"If we knew what it was we were doing, it would not be called research, would it?"

-- Albert Einstein

University of Alberta

Characterization of *Clostridium* spp. from "blown-pack", chill-stored, vacuum packaged beef

by

Linda Ho

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the degree of

Master of Science
in
Food Science and Technology

Agricultural, Food and Nutritional Science

Fall 2009
Edmonton, Alberta

ISBN: 978-0-494-52398-8

Examining Committee

Lynn M. McMullen, Agricultural, Food and Nutritional Science

Michael G. Gänzle, Agricultural, Food and Nutritional Science

Phillip M. Fedorak, Biological Sciences

Abstract

The objectives of this study were to determine the microbial ecology of "blown-pack" fresh beef obtained from a federally inspected facility and to use biopreservation to prevent spoilage of vacuum packaged chilled beef. Organic acids and alcohols in the purge obtained from the "blown" beef packages were detected using HPLC. PCR analysis indicated that *Clostridium* spp. were present in the purge of the commercial sample. Biochemical tests, RFLP and 16S rDNA sequencing were used to identify organisms isolated from the meat. Out of 66 isolated strains, 26 isolates were strict anaerobes and RFLP indicated that all were clonal isolates. Based on sequence analysis, the isolate was identified as *Clostridium putrefaciens*. The isolate caused "blown-pack" spoilage and produced butyric and propionic acids when inoculated onto fresh meat and *Carnobacterium maltaromaticum* UAL307 prevented the production gas and obvious signs of spoilage.

Acknowledgements

This project was supported by funding from NSERC and the Value Added Meat Program.

I would like to thank Lena, Emefa, Ken, Kamila and James for all the help provided in the lab and with my experiments.

Thank you to the inhabitants of 2-50. Thank you for listening to me talk about nothing and everything; and for all the great times. Here's to more!

I would also like to thank my supervisor and supervisory committee. Thank you to Dr. Michael Gänzle for being a sounding board to bounce ideas off of.

Thank you to Dr. Lynn McMullen for giving me a chance and taking me on as a student and for letting me loose in the lab to find my own way. Thank you for being so understanding when mistakes were made, for providing a critical eye in the kindest way possible and for providing guidance and nurturing the scientist in me.

Lastly, I would like to thank my family and Rich for their love and support through this endeavour. Thank you for the patience and love; for the understanding and comfort when things were not going right. Thank you for loving me when I wasn't my best and being proud of me regardless of how I felt. Thank you, mom and dad, for being great role models for Nancy and me. We learned that education is something that nobody could ever take from us.

Without all of you, I would not be who I am and for that I am forever grateful.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

