this type of spoilage occurs within 4 to 6 weeks of storage at refrigeration temperatures, with no temperature abuse. With vacuum packaging, the storage life of meat can be extended to up to 3 months (3,4). This is especially beneficial for long distance shipment of products to different countries. Packages that have not been temperature abused, but still exhibit "blown-pack" spoilage are thought to be due to the growth of psychrophilic *Clostridium* spp. In particular, *Clostridium estertheticum* produces sufficient carbon dioxide and hydrogen (2) to distend the package. With the exception of the *Clostridium* spp. present in the meat, the microflora of a "blown-pack" spoiled package of meat is similar to that of a package of unspoiled meat. *Lactobacillus* will dominate the microbiota and gas-producing organisms from the *Enterobacteriaceae* family will be present in very low numbers (2). Lower numbers of *Enterobacteriaceae* generally indicates that the package has not undergone temperature abuse and therefore the gas production is from a different source of bacterial growth(2). What makes the *Clostridium* spp. unique is that they grow

optimally at refrigeration temperatures and some can produce gas at temperatures slightly below 0°C (5).

Isolation of these organisms can be difficult and they are generally not recovered using conventional techniques. Common enumeration media (such as plate count agars) cannot be used to isolate these organisms (6,7). It is also best to keep the spoiled packages at refrigeration temperature to achieve the best results when isolating these organisms (8). Even when enumerating on blood agar plates, most of the colonies detected will be lactic acid bacteria. Despite the large amount of gas produced in the packages, very few colonies are generally isolated when using solid media. Even when plating pure cultures in liquid medium, very few or no colonies are observed when transferred to a solid medium (2).

The *Clostridium* spp. that causes "blown-pack" spoilage is thought to be found in the soil (9-11). The organisms most likely contaminate the meat carcass from the hide of the animal during the slaughter process. During the slaughtering process, a variety of interventions are used to decrease the microbial load of the carcass. Unfortunately, these interventions do not inactivate or prevent the outgrowth of endospores. "Blown-pack" spoilage has been observed in many places worldwide including Canada (12-14), the United States (6,15,16), Australia and New Zealand (2,5,17,18). "Blown-pack" spoilage has been reported in both vacuum packaged beef and lamb. The incidence of "blown-pack" spoilage of vacuum packaged beef is considered to be sporadic because spoilage only occurs in a fraction of the packages within each shipment (6).

This study reports the isolation and characterization of organisms from a spoiled package of vacuum-packed beef loin obtained from a commercial processor.

2.2 Material and Methods

2.2.1 Isolation of Psychrophilic *Clostridium* spp. From "Blown-pack" Beef

A spoiled vacuum packaged whole raw beef loin was received from a local Alberta processing plant. For comparison, an unspoiled beef loin was also obtained from the same facility. The processor also provided an environmental swab of the facility. The "environmental" swab was from a dusty area in the processing plant.

The loins had been stored at <1.0°C for 6 weeks. The sample was visually inspected for leaks and stored at 4°C until further processing. Processing was completed within two weeks. A silicon plug was placed onto the package, allowed to dry and a sterile needle attached to an aspirator was used to remove the gas. After the gas was removed, the package was transferred into an anaerobic hood (Forma Scientific, Model 1025 Anaerobic System Incubator,Waltham, MA) that was in a processing room held at 10°C. This was to ensure that any vegetative psychrophilic clostridia present on the surface of the meat would survive the isolation process. The surface of the package was cleaned with 70% ethanol prior to opening with a sterile blade. A 2 x 5 cm surface sample of meat was obtained using sterile blades and tweezers. The sample was transferred to a sterile lab stomacher bag (Fisherbrand; Fisher Scientific, ON, Canada) and 10 mL of sterile 0.85% saline was added. The sample was massaged by hand for at least 3 min. The purge that had accumulated in the package was also collected for sampling. Dilutions were made in sterile

0.85% saline and samples were plated onto Reinforced Clostridial Medium agar [RCM; Difco; Becton, Dickinson and Company, MD, US, (2,5)] with 0.5 % glucose and 5% defibrinated sheep blood (Oxoid SR51; MT, USA). Plates were incubated at 7°C for 3 wk in an anaerobic jar. Colonies were chosen based on difference in morphology and streaked for purity on RCM blood agar and incubated anaerobically at 7°C for 3 wk(2,5).

The microflora of both the "blown-pack" and unspoiled beef loins were enumerated using different media. Plate count agar (PCA, Difco) was used to determine the total aerobic mesophilic count. All Purpose Tween agar (APT, Difco) was used to enumerate lactic acid bacteria (LAB). Violet, red, bile agar (VRBG, Difco) with 10% glucose was used to enumerate *Enterobacteriaceae*. Streptomycin thallous acetate actidione (STAA, Difco) was used to enumerate *Brochothrix*spp. PCA, APT, STAA agars were incubated at room temperature (21°C) for 48 h. VRBG agar was incubated at 35°C for 24 h. Samples were spot-plated onto the surface of all of the agars with the exception of VRBG agar which were prepared as pour plates. All of these agars were incubated aerobically.

Shahihi-Ferguson perfringens (SFP, Difco) and Trptose Sulfite Cycolserine (TSC, Difco) agars were used to enumerate endospores of *Clostridium* spp. Samples of purge were treated with equal amounts of alcohol for 10 min at room temperature before plating(2,5). All meat samples were treated for 80°C for 10 minutes before plating. Both of these treatments are meant to inactivate vegetative cells that may be present in the sample(2,5). The endospores should survive the ethanol and/or heat treatments. These agars were incubated at 7°C for 3 wk in an anaerobic jar.

The "environmental" swab obtained from the processor was enumerated at the same time as the "blown-pack" spoiled beef was sampled. Sterile saline was added (10 mL) directly to the Whirlpak™ bag containing the swab. The sample was stomached for 1 min before plating. Appropriate dilutions were made and the sample was plated on to PCA, APT, RCM, STAA and VRBG agars and incubated as described above.

All isolates from the "blown-pack" meat samples that were strict anaerobes were given the designation "BP09". Isolates from the environmental samples that were strict anaerobes were designated with "En".

2.2.2 Molecular Identification of "Blown-pack" Spoilage Organisms

The reference strains used for molecular comparison included *Cl. frigidicarnis* ATCC BAA154 and ATCC BAA155, *Cl. estertheticum* ATCC 51377, and *Cl. putrefaciens* ATCC 25786. All anaerobic isolates from the "blown-pack" meat samples, anaerobic isolates from the environmental swab and BP-1, a "blown-pack" isolate identified as *Clostridium putrefaciens* (8), were subjected to molecular analysis and compared to the reference strains. Genomic DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen; MD, USA).*Clostridium* spp. were grown in peptone-yeast-glucose-starch (PYGS) broth at 7°C for 1 wk to acquire sufficient numbers of vegetative cells. The following modifications was applied to improve the lysis procedure from the manufacturer's protocol booklet: lysis, the cells were resuspended in enzymatic lysis

buffer (50 mg/mL lysozyme; Sigma, MO, USA) and incubated at 37°C for 90 min. The manufacturer's recommended protocol was followed for the remainder of the procedure.

These samples included organisms isolated from purge samples of "blown-pack" spoiled meat from 2009 that were enriched in PYGS broth (these were designated as ERP-spoiled and –clean) and a purge sample obtained from the Lacombe Meat Research center (designated LacP). LacP was obtained in 2006 and was from meat that also exhibited "blown-pack" spoilage (12).

Species-specific PCR was completed to confirm the presence of *Cl. estertheticum* in the meat purge and to presumptively identify the anaerobic isolate in both meat and environmental samples). The species specific primers were 16 SEF/SER and 16EISRF/EISRR with an expected size of 790 bp and 230 bp respectively (10). PCR was completed using the modified protocol suggested by Broda et al. (19).

Restriction fragment length polymorphism (RFLP) was completed on all anaerobic isolates using the following protocol. References strains used included: *Clostridium frigidicarnis*ATCC BAA154, *Cl. frigidicarnis* ATCC BAA155, *Cl. estertheticum* ATCC 51254 and *Cl. estertheticum* ATCC 51377. Using universal (eu) bacterial primers, were used to analyze the 16S rDNA gene by PCR. The primer sequences were: pA(forward) 5'-AGA GTT TGA TCC TGG CTC AG-3' and pH* (reverse) 5'-AAG GAG GTG ATC CAG CCG CA-3' (20). These primers are complementary to the conserved region of the 16S rDNA gene of the *Clostridium* spp. PCR was completed using the protocol of Broda

et al. (19). PCR-amplified 16S rDNA of the reference (type strains) and meat strains were digested with *Alu*I, *Hha*I, and *Hae*III endonucleases (Invitrogen, CA, USA). Restriction digests contained the following: 10 µL of PCR product, 2 µL of the appropriate buffer and 10 U of restriction endonuclease and balance Milli-Q water for a total volume of 20 µL (19). The digest mixtures were incubated overnight at 37°C. Products of the restriction digest were separated by electrophoresis using a 2.0% (w/v) agarose gel at 90 V for 1.5 h. A 1 Kb Plus DNA molecular weight marker (Invitrogen) was used as a size marker. The gel was stained with SYBR Green (Invitrogen) and banding patterns were visualized using a UV transilluminator. Restriction and visualization of PCR products was replicated three times.

2.2.3 Analysis of purge samples

Organic acids and alcohols in the purge were identified to determine the metabolites produced by the spoilage microorganism present in the "blown-pack" spoiled meat.Purge samples were incubated overnight at 4°C with equal amounts of 70% perchloric acid to remove particulates. Precipitate was removed by centrifugation at 6000 x*g* for 5 min. The supernatant (1 mL) was transferred to a high performance liquid chromatograph (HPLC) vial and stored at -20ºC until analysis by HPLC. Milli-Q water was used to make all solutions. Standard solutions were prepared in concentrations of 1 mM, 5 mM and 10 mM. Acetic acid (Fisher Scientific; Edmonton, AB), butyric acid (Sigma Aldrich, St. Louis, MO), butanol (Fisher Scientific), propan-1-ol (Fisher Scientific) and propan-2-ol (Fisher Scientific) were chosen as standard solutions based on gas chromatography profiles of headspace volatiles collected from "blown" vacuum packed venison (5).

Standard solutions of lactic acid (Fisher Scientific), valeric acid (Sigma Aldrich), propionic acid (Sigma Aldrich) were also prepared used to determine unknown peaks.

Standard solutions were made from analytical grade acids and alcohols listed above. Standard solutions (1 mL) were transferred to a HPLC vial and stored at -20ºC until it was used for analysis. The acids and alcohols were separated in a single analytical run. The organic acids and alcohols were separated using a Bio-Rad HPLC (1200 series; Agilent, Mississauga, ON) on an Aminex HPX-87H column (300 x 7.8 mm; Bio-Rad, Mississauga, ON) with a constant flow of 5 mM sulphuric acid and 5% acetonitrile (flow rate 0.4 mL/min). Samples (20 μL) of all standard solutions and supernatants were injected into the column and the presence of organic acids and alcohols was identfied with a refractive index detector and a UV detector at 210 nm, respectively. The total run for each sample was 80 min. SigmaPlot was used to obtain chromatographs.

2.2.4 Biochemical Analysis toIdentify "Blown-pack" Organisms

Clostridium isolates that were subjected to biochemical analysis were chosen based on RFLP patterns. *Clostridium* isolates and reference organism were used for biochemical testing included BP-1, BP09-01, BP09-13 and *Cl. estertheticum* ATCC 51377. All strains were grown anaerobically for 7 d in PYGS broth at 7°C. The biochemical tests were also performed at these temperatures. An API Anaerobe Identification system was used (API20A; bioMérieux, Marcy l'Etoile, France to determine which carbohydrates were fermented, indole production, and esculin hydrolysis. The manufacturer's protocols were followed and the strips were incubated at 7°C for 7 d.

2.2.5 Identification of Psychrophilic *Clostridium* spp.

Identification of the cultures was completed by sequence analysis of 16S rDNA amplification obtained by PCR as described above. Sequencing of the 16S rDNA of the isolates from RCM blood agar was done by Macrogen (MD, USA). Primers (pH* and pA) were diluted to a concentration of 1.6 pmol/μL. Sequencing was completed by Macrogen (Seoul, South Korea). Sequencing with the forward and reverse primers was done separately and aligned using National Center for Biotechnology Information (NCBI) alignment tool (blastn). The sequence was compared to gene databases.

2.3 Results

2.3.1 Isolation of Psychrophilic *Clostridium* spp. From "Blown-pack" Beef

The package of meat obtained from processor was approximately 6 weeks old and was extremely distended. A large amount of purge was collected from the package and the odour was described as "cheesy", "dairy" and "putrid". The unspoiled sample had minimal purge, no off odours, and there was no gas present in the package. The pH of the purge obtained from the "blown-pack" meat was 5.4.

Colonies (100) were chosen based on morphology. Of these colonies, 10 were strict anaerobes and were streaked for purity. These 10 anaerobes were subjected to species specific PCR and restriction fragment length polymorphism (RFLP) analysis.

The total aerobic mesophilic count, presumptive LAB counts, and total anaerobic counts of all samples were similar between the "blown-pack" spoiled meat and the unspoiled meat (Table 2.1). There was a 2 log difference between surface and purge samples for total and presumptive LAB counts on unspoiled beef. With "blown-pack" spoiled beef, there was no difference between the surface and purge samples. *Enterobacteriaceae* counts were about 1 or 2 logs less than the total and lactic acid bacteria counts (Table 2.1). *Brochothrix thermosphacta* was below 2 log CFU/cm^2 in all types of samples. Endospores were not detected after heat and ethanol treatment of the purge on media used for isolation of *Clostridium* spp. (SFP and TSC agars). Total aerobic, presumptive lactic acid bacteria, *Enterobacteriaceae*, *Br. thermosphacta*, anaerobic, and endospore counts were below detection limits for the environmental sample (Table 2.1). There was approximately 6 CFU/cm^2 (or ml for purge samples) of *Enterobacteriaceae* detected on "blown-pack" spoiled beef. This could indicate the presence of psychrotrophic *Enterobacteriaceae*. The total anaerobic count was higher than the *Enterobacteriaceae* count which suggests that the spoilage could be caused by *Clostridium* spp. It is more likely that the spoilage of the loin was caused by *Clostridium* spp. because of the odour present, temperature at which spoilage occurred and the time period in which the spoilage occurred.

Table 2.1: Enumeration of total aerobic bacteria, presumptive lactic acid bacteria, *Enterobacteriaceae*, *Br. thermosphacta* and anaerobic countsin commercial "blown-pack" and unspoiled beef on various media

	Environmental Sample (log CFU/cm^2)	Unspoiled Beef		"Blown-pack" Beef	
		Surface (log CFU/cm^2)	Purge (log CFU/mL)	Surface (log CFU/cm^2)	Purge (log CFU/mL)
Total plate count	<2	4.2	6.2	6.8	6.4
LAB	<2	5.9	7.7	6.7	8.2
Enterobacteriaceae	<1	<2	<2	5.7	5.7
Br. thermosphacta	<1	<2	<2	<2	<2
Anaerobic count	<2	7.04	7.6	7.7	8.7
Clostridium spp. counts	<2	ND	<2	ND	<2

ND = not determined; n=1.

2.3.2 Molecular Identification of "Blown-pack" Spoilage Organisms

The organisms tested were isolated from "blown-pack" spoiled meat as well as samples of purge collected from both unspoiled and spoiled packages. Using 16SEF/R and 16EISF/R primers, detection of *Cl. estertheticum* was completed. Bands were present at 790 bp using primers 16SEF/R for BP09-01, -03, -04, -06, -09, -13 and -14 and the reference strain *Cl. estertheticum* ATCC 51337 (Figure 2.1a). Bands were also observed at 230 bp using primers 16EISF/R for BP09-01, -03, -04, -06, -13 and *Cl. estertheticum* ATCC51377 (Figure 2.1b). No bands were detected for all of the anaerobic environmental isolates. No bands were observed for *Cl. frigidicarnis* ATCC BAA154, *Cl. frigidicarnis* ATCC BAA155, *Cl. estertheticum* ATCC 51524, BP-1 and the negative control. Bands were not expected for the reference strains except for *Cl. estertheticum* ATCC 51524 and *Cl. estertheticum* ATCC51377.

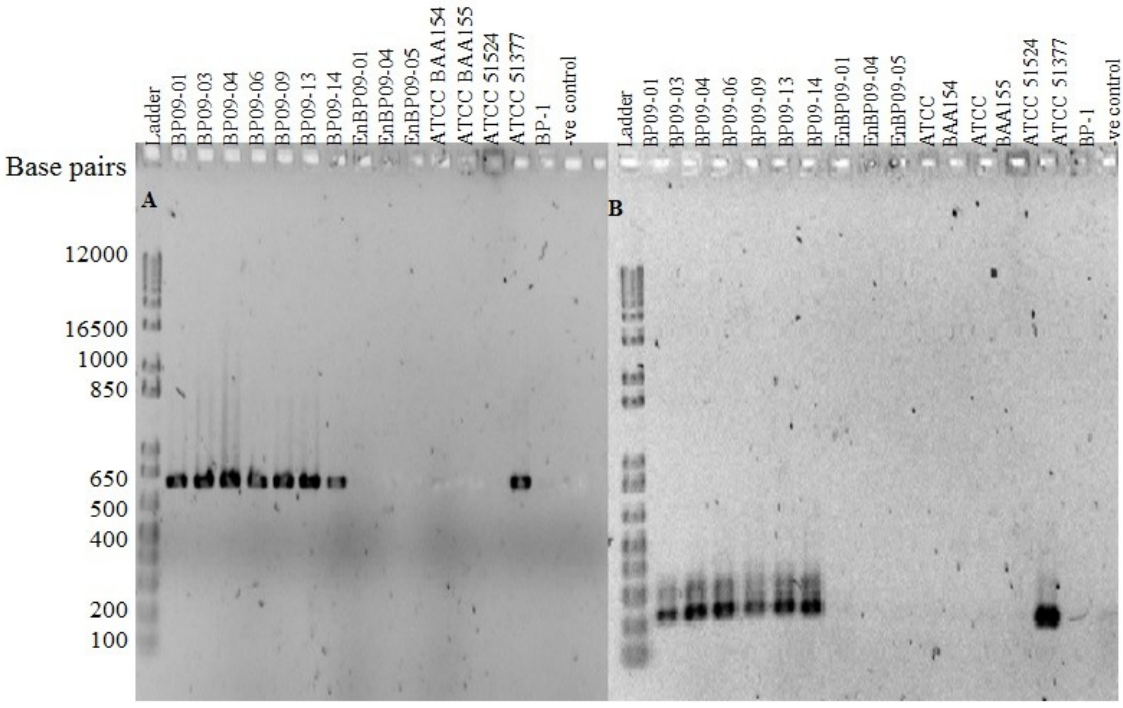


Figure 2.1: Agarose gel with bands using species-specific primers for *Cl. estertheticum*. Primers (A) 16SEF/R and (B) 16ESIF/F were used to detect *Cl. estertheticum* using genomic DNA isolated from each isolate. The designation "En" stands for environmental sample. *Clostridium frigidcarnis* ATCC BAA154, *Cl. frigidicarnis* ATCC BAA155, *Cl. estertheticum* ATCC 51377 and *Cl. estertheticum* ATCC 51254 were used as a reference strain and the negative control was a sample with PCR reaction mixture and water.

Restriction fragment length polymorphism (RFLP) patterns using *Alu*I, *Hae*III and *Hha*I were able to differentiate between different isolates and DNA obtained from the purge samples. With *Alu*I (Figure 2.2a), BP09-01 and BP09-14 had different banding patterns compared to the rest of the BP09 isolates. BP09-14 banding pattern was similar to that of BP-1, while BP0903, -04, -06, -09, and -13 had similar banding patterns to *Cl. estertheticum* ATCC 51377. All the environmental samples had similar banding patterns which did not match that of any of the reference strains. The enriched purge from the spoiled sample had banding patterns that were similar to the purge that was obtained from the Lacombe Research Center.

Similar results were observed with restriction enzyme *Hae*III (Figure 2.2b). BP09-01 and BP09-14 exhibited different banding compared to the other BP09 isolates. However, the banding pattern for BP09-14 was not similar to that observed for BP-1. The banding patterns for BP09-03, -04, -06, -09 and -13 were similar to *Cl. estertheticum* ATCC 51377 and were similar to the banding patterns obtained for BP-1. The banding patterns for the isolates from the environmental sample were not the same. EnBP09-05 had a different banding pattern compared to the other isolates from the environmental sample and it was similar to that detected in the enriched purge obtained from the "blown-pack" spoiled meat.

With the restriction endonuclease *Hha*I (Figure 2.2c), BP09-01 and -14 were different compared the other BP09 isolates. Isolates BP09-03, -04, -06, -09, and -13 had different banding patterns from that of *Cl. estertheticum* ATCC 51377 and did not match any of the other reference strains. The banding patterns from BP09-01 and -14 also did not match any of the reference strains. The enriched purge samples from both the spoiled and clean meat were similar to each other and were similar to purge that was obtained from Lacombe. The environmental sample exhibited different banding patterns, with EnBP09-01 having the different banding pattern compared to the other two environmental isolates. BP09-14 had similar banding patterns to that of the other two environmental isolates

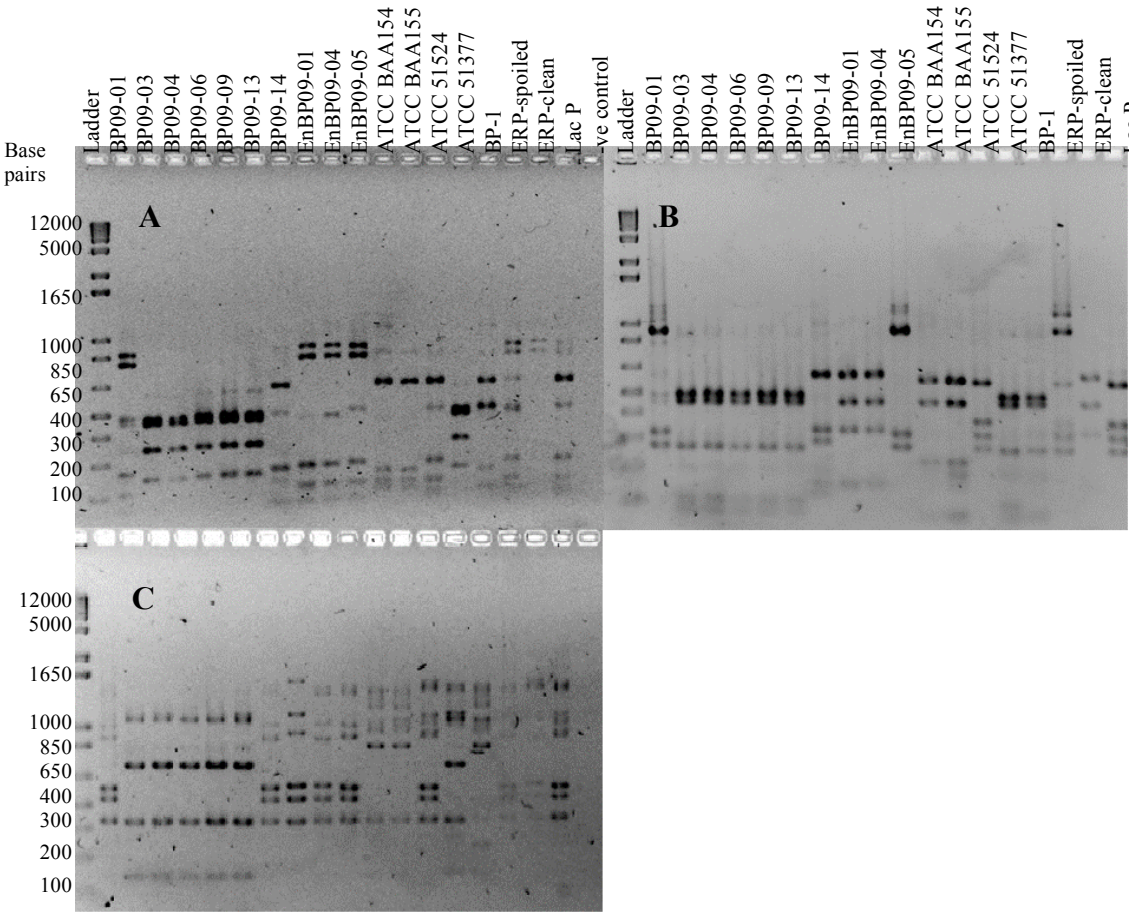


Figure 2.2: Restriction patterns of 16S DNA from anaerobic isolates using (A) *Alu*I,(B) *Hae*III, and (C) *Hha*I restriction endonuclease. *Cl. frigidicarnis* ATCC BAA154 and BAA155, *Cl. estertheticum* ATCC 51254 and ATCC 51377 were used as reference strains. BP- designates isolates from "blown-pack" spoiled meat and 09 designates that they were isolated in meat from 2009. En- designates isolates from the environmental samplethat were obtained from the processing plant. ERP-spoiled and –clean indicates purge samples from the spoiled meat and unspoiled meat that were enriched in PYGS broth. LacP was purge obtained from the Lacombe Meat Research center. The negative control was a sample with PCR reaction mixture and water without DNA template.

2.3.3 Analysis of Purge

Quantitative analysis of organic acids and alcohols showed that lactic acid, acetic acid and propionic acid were present in the purge collected from the distended package

(Fig. 2.3). The major difference between the "blown-pack" spoiled meat compared to the unspoiled meat is the presence a large amount of propionic acid. Lactic acid, acetic acid were present in both samples of purge. Valeric acid was also present in the purge collected from the unspoiled meat and was not present from the purge collected from the distended package.

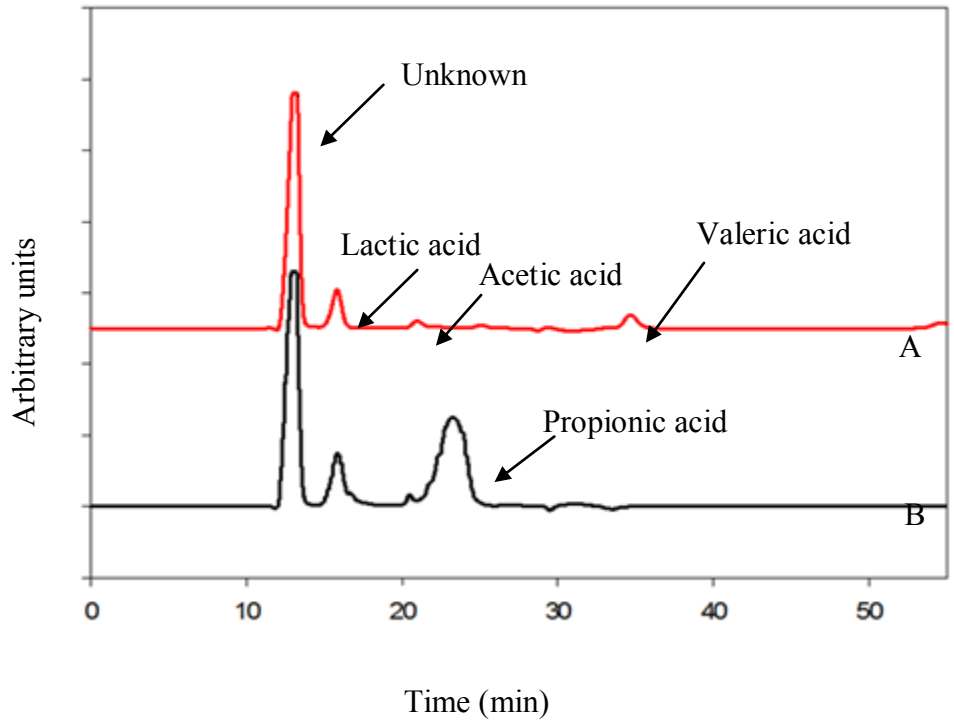


Fig. 2.3 HPLC profile using UV detection of organic acids dissolved in purge obtained from purge from (A) unspoiled vacuum packaged beef and; (B) "blown" vacuum packaged beef.

2.3.4 Biochemical Analysis of "Blown-pack" Organisms

Cl. putrefaciens BP-1 and *Cl. estertheticum* BP09-01 and BP09-13 had different biochemical characteristics (Table 2.2). All isolates were Gram positive, rods that formed endospores. The cells were generally single or in short chains. All of the colonies on PYGS agar were small, smooth and convex. All of the isolates were strictly anaerobic. BP-1 has the ability to grow at temperatures between 7 to 20°C. BP09-01 and

BP09-13 grew optimally at 7°C. These isolates did not grow at temperatures above 15°C.

2.3.5 Identification of psychrophilic *Clostridium* spp.

After RFLP was completed, 16S rDNA of BP09-01, -13 and BP-1 were sent for sequencing. The sequences were compared to type strains using gene databases (NCBI and Ribosome Project). Isolates BP09-01 and -13 were *Cl. estertheticum* and BP-1 was *Cl. putrefaciens*. The 16S rDNA sequence of BP-1 matched the sequences for *Cl. putrefaciens* with a 97% identity (Accession number NR_024995.1). The BP09-01 and BP09-13 sequences matched the sequences for *Cl. estertheticum* with a 93% and 97% identity, respectively (Accession number NR_042153.1). Isolates BP09-01 and -13 were chosen for continued work because of the different RFLP banding patterns. Isolate BP09-14 and the isolates from the environmental samples were not *Clostridium* spp. Most of the organisms were LAB and on further investigation, the organisms were not completely anaerobic. Some of the other organisms that were anaerobic were tested using sequencing of the 16S rDNA and they were mostly LAB with *Carnobacterium* spp. being one of the more predominant organisms.