°C: degree Celsius

µL: microlitre

16S rDNA: 16S ribosomal deoxyribonucleic acid

AAFC: Agriculture and Agri-Food Canada

ATCC: American Type Culture Collection

ATP: adenosine triphosphate

BoTN: Botulinal toxin

bp: base pairs

BP isolate: “blown-pack” isolate

c.: approximately

CBA: Columbia blood agar

cc: cubic centimetre

CFU: colony forming units

cm^2: centimetre squared

d: day

DNA: Deoxyribonucleic acid

DPA: dipicolinic acid

HPLC: high performance liquid chromatography

h: hour

ITS: internal transcribed region

kg: kilogram

kGy: kiloGray

LAB: lactic acid bacteria

MAL: muramic acid-δ-lactam

m: metre

mg: milligram

min: minutes

mL: millilitre

mM: millimolar

mm: millimetre

MRS: de Man, Rogosa Sharpe

NCBI: National Centre for Biotechnology Information

NR: Not reported

ND: not detected

nm: nanometre

PCA: plate count agar

PCR: polymerase chain reaction

POAA: peroxyacetic acid

PYGS: peptone-yeast-broth

RAPD: random amplified polymorphic DNA

RCM: reinforced Clostridial medium

RFLP: restriction fragment length polymorphism

r.h.: relative humidity

RNA: ribonucleic acid

SAS: Statistical Analysis Software

SASP: small acid soluble protein

SFP: Shahidi Ferguson Perfringens

Spl: endospore photoproduct lyase

SpoA: Surface presentation of antigens

spp.: species

STAA: Streptomycin Sulphate-Thallous Acetate-Acitidone

UAL: University of Alberta lactics

UV: ultraviolet

VRBG: violet red bile glucose

x *g*: Centrifugal force

1. Introduction

Packaging technology, combined with refrigerated storage, extends the storage life of fresh meat. Most spoilage organisms found on fresh meat grow aerobically and have the ability to grow at refrigeration conditions. The combination of vacuum packaging and chill storage modifies the environment so that it is unsuitable for the growth of "typical" spoilage microorganisms, thereby extending the storage life of the food product. Spoilage microorganisms include *Enterobacteriaceae*, *Brochothrix* spp. and lactic acid bacteria (LAB). Spoilage that typically occurs with fresh meat stored under low temperature and O_2-deprived conditions is primarily caused by the growth and metabolism of LAB. However, there have been reported incidences of "blown-pack" spoilage that are uncharacteristic of LAB. In these incidences, *Clostridium* spp. are known to have been implicated as the primary cause of "blown-pack" spoilage because of the ability to produce gas when they grown on vacuum packaged meat.

1.1 Characteristics and Causes of "Blown-pack" Spoilage

"Blown-pack" spoilage is characterised by distension of the package (due to gas production by the microorganism) and production of off odours within four to six weeks at refrigeration storage (-1.5 ºC to 2 ºC; Broda *et al.*, 2000a). Some of the off odours are due to the production of volatile compounds such as 1-butanol, butyl butyrate, butyl acetate and butyric and acetic acids (Broda *et al*., 1996). Different volatile profiles can be obtained from different types of meat. Different types of spoilage microorganisms will also produce different volatile profiles.

Although O_2-deprivation and low temperatures inhibit the growth of most spoilage microorganisms, there are still bacteria that will grow under these conditions. Psychrophilic *Clostridium* species have been implicated in "blown-pack" spoilage with two specific species, *Clostridium estertheticum* (Collins *et al*., 1992) and *Clostridium laramiense* (Kalchayanand *et al*., 1989), confirmed as causative agents. Not all incidences of "blown-pack" spoilage are due the presence and growth of these two species of clostridia. Eight other psychrotolerant *Clostridium* spp. have been isolated and associated with "blown-pack" meat products in New Zealand (Broda *et al.*, 1996a). *Clostridium* spp. are most likely to be found in soil, animal feed and on processing plant surfaces (Lund, 1986). Any surface contamination of a carcass could occur during dressing and evisceration (Broda *et al*., 2000; Boerema *et al.*, 2002). Dressing includes the removal of the head, hide, and fore and hind feet. Soil is most likely present on the hide and feet. The feet are removed to prevent the possibility of manure and dirt dropping from the hooves and contaminating the carcass. During evisceration, the carcass is opened from top to bottom to remove the internal organs (Purnell and Loeffen, 2006). Opening of the carcass leaves it vulnerable to contamination from processing plant surfaces and from the stomach and intestines. The stomach and intestines are emptied of manure and cleaned for further processing. If these organs are punctured during evisceration, further contamination of the carcass can occur. *Clostridium estertheticum*, a microorganism implicated in "blown-pack" spoilage, has been isolated from the gastrointestinal tract of farm animals (Broda *et al.*, 2002). What remains of the

carcass is rinsed using high pressure water or steam which facilitates the removal of most but not all the endospores. In some cases, chemical decontaminants (ex. lactic acid or inorganic acid washes) are used to further decrease numbers of bacteria on the carcass. Endospores of *Clostridium* spp. are resistant to most chemical decontaminants (ex. peroxyacetic acid) treatments used in slaughterhouses. Another process that increases the likelihood of the occurrence of "blown-pack" spoilage is post packaging heat shrink treatments (Bell *et al.*, 2001). Heat shrinkage is used to improve the appearance of the package meat but also prevents drip loss. When the package undergoes heat shrinkage, the package is more closely applied to the meat and therefore creating an anaerobic environment. As well, the heat applied during heat shrinkage may be enough to activate or induce germination of the endospores (Bell *et al.*, 2001). The climate of the countries in which the animal is raised and slaughtered may play a role in the psychrotrophic *Clostridium* spp. isolated. In more temperate climates, psychrotolerant species will dominate over mesophilic species due to the adaptation to lower temperatures (Lund, 1986). To prevent the occurrence of "blown-pack" spoilage, *Clostridium* spp. should be isolated and characterised in order to determine proper interventions.