Table 2.2
Characteristics of three strains of psychrotrophic *Clostridium* spp. isolated from spoiled meat

	Isolates		
	BP-1	BP09-01	BP09-13
Indole production	-	-	-
Urease presence	-	-	+
Hydrolysis of			
Gelatin	-	-	-
Esculin	-	-	+
Catalase	-	-	-
Acid produced from:			
Glucose	-	-	+

Mannitol	+	+	+
Lactose	+	+	-
Saccharose	+	-	+
Maltose	+	-	+
Salicin	+	-	+
Xylose	+	+	-
Arabinose	+	+	-
Glycerol	+	-	-
Cellobiose	+	-	-
Mannose	+	-	-
Melezitose	+	-	-
Raffinose	+	+	+
Sorbitol	+	+	+
Rhamnose	+	+	+
Trehalose	+	-	-
Gram stain	+	+	+
Presence of spores	+	+	+
Morphology	rods	rods	rods

2.4 Discussion

"Blown-pack" spoiled meat caused by *Clostridium* spp. is not a new problem for the food industry. It is just a sporadic issue that occurs in a fraction of the packages within a same time period. However, with increased movement of food products for domestic and international trade, these issues may arise more often. Along with potential product loss, the loss of confidence with companies if they have shipments with "blown-pack" spoiled meat can result. The loss of confidence has other unwanted effects such as losing contracts or loss of market access. The cases of "blown-pack" spoilage have been reportedfor vacuum packaged lamb in Australia and New Zealand (2), and in vacuum packaged beef in southern Africa, northern Europe and North America (6,7,12,16,21,22). Reports of "blown-pack" spoilage in Canada has been rare (8,12-14) and generally only occur in vacuum package beef. One possibility that may explain the sporadic nature of this type of spoilage is the variability in contamination of the meat product (23). Not all the meat product in one production cycle will be contaminated by *Clostridium* spp.

Availability of meat substrates, such as limited glucose, could also play a role in the sporadic nature of "blown-pack" spoilage (13).

Enterobacteriaceae were detected on the loin from the "blown-pack", but not on the unspoiled loin, which was expected. In some cases, especially where meat packages have been temperature abused, *Enterobacteriaceae* produce enough gas to cause the packages to blow up(1). The counts of *Enterobacteriaceae* on the spoiled loin do not indicate that spoilage was caused by this group of organisms (2). To produce enough gas to cause the type of spoilage that was observed, counts greater than 6 logs are required. Coupled with the spoilage observed at refrigeration temperature and the odour that was produced, the production of gas was more likely caused by *Clostridium* spp.

The total, lactic acid bacteria, and anaerobic counts were quite similar in thecommercially spoiled package of meat. Since all the counts were similar, it is likely that most of the bacteria that were isolated from the commercially spoiled package were lactic acid bacteria. Only approximately 10% of the colonies that were chosen were strictly anaerobic. Although *Clostridium* spp. should be present in relatively high numbers due to the type of spoilage observed, it is not always possible to isolate these organisms or grow a pure culture on solid media (2,5). Using specialized media still results in low numbers of psychrophilic *Clostridium* spp. (6,7). One way to increase the likelihood of isolation of *Cl. estertheticum* from "blown-pack" spoiled is to complete the isolation process in a cold room, which was done in the current experiment. *Cl. estertheticum* is

isolated more readily at 10°C compared to when the protocol was completed at room temperature (8).

The presence of *Brochrothrix thermosphacta* is indicative of spoiled meat. However, these organisms were not detected in the "blown-pack" spoiled meat or in the unspoiled meat. This is expected for the unspoiled meat and indicates that the "blown-pack" spoiled meat was not an older sample (2). *Clostridium* spp. was not detected in selective media, SFP and TSC, in both the spoiled and unspoiled meat. The addition of pre-treatment (heat or alcohol) should have resulted in the isolation of endospores. This could indicate that there was no endospore present or that the heat or alcohol treatment were too severe to isolate the endospores. The plates that were used were pre-reduced but there may have been some oxygen that was dissolved within the plastic petri plates (Phil Fedorak, personal communication, August 7, 2009). If some of the packages exhibited leakage before isolation, oxygen could have been introduced and therefore made it more difficult to isolate viable cells.

No strict anaerobic organisms were isolated from the unspoiled meat. The anaerobic or microaerophilic organisms isolated from the environmental samples were not *Clostridium* spp. Species specific PCR using primers to detect *Cl. estertheticum* in the isolates from the "blown-pack" spoiled meat. All of the isolates from the "blown-pack" were positive using the primers specific for *Cl. estertheticum*. However, the banding patterns from the RFLP analysis were different for two "blown-pack" isolates. Isolate BP09-01 had a different banding pattern for all three restriction enzymes used compared to the rest of the

BP09-isolates. The banding pattern of BP09-01 did not match the banding patterns for *Cl. estertheticum* ATCC51524 and ATCC51377 even though with species specific PCR, the bands were present for both *Cl. estertheticum* primers used. For all three restriction enzymes used, there were bands that were present for BP09-13 that were not present for the other BP09 isolates or for the reference strains *Cl. estertheicum* ATCC51524 and ATCC51377. Sequencing of the 16S rDNA of BP09-01 and some of the BP09 isolates indicated that these organisms were *Cl. estertherticum*. Therefore, the differences in banding patterns could indicate a different strain rather than clonal isolates.

The major metabolite that differed between the spoiled and unspoiled package of meat was propionic acid. This particular metabolite is quite pungent and could be partially responsible for the difference in smell. The use of HPLC on purge does not give a comprehensive view of all the metabolites that result from spoilage due to *Clostridium* spp. Headspace gas provides a different understanding of what is resulting from *Clostridium* spp. fermentation(5,6). Headspace gas analysis of "blown-pack" spoiled meat indicates that butyl esters, butyric acid and butanol are present and results in a distinctive cheesy odour (5,6).

Each isolate tested utilized glucose which was expected (12). There were differences between BP09-01 and BP09-13, even though both organisms were presumptively identified as *Cl. estertheticum*. This could explain why the banding patterns were slightly different between the two isolates and therefore the primers used for species specific PCR are not sensitive enough to detect strain differences. Also, differences in sugar utilization could be important in determining better growth mediums to isolate these organisms.

Overall, when comparing the spoiled versus unspoiled meat, as well as the environmental samples, there were different organisms detected in the three types of samples. The "blown-pack" spoiled beef did not have the same microflora as the unspoiled beef as expected. The environmental sample did not yield any organisms that were similar to those isolated from the "blown-pack" spoiled beef. This indicates that the dust in the processing plant (16) was not likely responsible for contaminating the beef during processing. It would have been beneficial to have received swabs from the hide or dirt in and around the area in which these animals graze to determine whether these organisms are present in the soil. Although judging by how difficult it was to isolate these organisms, it may not have been easy to detect these endospores in the environment. To isolate *Cl. estertheticum* it is best to maintain the temperature below 10°C.

2.5 References

(1) Hanna MO, Smith GC, Hall LC, Vanderzant C. Role of *Hafnia alvei* and a *Lactobacillus* species in the spoilage of vacuum-packaged strip loin steaks. J Food Prot 1979 Jul;42(7):569-571.

(2) Broda DM, DeLacy KM, Bell RG, Braggins TJ, Cook RL. Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Int J Food Microbiol 1996 Apr;29(2-3):335-352.

(3) Egan AF, Roberts TA. Microbiology of meat and meat products. 1st ed. New York: Wiley; 1987.

(4) Shay B, Egan A. The packaging of chilled red meats. Food Technology in Australia 1987 Jun;39(6):283-285.

(5) Broda DM, DeLacy KM, Bell RG, Penney N. Association of psychrotrophic *Clostridium* spp., with deep tissue spoilage of chilled vacuum-packed lamb. Int J Food Microbiol 1996 Apr;29(2-3):371-378.

(6) Dainty RH, Edwards RA, Hibbard CM. Spoilage of vacuum-packed beef by a *Clostridium* sp. J Sci Food Agric 1989 Apr;49:473-486.

(7) Kalchayanand N, Ray B, Field RA, Johnson MC. Spoilage of vacuum-packaged refrigerated beef by *Clostridium*. J Food Prot 1989 Jun;52:424-426.

(8) L. Ho. Characterization of "blown-pack" spoiled beef. Edmonton, Alberta: University of Alberta; 2009.

(9) Broda DM, Boerema JA, Bell RG. A PCR survey of psychrotrophic *Clostridiumbotulinum*-like isolates for the presence of BoNT genes. Lett Appl Microbiol 1998 Oct;27(4):219-223.

(10) Broda DM, Boerema JA, Bell RG. PCR detection of psychrophilic *Clostridium* spp. causing 'blown pack' spoilage of vacuum-packed chilled meats. J Appl Microbiol 2003 Feb;94(3):515-522.

(11) Broda DM, DeLacy KM, Cook RL, Bell RG. Prevalence of cold-tolerant clostridia associated with vacuum-packed beef and lamb stored at abusive and chill temperatures. N Z J Agric Res 1997 Mar;40(1):93-98.

(12) Yang X, Balamurugan S, Gill CO. Substrate utilization by *Clostridium estertheticum* cultivated in meat juice medium. Int J Food Microbiol 2009 Jan;128(3):501-505.

(13) Yang X, Gill CO, Balamurugan S. Products of glucose and lactate fermentation, and utilization of amino acids by *Clostridium estertheticum* subspp. *laramiense* and estertheticum growing in meat juice medium. J Food Prot 2010 Jul;73(7):1348-1352.

(14) Yang X, Balamurugan S, Gill CO. Effects on the development of blown pack spoilage of the initial numbers of *Clostridium estertheticum* spores and *Leuconostoc mesenteroides* on vacuum packed beef. Meat Sci 2011 Jul;88(3):361-367.

(15) Kalchayanand N, Ray B, Field RA. Characteristics of psychotrophic *Clostridium laramie* causing spoilage of vacuum-packaged refrigerated fresh and roasted beef. J Food Protein 1993 Jan;56:13-17.

(16) Helps CR, Harbour DA, Corry JEL. PCR-based 16S ribosomal DNA detection techniques for *Clostridium estertheticum* causing spoilage in vacuum-packed chill-stored beef. Int J Food Microbiol 1999 Nov;52:57-65.

(17) Broda DM, Lawson PA, Bell RG, Musgrave DR. *Clostridium frigidicarnis* sp nov., a psychrotolerant bacterium associated with 'blown pack' spoilage of vacuum-packed meats. Int J Syst Bacteriol 1999 Oct;49:1539-1550.

(18) Broda DM, Saul DJ, Lawson PA, Bell RG, Musgrave DR. *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum-packed meat. Int J Syst Evol Microbiol 2000 Jan;50:107-118.

(19) Broda DM, Bell RG, Boerema JA, Musgrave DR. The abattoir source of culturable psychrophilic *Clostridium* spp. causing 'blown pack' spoilage of vacuum-packed chilled venison. J Appl Microbiol 2002 Oct;93(5):817-824.

(20) Hutson RA, Thompson DE, Collins MD. Genetic interrelationships of saccharolytic *Clostridium botulinum* types B,E and F and related clostridia as revealed by small-subunit rRNA sequences. FEMS Microbiology Letters 1993 Mar;108:103-110.

(21) Byrne B, Monaghan AM, Lyng JG, Sheridan JJ, Bolton DJ. A case of "blown pack" meat linked to *Clostridium estertheticum* in Ireland. J Food Saf 2009 Nov;29(4):629-635.

(22) Moschonas G, Bolton DJ, Sheridan JJ, McDowell DA. Isolation and sources of 'blown pack' spoilage clostridia in beef abattoirs. J Appl Microbiol 2009 Aug; 107(2):616-624.

(23) Boerema JA, Broda DM, Bell RG. PCR detection of psychrotolerant clostridia associated with deep tissue spoilage of vacuum-packed chilled meats. Lett Appl Microbiol 2002 Oct;35:446-450.

3. Effect of Heat and Pressure on Resistance of Endospores of Psychrophilic *Clostridium* spp.

3.1 Introduction

Clostridium spp. have not traditionally been implicated as a major cause of meat spoilage; however, the causative agent for "blown-pack" spoilage of vacuum packaged meat has been identified as *Clostridium* spp. (1-4). "Blown-pack" spoilage is characterized by gross distention of the package due to gas production by *Clostridium* spp. and by the production of offensive odours within 4 to 6 wk of refrigeration (5). There are at least 10 different species of psychrotrophic and psychrophilic *Clostridium* spp. that have been isolated or associated with "blown-pack" vacuum packaged beef (6, 7). The most common of these organisms include *Clostridium estertheticum*, *Cl. estertheticum* subsp. Laramie and *Clostridiumgasigenes*. Both *Cl. estertheticum* and *Cl. estertheticum* subsp. Laramie, have been implicated in "blown-pack" spoilage of beef from southern Africa, northern Europe and North America (2, 8-10), and in vacuum packaged lamb from New Zealand (4). *Cl. gasigenes*, a psychrotroph, has only been implicated in "blown-pack" spoilage in vacuum packaged lamb from New Zealand (11). Recently, there have been cases of "blown-pack" spoilage of fresh beef in Canada (1, 10) by *Cl. estertheticum*.

Endospores are found in soil and can contaminate the carcass via contact with the hide or contaminated equipment. Interventions, such as steam pasteurization, acid washes, and peroxyacetic acid (12-14), are used to limit contamination of carcasses with pathogens. Endospores of *Clostridium* spp. have the ability to survive most, if not all, the

interventions that are used during the slaughter and fabrication processes (13). Thermal processing during slaughter and fabrication of carcasses are not harsh enough to inactivate endospores but instead may induce germination. Mild heat can induce germination of endospores (15-17), although the exact amount of heat required to induce germination of endospores is unknown. Exposure to a heatshrink process during packaging may play a role in the induction of germination of endospores which will lead to the onset of "blown-pack" spoilage (18, 19).

The use of heat to kill endospores of psychrophilic *Clostridium* spp. isolated from blown pack spoiled meat has been investigated to a limited extent. *Cl. estertheticum* was exposed to 80, 90, 95 and 100°C for up to 240s (14). A 6 log reduction was observed for 100°C after 240s of treatment (14). There was no significant reduction at all other heat treatments (14). Heat can reduce populations of endospores of type II *Clostridium botulinum*(20, 21). Heating psychrotrophic *Clostridiumbotulinum* at 90°C for 30 min is sufficient to decrease numbers by 6 log CFU/mL(22).

Compared to mesophilic endospores, psychrotrophic *Cl. botulinum* requires lower temperatures for inactivation (21). A combination of heat and pressure is used to inactivate endospores, however, these interventions are not suited for fresh meat. Little work has been completed with psychrotrophic endospores at refrigeration temperature for extended periods of time.

A promising approach for control of endospores in food is high pressure in combination with other interventions. Most of the research on the application of high pressure as an intervention for endospores in food has been completed in a liquid or puree. Very few studies have investigated the effects of high pressure on endospores in raw beef. There is a limitation on the pressure that can be used with fresh meat before the quality of the meat becomes unacceptable. High hydrostatic pressure (HHP) above 400 MPa denatures myoglobin, which results in an overall whitening effect of the meat (23, 24). When heat is added to the pressure treatment, the colour of the meat is further degraded. Therefore, pressure treatment at 400 MPa or lower and below ambient temperatures would be more suitable for raw meat processing.

At 400 MPa, inactivation of endospores is generally not achieved (25-27), but it will have an impact on the endospores. High pressure (>400 MPa) stimulates non-nutrient germination pathways of endospores (26, 28-32). High pressure stimulates the first stage of germination (26, 28-32), which simultaneously results in loss of dipicolinic acid (DPA) and some core hydration (33). The second stage of germination involves complete core hydration and loss of resistance to heat, chemicals or bacteriocins (33). Pressure treatment at 100 to 400 MPa, combined with temperatures of 20 to 50°C, stimulates nutrient germination pathways in *Bacillus subtilis* and *Bacillus cereus* (26, 28-32). This stimulation of germination pathways may offer an opportunity for other interventions to kill endospores. In food applications, high pressure may be used to induce germination of the endospore which may then allow bacteriocins or other antimicrobials to pass through all the protective layers and have an effect on the inner

membrane. Most bacteriocins will interact with the inner membrane of viable cells to produce pores that lead to the disruption of the electron gradient and result in cell death (34-37). Most of the research has focused on nisin and HHP and its effect on *Clostridium* spp. (38, 39). Reutercyclin and nisin in combination with HHP, and its effect on *Clostridium* and *Bacillus* endospores, has been explored with varying success (38). When choosing antimicrobials in combination with HHP, some antimicrobials may not increase reduction but instead provide protection. Reutercyclin enhanced survival of mesophilic *Clostridium* and *Bacillus* spp. (38). No work has been completed with other bacteriocins or bacteriocin-containing antimicrobial preparations, such as Micocin X™, and psychrophilic *Clostridium* spp. In addition, very little work has been done on the effect of HHP at refrigeration temperatures.

Bacterial endospores are extremely resistant to a variety of different environmental pressures. This can include temperature, pressure, radiation and chemicals. The multiple layers of a bacterial endospore play a major role in its resistance. However, small acid soluble proteins (SASPs) are essential for the resistance to stress such as temperature, radiation and high pressure processing (HHP). SASPs are unique to endospores and are synthesized during late sporulation and degraded during germination (40). SASPs bind tightly to DNA in the endospore core which causes conformational changes to the DNA that ensures that DNA is protected from UV radiation, desiccation, and dry heat (41-43).

Endospores lacking α/β-type SASPs are more sensitive to heat, UV radiation and chemicals (40, 44-47). Generally wet heat, hydrogen peroxide, or desiccation do not kill

endospores by DNA damage, but when endospores lack α/β-type SASP, DNA damage is detected. There are high levels of mutations in survivors and large decreases in endospore resistance due to the loss of DNA repair mechanisms (48-52). Mechanisms by which SASPs protects DNA from heat, chemicals and UV are known. SASPs enables DNA to withstand a variety of different interventions because of conformational changes that occur when SASPs bind to the DNA. Instead of B-DNA conformation, the α/β-type SASP forces the DNA into a A-like conformation (53, 54). The A-like structure is more stable than the B-DNA structure. The structure also makes it difficult for penetration of chemicals and radiation to the DNA. Few studies have discussed the role or mechanism of SASPs in pressure resistance (28, 55). In *Bacillus* spp., the presence of SASPs may actually increase pressure inactivation at 100 to 300 MPa (55).

The objectives of this study were to: 1) investigate the heat and pressure resistance of psychrophilic *Clostridium* spp. that are responsible for "blown-pack" spoilage; 2) investigate the use of HHP in combination with bacteriocins to destroy psychrophilic endospores in fresh meat; 3) understand the effects of peptidoglycan on the survival of endospores after HHP; and 4) to determine whether α/β-type SASPs are present in psychrophilic *Clostridium* spp. isolated from "blown-pack" spoiled beef.

3.2 Material and Methods

3.2.1 Strains, Growth and Sporulation Conditions

The bacterial strains used in this study included *Clostridium putrefaciens* BP-1 [BP – "blown-pack" isolate from commercially spoiled "blown-pack" vacuum packaged

beef; (1)], *Clostridium estertheticum* BP09-01, *Cl. estertheticum* BP09-13 (BP09 – indicates organisms isolated from commercially spoiled "blown-pack" beef from 2009), and *Cl. estertheticum* ATCC 51377. For growth, endospores (maintained at -80°C in growth media with 50% glycerol) were inoculated into peptone (Difco, Becton, Dickinson and Company, Sparks, MD), yeast extract (Difco), glucose, starch (PYGS) broth (56) and incubated under anaerobic conditions at 7°C for 3 weeks. Anaerobic handling was done in an anaerobic chamber (Forma Scientific, Model 1025 Anaerobic System Incubator, Marietta, OH). Before incubation, cultures were transferred to an aerobic jar containing GasPak™ EZ pouch(Becton, Dickinson and Company, Mississauga, ON). The jars were filled in the anaerobic hood to ensure no oxygen remained in the jar. Viable cells were sporulated by inoculating PYGS broth and incubating the media at 7°C for 3 to 6 months. Endospores were harvested by centrifugation at 7500 x *g* for 30 min. The harvested endospores were washed 10 times with 50 mL of cold saline (0.85%) and resuspended in 25 mL of saline (final concentration 6 log CFU/mL).

For SASPs analysis, *Clostridium perfringens* ATCC 13124 were inoculated from endospore stocks, maintained at -80°C in 50% glycerol, into PYGS broth. The isolates were incubated anaerobically in anaerobic jars with one to three (depending on the size of the jar) GasPak™ EZ sachet (Becton, Dickinson and Company, Mississauga, ON) before incubation. *Cl. perfringens* ATCC 13124 was incubated at 37°C for 24 h. *Cl. perfringens* ATCC 13124 was incubated anaerobically at 37°C for 1 week because *Cl. perfringens* does not require as much time to sporulate. The presence of endospores was

confirmed by phase-contrast microscopy. Endospores were harvested from the broth by centrifugation at 2700 x *g* for 30 min. The harvested endospores were washed with sterile 0.9% saline 10 times. The washed endospores were resuspended into sterile 0.9% saline.

For SDS-Page determination of SASPs, *Pseudomonas aeruginosa* ATCC 27853 and cells of *Cl. perfringens* ATCC 13124 were used as reference strains. *P. aeruginosa* ATCC 27853 was inoculated from stocks, maintained at -80°C in 50% glycol in all-purpose tween (APT; Difco, Becton, Dickinson and Company, Sparks, USA) broth and incubated at 21°C for 24 h.

3.2.2 Thermal Treatment of Endospores

Endospores were tested for heat resistance under anaerobic and aerobic conditions. Endospores (1 mL of 5 log CFU/mL) were placed into sterile Eppendorf tubes. For heating under anaerobic conditions, tubes were transferred into an anaerobic hood and heated in a dry bath incubator (Isotemp Digital 2-Block Model 125DQ, Fisher Scientific, Edmonton, AB) at 70, 80 and 90°C for 0, 4, 8, 15, 30, and 60 min. Immediately after heating, samples were put on ice before plating within 60 min after heating. Heated endospores were diluted in pre-reduced saline (0.85%; pre-reduced leaving saline in the anaerobic hood prior to use) and spot-plated onto pre-reduced PYGS agar. Plates were transferred into an anaerobic jar containing GasPak™ sachets and incubated at 7°C for 3 wk. The identical protocol was used to heat endospores in an aerobic environment. The endospores were plated aerobically and transferred to an anaerobic jar containing a

GasPak™sachet and incubated at 7°C for 3 wk prior to enumeration. All experiments were done in triplicate.

3.2.3 High Pressure Treatment of Endospores

Endospores (150 µL) were transferred to 0.42 cm Tygon S3™ E-3603 tubing (Application Specific Tubing, Saint-Gobain Performance Plastics, Pittsburgh, PA). The tubes were sealed using a hair-straightener (TONI&GUY®, Model TGST2976F, London, UK). Tubes were placed in a cryogenic vial (Corning Life Sciences, Pittsburgh, PA) and held on ice until treatment. Tubes for heating were prepared the same day as testing for pressure treatment, samples were placed into vessels of a U111 Unipress (Warsaw, Poland) conditioned at different temperatures. Samples were pressurized at 400 MPa at 4, 40 or 70°C for 0, ramp, 4, 8, 15, 30 and 60 min. Ramp is the time it took for the machine to reach desired pressure. Pressure vessels were 12 X 58 mm and temperature control was achieved using an external circulating propylene glycol bath (LAUDA Proline, Delran, NJ). Immediately after depressurization, samples were stored on ice until plating within 60 min. The treated endospores were diluted in saline and spot-plated onto PYGS agar. Plates were transferred to an anaerobic jar and incubated at 7°C for 3 wk prior to enumeration. All experiments were done in triplicate.

3.2.4 Endospore Inactivation by High Pressure and Antimicrobials in Meat

Beef was obtained from a local, federally inspected beef processing plant. Beef was cubed using a sterile knife and a cutting board that was sprayed with 70% ethanol. The cubed beef was transferred to a food processor (Model KFP0711CU, KitchenAid,

Mississauga, ON) that was cleaned with 70% ethanol. The beef was minced and portioned (50 g). The following treatments were prepared in beef: endospores alone; endospores and Micocin X™ (spray dried culture supernatant *Carnobacterium maltaromaticum* ATCC PTA-5313, Griffith Laboratories, Toronto, ON); endospores and the supernatant of *Carnobacterium maltaromaticum* UAL8C2; endospores and fresh APT broth; saline and Micocin X™; the supernatant of *C. maltaromaticum* UAL8C2; and beef with APT broth. Endospores were inoculated into the meat at 5log CFU/g. The Micocin X™ and APT broth were added at 1% (w/v) to the meat. Sterile saline was added to ensure that the amount of liquid added to every treatment was the same.

The inoculated meat (approximately 3 g) was transferred to sterile 1.5 mL pressure tubes (Corning Life Sciences, Pittsburgh, PA). The tubes were filled using sterile tweezers and packed so that no air bubbles were present. Samples were placed into vessels of the U111 Unipress and processed as described above. The samples were transferred to sterile saline and appropriate dilutions were made. All samples were spot-plated onto plate count agar (PCA; Difco), APT agar, PYGS agar, and violet, red bile, agar with 1% (w/v) glucose added (VRBG; Difco). The PCA and APT agars were incubated aerobically at 21°C for 48 h; VRBG was overlayered and incubated aerobically at 37°C for 24 h; and PYGS agar was placed in anaerobic jars with GasPak™ EZ Sachets and incubated at 7°C for 21 d before enumeration. All experiments were done in triplicate.

3.2.5 Inactivation of Endospores by High Pressure in the Presence of Peptidoglycan

Peptidoglycan was added to endospores in saline to determine whether cell wall fragments in meat or the Micocin X™ supernatant had a germinant effect on endospores.

Saline and endospore mixtures were prepared using an endospore suspension and 1% (w/v) of Micocin X™ and peptidoglycan isolated from *Staphylococcus aureus* (Sigma-Aldrich Canada, Oakville, ON)or from *Bacillus* spp. (Sigma-Aldrich) were added to the solution as follows: endospores; endospores with Micocin X™; endospores with peptidoglycan; and endospores with peptidoglycan and Micocin X™.

Solutions (150 µL) were transferred to 0.42 cm Tygon S3™ E-3603 tubing and treated in the U111 Unipress as described above. Immediately after depressurization, samples were stored on ice until plating (less than 60 min). The samples were transferred to a sterile eppendorf tube and diluted using sterile 0.85% saline. Diluted samples were spot-plated onto PYGS agar and plates were transferred to an anaerobic jar with GasPak™ EZ Sachets. Treatments that did not include peptidoglycan were plated onto both PYGS agar and PYGS with 0.1% peptidoglycan spread onto the plate. The samples were incubated at 7°C for 21 d before enumeration. All experiments were completed in triplicate.

3.2.6 Statistical Analysis

The bacterial counts of psychrotrophic *Clostridium* spp. were calculated as Log CFU/mL. The statistical analyses were completed using SAS software, SAS University Edition for Windows (Copyright © 2015 SAS Institute Inc., Cary NC, USA). Two way analysis of variance (ANOVA) was completed using the PROC GLM program. Difference between the means of the different heat and pressure treatments were identified using Student-Newman-Keuls (SNK) multiple range test.

3.2.7 Molecular Determination of SASPs in Endospores

Detection of some genes responsible for the production of SASPs was completed using polymerase chain reaction (PCR) with three gene specific primers (Table 3.1). Genonic DNA was isolated from vegetative cells of psychrophilic *Clostridium* spp using DNeasy Blood and Tissue Kit (Qiagen; Maryland,USA). The manufacturer's recommended protocol for isolation of nucleic acid from Gram positive bacterial was followed, with modification to the lysis procedure. To improve lysis, the cells were resuspended in enzymatic lysis buffer (50 mg/ml lysozyme; Sigma, St. Louis, MO) and incubated at 37ºC for 90 min. From this step the recommended protocol was followed. To confirm the presence of DNA, an aliquot of the eluted DNA was electrophoresed on a 1% (w/v) agarose gel (Invitrogen, Carlsbad, CA) in 0.5 X TE buffer at 90 V for 90 min. A 1 Kb Plus DNA ladder (Invitrogen, Carlsbad, CA) was used. The eluted DNA was stored at -20ºC pending PCR amplification.

PCR amplification was performed with the three primers used by Raju and Sarker (Table 3.1; 57). The PCR protocol of Raju *et al.*(58) was used with a modifiedthe PCR reaction mixture. Each PCR reaction mix consisted of 8.3µl of 10X PCR buffer, 2.5µl of $MgCl_2$ (50 nM), 0.1 µl of each primer (forward and reverse), 0.25 µl of each deoxynucleoside triphosphate (dNTPs; Invitrogen, Carlsbad, CA), 0.1 µl of Taq polymerase (Invitrogen, Carlsbad, CA), 1 µl of template DNA, and a balance Milli-Q water for a total volume of 50 µl. Amplifications were performed in a thermocycler (GeneAmp, Applied Biosystems, Foster City, CA). The initial denaturation was done at 94ºC for 5 min. Each DNA amplification cycle consisted of denaturation for 1 min at 94ºC, annealing for 1 min

at 43ºC, and extension for 1 min at 72ºC. Target DNA was amplified in 28 cycles. The final extension was completed at 72ºC for 10 min. PCR reaction tubes were held at 4ºC until further analysis.

PCR products were separated by gel electrophoresis in a 1.0 % (w/v) agarose gel at 90 V for 1 h. A 1 Kb Plus DNA molecular weight marker was used as a size marker (Invitrogen, Carlsbad, CA). The gel was stained with SYBR® Green (Invitrogen, Carlsbad, CA) and was visualized using a UV transilluminator. Three replicates of the DNA isolation and PCR confirmation were completed.

Table 3.1: Gene specific primers used to detect SASPs (α/β-type and γ-type) in psychrophilic *Clostridium* spp. (57)

Gene	Sequence	Expected size
*ssp*1	CPP7: 5'-GCTTACAAATTACCAAAGCC-3' CPP8: 5'-CAGTATTAGCGAAAGGTTTG-3'	239 bp
*ssp*2	CCP9: 5'-CTCCTATAATTCCCTCTCAT-3' CCP10: 5'-GTAGACTTTAATAGGTTCAGG- 3'	306 bp
*ssp*3	CPP11: 5'-CTGCACATCATAATATTGAAAGG-3' CPP34:5'-GCTATGGATCTTATGGAAGG-3'	522 bp

3.2.8 Dry Breakage of Endospores and Extraction of SASPs

Dry breakage of the endospores was completed using a modified protocol from Johnson and Tipper (59, 60). Lyophilized endospores (100 mg) were broken by dry rupture in a Mini-BeadBeater-8 homogenizer (BioSpec Products, Bartlesville, OK) with 100mg 0.1 mm glass beads (BioSpec Products). Eight period of one- minute shaking were used. Disrupted endospores were then extracted with cold 3% acetic acid (5 mL/100 mg endospores) and placed on ice for 30 min. The endospores and cells were pelleted via centrifugation at 5000 x*g* for 10 minutes. Supernatant was kept and pellet was re-

extracted with an additional 3 mL of acetic acid per 100 mg endospores and placed on ice for another 30 min. Supernatant fluids were pooled and dialyzed in cold sterile Milli-Q water for 24 h in regenerated cellulose dialysis tubing (Nominal MWCO 3500; Fisher Scientific, Nepean, ON). The water was changed three times. The fluid in the dialysis tube was lyophilized and stored at 4°C until further analysis.

3.2.9 SDS-PAGE

For analysis by gel electrophoresis, the dry residue from the endospores (100 mg) was reconstituted in 600 μL sterile Milli-Q water. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE)was completed using a Mini-PROTEAN® precast 10 % SDS-PAGE gel (BioRad Laboratories, Mississauga, ON). The gel was run for 30 min at 160 V. The gel was stained using a staining solution of 1g/L of 0.1% Comassie blue R-250 (BioRad Laboratories), 450 mL methanol, 100 mL acetic acid and 450 mL distilled water. The gel was destained in a destaining solution composed of 200 mL methanol, 100 mL acetic acid, and 700 mL water for a total of 60 min with three changes of destaining solution. A Precision Plus Protein Standard (BioRad Laboratories) was used to estimate the molecular weight of the bands.

3.3 Results

3.3.1 Inactivation of Endospores by Heat

To determine the impact of atmospheric conditions on heat resistance of endospores of psychrophilic and psychrotrophic *Clostridium* spp., endospores where treated under aerobic and anaerobic conditions. Under aerobic conditions, endospores of all strains tested did not survive more than 4 min at 70, 80, or 90°C (data not shown).

Under anaerobic conditions, the heat resistance of the endospores of *Cl. estertheticum* ATCC 51377 and BP09-13, and *Cl. putrefaciens* BP-1 were similar (Fig 3.1). At 70 and 80°C, counts of *Cl. estertheticum* BP09-01 reduced rapidly compared to counts of other psychrotrophic *Clostridium* spp. At 70°C, endospores of *Cl. estertheticum* ATCC 51377 and BP09-13, and *Cl. putrefaciens* BP-1 were detected after 60 min of treatment (Fig 3.1A); however, endospores of *Cl. estertheticum* BP09-01 were not detected after 15 min of heating. After heating at 80°C for 4 min (Fig 3.1B), endospores of *Cl. estertheticum* BP09-01 were not detected but all other strains were detected after 60 min of heating. Only *Cl. estertheticum* ATCC 51377 survived 15 min of heating at 90°C (Fig 3.1C). There were no viable endospores detected for the other psychrotrophic *Clostridium* spp. after 15 min of heating at 90°C.

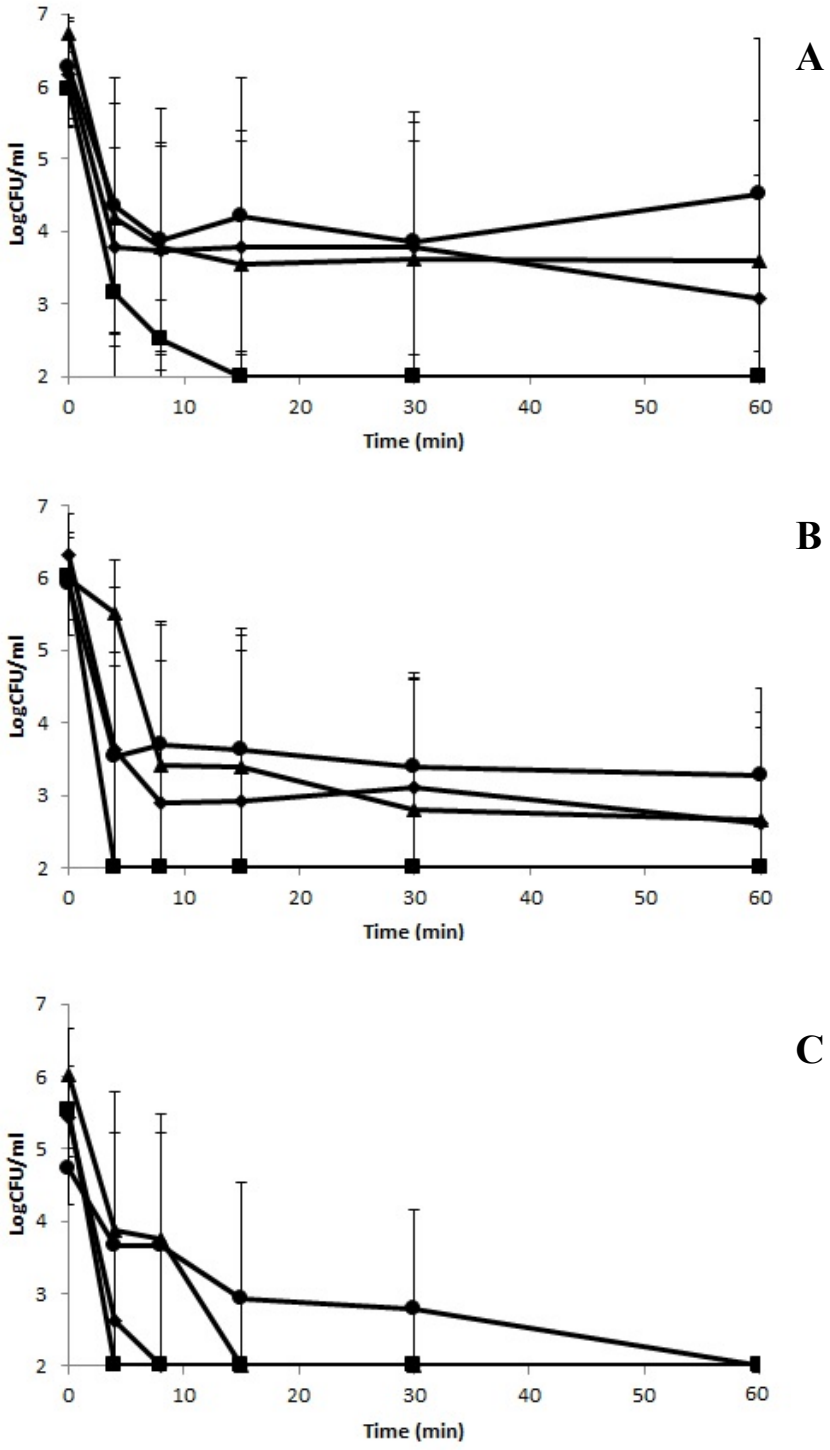


Figure 3.1: Mean log counts of psychrophilic *Clostridium* spp. after heating endospores at 70°C (A), 80°C (B) and 90°C (C) under anaerobic conditions. *Cl. putrefaciens* BP-1 (♦),*Cl. estertheticum* BP09-01 (■), BP09-13 (▲), andATCC 51377 (●). n=3.

3.3.2 Inactivation of Endospores by Pressure Treatment in Saline

Clostridium strains were tested for resistance to high pressure in saline to determine initial susceptibility to pressure treatment. Endospores of *Cl. estertheticum* BP09-01 were significantly less pressure tolerant compared to the psychrophilic endospores tested at 4 and 40°C (P-value < 0.0001). At 400 MPa and 4°C, *Cl. estertheticum* BP09-13 was the most resistant with survivors detected after 30 min of pressure treatment (Fig 3.2A). *Cl. putrefaciens* BP-1 and *Cl. estertheticum* ATCC 51377 were not detected after 15 min of pressure treatment. When subjected to 400 MPa at 40°C, the endospores of *Cl. estertheticum* ATCC 51377 and BP09-01 were not detected after ramp and endospores of *Cl. estertheticum* BP-1 were not detected after 4 min of treatment (Fig 3.2B). Endospores of *Cl. estertheticum* BP09-13 survived for 4 min, but where not detected after 8 min of pressure treatment. After ramp at 70°C and 400 MPa, there were no endospores detected (Fig 3.2C).

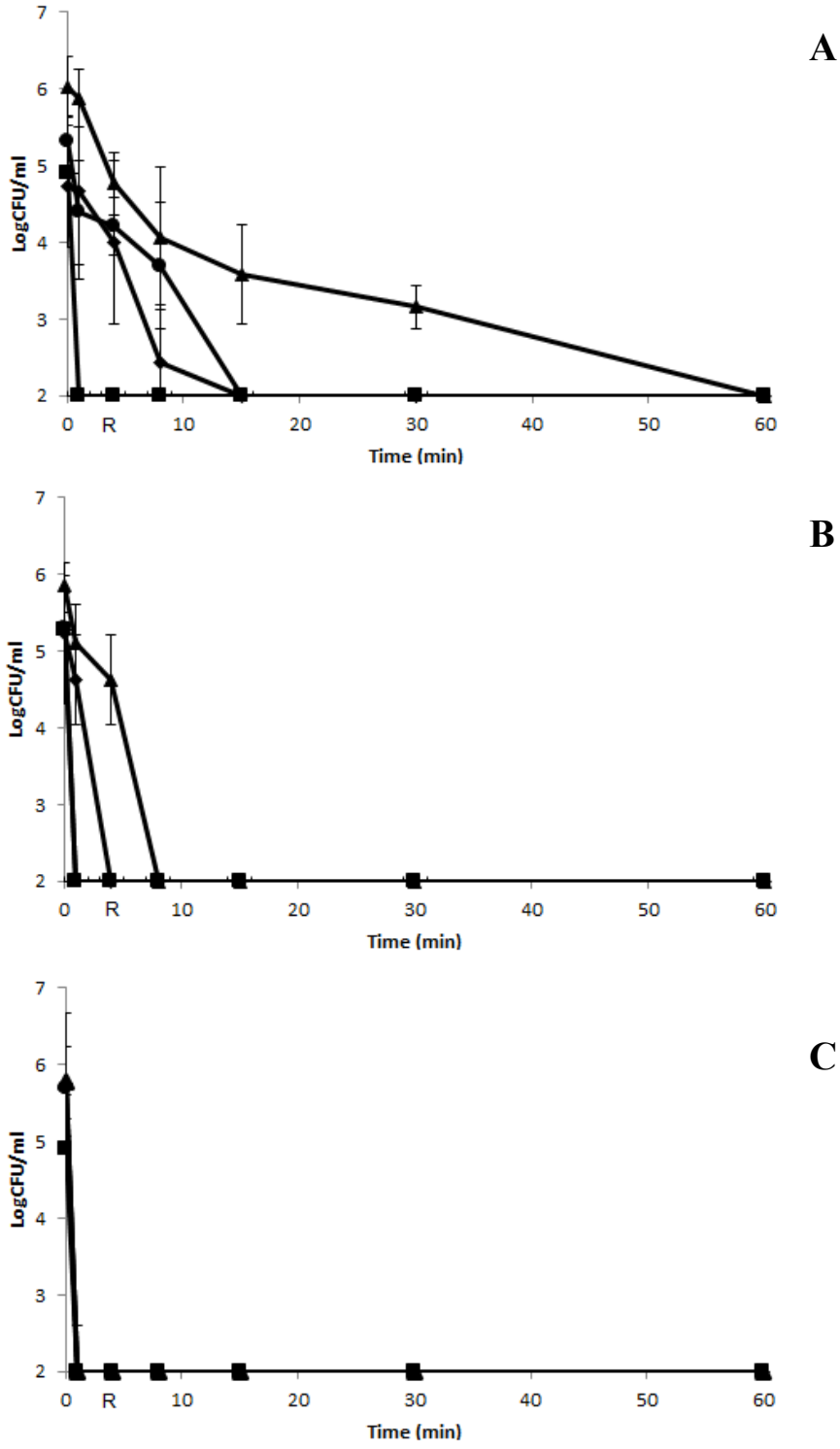


Figure 3.2: Mean log counts of psychrophilic *Clostridium* spp. after endospores were treated at 400 MPa at 4°C (A), 40°C (B), and 70°C (C); *Cl. putrefaciens* BP-1 (♦), *Cl. estertheticum* BP09-01 (■), BP09-13 (▲), and ATCC 51377 (●). R denotes ramp, the time required to reach pressure. All subsequent values are pressure holding times. n=3.

3.3.3 Endospore Inactivation by High Pressure and Antimicrobials in Meat

To determine the impact of the combination of high pressure with bacteriocins present in Micocin X™, ground meat with and without Micocin X™ was subjected to high pressure treatment at 400 MPa at 4°C (Fig 3.3A). Counts of total bacteria, presumptive lactic acid bacteria, presumptive *Enterobacteriaceae*, and endospores were below detection limit on control samples that had not been inoculated with endospores of *Clostridium* spp. (data not shown).

In minced beef, *Cl. putrefaciens* BP-1 was significantly less resistant to 400 MPa pressure compared to the other two psychrotrophic endospores tested (P-value <0.0001; Fig 3.3A). Endospores of *Cl. putrefaciens* BP-1 were below the detection limit after 4 min of pressure; however, endospores of *Cl. estertheticum* BP09-13 and *Cl. estertheticum* ATCC 51377 were detected after 4 min of pressure treatment. *Cl. estertheticum* BP09-13 survived 30 min of pressure treatment, but was not detected after 60 min.

Addition of Micocin X™ enhanced the survival of the psychrophilic endospores. Endospores of *Cl. putrefaciens* BP-1 and *Cl. estertheticum* ATCC 51377 survived pressure treatment for 15 min, but no endospores were detected after 30 min of treatment (Fig 3.3B). The presence of Micocin X™ in minced beef did not affect the survival of endospores of *Cl. estertheticum* BP09-13. To determine if the presence of media (Fig 3.3C) or spent bacterial supernatant (Fig 3.3D) would impact survival and recovery of endospores, APT or the supernatant of a bacteriocin-negative culture *C. maltaromaticum* UAL8C2 was added to the meat prior to pressure treatment. The addition of the APT to

meat prior to pressure treatment increased the recovery of endospores after pressure treatment for all three *Clostridium* spp. used in this study. When the culture supernatant of *C. maltaromaticum* UAL8C2 was added to meat, all of the endospores survived 30 min of pressure treatment.

3.3.4 Inactivation of Endospores by High Pressure in the Presence of Peptidoglycan

To determine if peptidoglycan from bacteria could enhance inactivation or recovery of endospores after high pressure processing, spores were subject to 400 MPa in the presence of peptidoglycan. Peptidoglycan from *Staphylococcus aureus* did not stimulate germination in the *Clostridium* spp. tested in this study (results not shown). When peptidoglycan from *Bacillus* spp. was added to the endospores, with or without Micocin X™, there was almost a 5 log reduction in both *Cl. estertheticum* BP09-13 and *Cl. estertheticum* ATCC 51377 (Fig 3.4 A, B) after 4 min of treatment. In contrast, endospores treated without peptidoglycan or in the presence of Micocin X had a much lower reduction after 4 min of pressure treatment. Plating endospores onto media containing peptidoglycan did not improve recovery.

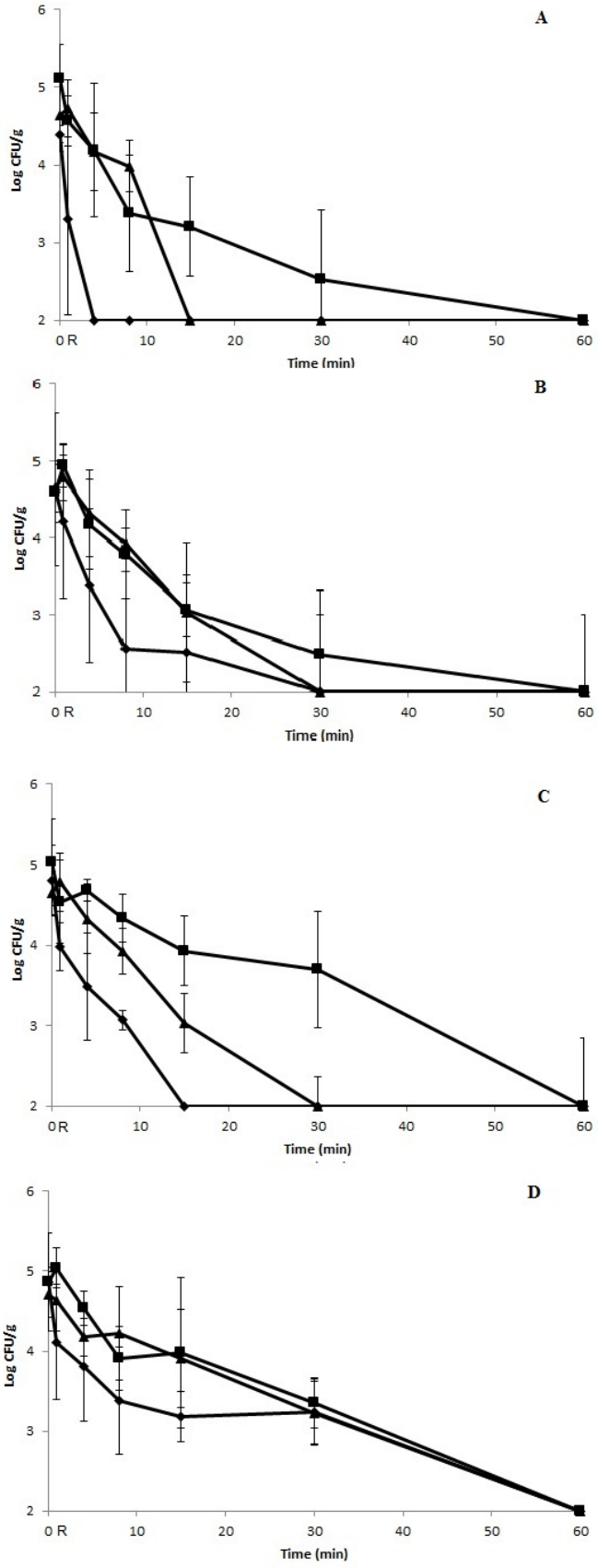


Figure 3.3: Mean log reduction of psychrophilic *Clostridium* spp.after pressurization at 400MPa and 4°C in minced beef (A), minced beef with Micocin X™ (B), with APT (C), and with supernatant of *C. maltaromaticum* UAL8C2 (D). BP-1 (♦), BP09-13 (■), *Clostridium estertheticum* ATCC51377 (▲) on PYGS agar; R denotes ramp, the time required to reach pressure. All subsequent values are pressure holding times.n=3.Error bars are standard deviations.

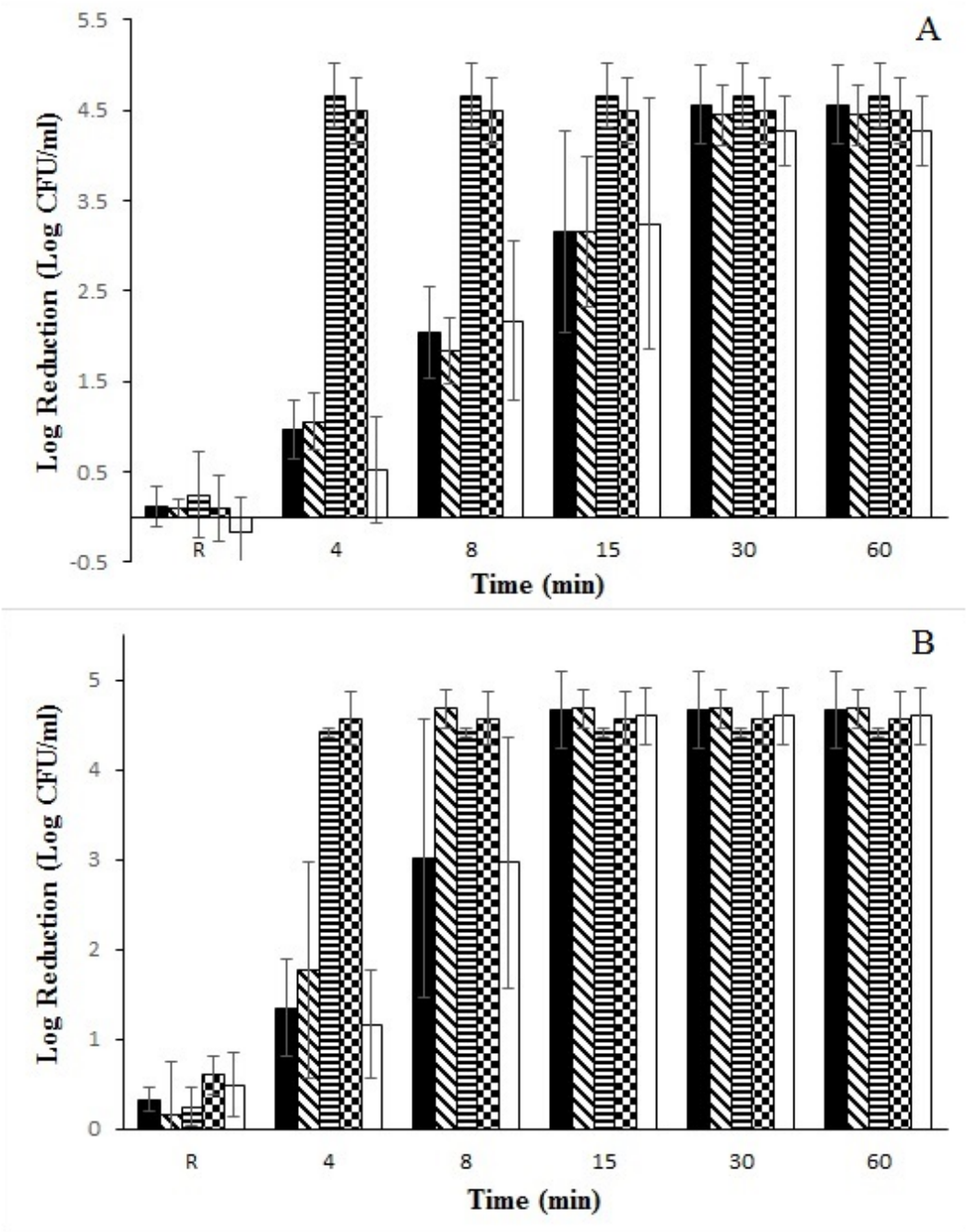


Figure 3.4: Mean log reduction of psychrophilic *Cl. estertheticum* BP09-13 (A) and *Cl. estertheticum* ATCC51377 (B) after pressurization of the endospores at 400 MPa and 4°C in saline. Endospores (black), endospores with Micocin X (diagonal), endospores with peptidoglycan (horizontal),endospores with peptidoglycan and Micocin X™ (checkers), endospores plated onto PYGS agarcontaining peptidoglycan (white); R denotes ramp, the time required to reach pressure. All subsequent values are pressure holding times. n=3. Error bars representstandard deviations.

3.3.5 Detection of Genes Responsible for SASPs in Psychrophilic Endospores of *Clostridium* spp.

PCR analysis was completed on DNA isolated from psychrophilic endospores ("blown-pack" isolates BP-1 and BP09-13 and *Cl. estertheticum* ATCC 51377) to determine the presence of the genes for SASPs. The primers pairs CCP7/8, CPP9/10, and CPP10/34 used were specific to SASPs of *Cl. perfringens* (57). *Cl. perfringens* ATCC 13124 was used as a positive control. When the template DNA of the endospores were subjected to the PCR analysis of *ssp* genes, PCR products of 239-, 306- and 522-bp (representing genes *ssp*1, *ssp*2, and *ssp*3, respectively) were not obtained for any of the psychrophilic endospores. No bands were detected for the three *ssp*1, 2 or 3 genes for the psychrophilic endospores (this included the reference strain *Cl. estertheticum* ATCC 51377). These three bands were detected for only*Cl. perfringens* ATCC 13124 (the positive control). Although the genes for *ssp*1, *ssp*2, and *ssp*3 were not detected, this does not indicate that SASPs were not present. The PCR primers used were specific to SASPs of *Cl. perfringens*, and thus if there were variations in SASPs sequences in psychrophilic *Clostridium* spp., then these primers would not be suitable.

3.3.6 Detection of SASPs using SDS-PAGE

SDS-PAGE was used to detect proteins that are similar in size to the SASPs proteins. The expected molecular weight of SASPs is 5.9 KDa for α/β-type SASPs and 11KDa for γ-type SASPs (59). SDS-PAGE analysis of the cell and endospore lysates produced banding patterns indicates the presence of SASPs (Fig 3.5). The gel used was not able to

separate between the α/β-type SASPs and γ-type SASPs. However, SASPs are present in all the psychrophilic and psychrotrophic endospores. There was a band around 10 to 11 KDa present in all of the supernatants isolated from *Clostridium* endospores. No bands were observed for the negative controls indicating that vegetative cells of *Cl.* perfringens ATCC 13124 and *P. aeruginosa* ATCC 27853 do not have α/β- or γ-type SASPs.

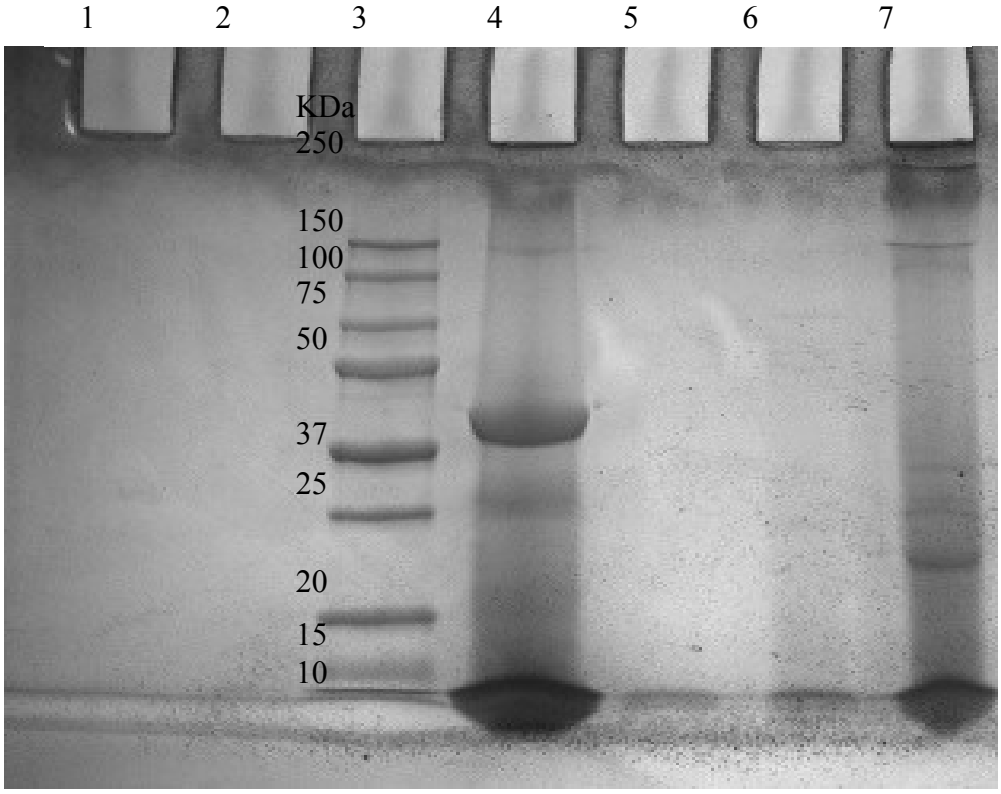


Figure 3.5: SDS-PAGE analysis of purified SASPs from vegetative cells and endospores. Lane 1, treated cells of *P. aeruginosa* ATCC 27853; lane 2, treated cells of *Cl. perfringens* ATCC 13124; lane 3, molecular weight markers; lane 4, treated endospores of *Cl. perfringens*; lane 5, treated endospores of *Cl. putrefaciens* BP-1; lane 6, treated endospores of *Cl. estertheticum* BP09-01; and lane 7, treated endospores of *Cl. estertheticum* BP09-13.