Clostridium spp. are Gram positive, rod shaped, obligate anaerobes that have the ability to produce endospores (Cato *et al*., 1986). Isolation of psychrophilic *Clostridium* spp. from raw meat involves the use of specialised media, anaerobic conditions, and temperatures between 10 and 21 °C (Dainty *et al.*, 1989;

Kalchayanand *et al.*, 1989). Specialised media includes peptone-yeast extract-glucose-starch (PYGS) broth (Lund *et al.*, 1990), Columbia Blood Agar (CBA), and Reinforced Clostridial Medium (RCM; Broda *et al.*, 1996) which are selected because they are highly nutritive media. Anaerobic conditions can be achieved through the use of anaerobic chambers and gas packs. Isolation should be done at approximately 15 ºC to minimise the stress on the psychrotrophic microorganisms. Depending on the strain and the species, it may take as long as 7 d for colonies to appear on the surface of the agar (Broda *et al.*, 1996). The meat may also be screened for the presence of other microorganisms, such as *Enterobacteriaceae*, LAB, and *Brochothrix thermosphacta* (Broda *et al*., 1996). Screening of microorganisms provides composition of the microbial community and may offer some insights on interactions between the microorganisms. Antagonists or synergistic effects due to the presence of a consortium of microorganisms may be studied.

A few microorganisms that have been implicated in "blown-pack" spoilage are described. One of the first "blown-pack" spoilage microorganisms isolated and described was *Clostridium estertheticum* (Collins *et al.*, 1992). Other "blown-pack" spoilage microorganisms that have been isolated include *Clostridium estertheticum* subsp. Laramiense (formally *Clostridium laramie*), *Clostridium frigidicarnis* (Spring *et al.*, 2003) and most recently *Clostridium gasigenes*. Phenotypic and genotypic characterisation of these microorganisms is complete (Spring *et al.*, 2003). Phenotypic characterisation includes morphology, ultra

structure, Gram reaction, physiological tests, biochemical tests and detection of botulinal neurotoxin (Broda *et al.*, 1999).

One particular strain of *Cl. frigidicarnis* grows on CBA, containing 5% sheep blood. These colonies are circular to irregular in shape with undulate to irregular margins, creamy grey in colour, raised, low convex, shiny and opaque (Broda *et al.*, 1999). Subterminal endospores were also observed. The rod shaped cells were motile with three to seven flagella per cell. *Cl. frigidicarnis* has a wide temperature range for growth. The temperature range was 3.8 to 40.5 ºC (Broda *et al.*, 1999). Since *Cl. frigidicarnis* has the ability to grow at 4 ºC, it can be classified as psychrotolerant. Although the optimal pH for growth is around neutral, these microorganisms have the ability to grow at pH levels of 4.5 to 9.2. The ultimate pH of meat is typically 5.8 to 6.3 (to minimize most bacterial growth and maintain quality), which allows these microorganisms to grow.

Comparing *Clostridium* spp. that cause "blown-pack" spoilage to other psychrotolerant *Clostridium* spp., distinct differences in metabolism, enzymatic activity and optimal growth temperatures are observed (Table 1.1). *Clostridium putrefaciens* and *Clostridium algidicarnis* are both psychrotolerant microorganisms isolated from spoiled vacuum packed meat. Compared with other isolated "blown-pack" *Clostridium* spp., the morphology, ultra structure, and physiological tests varied. Even within "blown-pack" *Clostridium* spp., the physiological tests were different. One noticeable difference was that the "blown-

pack" *Clostridium* spp. have the ability to ferment more carbohydrates than other psychrotrophic spoilage *Clostridium* spp. (Broda *et al*., 1996).