3.4 Discussion

Psychrotrophic *Clostridium* spp. have been implicated in "blown-pack" spoilage of beef and other red meat species (3, 4, 61-65). Interventions used in the meat industry to control this defect include carcass pasteurization and application of antimicrobials. In spite of the use of these interventions, cases of blown pack spoilage continue to occur and have resulted in severe economic consequences for processors (personal communication, meat industry personnel). This study investigated the role of heat and pressure and their interaction on the survival of psychrophilic endospores as well as interactions with antimicrobials during pressure treatment.

Most of the psychrotrophic endospores tested in this study were not resistant to 80 or 90°C for 30 min; however, some did survive a short heating time which explains why they survive steam pasteurization where the surface of a carcass reaches 80 to 90°C (66). These temperatures are generally adequate to reduce numbers of vegetative cells; however, the short residence time of the steam pasteurization does not impact the survival of endospores. Superdormant endospores, which are more resistant to higher temperatures, may also persist on the meat (67). Superdormant endospores could account for variability in thermal inactivation as there is generally a population of these endospores in any given endospore crop (38, 68, 69). The endospores used in the current study were less heat resistant than other psychrotrophic *Clostridium* spp. (21, 70). Endospores of *Cl. botulinum* survive 90°C for 30 min (22) whereas the psychrotrophic endospores used in this study were destroyed by this time/temperature combination. The variability of survival of the endospores could also explain variability of occurrence of

"blown-pack" spoilage. Other types of interventions may be required to control the sporadic occurrence of "blown-pack" spoilage.

Although the temperatures used in carcass pasteurization do not destroy the endospores, application of heat, during packaging, may play a role as a germinant (7, 18). Germination usually starts within minutes of a trigger (30, 31) but the presence of residual oxygen may disrupt the growth of *Clostridium* spp. There may be residual oxygen in the packages immediately after packaging (71). The current research demonstrated that the presence of oxygen after heat treatment interferes with recovery of endospores. This may also contribute to the variability in the incidence of "blown pack" spoilage.

High pressure processing may be an alternative technology to control the sporadic "blown pack" spoilage. Treatment at 400 MPa and 70°C inactivated the psychrotrophic endospores used in this study. Pressure, after packaging or during heat shrinkage, could be an added stressor that weakened the endospore initially making it more susceptible to heat treatment. One theory is that pressure stimulates germination by activating non-nutrient germination receptors in the endospore coat. When stimulated, these receptors activate a cascade of reactions which leads to hydration of the endospore core (13, 72) and weakening of the defenses to heat and pressure. The psychrophilic endospores used in this study were more susceptible to 70°C when pressure treatment was combined with heat treatment. Many authors have reported synergistic effects when combining heat and pressure treatments (27, 73-77). Pressure treatment at 70°C would not be applicable to

control “blown-pack” spoilage although they lower temperatures may be more applicable to fresh meat but may not provide sufficient inactivation to eliminate “blown-pack” spoilage.

Several factors can affect the heat and/or pressure resistance of endospores. Sporulation media or temperatures influence the fitness of the endospores. Cold tolerant *Clostridiumbotulinum*, when sporulated at their optimal temperatures, showed greater resistance to heat and pressure treatment compared to the endospores used in this study (21, 70, 78). *Cl. botulinum* type E strains were detected after 800 MPa at 40°C for 10 min and 800 MPa at 80°C for 5 min. The pressures in this study were lower, but the endospores did not survive 400 MPa at 70°C. The endospores used in this study were also grown and sporulated at optimal temperatures and therefore difference in sporulation temperatures should not have an effect on the resistance to heat and pressure.

Pressure induced germination can render an endospore more sensitive towards other treatments, such as heat (26, 29-31, 77) and potentially other antimicrobials. Generally, 400 MPa is the limit at which pressure can be used for treatment of fresh meat that is not marinated due to degradation in colour. Therefore, a combination of antimicrobials in addition to pressure treatment was explored in the current research.

The addition of Micocin X™, a bacteriocin-containing preparation, did not augment the effects of pressure treatment as predicted. It was thought that the addition of Micocin X™ would work synergistically with pressure to decrease the numbers of endospores

more rapidly or efficiently. Enhanced inactivation has been observed when nisin is added in combination with high pressure and high heat (38, 39, 79, 80), but this was not the case with the addition of Micocin X™. The bacteriocins present in Micocin X™ are carnocyclin A (CclA), carnobacteriocin BM1 (CbnBM1) and piscicolin 126 (PisA; (81). Although nisin, CclA, CbnBMI and PisA all interact with the cell membranes to form pores, each bacteriocin will have a different range of activity due to their structure (81). The class II bacteriocins generally interact with the cell membranes transporters and thus forming pores; whereas nisin interacts with lipid II to form pores. Some studies have implied that when nisin interacts with the endospore membrane, it facilities the release of dipicolinic acid (DPA) and thereby sensitizes the endospore to further treatment (38). Other antimicrobials have been reported to have an antagonistic effect when combined with high pressure. Reutericyclin increases the resistance of *Clostridium sporogenes* to a combination of high pressure and temperature (38). Reutericyclin is an inhibitory compound produced by *Lactobacillus reuteri* and has bacteriostatic or bactericidal effects on Gram positive organisms (82, 83). It is a proton ionophore that disrupts the proton gradient across a membrane. While nisin has the ability to produce pores in the endospore membrane that are large enough for water and other large compounds to move freely, reutericyclin acts as an ionophore, interacts with the cell membrane to translocate ions across all of which results in a leaky cell (38, 82, 83) and disrupts the ion gradient. CcIA has a different mechanism compared to the class II bacteriocins as it forms channels that are ion selective (84). Since these channels are ion selective, CcIA may not have the ability to trigger endospore germination or weaken the endospore so that it may be more susceptible to further interventions.

One of the unique aspects of the current research is that experiments with antimicrobials were completed in a meat matrix. A variety of different components of the meat could either be protective or act as germinants. These could include metabolites of other bacteria, peptidoglycan fragments from dead cells, or nutrients that may be present in the meat (85). Addition of Micocin X™ may have an effect on endospore inactivation not just because of the bacteriocin but the nutrients in the supernatant may act as germinants. The supernatant of *C. maltaromaticum* UAL 8C2 was used to determine whether nutrients or metabolites produced by *C. maltaromaticum* would have a protective effect on endospores. *C. maltaromaticum* UAL 8C2 was chosen because it does not produce bacteriocins, which would have interfered with the results. Components in the broth or metabolites of the *C. maltaromaticum* UAL 8C2 might protect the endospores during HHP or it could aid in germination during plating. *C. maltaromaticum* UAL 8C2 and the strain of *Carnobacterium* used to produce Micocin X will produce lactate as a result of breakdown of glucose (86), Lactate is generally not considered to be a germinant (87) but generally acts a co-germinant for other psychrophilic *Clostridium* spp. (88). However, *Cl. estertheticum* endospores were able to germinate in the presence of lactate (87). As the *C. maltaromaticum* UAL 8C2 was cultured in APT, endospores with APT were also included in the experiment as an additional control. In this study, the addition of APT, spent supernatant or Micocin X™ to meat improved the survival or recovery of endospores that were pressured at 400 MPa at 4°C. It is possible that the presence of cellular debris in the spent media or the meat could have provided some protective effect.

To determine if peptidoglycan enhances the recovery of endospores, it was added to the media used for enumeration of endospores pressured in saline; however, adding it to the media had no impact on recovery of endospores. However, peptidoglycan from *Bacillus* spp. enhanced inactivation of endospores that were pressurized with or without Micocin X™. Others have found that peptidoglycan can enhance germination of spores. *Bacillus* spp. germinates in the cell free supernatant of other *Bacillus* spp. (85) and peptidoglycan from *Escherichia coli* stimulates germination (85, 89) of *Bacillus subtilis.* In contrast, the cell free supernatant of *S. aureus,* which would contain peptidoglycan, does not stimulate germination (85). It is specifically the muropeptide in the peptidoglycan that stimulates germination (85). Some peptidoglycan fragments do not induce germination because of their structure, specifically the third position of the stem peptide (85). If peptidoglycan were to be used to induce germination during or slightly before pressurization, mild pressure treatment could be used to inactivate endospores in meat. Conceivably, in the presence of peptidoglycan, pressures below 400 MPa and temperatures at around 4°C could be used successfully to inactivate psychrophilic endospores in fresh meat.

Small acid soluble proteins, which may be present in endospores, are known to confer resistance to a variety of different environmental stressors (13, 42, 90, 91) including heat and pressure (58, 90, 92-94). SASPs protect endospores from heat by limiting DNA damage (43) from the change in conformation. In the current research, the genes for SASPs were not detected in the psychrophilic endospores. This could have been due to the use of primers that were specific to *ssp* genes found in *Cl. perfringens*. It is possible that the primers based on *Cl. perfringens* may not have enough variation to detect SASPs in the psychrophilic endospores of a different species. To confirm if SASPs were

present, analysis of SASPs was done using SDS-PAGE. This analysis confirmed that SASPs were present in the psychrophilic endospores. These proteins were not present in the vegetative cells (the negative controls), which was as expected. The presumptive presence of SASPs may indicate why some of these organisms are resistant to heat and pressure. Presence of SASPs could indicate better fitness during pressure because during pressurization, germination is induced and the SASPs provide essential amino acids that may protect the *Clostridium* spp. (77, 95). Presences of the amino acids provide essential building blocks for repair.

3.5 Conclusions

Sporadic incidences of "blown-pack" spoilage, caused by psychrophilic *Clostridium*, are unpredictable, which makes it difficult to implement interventions. Psychrophilic endospores isolated from "blown-pack" spoiled beef are resistant to heat so they will survive thermal interventions used by the meat industry. The psychrophilic endospores used in this study survived HHP, but only when pressure was combined with 70°C. This combination of temperature and pressure would not be applicable to raw meat to prevent "blown-pack" spoilage as it would result in changes in meat colour. However, high pressure may be useful to inactivate psychrophilic endospores when used together with other interventions. Micocin X™ did not have the desired inactivation effect on the endospores subjected to HHP; however, peptidoglycan, even in small amounts, in combination with pressure was effective in inactivating endospores. Addition of peptidoglycan could be used in conjunction to HHP to inactivate endospores but would be subject to regulatory approval.

Presumptive confirmation of SASPs in psychrophilic and psychrotrophic endospores suggests a mechanism of protection from stressors similar to what is observed in other *Clostridium* spp. These SASPs may not match SASPs of *Cl. perfringens* and small changes in SASPs could modulate the level of resistance to heat and pressure compared to mesophilic endospores.

3.6 References

(1) L. Ho. Characterization of "blown-pack" spoiled beef. Edmonton, Alberta: University of Alberta; 2009.

(2) Kalchayanand N, Ray B, Field RA, Johnson MC. Spoilage of vacuum-packaged refrigerated beef by *Clostridium*. J Food Prot 1989 Jan;52:424-426.

(3) Broda DM, DeLacy KM, Bell RG, Braggins TJ, Cook RL. Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Int J Food Microbiol 1996 Apr;29(2-3):335-352.

(4) Broda DM, DeLacy KM, Bell RG, Penney N. Association of psychrotrophic *Clostridium* spp., with deep tissue spoilage of chilled vacuum-packed lamb. Int J Food Microbiol 1996 Apr;29(2-3):371-378.

(5) Broda DM, Musgrave DR, Bell RG. Use of restriction fragment length polymorphism analysis to differentiate strains of psychrophilic and psychrotrophic clostridia associated with 'blown pack' spoilage of vacuum-packed meats. J Appl Microbiol 2000 Jan;88:107-118.

(6) Adam KH, Flint SH, Brightwell G. Psychrophilic and psychrotrophic clostridia: sporulation and germination processes and their role in the spoilage of chilled, vacuum-packaged beef, lamb and venison. Int J Food Sci Tech 2010 Aug;45(8):1539-1544.

(7) Silva AR, Tahara ACC, Chaves RD, Sant'Ana AS, Faria JdAF, Massaguer PR. Influence of different shrinking temperatures and vacuum conditions on the ability of psychrotrophic *Clostridium* to cause 'blown pack' spoilage in chilled vacuum-packaged beef. Meat Sci 2012 Dec;92(4):498-505.

(8) Dainty RH, Edwards RA, Hibbard CM. Spoilage of vacuum-packed beef by a *Clostridium* sp. J Sci Food Agric 1989 Apr;49:473-486.

(9) Helps CR, Harbour DA, Corry JEL. PCR-based 16S ribosomal DNA detection techniques for *Clostridium estertheticum* causing spoilage in vacuum-packed chill-stored beef. Int J Food Microbiol 1999 Nov;52:57-65.

(10) Yang X, Balamurugan S, Gill CO. Substrate utilization by *Clostridium estertheticum* cultivated in meat juice medium. Int J Food Microbiol 2009 Jan;128(3):501-505.

(11) Broda DM, Bell RG, Boerema JA, Musgrave DR. The abattoir source of culturable psychrophilic *Clostridium* spp. causing 'blown pack' spoilage of vacuum-packed chilled venison. J Appl Microbiol 2002 Oct;93(5):817-824.

(12) Boerema JA, Broda DM, Penney N, Brightwell G. Influence of peroxyacetic acid-based carcass rinse on the onset of "blown pack" spoilage in artificially inoculated vacuum-packed chilled beef. J Food Prot 2007 Jun;70(6):1434-1439.

(13) Setlow P. I will survive: DNA protection in bacterial spores. Trends Microbiol 2007 Apr;15(4):172-180.

(14) Broda DM. The effect of peroxyacetic acid-based sanitizer, heat and ultrasonic waves on the survival of *Clostridium estertheticum* spores in vitro. Lett Appl Microbiol 2007 Sep;45(3):336-341.

(15) Adam KH, Flint SH, Brightwell G. Psychrophilic and psychrotrophic clostridia: sporulation and germination processes and their role in the spoilage of chilled, vacuum-packaged beef, lamb and venison. Int J Food Sci Tech 2010 Aug;45(8):1539-1544.

(16) Black E, Koziol-Dube K, Guan D, Wei H, Setlow B, Cortezzo D, et al. Factors influencing germination of *Bacillus subtilis* spores via activation of nutrient receptors by high pressure. Appl Environ Microbiol 2005 Oct;71(10):5879-5887.

(17) Setlow P. Germination and outgrowth. In: Hurst A, Gould GW, editors. The Bacterial Spore. Vol. II ed. London: Academic Press; 1983. p. 211-254.

(18) Bell RG, Moorhead SM, Broda DM. Influence of heat shrink treatments on the onset of clostridial "blown pack" spoilage of vacuum packed chilled meat. Food Res Int 2001 Jul;34(4):271-275.

(19) Moschonas G, Bolton DJ, McDowell DA, Sheridan JJ. Diversity of culturable psychrophilic and psychrotrophic anaerobic bacteria isolated from beef abattoirs and their environments. Appl Environ Microbiol 2011 Jul;77(13):4280-4284.

(20) Schnabel J, Lenz CA, Vogel RF. High pressure inactivation of *Clostridium botulinum* type E endospores in model emulsion systems. High Pressure Research 2015 Jan;35(1):78-88.

(21) Lenz CA, Vogel RF. Differential effects of sporulation temperature on the high pressure resistance of *Clostridium botulinum* type E spores and the interconnection with sporulation medium cation contents. Food Microbiol 2015 Apr; 46:434-442.

(22) Stringer SC, Fairbairn DA, Peck MW. Combining heat treatment and subsequent incubation temperature to prevent growth from spores of non-proteolytic *Clostridium botulinum*. J Appl Microbiol 1997 Jan;82(1):126-136.

(23) Campus M. High pressure processing of meat, meat products and seafood. Food Engineering Reviews 2010 Dec;2(4):256-273.

(24) Defaye AB, Ledward DA, Macdougall DB, Tester RF. Renaturation of metmyoglobin subjected to high isostatic pressure. Food Chem 1995 Dec;52(1):19-22.

(25) Gould GW. History of science - spores - Lewis B Perry Memorial Lecture 2005. J Appl Microbiol 2006 Sep;101(3):507-513.

(26) Reineke K, Doehner I, Schlumbach K, Baier D, Mathys A, Knorr D. The different pathways of spore germination and inactivation in dependence of pressure and temperature. Innov Food Sci 2012 Jan;13:31-41.

(27) Reineke K, Mathys A, Knorr D. The impact of high pressure and temperature on bacterial spores: Inactivation mechanisms of *Bacillus subtilis* above 500 MPa. J Food Sci 2011 Apr;76(3):M189-M197.

(28) Black EP, Setlow P, Hocking AD, Stewart CM, Kelly AL, Hoover DG. Response of spores to high-pressure processing. Compr Rev Food Sci Food Saf2007 Oct;6(4):103-119.

(29) Gould G, Sale A. Initiation of germination of bacterial spores by hydrostatic pressure. J Gen Microbiol 1970 Jan;60:335-46.

(30) Wuytack E, Boven S, Michiels C. Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. Appl Environ Microbiol 1998 Sep;64(9):3220-3224.

(31) Wuytack EY, Soons J, Poschet F, Michiels CW. Comparative study of pressure- and nutrient-induced germination of *Bacillus subtilis* spores. Appl Environ Microbiol 2000 Jan;66(1):257-261.

(32) Reineke K, Ellinger N, Berger D, Baier D, Mathys A, Setlow P, et al. Structural analysis of high pressure treated *Bacillus subtilis* spores. Innov Food Sci Emerg Tech 2013 Jan;17:43-53.

(33) Markland SM, Farkas DF, Kniel KE, Hoover DG. Pathogenic psychrotolerant sporeformers: An emerging challenge for low-temperature storage of minimally processed foods. Foodborne Pathog Dis 2013 May;10(5):413-9.

(34) Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J. Applications of the bacteriocin, nisin. Anton Leeuw Int J G 1996 Feb;69(2):193-202.

(35) Garriga M, Aymerich MT, Costa S, Monfort JM, Hugas M. Bactericidal synergism through bacteriocins and high pressure in a meat model system during storage. Food Microbiol 2002 Oct;19:509-518.

(36) Deegan LH, Cotter PD, Hill C, Ross P. Bacteriocins: Biological tools for bio-preservation and shelf-life extension. Int Dairy J 2006 Sep;16(9):1058-1071.

(37) Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H. Biosynthesis of bacteriocins in lactic acid bacteria. Anton Leeuw Int J M S 1996 Oct;70(2-4):113-128.

(38) Hofstetter S, Gebhardt D, Ho L, Gänzle M, McMullen LM. Effects of nisin and reutericyclin on resistance of endospores of *Clostridium* spp. to heat and high pressure. Food Microbiol 2013 May;34(1):46-51.

(39) Gao Y, Ju X. Exploiting the combined effects of high pressure and moderate heat with nisin on inactivation of *Clostridium botulinum* spores. J Microbiol Methods 2008 Jan;72(1):20-28.

(40) Setlow P. Small, Acid-Soluble Spore Proteins of *Bacillus* Species - Structure, Synthesis, Genetics, Function, and Degradation. Annu Rev Microbiol 1988 Oct;42:319-338.

(41) Nicholson WL, Setlow B, Setlow P. Ultraviolet-irradiation of DNA complexed with alpha-beta-type small, acid-soluble proteins from spores of *Bacillus* or *Clostridium* species makes spore photoproduct but not thymine dimers. Proc Natl Acad Sci U S A 1991 Oct;88(19):8288-8292.

(42) Setlow P. I Will Survive - Protecting and Repairing Spore DNA. J Bacteriol 1992 May;174(9):2737-2741.

(43) Leyva-Illades JF, Setlow B, Sarker MR, Setlow P. Effect of a small, acid-soluble spore protein from *Clostridium perfringens* on the resistance properties of *Bacillus subtilis* spores. J Bacteriol 2007 Nov;189(21):7927-7931.

(44) Fairhead H, Setlow B, Setlow P. Prevention of DNA damage in spores and invitro by small, acid-soluble proteins from *Bacillus* species. J Bacteriol 1993 Mar;175(5):1367-1374.

(45) Loshon CA, Kraus P, Setlow B, Setlow P. Effects of inactivation or overexpression of the *ssp*F gene on properties of *Bacillus subtilis* spores. J Bacteriol 1997 Jan;179(1):272-275.

(46) Setlow B, Setlow P. Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen-peroxide. Appl Environ Microbiol 1993 Oct;59(10):3418-3423.

(47) Tennen R, Setlow B, Davis KL, Loshon CA, Setlow P. Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. J Appl Microbiol 2000 Aug;89(2):330-338.

(48) Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol R 2000 Sep;64(3):548-72.

(49) Setlow P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol 2006 Sep;101(3):514-525.

(50) Salas-Pacheco J, Setlow B, Setlow P, Pedraza-Reyes M. Role of the Nfo (YqfS) and ExoA apurinic/apyrimidinic endonucleases in protecting *Bacillus subtilis* spores from DNA damage. J Bacteriol 2005 Nov;187(21):7374-7381.

(51) Setlow P. Resistance of spores of *Bacillus* species to ultraviolet light. Environ Mol Mutagen 200138(2-3):97-104.

(52) Wang S, Setlow B, Conlon E, Lyon J, Imamura D, Sato T, et al. The forespore line of gene expression in *Bacillus subtilis*. J Mol Biol 2006 Apr 21;358(1):16-37.

(53) Hayes CS, Setlow P. An alpha/beta-type, small, acid-soluble spore protein which has very high affinity for DNA prevents outgrowth of *Bacillus subtilis* spores. J Bacteriol 2001 Apr;183(8):2662-2666.

(54) Hayes DS, Peng ZY, Setlow P. Equilibrium and kinetic binding interactions between DNA and a group of novel, nonspecific DNA-binding proteins from spores of *Bacillus* and *Clostridium* species. J Biol Chem 2000 Nov;275(45):35040-35050.

(55) Lee J, Movahedi S, Harding SE, Mackey BM, Waites WM. Effect of small, acid-soluble proteins on spore resistance and germination under a combination of pressure and heat treatment. J Food Prot 2007 Sep;70(9):2168-2171.

(56) Lund BM. Anaerobes in relation to foods of plant origin. In: Barnes EM, Mead GC, editors. Anaerobic Bacteria in Habitats Other Than Man. 13th ed. Oxford: Blackwell Scientific; 1986. p. 351-372.

(57) Raju D, Sarker MR. Production of small, acid-soluble spore proteins in *Clostridium perfringens* nonfoodborne gastrointestinal disease isolates. Can J Microbiol 2007 Apr;53(4):514-518.

(58) Raju D, Waters M, Setlow P, Sarker MR. Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. BMC Microbiology 2006 Jun8;6:1-13.

(59) Johnson W, Tipper D. Acid-soluble spore proteins of *Bacillus subtilis*. J Bacteriol 1981 Jun;146(3):972-982.

(60) Cabrera-Martinez RM, Mason JM, Setlow B, Waites WM, Setlow P. Purification and amino acid sequence of two small, acid-soluble proteins from *Clostridium bifermentans* spore**s.**. FEMS Microbiol Let 1989 Oct;52(1-2):139-143.

(61) Byrne B, Monaghan AM, Lyng JG, Sheridan JJ, Bolton DJ. A case of "blown pack" meat linked to *Clostridium estertheticum* in Ireland. J Food Saf 2009 Nov 2009;29(4):629-635.

(62) Yang X, Gill CO, Balamurugan S. Products of glucose and lactate fermentation, and utilization of amino acids by *Clostridium estertheticum* subsp. *laramiense* and estertheticum growing in meat juice medium. J Food Prot 2010 Jul; 73(7):1348-1352.

(63) Yang X, Balamurugan S, Gill CO. Effects on the development of blown pack spoilage of the initial numbers of *Clostridium estertheticum* spores and *Leuconostoc mesenteroides* on vacuum packed beef. Meat Sci 2011 Jul; 88(3):361-367.

(64) Broda DM, Saul DJ, Lawson PA, Bell RG, Musgrave DR. *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum-packed meat. Int J Syst Evol Microbiol 2000 Jan;50:107-118.

(65) Broda DM, Lawson PA, Bell RG, Musgrave DR. *Clostridium frigidicarnis* sp nov., a psychrotolerant bacterium associated with 'blown pack' spoilage of vacuum-packed meats. Int J Syst Bacteriol 1999 Oct;49:1539-1550.

(66) Dlusskaya EA, McMullen LM, Gänzle MG. Characterization of an extremely heat-resistant *Escherichia coli* obtained from a beef processing facility. J Appl Microbiol 2011 Mar;110(3):840-849.

(67) Setlow P. Summer meeting 2013-when the sleepers wake: the germination of spores of *Bacillus* species. J Appl Microbiol 2013 Dec;115(6):1251-1268.

(68) Hyytia E, Hielm S, Korkeala H. Prevalence of *Clostridium botulinum* type E in Finnish fish and fishery products. Epidemiol Infect 1998 Jun;120(3):245-250.

(69) Hyytia E, Bjorkroth J, Hielm S, Korkeala H. Characterisation of *Clostridium botulinum* groups I and II by randomly amplified polymorphic DNA analysis and repetitive element sequence-based PCR. Int J Food Microbiol 1999 Jun;48:179-189.

(70) Reddy NR, Tetzloff RC, Solomon HM, Larkin JW. Inactivation of *Clostridium botulinum* nonproteolytic type B spores by high pressure processing at moderate to elevated high temperatures. Innov Food Sci Emerg Tech 2006 Sep;7(3):169-175.

(71) Smith GC, Seidman SC, Savell JW, Dill CW, Vanderzant CV. Vacuum packaging versus modified atmosphere packaging of lamb loins. J Food Prot 1982 Jan;46(4):47-51.

(72) Setlow P. Dormant spores receive an unexpected wake-up call. Cell 2008 Oct 31;135(3):410-412.

(73) Bull MK, Olivier SA, van Diepenbeek RJ, Kormelink F, Chapman B. Synergistic inactivation of spores of proteolytic *Clostridium botulinum* strains by high pressure and heat is strain and product dependent. Appl Environ Microbiol 2009 Jan;75(2):434-445.

(74) Margosch D, Ehrmann MA, Buckow R, Heinz V, Vogel RF, Gänzle MG. High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. Appl Environ Microbiol 2006 May;72(5):3476-3481.

(75) Mathys A, Reineke K, Heinz V, Knorr D. High pressure thermal sterilization - development and application of temperature controlled spore inactivation studies. High Pressure Research 2009 Apr;29(1):3-7.

(76) Rajan S, Ahn J, Balasubramaniam VM, Yousef AE. Combined pressure-thermal inactivation kinetics of *Bacillus amyloliquefaciens* spores in egg patty mince. J Food Prot 2006 Apr;69(4):853-860.

(77) Reineke K, Mathys A, Heinz V, Knorr D. Mechanisms of endospore inactivation under high pressure. Trends Microbiol 2013 Jun;21(6):296-304.

(78) Marshall KM, Nowaczyk L,II, Morrissey TR, Loeza V, Halik LA, Skinner GE, et al. Effect of sporulation temperature on the resistance of *Clostridium botulinum* Type A spores to thermal and high pressure processing. J Food Prot 2015 Jan;78(1):146-150.

(79) Lopez-Pedemonte TJ, Roig-Sagues AX, Trujillo AJ, Capellas M, Guamis B. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. J Dairy Sci 2003 Oct;86(10):3075-3081.

(80) Roberts CM, Hoover DG. Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity and nisin. J Appl Bacteriol 1996 Oct;81(4):363-368.

(81) Martin-Visscher LA, Yoganathan S, Sit CS, Lohans CT, Vederas JC. The activity of bacteriocins from *Carnobacterium maltaromaticum* UAL307 against Gram-negative bacteria in combination with EDTA treatment. FEMS Microbiol Lett 2011 Apr;317(2):152-159.

(82) Gänzle MG. Reutericyclin: biological activity, mode of action, and potential applications. Appl Microbiol Biotechnol 2004 Apr;64(3):326-332.

(83) Hofstetter SC. Resistance and membrane fluidity of endospores of *Clostridium* spp. during pressure-assisted thermal processing in teh presence of antimicrobials. Edmonton: University of Alberta; 2012.

(84) Gong X, Martin-Visscher LA, Nahirney D, Vederas JC, Duszyk M. The circular bacteriocin, carnocyclin A, forms anion-selective channels in lipid bilayers. Biochimica Et Biophysica Acta-Biomembranes 2009 Sep;1788(9):1797-1803.

(85) Shah IM, Laaberki M, Popham DL, Dworkin J. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell 2008 Oct;135(3):486-496.

(86) Leisner JJ, Laursen BG, Prevost H, Drider D, Dalgaard P. *Carnobacterium*: positive and negative effects in the environment and in foods. FEMS Microbiol Rev 2007 Sep;31(5):592-613.

(87) Rajagopal, S. Characterization of germination of *Clostridium estertheticum* spores. Edmonton, Alberta: University of Alberta; 2014.

(88) Alberto F, Broussolle V, Mason DR, Carlin F, Peck MW. Variability in spore germination response by strains of proteolytic *Clostridium botulinum* types A, B and F. Lett Appl Microbiol 2003 Dec;36(1):41-45.

(89) Popham DL. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. Cellular and Molecular Life Sciences 2002 Mar;59(3):426-433.

(90) Setlow B, Setlow P. Heat inactivation of *Bacillus subtilis* spores lacking small, acid-soluble spore proteins is accompanied by generation of abasic sites in spore DNA. J Bacteriol 1994 Apr;176(7):2111-2113.

(91) Gerhardt P, Marquis RE. Spore thermoresistance mechanisms. In: Smith I, Slepecky RA, Setlow P, editors. Regulation of Prokaryotic Development. 1st ed. Washington, DC: American Society for Microbiology; 1989. p. 43-63.

(92) Raju D, Setlow P, Sarker MR. Antisense-RNA-mediated decreased synthesis of small, acid-soluble spore proteins leads to decreased resistance of *Clostridium perfringens* spores to moist heat and UV radiation. Appl Environ Microbiol 2007 Apr;73(7):2048-2053.

(93) Mason JM, Setlow P. Different small, acid-soluble proteins of the alpha-beta-type have interchangeable roles in the heat and UV-radiation resistance of *Bacillus subtilis* spores. J Bacteriol 1987 Aug;169(8):3633-3637.

(94) Popham DL, Sengupta S, Setlow P. Heat, hydrogen peroxide, and UV resistance of *Bacillus subtilis* spores with increased core water-content and with or without major DNA-binding proteins. Appl Environ Microbiol 1995 Oct;61(10):3633-3638.

(95) Lenz CA, Reineke K, Knorr D, Vogel RF. High pressure thermal inactivation of *Clostridium botulinum* type E endospores - kinetic modeling and mechanistic insights. Frontiers in Microbiology 2015 Jul;6:652.

4. Whole Genome Sequence of Psychrotrophic and Psychrophilic *Clostridium* spp. Involved in "Blown-pack" Spoilage

4.1 Introduction

Clostridium estertheticum BP09-01 and BP09-13, and *Clostridium putrefaciens* BP-1 isolated from "blown-pack" vacuum packaged meat, are psychrophilic and psychrotrophic anaerobes, respectively (Chapter 2 of this thesis). These organisms are important to the meat industry because of their ability to cause spoilage of meat at refrigeration temperatures. Vacuum packaging and refrigeration temperatures are used in the meat industry to mitigate spoilage during transportation and storage, but psychrophilic *Clostridium* spp. have been implicated in sporadic occurrence of "blown-pack" spoilage of variety of different red meats (1-5). A four year survey was completed on the presences of *Cl. estertheticum* and *Clostridium gasigenes* in Ireland and found that "blown-pack" spoilage organisms were more likely to be isolated during July and November and seasonal differences were not significant (6). Inability to predict these outbreaks could lead to decreased confidence in the supply chain and could lead to loss of national and international market access.

Initial analysis of 16S rDNA indicated that the *Clostridium* spp. that had caused "blown-pack" spoilage were psychrophilic *Cl. estertheticum* and psychrotrophic *Cl. putrefaciens* (5). However, there were differences in restriction fragment length polymorphism (RFLP) patterns (Chapter 2 of this thesis) and resistance to heat and pressure (Chapter 3 of this thesis) between the strains of *Cl. estertheticum* isolated from commercially spoiled "blown-pack" meat. In addition, the *Cl. putrefaciens* strain had an optimal growth

temperature of 15°C (5), which differs from the growth temperatures described in the literature for *Cl. putrefaciens* (7).

Heat and pressure resistance is thought to be determined by the presence of small acid soluble proteins [SASPs; (8-12)]. Initial screening for the presence of SASP genes, using primers specific to SASPs found in *Cl. perfringens,* did not confirm the presence of SASPs in the isolates from "blown-pack" meat; however, presence of SASPs was presumptively elucidated by isolation of proteins of similar size (Chapter 3 of this thesis).

Differences were observed in sugar metabolism and the subsequent acid production in all three "blown-pack" isolates (Chapter 2 of this thesis). Glucose was consumed by all organisms tested. However, relatively common sugars such as lactose, saccharose, and maltose were not metabolized by any of the psychrotrophic and psychrophilic *Clostridium* tested. *Cl. estertheticum* BP09-13 was unique in that was able to metabolise urea and to hydrolyze esculin.

The purpose of this research was to use whole genome sequencing to understand the differences among the *Clostridium* spp. isolated from "blown-pack" spoiled meat. Whole genome sequencing was also used to confirm if SASPs are present in psychrophilic and psychrotrophic *Clostridium* spp.

4.2Material and Methods

4.2.1 Genomic DNA Isolation, Sequencing, Assembly and Annotation

For growth of vegetative cells of *Cl. putrefaciens* BP-1, *Cl. estertheticum* BP09-01, *Cl. estertheticum* BP09-13, endospores (maintained at -80°C in growth media with 50% glycerol) were inoculated onto Columbia Blood agar (Oxoid, Nepean, ON) with 5% defibrinated sheep blood (Oxoid) and incubated under anaerobic conditions at 7°C for one week. To increase biomass for isolation of genomic DNA, colonies were transferred to modified peptone (Difco™, Becton, Dickinson and Company, Sparks, MD), yeast (Difco™), glucose (Difco™) broth (13) and incubated under anaerobic conditions for one week at 7°C. Genomic DNA was isolated using the Wizard ®Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA) following the manufacturer's guidelines. The quality and quantity of each sample was assessed by gel electrophoresis and a NanoDrop® 2000c spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). DNA samples were sequenced by Axeq Technologies (Seoul, South Korea) using Illumina Hi-Seq 2000 paired-end sequencing with 100 bp read length. Raw reads were assembled into contigs using ABySS 1.3.4 [Assembly By Short Sequence; (14)] using optimal k-mer values for each genome. Repetitive sequences and short assemblies were removed by filtering out contigs < 500 bp in size. The remaining sequences were annotated automatically by the Rapid Annotation using Subsystem Technology [RAST; (15)] server.

4.2.2 Genome Bioinformatics Analyses

Gene products for each psychrophilic *Clostridium* strain were identified and categorized into The Institute of Genomic Research (TIGR) categories (16). TIGR categories are divided into 17 separate categories and are based on gene function and processes (Table 4.2).

Geneious (Biomatters, Auckland, New Zealand) software was used to identify sequence differences between *Cl. estertheticum* BP09-01 and BP09-13. An all-against-all BLAST of coding and non-coding regions was carried out. Sequence differences generating changes in protein-coding reading frames were recorded. Additionally, a whole-genome MAUVE alignment was performed between *Cl. estertheticum* BP09-01 and BP09-13 to determine differences in large genetic regions and contigs. Whole-genome local BLAST searches for candidate genes were performed in Geneious.

4.2.3 16s Phylogenetic Tree

For phylogenetic analysis, 88 16S rRNA gene sequences from *Clostridium* genera type strains were obtained from the Ribosomal Database Project (17); See Appendix 3. These sequences, along with the 16S rRNA gene sequences from *Cl. estertheticum* BP09-01, *Cl. estertheticum* BP09-13, *Cl. putrefaciens* BP-1, and a strain of *Bacillus subtilis* [strain 168, National Center for Biotechnology Information (NCBI), (18)], were aligned by ClustalW [implemented in Molecular Evolutionary Genetics Analysis Version 6, (19)]. Aligned sequences were trimmed to an equal length of 1261-bp and a maximum-likelihood tree was constructed under default parameters and a bootstrap value of 1000.

4.2.4 Comparison of Small Acid Soluble Protein (SASPs) Genes

SASPs genes were compared among the psychrophilic and psychrotrophic *Clostridium* spp. sequenced in this study. Genes with SASPs functions were determined from annotation of the genome. Sequences from *Cl. putrefaciens* BP-1, *Cl. estertheticum* BP09-01, and *Cl. estertheticum* BP09-13 corresponding to SASPs were compared and aligned to sequences obtained for *Cl. perfringens*(20) by ClustalW.

4.3Results

4.3.1 Genome Bioinformatics Analysis

Whole genome sequencing was completed to verify the presence of SASPs and to elucidate genetic differences that could explain phenotypic differences between psychrophilic and one psychrotrophic strain of *Clostridium* isolated from "blown-pack" spoiled meat. The genome size of *Cl. putrefaciens* BP-1 was smaller by almost half, than that of the two *Cl. estertheticum* (Table 4.1). Both *Cl. estertheticum* BP09-01 and BP09-13 had comparable sized genomes, with similar numbers of contigs, and differed by only one putative gene. Genomic GC content was similar among all the *Clostridium* spp. tested with the two strains of *Cl. estertheticum* being exactly the same.

Table 4.1 Description of the whole genome sequences of psychrophilic and psychrotrophic *Clostridium* spp. isolated from "blown-pack" spoiled meat.

Strain	Genome size (bp)	Number of contigs	Max contig size	Number of putative genes	%GC
Cl. putrefaciens BP-1	2,876,364	29	673,788	2641	30.1
Cl. estertheticum BP09-01	5,021,511	114	335,304	4733	30.6
Cl. estertheticum BP09-13	5,013,181	100	335,637	4732	30.6

Comparison of the number of genes and their classification indicates that hypothetical proteins and proteins or genes with unknown and unclassified function make up the majority of the genome (Table 4.2). The majority of the classified genes are responsible for metabolism, be it energy or central intermediary metabolism. Although the *Cl. putrefaciens* BP-1 has an overall smaller genome, the proportion of genes within each category is distributed similarly to that of the *Cl. estertheticum* strains (see Appendix 1 for visual comparison).

Whole-genome MAUVE alignment was used to compare differences between *Cl. estertheticum* BP09-01 andBP09-13 (See Appendix 2). The MAUVE alignment showed that the contigs present in both strains were the same and that the genome of *Cl. estertheticum* BP09-13 is smaller than BP09-01.

Two methods were used to compare differences between the genomes of the two strainsof *Cl. estertheticum*. An all-against-all BLAST of coding and non-coding regions was done first and indicated that there were differences in 15 genes and these differences

were single nucleotide polymorphism (SNP; Table 4.3). The majority of the SNPs were in transport and membrane proteins.

Table 4.2 Categorization of genes from whole genome sequences of *Cl. putrefaciens*, *Cl. estertheticum* BP09-01, and *Cl. estertheticum* BP09-13. Classification scheme is summarized based on TIGR categories (186).

Functional TIGRCategory	**Number of genes**		
	Cl. putrefaciens BP-1	*Cl. estertheticum* BP09-01	*Cl. estertheticum* BP09-13
Amino acid biosynthesis	145	225	222
Biosynthesis of cofactors, prosthetic groups and carriers	40	71	71
Cell envelope	126	232	229
Cellular process	278	480	478
Central intermediary metabolism	156	257	261
DNA metabolism	209	252	252
Energy metabolism	248	448	446
Fatty acid and phospholipid metabolism	29	44	43
Hypothetical proteins	650	1384	1389
Protein fate	27	52	54
Protein synthesis	124	154	135
Purine, pyrimidines, nucleosides, and nucleotides	38	56	57
Regulatory function	193	300	297
Transcription	81	224	225
Transport and binding proteins	292	518	519
Unclassified	3	19	19
Unknown function	29	65	64

Table 4.3 Differences between whole genome sequences by comparison of classfied SNPs of *Cl. estertheticum* BP09-01 and *Cl. estertheticum* BP09-13.

Description	Size (aa)	Contig of		Location of contig (bp designation)		Amino acid sequence differences	Top BLAST hit (% ID)	Specific Conserved Domains
		BP09-01	BP09-13	BP09-01	BP09-13	(BP09-01 vs BP09-13)		
Multi antimicrobial extrusion protein, (Na(+)/drug antiporter), MATE family of MDR efflux pumps	457	16	1	32762-31389	32762-31389	E219G	MATE family efflux transporter, *Clostridiaceae* bacterium mt12 (66%)	MATE_MepA_like
	455	16	1	23-1390	23-1390	E219G		
Magnesium and cobalt transport protein CorA	316	22	86	46364-47314	46364-47314	E9A	Magnesium and cobalt transport protein CorA, *Clostridium autoethanogenum* (59%)	TmCorA-like
ABC transporter ATP-binding protein	296	86	89	107378-106488	107378-106488	S57R	ABC transporter, *Clostridium tetani* (60%)	ABC_DR_subfamily_A
Pyruvate-flavodoxin oxidoreductase	1170	36	54	3523-11	23-3535	A266P, I275M, T290I, G710D	Pyruvate-flavodoxin oxidoreductase, *Clostridium argentinense* (76%)	TPP_pFOR_PNO, TPP_PYR_PFOR_IOR-alpha_like, PorG, EKR, Fer4_8, Fer4_16
Putative transporter, trans-membrane domain bacteriocin immunity protein	247	13	81	12804-13547	34937-35680	P136S	Permease, *Clostridium carboxidivorans* (64%)	EfiE (non-specific hit)
ABC-type multidrug transport system, ATPase component CDS	303	13	81	1141-12052	33274-34185	N74K, E291D	Bacitricin ABC transporter ATP-binding protein, *Clostridium carboxidivorans* (76%)	ABC_BcrA_bacitricin_resist (non-specific hit)
Putative transporter, trans-membrane domain bacteriocin immunity protein	244	13	81	12053-12787	34186-34920	V4I, V168G	ABC transporter permease, *Clostridium carboxidivorans* (56%)	ABC2_membrane_4
Alcohol dehydrogenase	340	99	83	180238-181260	180113-181135	S127C, T164N, K197R, Q203E	Zn-dependent alcohol dehydrogenase, *Clostridium beijerinckii* (92%)	Zn_ADH7

Table 4.3 continued

Branched Chain amino acid transport system carrier	419	28	33	54863-56121	11983-10725	Insertion - V and I after amino acid L109 in BP09-01; V112C, A114H	Branched-chain amino acid ABC transporter, Branch_AA_trans, BrnQ (non-specific hits) (56%)	Branch_AA_trans, BrnQ (non-specific hits)
ABC-type multidrug transport system, ATPase component	153	41	20	202-663	202-663	D99E, E124D	Multidrug ABC transporter ATP-binding protein, *Pelosinus* sp. UFO1	ABC_DR_subfamily_A, ABC_tran (non-specific hits)
Hypothetical protein	76	29	2	200777-200547	201250-201020	C50S	Transposase, *Clostridium cellulovorans* (54%)	DUF4351
Alcohol dehydrogenase (EC 1.1.1.1)	279	86	89	212298-211459	212415-211576	S66C, T103N, K136R, Q142E	Zn-dependent alcohol dehydrogenase, *Clostridium beijerinckii* (92%)	Zn_ADH7
Conserved membrane spanning protein	144	111	52	123383-122949	130-564	E18R, H20D, Y129C	Membrane protein, *Clostridium botulinum* (35%)	Near endospore forming operon
Hypothetical protein	200	58	99	1305-703	1585-983	D9N, P29L, E38K, I46T, Y53F, S67P, T75K, E79D	Hypothetical protein, *Clostridium scatologenes* (66%)	None

4.3.2 16s rRNA Phylogenetic Tree

A phylogenetic tree was constructed using 88 16S rRNA sequences of various *Clostridium* spp. (Fig 4.1) and the "blown-pack" spoilage isolates were included in the construction of the tree. See Appendix 3 for all strains and accession numbers. Both *Cl. estertheticum* BP09-01 and BP09-13 were identified as *Cl. estertheticum* using 16S DNA gene sequencing (Chapter 2 of this thesis) and were located within a tight cluster comprised of *Cl. estertheticum*, *Clostridium lacusfryxellense*, *Clostridium frigoris* and *Clostridiumpsychrophilum*.

Cl. putrefaciens BP-1 was identified as *Cl. putrefaciens* using 16S rRNA gene sequencing (5) and was located within a tight clustered that included *Cl. putrefaciens* and *Cl. algidicarnis*. The bootstrap value of *Cl. putrefaciens* BP-1 and *Cl. algidicarnis* was 99. *Cl. putrefaciens* and *Cl. estertheticum* BP09-01 and BP09-13 were not closely clustered.

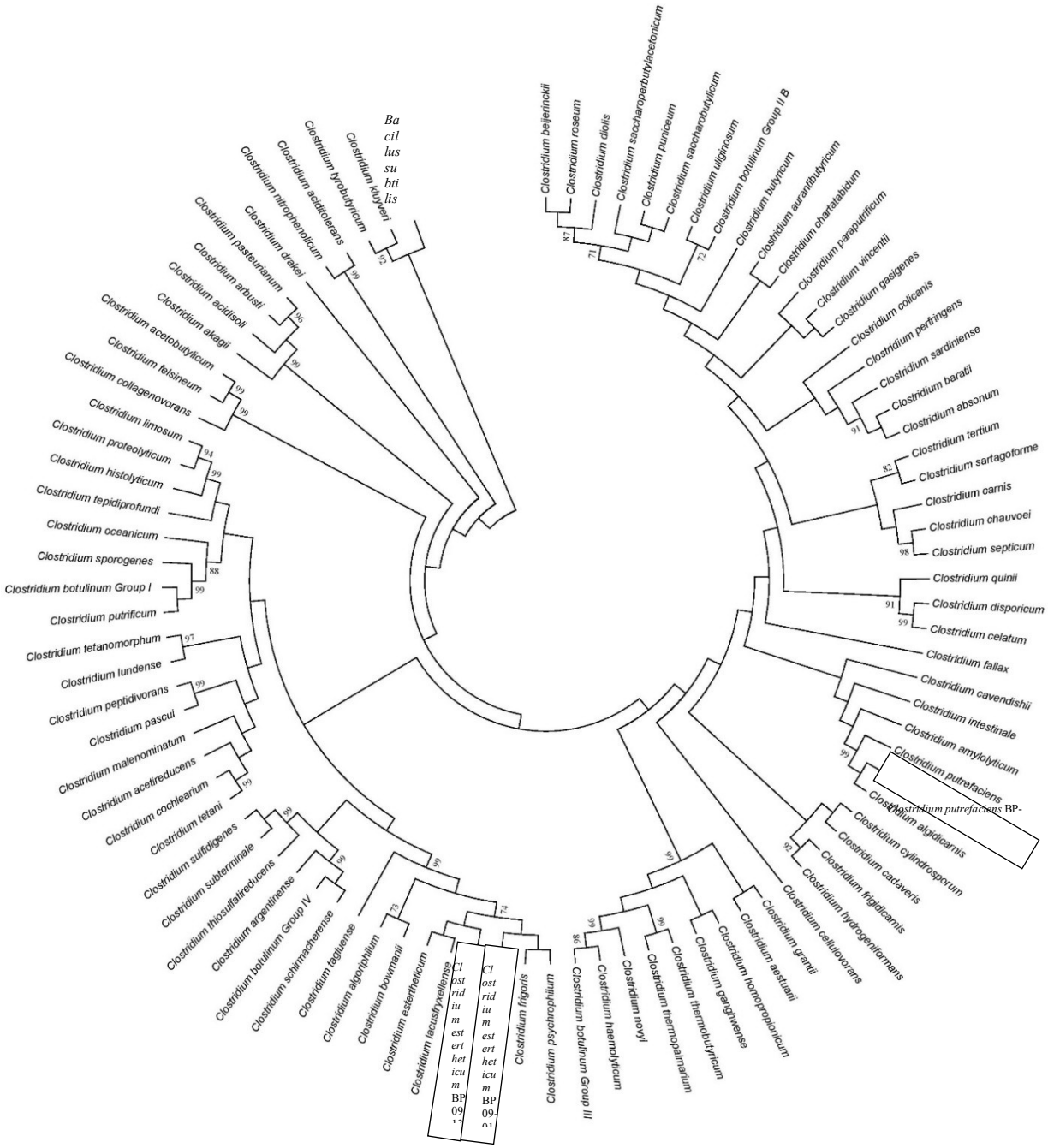


Figure 4.1 Phylogenetic analysis based on 88 16s rRNA sequences. Clusters were accessed by bootstrap analysis (1000 replicates) and the percentages are indicated on the branches. Only bootstrap values of >70% are shown. Organisms in boxes were isolated from "blown-pack" spoiled meat for this thesis.

4.3.3 Comparison of Genes for Small Acid Soluble Proteins (SASPs)

From gene annotations, there were at least 7 different putative genes for SASPs in *Cl. estertheticum* BP09-01and BP09-13, with most of them identified as beta-type SASPs. *Cl. putrefaciens* BP-1 only had two genes that were annotated as genes for SASPs.

To compare genes for SASPs in *Cl. estertheticum* BP09-01 and BP09-13 and *Cl. putrefaciens* BP-1 a nucleotide alignment was done using the SASP genes from *Cl. perfringens* ATCC 13124. A nucleotide alignment of the SASP *ssp*2 gene of *Cl. perfringens* ATCC 13124 with putative genes from *Cl. estertheticum* BP09-01 and BP09-13 yielded a coding region alignment over 189 bp with a 72.5% sequence identity to the *ssp*2 gene of *Cl. perfringens*. No alignment with *ssp*1 or *ssp*3 was observed. The flanking regions of the SASP *ssp*2 gene in *Cl. estertheticum* BP09-01 and BP09-13 did not match that of the *ssp*2 gene of *Cl. perfringens* (See Appendix 4). A comparison of amino acid sequences of the SASPs from *Cl. estertheticum* BP09-01 and BP09-13 (Fig 4.2), revealed that the sequences have 66.7% sequence identity with the reference sequence of *Cl. perfringens* ATCC 13124. The reference sequence was 59 amino acids and 6.437 kDa whereas the protein in *Cl. estertheticum* BP09-01 and BP09-13 was 60 amino acids and 6.751 kDa. When the nucleotide sequences for the SASPs of *Cl. putrefaciens* BP-1 were compared to that of the reference strain, no matches were observed for the genes from *Cl. perfringens* for *ssp*1, *ssp*2 and *ssp*3.

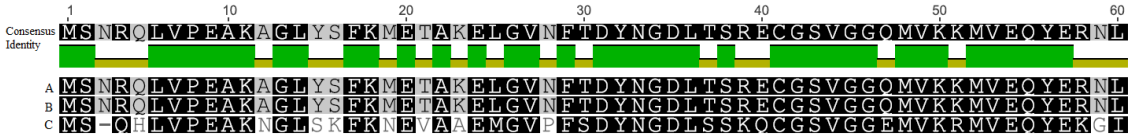


Figure 4.2 Alignment of amino acid sequences of SASPs of *Cl. estertheticum* BP09-01 (A), *Cl. estertheticum* BP09-13 (B) with that of the *ssp*2 of *Cl. perfringens* (C). Similarities are described by the consensus identity and are highlighted in black. Green denote conserved regions.

4.4Discussion

Whole genome sequences of *Clostridium* spp. have generally been completed on strains involved in disease. Comparison of *Clostridium* spp. genomes was especially difficult because of the great diversity within the genus. Even within species, there can be great genetic diversity in toxin production, motility and overall genome size (21-23). Comparison of genomes of *Clostridium* spp. that cause "blown-pack" spoilage is especially difficult because of the lack of published genomes for these organisms. There were several differences among the psychrophilic and psychrotrophic *Clostridium* spp., such as resistance to heat and pressure, which could be resolved by whole genome sequencing. Analysis of the genome of psychrophilic and psychrotrophic *Clostridium* spp. allowed for some comparison among other *Clostridium*. Verification of proteins, changes in species designation, and comparisons of differences among strains used in this study were completed.

The initial comparison of the genomes of the three *Clostridium* spp. revealed that *Cl. putrefaciens* BP-1 had a smaller genome compared to that of the two strains of *Cl. estertheticum*. This is not surprising as the size of clostridial genomes can range from 2 to 6.5 mega base pairs [Mbp; (24)]. The %GC content of the *Clostridium* genus is

generally within the range of 23 to 68% (25,26). All of the strains sequenced in this study were within range for %GC of *Clostridium* spp. Generally psychrophilic and psychrotrophic *Clostridium* are described as having a lower %GC content (27), which was the case for the psychrophilic and psychrotrophic *Clostridium* spp. used in this study. The GC content (28%) of the strains isolated in this study was comparable to that of the strains described in literature(28). The GC content of bacteria has been postulated to be related to thermal stability but there is a debate as to the validity of this postulate. Hurst and Merchant (29) indicated that GC content was not related to thermal adaptation. However, their comparisons were only done among thermophiles and mesophiles. No psychrotrophic or psychrophilic organisms or *Clostridium* spp. were included in their study. Although there may not be a correlation between GC content level and thermal adaptation, there may be some evidence that indicates GC contents in certain genes correlate to optimal growth temperature (30).

Comparison and categorization of the genomes was completed for all three strains of *Clostridium* spp. and for all three, nearly half of the genome was categorized as hypothetical, undefined or unknown. Some of those genes could have some function but it could be that these sequences are noncoding areas. The large number of hypothetical, undefined or unknown genes was expected because generally *Clostridium* genomes that are sequenced are generally disease-causing species. Genome diversity in psychrophilic and psychrotrophic *Clostridium* species, that cause "blown-pack" spoilage, cannot be compared to other species as there are few genomes that have been annotated for this genus. The large number of hypothetical proteins indicates how little is known about

psychrotrophic and psychrophilic *Clostridium* spp. The bulk of the known genes were described as being responsible for metabolism, be it energy or central intermediary metabolism, which is expected as these processes are crucial to survival. The sequences of the *Cl. estertheticum* were very similar and points to the strains being related or derived from each other.

Whole genome comparison of the two *Cl. estertheticum* strains was also completed using MAUVE alignment. This alignment differs from other alignments because it ensures that regions that do not completely align are not discarded. MAUVE alignment indicated that there were no contigs missing and that the genome of *Cl. estertheticum* BP09-01 was slightly longer than that of *Cl. estertheticum* BP09-13. AsSince no contigs were missing, phenotypic differences are most likely to be a result of SNPs.

Despite the similarities in the overall genomes of the two *Cl. estertheticum,* there are some phenotypic differences that could be accounted for based on differences in the genomes. The major difference between *Cl. estertheticum* BP09-01 and BP09-13 is the resistance to heat and/or pressure treatment with *Cl. estertheticum* BP09-13 being more resistant to heat and pressure treatment (Chapter 3 of this thesis). SNP comparison was used to determine possible genetic differences between *Cl. estertheticum* BP09-01 and BP09-13 as a cause for differences in resistance to these interventions. A list of 15 SNPs was analyzed as possible candidates for phenotypic differences. Most of the SNPs analyzed were responsible for transport proteins, which may be responsible for some of the difference in heat and pressure resistance observed between *Cl. estertheticum*

BP09-01 and BP09-13. Under heat stress, ABC transporters and hypothetical proteins have been differentially expressed or upregulated in *Cl. botulinum* (31). Genes responsible for insertion of integral membrane proteins have also been upregulated during heat stress (31). The hypothesis is that upregulation of the expression of membrane proteins is required for membrane stabilization during heat stress (31). The rigidity of a membrane is related to the presence of unsaturated fatty acids and protein content which may play a role in resistance to heat and pressure (32-36). Differences in membrane transport proteins between the two strains of *Cl. estertheticum* may account for the differences observed in the resistance to heat and pressure.

The organisms that were isolated from commercially spoiled "blown-pack" spoiled meat were compared to 88 other *Clostridium* spp. to determine their relatedness. The two presumptive *Cl. estertheticum* strains were clustered closely with other *Cl. estertheticum* strains verifying the identity of BP09-01 and BP09-13. It is interesting to note that *Cl. estertheticum* BP09-01 and *Cl. estertheticum* BP09-13 were not more closely related which could also explain the differences in restriction length polymorphism patterns (Chapter 2 of this thesis) as well as the phenotypic differences observed in sugar utilization and resistance to heat and pressure (Chapters 2 and 3 of this thesis).

Cl. putrefaciens BP-1 was initially identified as a *Cl. putrefaciens* using 16S rDNA sequencing. However when compared to other *Clostridium* spp., *Cl. putrefaciens* BP-1 is more closely related to *Cl. algidicarnis*. *Cl. putrefaciens* is not generally known to be involved with "blown-pack" spoilage and is not known to be a psychrotroph (37,38). *Cl.*

putrefaciens and *Cl. algidicarnis* are so similar and cannot be differentiated using restriction fragment length polymorphism (RFLP) patterns (41,42). *Cl. algidicarnis* has been implicated in "blown-pack" spoilage in vacuum packaged beef from Brazil and United States (27,39,40). Based on the phylogenetic analysis, the presumptive identification of *Cl. putrefaciens*forBP-1 should be corrected to*Cl. algidicarnis* BP-1.

Comparison of the sequences for SASPs from the psychrophilic and psychrotrophic *Clostridium* spp. was also completed in this study. Initial PCR with primers specific for SASPs genes from *Cl. perfringens* indicated that there were no SASPs present. However, protein isolation identified the presence of presumptive SASPs (Chapter 3 of this thesis). Gene annotation verified that SASPs were present in all three organisms used in this study. These genes were compared to the *ssp*2 genes for *Cl. perfringens* and the flanking regions were very different from that of *Cl. perfringens*. *Cl. algidicarnis*BP-1 did not have SASPs that were similar to those of *C. perfringens*. This would explain why the primers that were used in the previous study did not allow the detection of genes for SASPs via PCR analysis. The presence of SASPs in the strains of *Clostridium* isolated from "blown-pack" meat does not explain why the strains were not as resistance to heat and pressure when compared to other *Clostridium* spp. From the gene annotations, differences are observed at how many genes encode SASPs. In some strains of *Cl. botulinum*, there are only four genes responsible for SASPs (43) and upwards of 8 genes have been described for *Cl. perfringens*(44-46). The differences in the number of genes that each *Clostridium* spp. has for the production of SASPs indicates great variation among species. These variations could account for some of the variation

observed for resistance to heat and pressure. The amino acid sequence of the SASPs of *Cl. estertheticum* were relatively different from that of *Cl. perfringens* and supports the observation of negative PCR results. These differences may or may not explain differences in heat and pressure resistance. The only way to understand whether or not these amino acid substitutions would inhibit or enhance resistance to heat and pressure would be through cloning or mutagenesis of the SASPs in either of the strains.