Table 1.1: Some phenotypic characteristics of some clostridia

Phenotypic properties	**1***	**2§**	**3¶**	**4†**	**5‡**
Maximum growth temperature (ºC)	15	20	40.5	<37	37
Optimum growth temperature (ºC)	10-12	15	30-38.5	15-22	25-30
Gelatin hydrolysis	NR	-	+	-	-
Meat digestion	NR	+	+	-	-
Lipase activity	NR	+	-	-	NR
Lecithinase activity	NR	-	+	-	-
Starch hydrolysis	+	+	-	-	-
Fermentation of:					
Cellobiose	+	-	-	-	-
Galactose	+	+	-	-	NR
Lactose	NR	-	-	-	-
Maltose	+	+	+	-	-
Mannitol	+	+	-	-	-
Sorbitol	+	NR	+	-	-
Trehalose	NR	-	+	-	-
Xylose	+	-	-	-	+

(**1**) *Cl. estertheticum* *from Collins *et al.* (1992); (**2**) *Cl. estertheticum* subsp. Laramiense §from Kalchayanand *et al.* (1993); (**3**) *Cl. frigidicarnis* ¶from Broda *et al.* (1999); (**4**) *Cl. putrefaciens* †from Cato *et al.* (1986); (**5**) *Cl. algidicarnis* ‡ from Lawson *et al.* (1994)
NR, not reported

Beta-haemolysis on blood agar is also indicative of clostridia species. Beta-haemolysis occurs when there is complete lysis of red blood cells. In blood agar, lysis is observed when areas in and around the colonies are lightened and transparent. *Cl. putrefaciens* have the ability to induce beta-haemolysis on blood agar, but *Cl. algidicarnis* does not (Cato *et al.*, 1986). Not all *Clostridium* spp. will induce beta-haemolysis on blood agar. *Clostridium perfringens*, a foodborne pathogen, will induce alpha-haemolysis as well as beta-haemolysis (Cato *et al.*,

1986). Alpha-haemolysis is observed as "greening" of the area in and around the colonies because of partial decomposition of haemoglobin. Haemolysis can be used as an indication of pathogenicity. The psychrotrophic microorganisms have not been observed to produce any haemolysis indicating that they are most likely not pathogenic (Broda *et al.*, 1998).

Cl. frigidicarnis do not produce botulinal neurotoxin as demonstrated by a negative reaction in a mouse bioassay (Broda *et al.*, 1999). The ability to produce botulinal neurotoxin can also be elucidated through the presence of botulinum toxin (BoTN) genes. Although the presence of these genes indicates the ability to produce botulinal toxin, it does not mean they will express the genes. A survey based on polymerase chain reaction (PCR) of these genes was conducted on psychrotrophic *Clostridium* spp. isolated from meat and it was determined that these species, obtained from a variety of sources, do not contain these genes (Broda *et al*., 1998). It was concluded that these "blown-pack" *Clostridium* spp. do not pose a food safety issue; rather they pose a food quality concern.

Although all of these tests can be used to characterise and identify *Clostridium* spp., the process is quite lengthy and can be very expensive. Not only are there variation of phenotypic characteristics among species, there is much diversity within strains. Phenotypic tests do not provide reproducible results, and results are dependent on when the microorganisms are harvested for testing. Growth phase can affect the outcome of these tests, making it can be difficult to determine

the identity of these microorganisms when solely relying on phenotypic tests. In terms of the food industry, the time required to identify these microorganisms using phenotypic tests is not feasible. Genotypic characterisation provides a quicker, reproducible and therefore, more reliable alternative to identification of *Clostridium* spp. in "blown-pack" spoiled meats.

1.2 Molecular Methods to Identify Clostridia from "Blown-pack" Spoiled Meats

PCR and Restriction Fragment Length Polymorphism (RFLP) are the primary methods used to rapidly identify *Clostridium* spp. that cause spoilage in vacuum packed chill-stored meat. PCR is used to amplify targeted DNA sequences. These sequences can then be further analysed to identify the microorganism. Other PCR based methods, such as DNA fingerprinting (Bassam *et al*., 1992), random amplified polymorphic DNA analysis, and repetitive element sequence-based PCR, have also been used to characterise inter-strain difference between *Clostridium botulinum* (Hyytia *et al.*, 1999). However, for the purpose of this study, DNA-RFLP provides sufficient discriminatory power, and was used as a rapid and reliable method for specific detection. In this study, one of the objectives was to isolate and identify the causative microorganism of "blown-pack" spoilage. DNA-RFLP was used to discriminate between clonal isolates and not differentiate between the strains.