4.5Conclusions

The objectives of this study were met using whole genome sequencing. The organisms isolated from commercially "blown-pack" spoiled vacuum packaged meats were *Cl. estertheticum*. The *Cl. putrefaciens* that was isolated was initially thought to be a variant of *Cl. putrefaciens* because of its ability to growth at refrigeration temperature. Genome comparison determined that *Cl. putrefaciens* BP-1 was more closely related to *Cl. algidicarnis*. This organism has been associated with "blown-pack" spoiled meat and therefore *Cl.putrefaciens* BP-1 should be renamed as *Cl. algidicarnis* BP-1. SASPs are present in psychrophilic and psychrotrophic *Clostridium* spp. but their role in heat and pressure resistance is still unclear. Since the genus *Clostridium* is so diverse, more data mining from the genome could elucidate the genetic determinants that render these organisms as psychrophiles, why they germinate sporadically and eventually how to prevent their germination and growth in meat.

4.6 References

(1) Broda DM, Saul DJ, Lawson PA, Bell RG, Musgrave DR. *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum-packed meat. Int J Syst Evol Microbiol 2000 Jan;50:107-118.

(2) Broda DM, Lawson PA, Bell RG, Musgrave DR. *Clostridium frigidicarnis* sp nov., a psychrotolerant bacterium associated with 'blown pack' spoilage of vacuum-packed meats. Int J Syst Bacteriol 1999 Oct;49:1539-1550.

(3) Byrne B, Monaghan AM, Lyng JG, Sheridan JJ, Bolton DJ. A case of "blown pack" meat linked to *Clostridium estertheticum* in Ireland. J Food Saf 2009 Nov 2009;29(4):629-635.

(4) Yang X, Balamurugan S, Gill CO. Effects on the development of blown pack spoilage of the initial numbers of *Clostridium estertheticum* spores and *Leuconostoc mesenteroide*s on vacuum packed beef. Meat Sci 2011 Jul;88(3):361-367.

(5) Ho L. Characterization of "blown-pack" spoiled beef. Edmonton, Alberta: University of Alberta; 2009.

(6) Bolton DJ, Carroll J, Walsh D. A four-year survey of blown pack spoilage *Clostridium estertheticum* and *Clostridium gasigenes* on beef primal cuts. Lett Appl Microbiol 2015 Aug;61(2):153-157.

(7) Sturges WS, Drake ET. A complete description of *Clostridium putrefaiens*. J Bacteriol 1927 Sep;14(3):175-179.

(8) Hayes CS, Setlow P. An alpha/beta-type, small, acid-soluble spore protein which has very high affinity for DNA prevents outgrowth of *Bacillus subtilis* spores. J Bacteriol 2001 Apr;183(8):2662-2666.

(9) Setlow P. DNA in dormant spores of *Bacillus* species is in an A-like conformation. Mol Microbiol 1992 Mar;6(5):563-567.

(10) Paredes-Sabja D, Setlow B, Setlow P, Sarker MR. Characterization of *Clostridium perfringens* spores that lack spoVA proteins and dipicolinic acid. J Bacteriol 2008 Jul;190(13):4648-4659.

(11) Setlow B, Setlow P. Heat inactivation of *Bacillus subtilis* spores lacking small, acid-soluble spore proteins is accompanied by generation of abasic sites in spore DNA. J Bacteriol 1994 Apr;176(7):2111-2113.

(12) Popham DL, Sengupta S, Setlow P. Heat, hydrogen peroxide, and UV resistance of *Bacillus subtilis* spores with increased core water-content and with or without major DNA-binding proteins. Appl Environ Microbiol 1995 Oct;61(10):3633-3638.

(13) Lund BM. Anaerobes in relation to foods of plant origin. In: Barnes EM, Mead GC, editors. Anaerobic Bacteria in Habitats Other Than Man. 13th ed. Oxford: Blackwell Scientific; 1986. p. 351-372.

(14) Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, Birol I. ABySS: a parallel assembler for short read sequence data. Genome Res 2009 Jun;19(6):1117-1123.

(15) Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, et al. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res 2014 Jan 1;42(Database issue):D206-14.

(16) Sacchettini T. WebTB - TIGR function roles of ORFs. 2016; Available at: http://webtb.org/GenomeBrowser/tigr_roles.php. Accessed January 30, 2016.

(17) Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucl Acids Res 2014(42):D633-D642.

(18) Geer LY, Marchler-Bauer A, Geer RC, Han L, He J, He S, et al. The NCBI BioSystems database. Nucleic Acids Res 2010 Jan;38(Database issue):D492-6.

(19) Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 2013 Dec 2013;30(12):2725-2729.

(20) Cabrera-Martinez RM, Setlow P. Cloning and nucleotide sequence of three genes coding for small, acid-soluble proteins of *Clostridium perfringens* spores. FEMS Microbiol Lett 1991 Jan;61(2-3):127-31.

(21) Peck MW, Stringer SC, Carter AT. *Clostridium botulinum* in the post-genomic era. Food Microbiol 2011 Apr;28(2):183-191.

(22) Hill KK, Xie G, Foley BT, Smith TJ. Genetic diversity within the botulinum neurotoxin-producing bacteria and their neurotoxins. Toxicon 2015 Dec;107:2-8.

(23) Marshall KM, Nowaczyk L,II, Morrissey TR, Loeza V, Halik LA, Skinner GE, et al. Effect of sporulation temperature on the resistance of *Clostridium botulinum* Type A spores to thermal and high pressure processing. J Food Prot 2015 Jan;78(1):146-150.

(24) Cato EP, George WL, Finegold SM. Genus *Clostridium* Prazmowski, 1880. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editors. Bergey's Manual of Systematic Bacteriology Baltimore: The Williams & Wilkins Co.; 1986. p. 1141-1200.

(25) Parish JH. Studies in Microbiology Vol. 1. Developmental Biology of Prokaryotes. USA: University of California Press; 1979.

(26) Johnson JL, Francis BS. Taxonomy of the Clostridia : Ribosomal ribonucleic acid homologies among the species. J Gen Microbiol 1974 Jan;88:229-244.

(27) Lawson P, Dainty RH, Kristiansen N, Berg J, Collins MD. Characterization of a psychrotrophic *Clostridium* causing spoilage in vacuum-packed cooked pork: description of *Clostridium algidicarnis* sp. nov. Lett Appl Microbiol 1994 Feb; 153-157.

(28) Dainty RH, Edwards RA, Hibbard CM. Spoilage of vacuum-packed beef by a *Clostridium* sp. J Sci Food Agric 1989 Apr;49:473-486.

(29) Hurst LD, Merchant AR. High guanine-cytosine content is not an adaptation to high temperature: a comparative analysis amongst prokaryotes. Proc R Soc Lond 2001;268:493-497.

(30) Zheng H, Wu H. Gene-centeric association analysis for the correlation between the guanine-cytogsine content levels and temperature range conditions of prokaryotic species. BMC Bioinformatics 2010 Dec; 11(Suppl 11):S7.

(31) Liang W, Bi Y, Wang H, Dong S, Li K, Li J. Gene expression profiling of *Clostridium botulinum* under heat shock stress. BioMed Res. Int. 2013 Sep; 2013:760904.

(32) Hofstetter S, Gebhardt D, Ho L, Gänzle M, McMullen LM. Effects of nisin and reutericyclin on resistance of endospores of *Clostridium* spp. to heat and high pressure. Food Microbiol 2013 May;34(1):46-51.

(33) Hofstetter SC. Resistance and membrane fluidity of endospores of *Clostridium* spp. during pressure-assisted thermal processing in teh presence of antimicrobials. Edmonton: University of Alberta; 2012.

(34) Hofstetter S, Denter C, Winter R, McMullen LM, Gaäzle MG. Use of the fluorescent probe LAURDAN to label and measure inner membrane fluidity of endospores of *Clostridium* spp. J Microbiol Methods 2012 Oct;91(1):93-100.

(35) Margosch D, Ehrmann MA, Buckow R, Heinz V, Vogel RF, Gänzle MG. High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. Appl Environ Microbiol 2006 Mar 2006;72(5):3476-3481.

(36) Yoon Y, Lee H, Lee S, Kim S, Choi K. Membrane fluidity-related adaptive response mechanisms of foodborne bacterial pathogens under environmental stresses. Food Res Int 2015 Jun 2015;72:25-36.

(37) Ross HE. *Clostridium putrefaciens* - a Neglected Anaerobe. J Appl Bacteriol 1965 Apr;28(1):49-51.

(38) Sturges WS, Drake ET. A complete description of *Clostridium putrefaciens* (McBryde). J Bacteriol 1927 Sep;14(3):175-179.

(39) Silva AR, Tahara ACC, Chaves RD, Sant'Ana AS, Faria JdAF, Massaguer PR. Influence of different shrinking temperatures and vacuum conditions on the ability of psychrotrophic *Clostridium* to cause 'blown pack' spoilage in chilled vacuum-packaged beef. Meat Sci 2012 Dec; 92(4):498-505.

(40) Silva AR, Paulo EN, Sant'Ana AS, Chaves RD, Massaguer PR. Involvement of *Clostridium gasigenes* and *C. algidicarnis* in 'blown pack' spoilage of Brazilian vacuum-packed beef. Int J Food Microbiol 2011 Aug 15;148(3):156-163.

(41) Broda DM, Boerema JA, Brightwell G. Sources of psychrophilic and psychrotolerant clostridia causing spoilage of vacuum-packed chilled meats, as determined by PCR amplification procedure. J Appl Microbiol 2009 Jul;107(1):178-186.

(42) Broda DM, Musgrave DR, Bell RG. Use of restriction fragment length polymorphism analysis to differentiate strains of psychrophilic and psychrotrophic clostridia associated with 'blown pack' spoilage of vacuum-packed meats. J Appl Microbiol 2000 Jan;88:107-118.

(43) Meaney CA, Cartman ST, McClure PJ, Minton NP. The role of small acid-soluble proteins (SASPs) in protection of spores of *Clostridium botulinum* against nitrous acid. Int J Food Microbiol 2016 Jan;216:25-30.

(44) Orsburn B, Melville SB, Popham DL. Factors contributing to heat resistance of *Clostridium perfringens* endospores. Appl Environ Microbiol 2008 Jun;74(11):3328-3335.

(45) Raju D, Waters M, Setlow P, Sarker MR. Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. BMC Microbiology 2006 Jun;6:1-13.

(46) Raju D, Sarker MR. Production of small, acid-soluble spore proteins in *Clostridium perfringens* nonfoodborne gastrointestinal disease isolates. Can J Microbiol 2007 Apr;53(4):514-518.

5. General Discussion and Conclusions

Meat spoilage is a concern for the fresh meat industry as it has significant economic consequences (1). Technologies that impact the microbial spoilage of meat, such as acid washes of carcasses, vacuum packaging, and temperature control are meant to control the microbiota and mitigate the growth of spoilage and pathogens organisms on fresh meat. Organisms that cause "blown-pack" spoilage of meat circumvent all of these interventions and are known to be sporadic in nature and unpredictable (2, 3). This makes the control of "blown-pack" spoilage difficult and ultimately leads to unforeseen economic losses. It is important to understand the microbiological cause of "blown-pack" spoilage so that viable interventions can be implemented to mitigate spoilage.

Bacterial endospores of *Clostridium* spp., which have been implicated in "blown-pack" spoilage, persist in meat because of their resistance to various interventions used in the meat industry (4-6). *Clostridium* spp. are generally not the organism that is first considered with regard to spoilage; but in the absence of oxygen and at cold temperatures, psychrophilic *Clostridium* spp. will grow and cause "blown-pack" spoilage in vacuum package meats. "Blown-pack" spoilage occurs in a fraction of packages within a shipment and has been observed in many places worldwide including Canada (7-9), the United States (10-12), Ireland (13-15), Brazil (16-18), Australia and New Zealand (5, 19-21). Characterization of the organisms that cause "blown-pack" spoilage helps bring insights to how to prevent the outgrowth and subsequent spoilage of meat.

Resistance to heat, pressure, chemicals, and antimicrobials poses a huge challenge in decontamination of fresh meat. Multiple hurdles using several technologies can result in

the maintenance of meat safety and quality. Although these help to mitigate problems with pathogens, they remove the bulk of the vegetative cells or background microflora, which may create an environment that is more suitable for survival and growth of bacteria that may not otherwise cause problems. In addition, some interventions may stimulate the germination of bacterial endospores, which may result in spoilage. However, if food processors can target their interventions to coincide with germination, they could decrease the survival of endospore–producing bacteria present on the meat. This research assessed the use of hurdle technology, including high pressure processing (HHP) in combination with antimicrobials, to reduce the risk of growth of *Clostridium* spp.

Isolation and detection of organisms responsible for "blown-pack" spoilage is important to understand why this type of spoilage occurs sporadically and how to prevent it. Psychrotrophic endospores are difficult to isolate and can take weeks to be detected using traditional culture methods. Sampling using an anaerobic hood held at 10°C and minimizing oxygen to the package increases the ability to isolate the "blown-pack" spoilage organisms. Compared to previous attempts of isolating psychrotrophic endospores from "blown-pack" spoiled meat, isolation of *Clostridium* spp. to a cooler environment increased the detection of cold tolerant *Clostridium* spp. tenfold (22). In the current research, endospores were isolated from commercially spoiled "blown-pack" meat using a nutrient rich microbiological medium and all work was done at <10°C. The isolated endospores were characterized and determined to be psychrophilic and identified as *Clostridiumestertheticum* using 16S DNA sequencing. These organisms, along with a psychrotrophic *Clostridium* spp., presumptively identified as *Clostridiumputrefaciens*,

were subjected to heat, pressure, and a combination of heat and pressure with antimicrobials.

In this research, PCR using species-specific primers (3, 23) was used to detect the presence of *Cl. estertheticum* in the purge meat from a package of spoiled meat. Quantitative PCR using the same primers as the one this study has also been used to detect growth of *Cl. estertheticum* during storage (7). Rapid methods such as these are more suitable for detection than culture and isolation methods as these are extremely time consuming and must be done under carefully controlled conditions.

Exposing psychrophilic *Clostridium* spp. to heat resulted in reduction in viability of endospores. Increasing temperatures from 70 to 80 or 90°C resulted in decreased viability of endospores. *Cl. estertheticum* isolated from the commercially spoiled meat was not as resistant to heat compared to *Cl. estertheticum* ATCC 51377 that had been implicated in "blown-pack" spoilage. Thus there is strain-to-strain variability in relation to temperature sensitivity. Compared to other psychrophilic *Clostridium* spp., *Clostridium* spp. that cause "blown-pack" spoilage are not as resistant to heat (24, 25). Cold tolerant *Clostridium botulinum* can survive heating to 80°C for 10 min; however, this was not observed with any of the *Cl. estertheticum* (BP09-01, BP09-13 or ATCC 51377) used in this study. The psychrophilic endospores were less resistant to heat and pressure compared to cold tolerant *Clostridium* in literature. Heating to 80°C for 8 min resulted in a 3-log reduction of *Cl. estertheticum*. The temperature at which organisms produce endospores may play a role in the overall resistance to heat. When endospores of psychrotrophic *Cl. botulinum* are produced at low temperatures, their resistance to heat is decreased (24). The endospores used in this study were sporulated at 7 °C as this is the

optimum temperature for growth of some strains of *Cl. estertheticum*. Understanding resistance to heat is the initial step in developing a thermal based intervention to inactivate psychrotrophic endospores.

Resistance to pressure was also investigated in the current research. Endospores survived heating to 70°C, but with the addition of pressure (400 MPa) rapid inactivation was observed, indicating that addition of pressure did aid in inactivation. When pressure was applied at lower temperatures, inactivation of endospores was still observed. At 4°C, endospores were inactivated when 400 MPa was applied for 30 min in saline. Psychrophilic endospores are not as pressure resistant compared to other endospores (24, 26) and therefore, pressure treatment could be an option for controlling the survival of endospores on meat. Pressure treatment at 400 MPa and 4°C for 30 min would be sufficient to reduce psychrophilic endospores to below detection limits in meat. Other researchers have investigated the impact of pressure and temperature on survival of endospores in other menstrums (27). When *Cl. botulinum* endospores were treated at 450 MPa and 45°C in a 70% oil and water emulsion, there was a lower log reduction observed compared to endospores treated in water (27). Endospores react differently to pressure in different media or when different components are added; which is why the pressure resistance of psychrotrophic endospores was assessed in meat and in saline. *Cl. estertheticum* BP09-13 survived longer when treated in meat at 400 MPa and 4°C as compared to treatment in saline. However, the same was not the case for *Cl. estertheticum* ATCC 51377 or *Cl. algidicarnis* BP-1. Thus, results for pressure inactivation in saline cannot be extrapolated to a meat matrix. Experiments assessing the

application of pressure and heat for potential use to control *Clostridium* spp. in meat should be done using a meat product.

All strains of psychrophilic and psychrotrophic *Clostridium* spp. in this study were confirmed to produce SASPs. SASPs play a role in resistance of endospores to heat (28-30). *Bacillus subtilis* endospores without α/β-type SASPs are less resistance to heat, but in some cases are more resistant to pressure (31). The psychrophilic endospores used in this study were less resistant to heat compared to what is reported in the literature for other cold-tolerant endospores (25, 32).

Often when intervention technologies are combined as multiple hurdles, greater antimicrobial effects are observed (31, 33). Thus, a combination of Micocin X™ and pressure treatment was evaluated to determine whether enhanced inactivation of endospores could be achieved. Micocin X™ did not have the desired effect of increasing inactivation of endospores, but appeared to improve survival in minced meat. Micocin X™ is the spray-dried supernatant of *Carnobacterium maltaromaticum* UAL 307 and may contain cellular debris. To determine if cellular debris may have improved survival, the supernatant of a bacteriocin-negative *C.maltaromaticum* UAL 8C2 and APT broth was added to the meat. These also improved the survival of endospores in minced meat. Initially, it was thought that the peptidoglycan in the Micocin X™ and the supernatant of *C. maltaromaticum* UAL 8C2 may have played a role in protecting the endospores from heat and pressure inactivation (Chapter 3 of this thesis). However, this was not the case as the addition of peptidoglycan from *Bacillus* spp. increased inactivation of endospores of *Cl. estertheticum* and *Cl. algidicarnis*. It is possible that peptidoglycan fragments

from *Bacillus* spp. may have induced germination of the endospores(34) and therefore increased the inactivation of the endospores in saline.

It would be interesting to understand why Micocin X™, the supernatant of *C. maltaromaticum* UAL 8C2 and APT broth actually improved the survival of endospores in minced meat. It could be because that the nutrients present in Micocin X™, the supernatant of *C. maltaromaticum* UAL 8C2 and APT broth had a protective effect on the endospores and may not have triggered nutrient germination receptors. Other research on germination of *Cl. estertheticum* has shown that lactate induces spore germination (35). Lactate should be present in Micocin X™; thus this does not explain why Micocin X™ protected the endospores from heat and pressure inactivation. Other bacteriocins, such as nisin, in combination with pressure and heat to have resulted in increased inactivation of endospores (36). Micocin X™ contains carnocyclin A, piscicolin JG126 and carnobacteriocin BM1. It is possible that the different modes of action of the bacteriocins could explain why some bacteriocins are effective in enhancing inactivation of endospores and why some are not(36).

Whole genome sequencing was used to verify the identity of the organisms isolated from commercially "blown-pack" spoiled vacuum packaged meats and to determine if the genes for SASPs were present in the strains isolated as part of this research. Early in this research, *Cl. estertheticum* and *Cl. putrefaciens* were presumptively identified using 16S DNA sequencing. The strain presumptively identified as *Cl. putrefaciens* had the ability to grow at refrigeration temperature which differs from *Cl. putrefaciens* described in literature. Genome comparison of *Cl. putrefaciens* BP-1 to other *Clostridium* genomes

revealed that this strain was more closely related to *Cl. algidicarnis*. *Cl. putrefaciens* has not generally been associated with "blown-pack" spoilage whereas *Cl. algidicarnis* has been (16, 17) and therefore *Cl.putrefaciens* BP-1 was renamed *Cl. algidicarnis* BP-1. The genes for SASPs were present in the psychrophilic and psychrotrophic *Clostridium* spp. studied in this research, but their role in heat and pressure resistance in these organisms is still unclear. Strategies to create isogenic knockouts could be used to elucidate the specific role of SASPs in heat and pressure resistance. Since the genus *Clostridium* is so diverse, additonal data mining from the genome could elucidate the genetic determinants that render these organisms as psychrophiles, the reason why they germinate sporadically and eventually how to prevent their germination and growth in meat.

The objectives of this research have been met. Inactivation of psychrophilic endospores can be completed using 400 MPa at 4°C for 30 min. This has not been previously reported in the literature for any wildtype *Clostridium*endospores (psychrophilic or otherwise). Addition of mild heat in combination with HHP resulted in increased inactivation of the endospores while still maintaining the visual quality of the meat; therefore making this a viable option for industry. The long processing time required may render the treatment economically prohibitative for the meat industry. However, addition of additives may alter the inactivation rates of the endospores in meat. Processors have to carefully choose the antimicrobials to ensure that there is not increased resistance to heat or pressure. Identification of psychrotrophic and psychrophilic *Clostridium* spp. was completed and differences in the genomes of the psychrophilic *Clostridium* spp. were compared. This was the first time that the genome of *Cl. estertheticum* had been

sequenced. *Clostridiumputrefaciens* BP-1 was renamed *Cl. algidicarnis* BP-1 because of its ability to growth at refrigeration temperature, produce gas under vacuum-packed storage, and it was more closely related when comparing the genome.

5.1 References

(1) Moschonas G, Bolton DJ, McDowell DA, Sheridan JJ. Diversity of culturable psychrophilic and psychrotrophic anaerobic bacteria isolated from beef abattoirs and their environments. Appl Environ Microbiol 2011 Jul;77(13):4280-4284.

(2) Boerema JA, Broda DM, Penney N, Brightwell G. Influence of peroxyacetic acid-based carcass rinse on the onset of "blown pack" spoilage in artificially inoculated vacuum-packed chilled beef. J Food Prot 2007 Jun; 70(6):1434-1439.

(3) Broda DM, Boerema JA, Bell RG. PCR detection of psychrophilic *Clostridium* spp. causing 'blown pack' spoilage of vacuum-packed chilled meats. J Appl Microbiol 2003 Feb; 94(3):515-522.

(4) Broda DM, Boerema JA, Bell RG. A PCR survey of psychrotrophic *Clostridium* botulinum-like isolates for the presence of BoNT genes. Lett Appl Microbiol 1998 Oct;27(4):219-223.

(5) Broda DM, DeLacy KM, Bell RG, Penney N. Association of psychrotrophic *Clostridium* spp., with deep tissue spoilage of chilled vacuum-packed lamb. Int J Food Microbiol 1996 Apr;29(2-3):371-378.

(6) Bell RG, Moorhead SM, Broda DM. Influence of heat shrink treatments on the onset of clostridial "blown pack" spoilage of vacuum packed chilled meat. Food Res Int 2001 Jul;34(4):271-275.

(7) Yang X, Balamurugan S, Gill CO. Substrate utilization by *Clostridium estertheticum* cultivated in meat juice medium. Int J Food Microbiol 2009 Jan;128(3):501-505.

(8) Yang X, Gill CO, Balamurugan S. Products of Glucose and Lactate Fermentation, and Utilization of Amino Acids by Clostridium estertheticum subspp. *laramiense* and estertheticum Growing in Meat Juice Medium. J Food Prot 2010 Jul;73(7):1348-1352.

(9) Yang X, Balamurugan S, Gill CO. Effects on the development of blown pack spoilage of the initial numbers of Clostridium estertheticum spores and Leuconostoc mesenteroides on vacuum packed beef. Meat Sci 2011 Jul;88(3):361-367.

(10) Kalchayanand N, Ray B, Field RA. Characteristics of psychotrophic *Clostridium laramie* causing spoilage of vacuum-packaged refrigerated fresh and roasted beef. J Food Protein 1993 Jun;56:13-17.

(11) Dainty RH, Edwards RA, Hibbard CM. Spoilage of vacuum-packed beef by a *Clostridium* sp. J Sci Food Agric 1989 Apr;49:473-486.

(12) Helps CR, Harbour DA, Corry JEL. PCR-based 16S ribosomal DNA detection techniques for *Clostridium estertheticum* causing spoilage in vacuum-packed chill-stored beef. Int J Food Microbiol 1999 Nov;52:57-65.

(13) Bolton DJ, Carroll J, Walsh D. A four-year survey of blown pack spoilage *Clostridium estertheticum* and *Clostridium gasigenes* on beef primal cuts. Lett Appl Microbiol 2015 Aug;61(2):153-157.

(14) Byrne B, Monaghan AM, Lyng JG, Sheridan JJ, Bolton DJ. A case of "blown pack" meat linked to *Clostridium estertheticum* in Ireland. J Food Saf 2009 Nov;29(4):629-635.

(15) Moschonas G, Bolton DJ, McDowell DA, Sheridan JJ. Diversity of culturable psychrophilic and psychrotrophic anaerobic bacteria isolated from beef abattoirs and their environments. Appl Environ Microbiol 2011 Jul;77(13):4280-4284.

(16) Silva AR, Tahara ACC, Chaves RD, Sant'Ana AS, Faria JdAF, Massaguer PR. Influence of different shrinking temperatures and vacuum conditions on the ability of psychrotrophic *Clostridium* to cause 'blown pack' spoilage in chilled vacuum-packaged beef. Meat Sci 2012 Dec; 92(4):498-505.

(17) Silva AR, Paulo EN, Sant'Ana AS, Chaves RD, Massaguer PR. Involvement of *Clostridium gasigenes* and *C. algidicarnis* in "blown pack" spoilage of Brazilian vacuum-packed beef. Int J Food Microbiol 2011 Aug;148(3):156-163.

(19) Broda DM, Lawson PA, Bell RG, Musgrave DR. *Clostridium frigidicarnis* sp nov., a psychrotolerant bacterium associated with 'blown pack' spoilage of vacuum-packed meats. Int J Syst Bacteriol 1999 Oct;49:1539-1550.

(20) Broda DM, Saul DJ, Lawson PA, Bell RG, Musgrave DR. *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum-packed meat. Int J Syst Evol Microbiol 2000 Jan;50:107-118.

(21) Broda DM, DeLacy KM, Bell RG, Braggins TJ, Cook RL. Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Int J Food Microbiol 1996 Apr;29(2-3):335-352.

(22) Ho L. Characterization of "blown-pack" spoiled beef. Edmonton, Alberta: University of Alberta; 2009.

(23) Boerema JA, Broda DM, Bell RG. PCR detection of psychrotolerant clostridia associated with deep tissue spoilage of vacuum-packed chilled meats. Lett Appl Microbiol 2002 Oct;35:446-450.

(24) Lenz CA, Vogel RF. Differential effects of sporulation temperature on the high pressure resistance of *Clostridium botulinum* type E spores and the interconnection with sporulation medium cation contents. Food Microbiol 2015 Apr;46:434-442.

(25) Reddy NR, Tetzloff RC, Solomon HM, Larkin JW. Inactivation of Clostridium botulinum nonproteolytic type B spores by high pressure processing at moderate to elevated high temperatures. Innov Sci Food Emerg Tech 2006 Sep;7(3):169-175.

(26) Nakayama A, Yano Y, Kobayashi S, Ishikawa M, Sakai K. Comparison of pressure resistances of spores of six *Bacillus* strains with their heat resistances. Appl Environ Microbiol 1996 Oct;62(10):3897-3900.

(27) Schnabel J, Lenz CA, Vogel RF. High pressure inactivation of *Clostridium botulinum* type E endospores in model emulsion systems. High Pressure Research 2015 Jan;35(1):78-88.

(28) Raju D, Waters M, Setlow P, Sarker MR. Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. BMC Microbiology 2006 Jun;6:1-13.

(29) Paredes-Sabja D, Sarker N, Setlow B, Setlow P, Sarker MR. Roles of dacB and spm proteins in *Clostridium perfringens* spore resistance to moist heat, chemicals, and UV radiation. Appl Environ Microbiol 2008 Jun;74(12):3730-3738.

(30) Gerhardt P, Marquis RE. Spore thermoresistance mechanisms. In: Smith I, Slepecky RA, Setlow P, editors. Regulation of Prokaryotic Development. 1st ed. Washington, DC: American Society for Microbiology; 1989. p. 43-63.

(31) Lee J, Movahedi S, Harding SE, Mackey BM, Waites WM. Effect of small, acid-soluble proteins on spore resistance and germination under a combination of pressure and heat treatment. J Food Prot 2007 Sep;70(9):2168-2171.

(32) Marshall KM, Nowaczyk L,II, Morrissey TR, Loeza V, Halik LA, Skinner GE, et al. Effect of sporulation temperature on the resistance of *Clostridium botulinum* Type A spores to thermal and high pressure processing. J Food Prot 2015 Jan;78(1):146-150.

(33) Stringer SC, Fairbairn DA, Peck MW. Combining heat treatment and subsequent incubation temperature to prevent growth from spores of non-proteolytic *Clostridium botulinum*. J Appl Microbiol 1997 Jan;82(1):126-136.

(34) Popham DL. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. Cellular and Molecular Life Sciences. 2002 Mar;59(3):426-33.

(35) Rajagopal S. Characterization of germination of *Clostridium estertheticum* spores. Edmonton, Alberta: University of Alberta; 2014.

(36) Hofstetter S, Gebhardt D, Ho L, Gänzle M, McMullen LM. Effects of nisin and reutericyclin on resistance of endospores of *Clostridium* spp. to heat and high pressure. Food Microbiol 2013May;34(1):46-51.

Bibliography

Ablett S, Darke AH, Lillford PJ, Martin DR. Glass formation and dormancy in bacterial spores. Int J Food Sci Tech 1999 Feb; 34(1):59-69.

Adam KH, Flint SH, Brightwell G. Psychrophilic and psychrotrophic clostridia: sporulation and germination processes and their role in the spoilage of chilled, vacuum-packaged beef, lamb and venison. Int J Food Sci Tech 2010 Aug;45(8):1539-1544.

Alberto F, Broussolle V, Mason DR, Carlin F, Peck MW. Variability in spore germination response by strains of proteolytic *Clostridium botulinum* types A, B and F. Lett Appl Microbiol 2003 Dec;36(1):41-45.

Aouadhi C, Rouissi Z, Mejri S, Maaroufi A. Inactivation of *Bacillus sporothermodurans* spores by nisin and temperature studied by design of experiments in water and milk. Food Microbiol 2014 Apr; 38:270-275.

Balciunas EM, Castillo Martinez FA, Todorov SD, Gombossy de Melo Franco,Bernadette Dora, Converti A, de Souza Oliveira RP. Novel biotechnological applications of bacteriocins: A review. Food Control 2013 Jul; 32(1):134-142.

Bell RG, Moorhead SM, Broda DM. Influence of heat shrink treatments on the onset of clostridial "blown pack" spoilage of vacuum packed chilled meat. Food Res Int 2001 Jul; 34(4):271-275.

Black E, Koziol-Dube K, Guan D, Wei H, Setlow B, Cortezzo D, et al. Factors influencing germination of *Bacillus subtilis* spores via activation of nutrient receptors by high pressure. Appl Environ Microbiol 2005 Oct; 71(10):5879-5887.

Black EP, Setlow P, Hocking AD, Stewart CM, Kelly AL, Hoover DG. Response of spores to high-pressure processing. Compr Rev Food Sci Food Saf2007 Oct;6(4):103-119.

Boerema JA, Broda DM, Bell RG. PCR detection of psychrotolerant clostridia associated with deep tissue spoilage of vacuum-packed chilled meats. Lett Appl Microbiol 2002 Oct; 35:446-450.

Boerema JA, Broda DM, Penney N, Brightwell G. Influence of peroxyacetic acid-based carcass rinse on the onset of "blown pack" spoilage in artificially inoculated vacuum-packed chilled beef. J Food Prot 2007 Jun;70(6):1434-1439.

Bolton DJ, Carroll J, Walsh D. A four-year survey of blown pack spoilage *Clostridium estertheticum* and *Clostridium gasigenes* on beef primal cuts. Lett Appl Microbiol 2015 Aug;61(2):153-157.

Breukink E, Wiedemann I, van Kraaij C, Kuipers OP, Sahl HG, de Kruijff B. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science 1999 Dec; 286(5448):2361-2364.

Broda DM. The effect of peroxyacetic acid-based sanitizer, heat and ultrasonic waves on the survival of *Clostridium estertheticum* spores in vitro. Lett Appl Microbiol 2007 Sep;45(3):336-341.

Broda DM, Bell RG, Boerema JA, Musgrave DR. The abattoir source of culturable psychrophilic *Clostridium* spp. causing 'blown pack' spoilage of vacuum-packed chilled venison. J Appl Microbiol 2002 Oct; 93(5):817-824.

Broda DM, Boerema JA, Bell RG. A PCR survey of psychrotrophic *Clostridiumbotulinum*-like isolates for the presence of BoNT genes. Lett Appl Microbiol 1998 Oct; 27(4):219-223.

Broda DM, Boerema JA, Bell RG. PCR detection of psychrophilic *Clostridium* spp. causing 'blown pack' spoilage of vacuum-packed chilled meats. J Appl Microbiol 2003 Feb; 94(3):515-522.

Broda DM, Boerema JA, Brightwell G. Sources of psychrophilic and psychrotolerant clostridia causing spoilage of vacuum-packed chilled meats, as determined by PCR amplification procedure. J Appl Microbiol 2009 Jul; 107(1):178-186.

Broda DM, DeLacy KM, Bell RG, Braggins TJ, Cook RL. Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Int J Food Microbiol 1996 Apr; 29(2-3):335-352.

Broda DM, DeLacy KM, Bell RG, Penney N. Association of psychrotrophic *Clostridium* spp., with deep tissue spoilage of chilled vacuum-packed lamb. Int J Food Microbiol 1996 Apr; 29(2-3):371-378.

Broda DM, DeLacy KM, Cook RL, Bell RG. Prevalence of cold-tolerant clostridia associated with vacuum-packed beef and lamb stored at abusive and chill temperatures. N Z J Agric Res 1997 Mar; 40(1):93-98.

Broda DM, Lawson PA, Bell RG, Musgrave DR. *Clostridium frigidicarnis* sp nov., a psychrotolerant bacterium associated with 'blown pack' spoilage of vacuum-packed meats. Int J Syst Bacteriol 1999 Oct; 49:1539-1550.

Broda DM, Musgrave DR, Bell RG. Use of restriction fragment length polymorphism analysis to differentiate strains of psychrophilic and psychrotrophic clostridia associated with 'blown pack' spoilage of vacuum-packed meats. J Appl Microbiol 2000 Jan; 88:107-118.

Broda DM, Saul DJ, Bell RG, Musgrave DR. *Clostridium algidixylanolyticum* sp. nov., a psychrotolerant, xylan degrading, spore-forming bacterium. Int J Syst Evol Microbiol 2000 Mar; 50:623-631.

Broda DM, Saul DJ, Lawson PA, Bell RG, Musgrave DR. *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum-packed meat. Int J Syst Evol Microbiol 2000 Jan; 50:107-118.

Buckow R, Sikes A, Tume R. Effect of high pressure on physicochemical properties of meat. Crit Rev Food Sci Nutr 2013 Jan; 53(7):770-786.

Bull MK, Olivier SA, van Diepenbeek RJ, Kormelink F, Chapman B. Synergistic inactivation of spores of proteolytic *Clostridium botulinum* strains by high pressure and heat is strain and product dependent. Appl Environ Microbiol 2009 Jan;75(2):434-445.

Byrne B, Monaghan AM, Lyng JG, Sheridan JJ, Bolton DJ. A case of "blown pack" meat linked to *Clostridium estertheticum* in Ireland. J Food Saf 2009 Nov; 29(4):629-635.

Cabrera-Martinez RM, Mason JM, Setlow B, Waites WM, Setlow P. Purification and amino acid sequence of two small, acid-soluble proteins from *Clostridium bifermentans* spore**s.**. FEMS Microbiol Let 1989 Oct;52(1-2):139-143.

Cabrera-Martinez RM, Setlow P. Cloning and nucleotide sequence of three genes coding for small, acid-soluble proteins of *Clostridium perfringens* spores. FEMS Microbiol Lett 1991 Jan;61(2-3):127-31.

Campus M. High pressure processing of meat, meat products and seafood. Food Engineering Reviews 2010 Dec; 2(4):256-273.

Carlez A, Veciananogues T, Cheftel JC. Changes in color and myoglobin of minced beef meat due to high-pressure processing. Lebensm Wiss Technol 1995 Mar; 28(5):528-538.

Cato EP, George WL, Finegold SM. Genus *Clostridium* Prazmowski, 1880. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editors. Bergey's Manual of Systematic Bacteriology Baltimore: The Williams & Wilkins Co.; 1986. p. 1141-1200.

Collins MD, Rodrigues UM, Dainty RM, Edwards RA, Roberts TA. Taxonomic studies on a psychrophilic *Clostridium* from vacuum-packed beef: description of *Clostridium estertheticum* sp. FEMS Microbiology Letters 1992 Sep; 96:235-240.

Clouston JG, Wills PA. Initiation of germination and inactivation of *Bacillus pumilus* spores by hydrostatic pressure. J Bacteriol 1969 Feb; 97(2):684-690.

Cortezzo DE, Setlow P. Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. J Appl Microbiol 2005 Dec; 98(3):606-617.

Cotter PD, Hill C, Ross RP. Bacteriocins: Developing innate immunity for food. Nature Reviews Microbiology 2005 Oct; 3(10):777-788.

Cowan AE, Koppel DE, Setlow B, Setlow P. A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: Implications for spore dormancy. Proc Natl Acad Sci USA 2003 Apr; 100(7):4209-4214.

Cowan AE, Olivastro EM, Koppel DE, Loshon CA, Setlow B, Setlow P. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. Proc Natl Acad Sci U S A 2004 Ma 18;101(20):7733-7738.

Dainty RH, Edwards RA, Hibbard CM. Spoilage of vacuum-packed beef by a *Clostridium* sp. J Sci Food Agric 1989 Apr;49:473-486.

Deegan LH, Cotter PD, Hill C, Ross P. Bacteriocins: Biological tools for bio-preservation and shelf-life extension. Int Dairy J 2006 Sep; 16(9):1058-1071.

Defaye AB, Ledward DA, Macdougall DB, Tester RF. Renaturation of metmyoglobin subjected to high isostatic pressure. Food Chem 1995 Dec; 52(1):19-22.

Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J. Applications of the bacteriocin, nisin. Anton Leeuw Int J G 1996 Feb;69(2):193-202.

Dlusskaya EA, McMullen LM, Gänzle MG. Characterization of an extremely heat-resistant *Escherichia coli* obtained from a beef processing facility. J Appl Microbiol 2011 Mar;110(3):840-849.

Drider D, Fimland G, Hechard Y, McMullen LM, Prevost H. The continuing story of class IIa bacteriocins. Microbiol Mol Biol R 2006 Jun; 70(2):564-582.

Egan AF, Roberts TA. Microbiology of meat and meat products. 1st ed. New York: Wiley; 1987.

Eijsink VGH, Skeie M, Middelhoven PH, Brurberg MB, Nes IF. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl Environ Microbiol 1998 Sep; 64(9):3275-3281.

Fairhead H, Setlow B, Setlow P. Prevention of DNA damage in spores and invitro by small, acid-soluble proteins from *Bacillus* species. J Bacteriol 1993 Mar;175(5):1367-1374.

Fimland G, Johnsen L, Dalhus B, Nissen-Meyer J. Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. Journal of Peptide Science 2005 Nov; 11(11):688-696.

Gänzle M, Vogel R. On-line fluorescence determination of pressure mediated outer membrane damage in *Escherichia coli*. Syst Appl Microbiol 2001 Dec; 24(4):477-485.

Gänzle MG. Reutericyclin: biological activity, mode of action, and potential applications. Appl Microbiol Biotechnol 2004 Apr;64(3):326-332.

Gao Y, Ju X. Exploiting the combined effects of high pressure and moderate heat with nisin on inactivation of *Clostridium botulinum* spores. J Microbiol Methods 2008 Jan;72(1):20-28.

Garriga M, Aymerich MT, Costa S, Monfort JM, Hugas M. Bactericidal synergism through bacteriocins and high pressure in a meat model system during storage. Food Microbiol 2002 Oct; 19:509-518.

Geer LY, Marchler-Bauer A, Geer RC, Han L, He J, He S, et al. The NCBI BioSystems database. Nucleic Acids Res 2010 Jan;38(Database issue):D492-6.

Gerhardt P, Marquis RE. Spore thermoresistance mechanisms. In: Smith I, Slepecky RA, Setlow P, editors. Regulation of Prokaryotic Development. 1st ed. Washington, DC: American Society for Microbiology; 1989. p. 43-63.

Gong X, Martin-Visscher LA, Nahirney D, Vederas JC, Duszyk M. The circular bacteriocin, carnocyclin A, forms anion-selective channels in lipid bilayers. Biochimica Et Biophysica Acta-Biomembranes 2009 Sep;1788(9):1797-1803.

Gould GW. History of science - spores - Lewis B Perry Memorial Lecture 2005. J Appl Microbiol 2006 Sep; 101(3):507-513.

Gould G, Sale A. Initiation of germination of bacterial spores by hydrostatic pressure. J Gen Microbiol 1970 Jan;60:335-46.

Gursky LJ, Martin NI, Derksen DJ, van Belkum MJ, Kaur K, Vederas JC, et al. Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. Arch Microbiol 2006 Oct; 186(4):317-325.

Hanna MO, Smith GC, Hall LC, Vanderzant C. Role of *Hafnia alvei* and a *Lactobacillus* species in the spoilage of vacuum-packaged strip loin steaks. J Food Prot 1979 Jul; 42(7):569-571.

Hayes DS, Peng ZY, Setlow P. Equilibrium and kinetic binding interactions between DNA and a group of novel, nonspecific DNA-binding proteins from spores of *Bacillus* and *Clostridium* species. J Biol Chem 2000 Nov;275(45):35040-35050.

Hayes CS, Setlow P. An alpha/beta-type, small, acid-soluble spore protein which has very high affinity for DNA prevents outgrowth of *Bacillus subtilis* spores. J Bacteriol 2001 Apr;183(8):2662-2666.

Hechard Y, Sahl HG. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie 2002 Jun; 84(5-6):545-557.

Helps CR, Harbour DA, Corry JEL. PCR-based 16S ribosomal DNA detection techniques for *Clostridium estertheticum* causing spoilage in vacuum-packed chill-stored beef. Int J Food Microbiol 1999 Nov; 52:57-65.

Hills GM. Chemical Factors in the Germination of Spore-Bearing Aerobes - the Effects of Amino-Acids on the Germination of *Bacillus anthracis*, with some Observations on the Relation of Optical Form to Biological Activity. Biochem J. 1949 Dec;45(3):363-70.

Hill KK, Xie G, Foley BT, Smith TJ. Genetic diversity within the botulinum neurotoxin-producing bacteria and their neurotoxins. Toxicon 2015 Dec;107:2-8.

Ho L. Characterization of "blown-pack" spoiled beef. Edmonton, Alberta: University of Alberta; 2009.

Hofstetter SC. Resistance and membrane fluidity of endospores of *Clostridium* spp. during pressure-assisted thermal processing in teh presence of antimicrobials. Edmonton: University of Alberta; 2012.

Hofstetter S, Denter C, Winter R, McMullen LM, Gänzle MG. Use of the fluorescent probe LAURDAN to label and measure inner membrane fluidity of endospores of *Clostridium* spp. J Microbiol Methods 2012 Oct; 91(1):93-100.

Hofstetter S, Gebhardt D, Ho L, Gänzle M, McMullen LM. Effects of nisin and reutericyclin on resistance of endospores of *Clostridium* spp. to heat and high pressure. Food Microbiol 2013 May; 34(1):46-51.

Huang S, Chen D, Pelczar PL, Vepachedu VR, Setlow P, Li Y. Levels of Ca(2+)-dipicolinic acid in individual *Bacillus* spores determined using microfluidic raman tweezers. J Bacteriol 2007 Jul;189(13):4681-4687.

Hurst, LD and Merchant AR. High guanine-cytosine content is not an adaptation to high temperature: A comparative analysis amongst prokaryotes. Proc R Soc Lond 2001 Nov 268:493-497.

Hutson RA, Thompson DE, Collins MD. Genetic interrelationships of saccharolytic *Clostridium botulinum* types B,E and F and related clostridia as revealed by small-subunit rRNA sequences. FEMS Microbiology Letters 1993 Mar; 108:103-110.

Hutton MT, Chehak PA, Hanlin JH. Inhibition of botulinum toxin production by *Pediococcus acidilactici* in temperature abused refrigerated foods. J Food Saf 1991 Apr; 11(4):255-267.

Hyytia E, Bjorkroth J, Hielm S, Korkeala H. Characterisation of *Clostridium botulinum* groups I and II by randomly amplified polymorphic DNA analysis and repetitive element sequence-based PCR. Int J Food Microbiol 1999 Jun;48:179-189.

Hyytia E, Hielm S, Korkeala H. Prevalence of *Clostridium botulinum* type E in Finnish fish and fishery products. Epidemiol Infect 1998 Jun;120(3):245-250.

Islam M, Inoue A, Igura N, Shimoda M, Hayakawa I. Inactivation of *Bacillus* spores by the combination of moderate heat and low hydrostatic pressure in ketchup and potage. Int J Food Microbiol 2006 Mar; 107(2):124-130.

Jack RW, Wan J, Gordon J, Harmark K, Davidson BE, Hillier AJ, et al. Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Appl Environ Microbiol 1996 Aug; 62(8):2897-2903.

Johnson JL, Francis BS. Taxonomy of the Clostridia : Ribosomal ribonucleic acid homologies among the species. J Gen Microbiol 1974 Jan;88:229-244.

Johnson W, Tipper D. Acid-soluble spore proteins of *Bacillus subtilis*. J Bacteriol 1981 Jun;146(3):972-982.

Jung S, Ghoul M, de Lamballerie-Anton M. Influence of high pressure on the color and microbial quality of beef meat. Lebens Wiss Technol 2003 May; 36(6):625-631.

Kaiser AL, Montville TJ. Purification of the bacteriocin bavaricin MN and characterization of its mode of action against *Listeria monocytogenes* Scott A cells and lipid vesicles. Appl Environ Microbiol 1996 Dec; 62(12):4529-4535.

Kalchayanand N, Dunne CP, Sikes A, Ray B. Inactivation of bacterial spores by combined action of hydrostatic pressure and bacteriocins in roast beef. J Food Saf 2003 Nov; 23(4):219-231.

Kalchayanand N, Ray B, Field RA. Characteristics of psychrotrophic *Clostridium laramie* causing spoilage of vacuum-packaged refrigerated fresh and roasted beef. J Food Protein 1993 Jan; 56:13-17.

Kalchayanand N, Ray B, Field RA, Johnson MC. Spoilage of vacuum-packaged refrigerated beef by *Clostridium*. J Food Prot 1989 Jun; 52:424-426.

Kato M, Hayashi R. Effects of high pressure on lipids and biomembranes for understanding high-pressure-induced biological phenomena. Biosci Biotechnol Biochem 1999 Aug;63(8):1321-1328.

Kato M, Hayashi R, Tsuda T, Taniguchi K. High pressure-induced changes of biological membrane - Study on the membrane-bound Na+/K+-ATPase as a model system. Eur J Biochem 2002 Jan; 269(1):110-118.

Kawai Y, Kemperman R, Kok J, Saito T. The circular Bacteriocins gassericin A and circularin A. Curr Protein Peptide Sci 2004 Oct; 5(5):393-398.

Kim H, Hahn M, Grabowski P, McPherson DC, Otte MM, Wang R, et al. The *Bacillus subtilis* spore coat protein interaction network. Mol Microbiol 2006 Jan; 59(2):487-502.

Klaenhammer TR. Genetics of bacteriocins produced by lactic-acid bacteria. FEMS Microbiol Rev 1993 Sep; 12(1-3):39-86.

Laursen BG, Leisner JJ, Dalgaard P. *Carnobacterium* species: Effect of metabolic activity and interaction with *Brochothrix thermosphacta* on sensory characteristics of modified atmosphere packed shrimp. J Agric Food Chem 2006 May; 54(10):3604-3611.

Lawson P, Dainty RH, Kristiansen N, Berg J, Collins MD. Characterization of a psychrotrophic *Clostridium* causing spoilage in vacuum-packed cooked pork: description of *Clostridium algidicarnis* sp. nov. Lett Appl Microbiol 1994 Feb; 19:153-157.

Lee KS, Bumbaca D, Kosman J, Setlow P, Jedrzejas MJ. Structure of a protein-DNA complex essential for DNA protection in spores of *Bacillus* species. Proc Natl Acad Sci U S A 2008 Feb; 105(8):2806-2811.

Lee J, Movahedi S, Harding SE, Mackey BM, Waites WM. Effect of small, acid-soluble proteins on spore resistance and germination under a combination of pressure and heat treatment. J Food Prot 2007 Sep;70(9):2168-2171.

Leggett MJ, McDonnell G, Denyer SP, Setlow P, Maillard J-. Bacterial spore structures and their protective role in biocide resistance. J Appl Microbiol 2012 Sep 2012; 113(3):485-498.

Leisner JJ, Laursen BG, Prevost H, Drider D, Dalgaard P. *Carnobacterium*: positive and negative effects in the environment and in foods. FEMS Microbiol Rev 2007 Sep; 31(5):592-613.

Lenz CA, Reineke K, Knorr D, Vogel RF. High pressure thermal inactivation of *Clostridium botulinum* type E endospores - kinetic modeling and mechanistic insights. Frontiers in Microbiology 2015 Jul;6:652.

Lenz CA, Vogel RF. Effect of sporulation medium and its divalent cation content on the heat and high pressure resistance of *Clostridium botulinum* type E spores. Food Microbiol 2014 Dec; 44:156-167.

Lenz CA, Vogel RF. Differential effects of sporulation temperature on the high pressure resistance of *Clostridium botulinum* type E spores and the interconnection with sporulation medium cation contents. Food Microbiol 2015 Apr; 46:434-442.

Leyva-Illades JF, Setlow B, Sarker MR, Setlow P. Effect of a small, acid-soluble spore protein from *Clostridium perfringens* on the resistance properties of *Bacillus subtilis* spores. J Bacteriol 2007 Nov;189(21):7927-7931.

Liang W, Bi Y, Wang H, Dong S, Li K, Li J. Gene expression profiling of *Clostridium botulinum* under heat shock stress. BioMed Res. Int. 2013 Sep: 760904.

Lopez-Pedemonte TJ, Roig-Sagues AX, Trujillo AJ, Capellas M, Guamis B. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. J Dairy Sci 2003 Oct;86(10):3075-3081.

Loshon CA, Kraus P, Setlow B, Setlow P. Effects of inactivation or overexpression of the *ssp*F gene on properties of *Bacillus subtilis* spores. J Bacteriol 1997 Jan;179(1):272-275.

Lucke FK. Utilization of microbes to process and preserve meat. Meat Sci 2000 Oct; 56(2):105-115.

Ludwig H, van Almsick G, Schreck C. The effect of hydrostatic pressure on the survival of microorganisms. In: Taniguchi, Yoshihiro Stanley, Harry E. Ludwig,Horst, editor. Biological and Medical Physics Series 175 Fifth Avenue, New York, NY, 10010-7858, USA Heidelberger Platz 3, D-14197, Berlin, Germany: Springer-Verlag New York Inc., 175 Fifth Avenue, New York, NY, 10010-7858, USA Heidelberger Platz 3, D-14197, Berlin, Germany; 2002. p. 239-256.

Lund BM. Anaerobes in relation to foods of plant origin. In: Barnes EM, Mead GC, editors. Anaerobic Bacteria in Habitats Other Than Man. 13th ed. Oxford: Blackwell Scientific; 1986. p. 351-372.

Ma B, Zhang KZ, Hendrie C, Liang CZ, Li M, Doherty-Kirby A, et al. PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. Rapid Commun Mass Sp 2003 Sep; 17(20):2337-2342.

Mancini, RA and Hung MC. Current research in meat color. Meat Sci. 2005 Sep: 71(1): 100-121.

Margosch D, Ehrmann MA, Buckow R, Heinz V, Vogel RF, Gänzle MG. High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. Appl Environ Microbiol 2006 May;72(5):3476-3481.

Markland SM, Farkas DF, Kniel KE, Hoover DG. Pathogenic psychrotolerant sporeformers: An emerging challenge for low-temperature storage of minimally processed foods. Foodborne Pathog Dis 2013 May; 10(5):413-9.

Marshall KM, Nowaczyk L,II, Morrissey TR, Loeza V, Halik LA, Skinner GE, et al. Effect of sporulation temperature on the resistance of *Clostridium botulinum* Type A spores to thermal and high pressure processing. J Food Prot 2015 Jan;78(1):146-150.

Martin-Visccher LA, van Belkum MJ, Garneau-Tsodikova S, Whittal RM, Zheng J, McMullen LM, Vederas JC. Isolation and characterization of carnocyclin a, a novel circular bacteriocin, produced by *Carnobacterium maltaromaticum* UAL307. Appl Environ Microbiol 2008 Aug; 74(15):4756-4763.

Martin-Visscher LA, Yoganathan S, Sit CS, Lohans CT, Vederas JC. The activity of bacteriocins from *Carnobacterium maltaromaticum* UAL307 against Gram-negative bacteria in combination with EDTA treatment. FEMS Microbiol Lett 2011 Apr;317(2):152-159.

Mason JM, Setlow P. Different small, acid-soluble proteins of the alpha-beta-type have interchangeable roles in the heat and UV-radiation resistance of *Bacillus subtilis* spores. J Bacteriol 1987 Aug;169(8):3633-3637.

Mathys A, Reineke K, Heinz V, Knorr D. High pressure thermal sterilization - development and application of temperature controlled spore inactivation studies. High Pressure Research 2009 Apr;29(1):3-7.

Meaney CA, Cartman ST, McClure PJ, Minton NP. The role of small acid-soluble proteins (SASPs) in protection of spores of *Clostridium botulinum* against nitrous acid. Int J Food Microbiol 2016 Jan;216:25-30.

Mejlholm O, Boknaes N, Dalgaard P. Shelf life and safety aspects of chilled cooked and peeled shrimps (*Pandalus borealis*) in modified atmosphere packaging. J Appl Microbiol 2005 Mar; 99(1):66-76.

Moir A. How do spores germinate? J Appl Microbiol 2006 Sep;101(3):526-530.

Moschonas G, Bolton DJ, Sheridan JJ, McDowell DA. Isolation and sources of 'blown pack' spoilage clostridia in beef abattoirs. J Appl Microbiol 2009 Aug; 107(2):616-624.

Moschonas G, Bolton DJ, McDowell DA, Sheridan JJ. Diversity of culturable psychrophilic and psychrotrophic anaerobic bacteria isolated from beef abattoirs and their environments. Appl Environ Microbiol 2011 Jul;77(13):4280-4284.

Nakayama A, Yano Y, Kobayashi S, Ishikawa M, Sakai K. Comparison of pressure resistances of spores of six *Bacillus* strains with their heat resistances. Appl Environ Microbiol 1996 Oct;62(10):3897-3900.

Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H. Biosynthesis of bacteriocins in lactic acid bacteria. Anton Van Lee J M S 1996 Oct; 70(2-4):113-128.

Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol R 2000 Sep; 64(3):548-72.

Nicholson WL, Setlow B, Setlow P. Ultraviolet-irradiation of DNA complexed with alpha-beta-type small, acid soluble proteins from spores of *Bacillus* or *Clostridium* species makes spore photoproduct but not thymine dimers. Proc Natl Acad Sci USA 1991 Oct; 88(19):8288-8292.

Orsburn B, Melville SB, Popham DL. Factors contributing to heat resistance of *Clostridium perfringens* endospores. Appl Environ Microbiol 2008 Jun;74(11):3328-3335.

Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, et al. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res 2014 Jan 1;42(Database issue):D206-14.

Paidhungat M, Setlow B, Daniels WB, Hoover D, Papafragkou E, Setlow P. Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. Appl Environ Microbiol 2002 Jun; 68(6):3172-3175.

Paidhungat M, Setlow B, Driks A, Setlow P. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. J Bacteriol 2000 Oct;182(19):5505-5512.

Paredes-Sabja D, Raju D, Torres JA, Sarker MR. Role of small, acid-soluble spore proteins in the resistance of *Clostridium perfringens* spores to chemicals. Int J Food Microbiol 2008 Mar; 122(3):333-335.

Paredes-Sabja D, Setlow B, Setlow P, Sarker MR. Characterization of *Clostridium perfringens* spores that lack spoVA proteins and dipicolinic acid. J Bacteriol 2008 Jul;190(13):4648-4659.

Paredes-Sabja D, Sarker MR. Germination response of spores of the pathogenic bacterium *Clostridium perfringens* and *Clostridium difficile* to cultured human epithelial cells. Anaerobe 2011 Apr;17(2):78-84.

Paredes-Sabja D, Setlow P, Sarker MR. Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and proteins involved. Trends Microbiol 2011 Feb;19(2):85-94.

Parish JH. Studies in Microbiology Vol. 1. Developmental Biology of Prokaryotes. USA: University of California Press; 1979.

Park SW, Sohn KH, Shin JH, Lee HJ. High hydrostatic pressure inactivation of *Lactobacillus viridescens* and its effects on ultrastructure of cells. Int J Food Sci Tech 2001 Oct 2001; 36(7):775-781.

Peck MW, Stringer SC, Carter AT. *Clostridium botulinum* in the post-genomic era. Food Microbiol 2011 Apr;28(2):183-191.

Popham DL. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. Cellular and Molecular Life Sciences 2002 Mar; 59(3):426-433.

Popham DL, Sengupta S, Setlow P. Heat, hydrogen peroxide, and UV resistance of *Bacillus subtilis* spores with increased core water-content and with or without major DNA-binding proteins. Appl Environ Microbiol 1995 Oct;61(10):3633-3638.