To identify a species with DNA-RFLP, a sequence of DNA that varies in different species is required. In most methods, the 16S rRNA gene sequence is used because of its highly conserved nature. There is some heterogeneity in the 16S rRNA gene sequence (Woese, 1987). Evidence of heterogeneity is visualised in the restriction patterns of PCR-amplified 16S rDNA genes. Other regions that have been investigated include the 16S to 23 S rDNA Internal Transcribed Spacer (ITS) regions. Polymorphism analysis has been applied to the ITS region; however, when comparing it against the 16S rDNA region, ITS analysis does not provide sufficient discrimination between meat isolates (Broda *et al*., 2003b).

For DNA-RFLP analysis of the 16S rDNA gene, genomic DNA serves as the PCR template. The primer sequences are universal (eu) bacterial primers that are complementary to the 5' and 3' ends of 16S rRNA gene (Broda *et al.*, 2002a). Primer sequences used were: pA (forward) 5'-AGA GTT TGA TCC TGG CTC AG -3' and pH* (reverse) 5'-AAG GAG GTG ATC CAG CCG CA -3' (Hutson *et al.*, 1993). After amplification of the 16S rDNA region, the purified DNA mixture is subjected to restriction endonucleases. Different species of *Clostridium* require different combinations of restriction endonucleases to differentiate among the strains. For example, the use of *Alu*I and *Apo*I restriction enzymes can distinguish between variant strains of *Cl. estertheticum* (Helps *et al.*, 1999). PCR and RFLP can be used to identify the microorganism if the restriction patterns are identical to the patterns of a known microorganism (generally a type strain). The patterns obtained from the restrictions are compared to each other

and to standards. Microorganisms that are the same should have the same restriction pattern.

Another rapid identification method is the use of species specific PCR. Primers sets can be designed specifically to detect the presence of various microorganisms. For this procedure to work, the targeted area of the DNA has to be unique to the microorganism. Different size of PCR products will differentiate between closely related species. Two primer sets have been developed to detect the presence of *Cl. estertheticum* (Broda *et al.*, 2002 a,b). Each of these primer sets produces PCR products of different sizes. The major advantage of species specific PCR compared to RFLP is that species-specific PCR is more rapid and can be used in mixed cultures. Isolation may not be required in order for identification of the microorganisms responsible for the "blown-pack" spoilage. Meat samples can be analysed using species specific PCR. Identification of *Cl. estertheticum* can be achieved with at least 10^4 cells/g of minced beef for non-enriched meat samples; with broth enrichment of the minced beef samples the detection level is 10^2 cells/g (Broda *et al.*, 2003a). Using multiplex PCR, more than one microorganism can be identified. Multiplex PCR utilises more than one primer set. Use of multiple primer sets allows for the simultaneous amplification of several gene targets of interests. However, multiplex PCR can be difficult to optimise. One disadvantage of species-specific PCR is that knowledge of the DNA sequence is required. If a microorganism has not been sequenced, specific primer sets cannot be developed. The 16S rDNA sequence is generally used to

differentiate genera of bacteria. Some microorganisms are described by specific genes. In some cases, the primers used may not be specific enough to differentiate between different species. Despite these disadvantages, species-specific PCR is useful for identification of "blown-pack" spoilage microorganisms in a meat sample. PCR and RFLP are tools that are extremely useful for the rapid identification of "blown-pack" microorganisms relative to phenotypic and physiological tests.

To develop strategies to control the occurrence of "blown-pack" spoilage, isolation, identification, and characterisation of growth conditions in vacuum packaged meat should be completed. Potential reservoirs can also be studied to further devise a method of preventing the contamination of meat with psychrotrophic *Clostridium*. Because these microorganisms produce endospores, they cannot be readily destroyed with use of mild heat, detergents or sanitation chemicals used in the food industry. Therefore, other methods may have to be applied to prevent "blown-pack" spoilage. Although identification and characterisation of these isolates from meat may indicate causation of "blown-pack" spoilage, without further confirmation, this conclusion cannot be made. Confirmation of the production of gas and volatile compounds must be completed. This can only be done through an applied study where the microorganisms are reintroduced into a meat product.