Quadri LEN, Sailer M, Roy KL, Vederas JC, Stiles ME. Chemical and Genetic-Characterization of Bacteriocins Produced by *Carnobacterium piscicola* Lv17b. J Biol Chem 1994 Apr; 269(16):12204-12211.

Rajagopal S. Characterization of germination of *Clostridium estertheticum* spores. Edmonton, Alberta: University of Alberta; 2014.

Rajan S, Ahn J, Balasubramaniam VM, Yousef AE. Combined pressure-thermal inactivation kinetics of *Bacillus amyloliquefaciens* spores in egg patty mince. J Food Prot 2006 Apr;69(4):853-860.

Raju D, Setlow P, Sarker MR. Antisense-RNA-mediated decreased synthesis of small, acid-soluble spore proteins leads to decreased resistance of *Clostridium perfringens* spores to moist heat and UV radiation. Appl Environ Microbiol 2007 Apr;73(7):2048-2053.

Raju D, Sarker MR. Production of small, acid-soluble spore proteins in *Clostridium perfringens* nonfoodborne gastrointestinal disease isolates. Can J Microbiol 2007 Apr;53(4):514-518.

Raju D, Waters M, Setlow P, Sarker MR. Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. BMC Microbiology 2006 Jun; 6:1-13.

Reddy NR, Tetzloff RC, Solomon HM, Larkin JW. Inactivation of *Clostridium botulinum* nonproteolytic type B spores by high pressure processing at moderate to elevated high temperatures. Innov Food Sci Emerg Tech 2006 Sep;7(3):169-175.

Reineke K, Doehner I, Schlumbach K, Baier D, Mathys A, Knorr D. The different pathways of spore germination and inactivation in dependence of pressure and temperature. Innov Food Sci Emerg Tech 2012 Jan; 13:31-41.

Reineke K, Ellinger N, Berger D, Baier D, Mathys A, Setlow P, et al. Structural analysis of high pressure treated *Bacillus subtilis* spores. Innov Food Sci Emerg Tech 2013 Jan;17:43-53.

Reineke K, Mathys A, Knorr D. The impact of high pressure and temperature on bacterial spores: Inactivation mechanisms of *Bacillus subtilis* above 500 MPa. J Food Sci 2011 Apr;76(3):M189-M197.

Reineke K, Mathys A, Heinz V, Knorr D. Mechanisms of endospore inactivation under high pressure. Trends Microbiol 2013 Jun;21(6):296-304.

Riemann H, Ordal ZJ. Germination of bacterial endospores with calcium and dipicolinic acid. Science 1961 May; 133(346):1703-1704.

Ritz M, Tholozan JL, Federighi M, Pilet MF. Morphological and physiological characterization of *Listeria monocytogenes* subjected to high hydrostatic pressure. Appl Environ Microbiol 2001 May; 67(5):2240-2247.

Roberts CM, Hoover DG. Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity and nisin. J Appl Bacteriol 1996 Oct;81(4):363-368.

Rode LJ, Foster JW. The action of surfactants on bacterial spores. Arch Microbiol 1960 Mar; 36(1):67-94.

Rodgers S. Preserving non-fermented refrigerated foods with microbial cultures - a review. Trends Food Sci Technol 2001 Aug; 12(8):276-284.

Rodgers S, Kailasapathy K, Cox J, Peiris P. Co-incubation of *Clostridium botulinum* with protective cultures. Food Res Int 2004 Mar; 37(7):659-666.

Rose NL, Palcic MM, Sporns P, McMullen LM. Nisin: A novel substrate for glutathione S-transferase isolated from fresh beef. J Food Sci 2002 Aug; 67(6):2288-2293.

Ross HE. *Clostridium putrefaciens* - a Neglected Anaerobe. J Appl Bacteriol 1965 Apr; 28(1):49-51.

Sacchettini T. WebTB - TIGR function roles of ORFs. 2016; Available at: http://webtb.org/GenomeBrowser/tigr_roles.php. Accessed January 30, 2016.

Salas-Pacheco J, Setlow B, Setlow P, Pedraza-Reyes M. Role of the Nfo (YqfS) and ExoA apurinic/apyrimidinic endonucleases in protecting *Bacillus subtilis* spores from DNA damage. J Bacteriol 2005 Nov;187(21):7374-7381.

Saleh MA, Ordal ZJ. Studies on growth and toxin production of *Clostridium botulinum* in a precooked frozen food. Food research 2006 Aug;20:332-339.

San Martin M, Barbosa-Canovas G, Swanson B. Food processing by high hydrostatic pressure. Crit Rev Food Sci Nutr 2002 Jun; 42(6):627-645.

Schillinger U, Geisen R, Holzapfel WH. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. Trends Food Sci Technol 1996 May; 7(5):158-164.

Schnabel J, Lenz CA, Vogel RF. High pressure inactivation of *Clostridium botulinum* type E endospores in model emulsion systems. High Pressure Research 2015 Jan;35(1):78-88.

Setlow B, Setlow P. Thymine-containing dimers as well as spore photoproducts are found in ultraviolet-irradiated *Bacillus subtilis* spores that lack small acid-soluble proteins. Proc Natl Acad Sci USA 1987 Jan; 84(2):421-423.

Setlow B, Setlow P. Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen-peroxide. Appl Environ Microbiol 1993 Oct;59(10):3418-3423.

Setlow B, Setlow P. Heat inactivation of *Bacillus subtilis* spores lacking small, acid-soluble spore proteins is accompanied by generation of abasic sites in spore DNA. J Bacteriol 1994 Apr;176(7):2111-2113.

Setlow P. Germination and outgrowth. In: Hurst A, Gould GW, editors. The Bacterial Spore. Vol. II ed. London: Academic Press; 1983. p. 211-254.

Setlow P. Small, acid soluble spore proteins of *Bacillus* species - Structure, synthesis, genetics, function, and degradation. Annu Rev Microbiol 1988 Oct; 42:319-338.

Setlow P. DNA in dormant spores of *Bacillus* species is in an A-like conformation. Mol Microbiol 1992 Mar;6(5):563-567.

Setlow P. I Will Survive - Protecting and repairing spore DNA. J Bacteriol 1992 May; 174(9):2737-2741.

Setlow P. Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. J Appl Bacteriol 1994 Jun; 76:S49-S60.

Setlow P. Resistance of spores of *Bacillus* species to ultraviolet light. Environ Mol Mutagen 2001;38(2-3):97-104.

Setlow P. Spore germination. Curr Opin Microbiol 2003 Dec;6(6):550-556.

Setlow P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol 2006 Sep; 101(3):514-525.

Setlow P. I will survive: DNA protection in bacterial spores. Trends Microbiol 2007 Apr; 15(4):172-180.

Setlow P. Dormant spores receive an unexpected wake-up call. Cell 2008 Oct 31;135(3):410-412.

Setlow P. Summer meeting 2013-when the sleepers wake: the germination of spores of *Bacillus* species. J Appl Microbiol 2013 Dec; 115(6):1251-1268.

Setlow B, McGinnis KA, Ragkousi K, Setlow P. Effects of major spore-specific DNA binding proteins on *Bacillus subtilis* sporulation and spore properties. J Bacteriol 2000 Dec; 182(24):6906-6912.

Shah IM, Laaberki M, Popham DL, Dworkin J. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell 2008 Oct;135(3):486-496.

Shay B, Egan A. The packaging of chilled red meats. Food Technology in Australia 1987 Jun; 39(6):283-285.

Shimada S, Andou M, Naito N, Yamada N, Osumi M, Hayashi R. Effects of Hydrostatic-pressure on the ultrastructure and leakage of internal substances in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 1993 Oct; 40(1):123-131.

Silva AR, Tahara ACC, Chaves RD, Sant'Ana AS, Faria JdAF, Massaguer PR. Influence of different shrinking temperatures and vacuum conditions on the ability of psychrotrophic *Clostridium* to cause 'blown pack' spoilage in chilled vacuum-packaged beef. Meat Sci 2012 Dec; 92(4):498-505.

Silva AR, Paulo EN, Sant'Ana AS, Chaves RD, Massaguer PR. Involvement of *Clostridium gasigenes* and *C. algidicarnis* in 'blown pack' spoilage of Brazilian vacuum-packed beef. Int J Food Microbiol 2011 Aug 15;148(3):156-163.

Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, Birol I. ABySS: a parallel assembler for short read sequence data. Genome Res 2009 Jun;19(6):1117-1123.

Sip A, Grajek W, Boyaval P. Enhancement of bacteriocin production by *Carnobacterium divergens* AS7 in the presence of a bacteriocin-sensitive strain *Carnobacterium piscicola*. Int J Food Microbiol 1998 Jun; 42(1-2):63-69.

Skinner GE, Solomon HM, Fingerhut GA. Prevention of *Clostridium botulinum* type A, proteolytic B and E toxin formation in refrigerated pea soup by *Lactobacillus plantarum* ATCC 8014. J Food Sci 1999 Aug; 64(4):724-727.

Smith GC, Seidman SC, Savell JW, Dill CW, Vanderzant CV. Vacuum packaging versus modified atmosphere packaging of lamb loins. J Food Prot 1982 Jan;46(4):47-51.

Smith LD. Inhibition of *Clostridium botulinum* by strains of *Clostridium perfringens* isolated from soil. Appl Microbiol 1975 Aug; 30(2):319-323.

Stringer SC, Fairbairn DA, Peck MW. Combining heat treatment and subsequent incubation temperature to prevent growth from spores of non-proteolytic *Clostridium botulinum*. J Appl Microbiol 1997 Jan;82(1):126-136.

Sturges WS, Drake ET. A complete description of *Clostridium putrefaiens*. J Bacteriol 1927 Sep;14(3):175-179.

Sunde EP, Setlow P, Hederstedt L, Halle B. The physical state of water in bacterial spores. Proc Natl Acad Sci U S A 2009 Nov ;106(46):19334-19339.

Suzuki M, Yamamoto T, Kawai Y, Inoue N, Yamazaki K. Mode of action of piscicocin CS526 produced by *Carnobacterium piscicola* CS526. J Appl Microbiol 2005 Feb; 98(5):1146-1151.

Tagg JR, Wannamaker LW. Streptococcin-A-Ff22 - nisin-like antibiotic substance produced by a Group-A-*Streptococcus*. Antimicrobial Agents Ch 1978 Jul;14(1):31-39.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 2013 Dec 2013;30(12):2725-2729.

Tanaka N, Traisman E, Lee MH, Cassens RG, Foster EM. Inhibition of botulinum toxin formation in bacon by acid development. J Food Prot 1980 Jun; 43(6):450-457.

Tennen R, Setlow B, Davis KL, Loshon CA, Setlow P. Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. J Appl Microbiol 2000 Aug;89(2):330-338.

Tintchev F, Bindrich U, Toepfl S, Strijowski U, Heinz V, Knorr D. High hydrostatic pressure/temperature modeling of frankfurter batters. Meat Sci 2013 Jul; 94(3):376-387.

Toepfl S, Mathys A, Heinz V, Knorr D. Review: Potential of high hydrostatic pressure and pulsed electric fields for energy efficient and environmentally friendly food processing. Food Rev Int 2006 Oct; 22(4):405-423.

Torres JA, Velazquez G. Commercial opportunities and research challenges in the high pressure processing of foods. J Food Eng 2005 Mar; 67(1-2):95-112.

Vercammen A, Vanoirbeek KGA, Lemmens L, Lurquin I, Hendrickx MEG, Michiels CW. High pressure pasteurization of apple pieces in syrup: Microbiological shelf-life and quality evolution during refrigerated storage. Innov Food Sci Emerg 2012 Oct; 16:259-266.

Wang S, Setlow B, Conlon E, Lyon J, Imamura D, Sato T, et al. The forespore line of gene expression in *Bacillus subtilis*. J Mol Biol 2006 Apr 21;358(1):16-37.

Wuytack EY, Boven S, Michiels CW. Comparative study of pressure-induced germination of Bacillus subtilis spores at low and high pressures. Appl Environ Microbiol 1998 Sep; 64(9):3220-3224.

Wuytack E, Boven S, Michiels C. Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. Appl Environ Microbiol 1998 Sep;64(9):3220-3224.

Wuytack EY, Soons J, Poschet F, Michiels CW. Comparative study of pressure- and nutrient-induced germination of Bacillus subtilis spores. Appl Environ Microbiol 2000 Jan; 66(1):257-261

Yang X, Balamurugan S, Gill CO. Substrate utilization by *Clostridium estertheticum* cultivated in meat juice medium. Int J Food Microbiol 2009 Jan; 128(3):501-505.

Yang X, Balamurugan S, Gill CO. Effects on the development of blown pack spoilage of the initial numbers of *Clostridium estertheticum* spores and *Leuconostoc mesenteroides* on vacuum packed beef. Meat Sci 2011 Jul; 88(3):361-367.

Yang X, Gill CO, Balamurugan S. Products of glucose and lactate fermentation, and utilization of amino acids by *Clostridium estertheticum* subspp. *laramiense* and estertheticum growing in meat juice medium. J Food Prot 2010 Jul; 73(7):1348-1352.

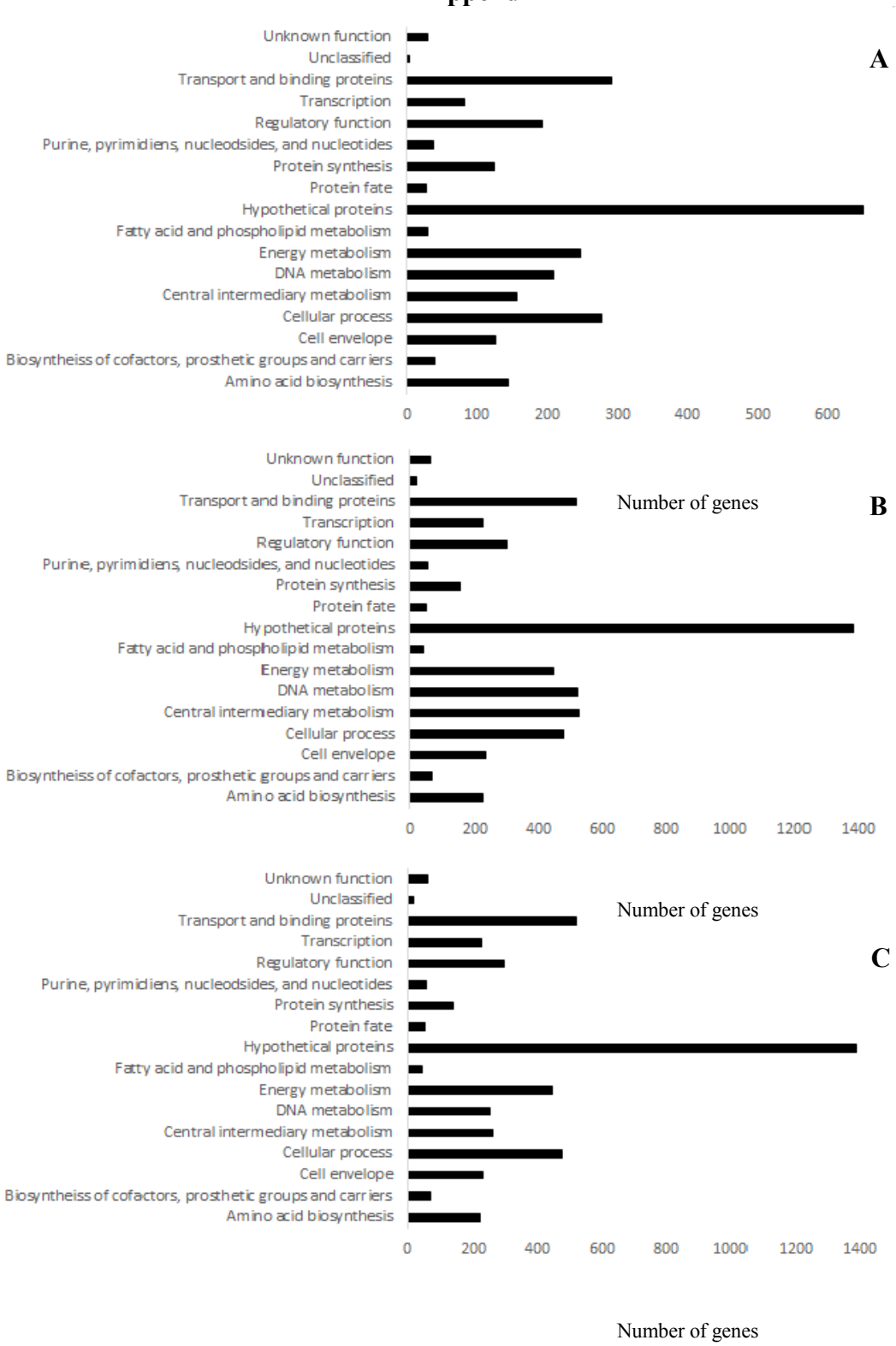
Yang X, Wang H, Badoni M. Effects of meat pH and the initial numbers of spores of *Clostridium estertheticum* on the development of blown pack spoilage of vacuum-packaged beef. Int J Food Sci Tech 2014 Jul 2014;49(7):1619-1625.

Yoon Y, Lee H, Lee S, Kim S, Choi K. Membrane fluidity-related adaptive response mechanisms of foodborne bacterial pathogens under environmental stresses. Food Res Int 2015 Jun 2015;72:25-36.

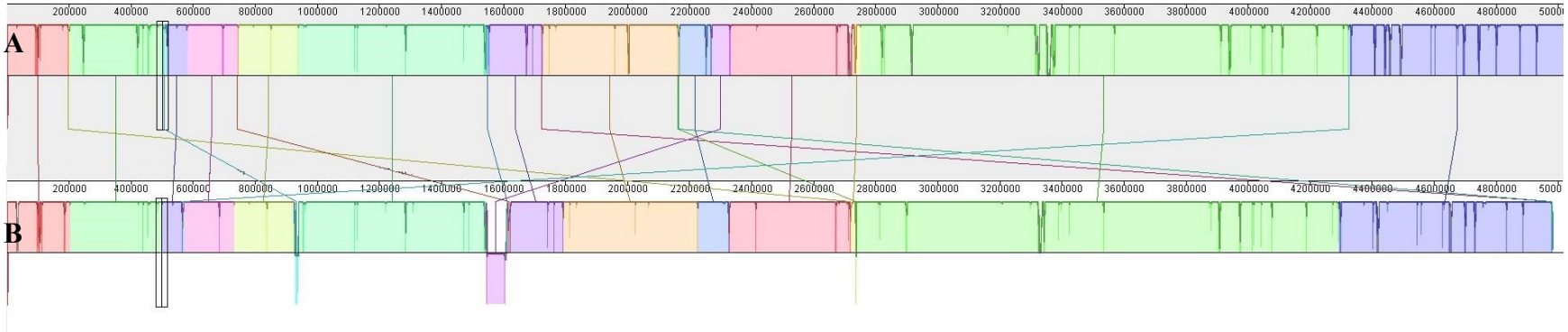
Yuste J, Mor-Mur M, Capellas M, Guamis B, Pla R. Microbiological quality of mechanically recovered poultry meat treated with high hydrostatic pressure and nisin. Food Microbiol 1998 Aug; 15(4):407-414.

Zheng H, Wu H. Gene-centeric association analysis for the correlation between the guanine-cytogsine content levels and temperature range conditions of prokaryotic species. BMC Bioinformatics 2010 Dec; 11(Suppl 11):S7.

Appendix



Appendix 1 Categorization of genes from whole genome sequences of*Cl. putrefaciens* (A), *Cl. estertheticum* BP09-01 (B), and *Cl. estertheticum* BP09-13. Classification scheme is summarized based on TIGR categories (186).Note that the scale for A is different from B and C.



Appendix 2 MAUVE alignment of *Cl. estertheticum* BP09-01 (A) and *Cl. estertheticum* BP09-13 (B).

Appendix 3 Organisms used for the phylogenetic tree analysis based on 88 16s rRNA sequences.

Accession Numbers	Strains
CP002410	*Clostridium botulinum* Group III BKT015925
NC 964	*Bacillus subtilis* subsp. subtilis str 168
NC 9495	*Clostridium botulinum* Group I ATCC3502
NC 10674	*Clostridium botulinum* Group II B str Eklund 17B
S000000413	*Clostridium tertium* (T) DSM 2485 Y18174
S000001564	*Clostridium aurantibutyricum* (T) NCIMB10659 X68183
S000002328	*Clostridium psychrophilum* (T) A-1/C-an/I AJ297443
S000003885	*Clostridium pascui* (T) DSM 10365 (cm19) X96736
S000004297	*Clostridium sartagoforme* (T) DSM 1292 Y18175
S000004716	*Clostridium acetireducens* (T) 30A X79862
S000004827	*Clostridium haemolyticum* (T) ATCC9650 AB037910
S000005084	*Clostridium cellulovorans* (T) DSM 3052 X71849
S000005089	*Clostridium drakei* (T) type strain: SL1 Y18813
S000007751	*Clostridium thermobutyricum* (T) X72868
S000008489	*Clostridium thermopalmarium* (T) X72869
S000009214	*Clostridium chartatabidum* (T) DSM 5482 X71850
S000009597	*Clostridium baratii* (T) ATCC27638 X68174
S000014235	*Clostridium vincentii* (T) X97432
S000014607	*Clostridium beijerinckii* (T) DSM791 X68179
S000015682	*Clostridium disporicum* (T) DSM 5521 Y18176
S000016169	*Clostridium novyi* (T) JCM1406 AB045606
S000016392	*Clostridium grantii* (T) A1 X75272
S000021696	*Clostridium puniceum* (T) DSM 2619 X71857
S000022312	*Clostridium roseum* (T) ATCC 17797 Y18171
S000022469	*Clostridium cylindrosporum* (T) DSM 605 Y18179
S000115736	*Clostridium diolis* (T) DSM 5431 AJ458418
S000116309	*Clostridium butyricum* (T) VPI3266 AJ458420
S000127491	*Clostridium akagii* (T) CK58 AJ237755
S000128907	*Clostridium paraputrificum* (T) ATCC 25780 X75907
S000130726	*Clostridium acidisoli* (T) CK74 AJ237756
S000131270	*Clostridium uliginosum* (T) CK55 AJ276992
S000137817	*Clostridium bowmanii* (T) type strain: DSM 14206 11 AJ506119
S000138148	*Clostridium frigoris* (T) type strain: DSM 14204 5 AJ506116
S000138431	*Clostridium lacusfryxellense* (T) type strain: DSM 14205 AJ506118
S000260209	*Clostridium putrificum* (T) DSM 1734 X73442

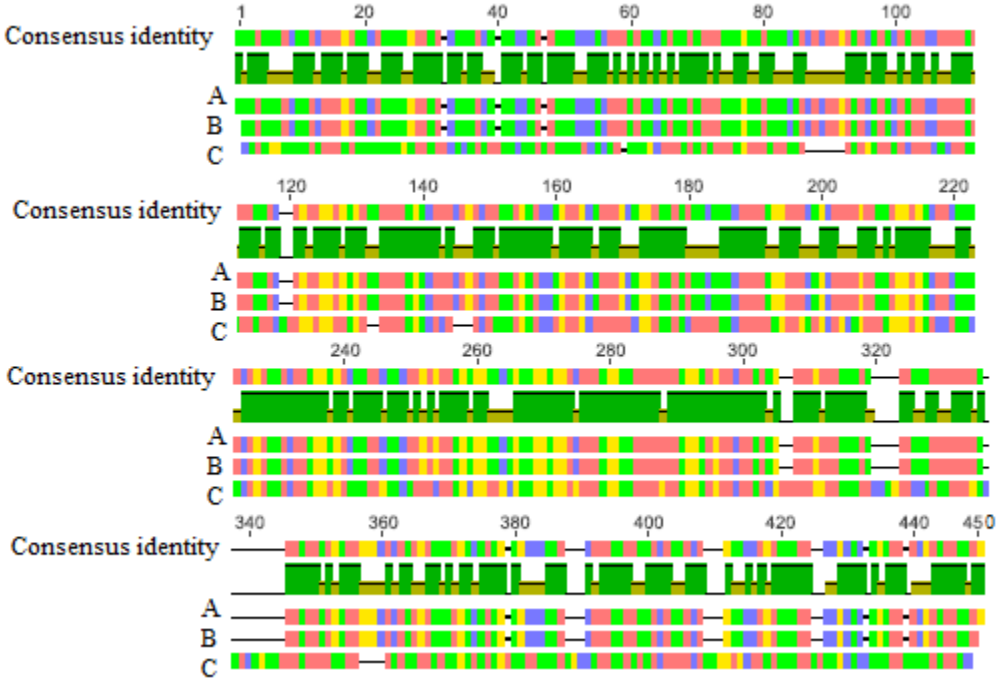
Appendix 3 continued.

S000260249	*Clostridium homopropionicum* (T) type strain: DSM5847 X76744
S000260250	*Clostridium quinii* (T) type strain: DSM6736 X76745
S000260369	*Clostridium intestinale* (T) type strain: DSM 6191 X76740
S000260454	*Clostridium collagenovorans* (T) DSM 3089 X73439
S000260489	*Clostridium absonum* (T) DSM 599 X77842
S000260539	*Clostridium sporogenes* (T) ATCC3584 X68189
S000260571	*Clostridium proteolyticum* (T) DSM 3090 X73448
S000260680	*Clostridium argentinense* (T) ATCC 27322 X68316
S000260778	*Clostridium tetani* (T) NCTC 279 X74770
S000260781	*Clostridium celatum* (T) DSM 1785 X77844
S000366397	*Clostridium colicanis* (T) 13634 AJ420008
S000387304	*Clostridium algidicarnis* (T) NCFB 2931 AF127023
S000387305	*Clostridium putrefaciens* (T) DSM 1291 AF127024
S000388299	*Clostridium* peptidivorans (T) TMC4 DSM 12505 AF156796
S000389872	*Clostridium subterminale* (T) DSM 6970 AF241844
S000390414	*Clostridium felsineum* (T) NCIMB 10690 AF270501
S000396287	*Clostridium algoriphilum* (T) 14D1 AY117755
S000428393	*Clostridium frigidicarnis* (T) SPL77A AF069742
S000428722	*Clostridium gasigenes* (T) DSM 12272 AF092548
S000434520	*Clostridium thiosulfatireducens* (T) LUP 21 AY024332
S000437203	*Clostridium saccharoperbutylacetonicum* (T) N1-4 U16122
S000437206	*Clostridium saccharobutylicum* (T) P262 U16147
S000437764	*Clostridium chauvoei* (T) ATCC 10092T U51843
S000437948	*Clostridium septicum* (T) ATCC 12464T U59278
S000539075	*Clostridium sardiniense* (T) DSM 2632 AB161367
S000576711	*Clostridium lundense* (T) DSM 17049 AY858804
S000608913	*Clostridium schirmacherense* (T) type strain: AP15 AM114453
S000620000	*Clostridium tetanomorphum* (T) DSM 4474 DQ241819
S000625977	*Clostridium tagluense* (T) A121 DQ296031
S000690535	*Clostridium nitrophenolicum* (T) type strain:1D AM261414
S000701420	*Clostridium aciditolerans* (T) JW/YJL-B3 DQ114945
S000805416	*Clostridium tepidiprofundi* (T) SG 508 EF197795
S000805548	*Clostridium sulfidigenes* (T) SGB2 EF199998
S000903067	*Clostridium amylolyticum* (T) SW408 EU037903
S001152393	*Clostridium cavendishii* (T) BL-28 DQ196621
S001152395	*Clostridium hydrogeniformans* (T) BL-20 DQ196623

Appendix 3 continued

S001153886	*Clostridium arbusti* (T) SL206 EU816420
S001792892	*Clostridium pasteurianum* (T) JCM 1408 AB536773

S001872426	*Clostridium cadaveris* (T) JCM 1392 AB542932
S002150813	*Clostridium histolyticum* (T) JCM 1403 AB566416
S002289858	*Clostridium perfringens* (T) ATCC 13124 CP000246
S002289912	*Clostridium kluyveri* (T) DSM 555 CP000673
S002350756	*Clostridium malenominatum* (T) type strain: DSM 1127 FR749893
S002350786	*Clostridium oceanicum* (T) type strain: DSM 1290 FR749923
S002761931	*Clostridium limosum* (T) type strain: CECT 4329 FR870444
S003610434	*Clostridium ganghwense* (T) HY-42-06 AY903294
S003610724	*Clostridium aestuarii* (T) HY-45-18 DQ126679
S004056798	*Clostridium fallax* (T) ATCC 19400 M59088
S004056800	*Clostridium carnis* (T) ATCC 25777 M59091
S004056801	*Clostridium cochlearium* (T) ATCC 17787 M59093
S004056802	*Clostridium tyrobutyricum* (T) ATCC 25755 M59113
S004056880	*Clostridium estertheticum* (T) NCIBM 12511 S46734
S004068337	*Clostridium acetobutylicum* (T) ATCC 824 AE001437
X68316	*Clostridium botulinum* Group IV ATCC27322



Appendix 4

Alignment of SASPs on a nucleotide basis of *Cl. estertheticum* BP09-01 (A), *Cl. estertheticum* BP09-13 (B) with *ssp*2 of *Cl. perfringens* (C). Similarities are described by the consensus identity. All similar nucleotides are indicated by the green blocks, dissimilar nucleotides are indicated by the gaps.