1.3 Endospores and Endospore Structure

The ability to produce endospores enables *Clostridium* spp. to survive treatments of wet and dry heat, ultraviolet (UV) radiation, γ-radiation, desiccation and toxic chemicals; treatments that would otherwise be very effective against growing bacterial cells (Kennedy *et al*., 1994; Vreeland *et al.*, 2000; Nicholson *et al.*, 2000). Endospores can also survive starvation and lysozyme treatments. Endospores are inactive structures produced by bacteria during periods of environmental stress. Bacterial endospores are described as refractile bodies in bacterial cells and were first studied approximately 130 years ago by Cohn (1876). During dormancy, endospores contain minimal amounts of high-energy compounds but they do contain a variety of metabolites and enzymes required for catabolism (Setlow., 1995; Setlow and Setlow., 1996; Setlow., 2007). Despite containing substantial levels of a variety of metabolites and enzymes for catabolism, there is virtually no measurable metabolic activity in endospores during dormancy. Lack of metabolic activity is assumed to aid in extreme longevity (Crook, 1952; Desser and Broda, 1969). Another important aspect of endospore dormancy is its low water content. The low water content is presumed to immobilise proteins (Cowan *et al.*, 2004) which results in low enzyme activity. Bacterial endospores have been known to survive for over a thousand years. Viable endospores have been isolated from insects that were trapped in amber. The amber was thought to be approximately 25 to 40 million years old (Cano and Borucki, 1995). The longevity of endospores is facilitated by its resistance to temperature, desiccation, toxic chemicals and UV radiation.

The general structure of an endospore includes an exosporium (particularly in *Bacillus* spp.), a cortex and an endospore core (see Figure 1.1). The exosporium may be responsible for pathogenesis (ex. *Bacillus* spp.), however, its function is not entirely known (Setlow, 2007). Underneath the exosporium, there is a layer that contains an abundance of proteins. The coat layer contains >40 different proteins, with most being specific to endospores (Kim, 2006). The coat layer protects the endospore from a variety of environmental stresses, including predation from phagocytic eukaryotes (Klobutcher, 2006). Chemical resistance is also a function of the endospore coat. Exogenous lytic enzyme is prevented from degrading the cortex via the barrier that the endospore coat provides (Setlow, 2006). Inside the coat layer is the outer membrane which is required for endospore formation but does not impart any protective or barrier in the mature endospore. The cortex is the following layer and contains primarily peptidoglycan. The structure of the peptidoglycan in the endospore cortex is similar to the peptidoglycan found in vegetative cells (Popham, 2002). There are modifications that are specific to endospores, including muramic acid-δ-lactam (MAL) and muramic acid linked to only alanine (Setlow, 2007). The formation of a dormant endospore requires the cortex to protect the inner membranes and core. The cortex also aids in the reduction of the water content of the endospore coat; however, the mechanism is unknown (Setlow, 2006). The germ cell wall is also composed of peptidoglycan and is thought to play no direct role in endospore resistance. However, during germination, the germ cell wall becomes the cell

wall of the outgrowing endospore (Setlow, 2006). Of all the layers, the inner membrane has the lowest permeability to small molecules. The inner membrane has strong permeability barriers that confer endospore resistance to a variety of chemicals, especially those that damage endospore DNA (Nicholson *et al.*, 2000; Cortezzo and Setlow, 2006). The lipid molecules in the inner membrane are immobile during dormancy but become fully mobile during germination (Cowan *et al.*, 2004). Immobile lipid corresponds with the compressed nature of the inner membrane during dormancy. The lipids present in the inner membrane are comparable to those that are found in the membrane of growing cells (Cortezzo and Setlow, 2006); therefore, the lipids are not responsible for the barrier that the inner membrane provides during dormancy. The innermost layer of the endospore is the endospore core. During endospore germination, the endospore core becomes the cell's protoplast. The core contains enzymes, DNA, RNA, and nucleic acids that are identical to those found in vegetative cells (Setlow, 1983; Setlow, 1994).

The low water content of endospores is one attribute that facilitates endospore resistance to desiccation. Generally the protoplast of a growing cell contains approximately 75 to 80% water. The endospore core contains 27 to 55% water (Gerhardt and Marquis, 1989) depending on the species. It is not known how water content is lowered during sporulation. An important molecule found only in the endospore core is pyridine-2,6-dicarboxylic acid (also known as dipicolinic acid, DPA) which contribute to the resistance of the endospore. DPA accounts for

5 to 15% of the endospore's dry weight (Gerhardt and Marquis, 1989). Chelation of DPA with divalent cations, such as Ca^{2+}, is common in the endospore core. The amount of DPA found in the endospore core is above its solubility (Setlow, 2006) which may account for the reduction in core water content during sporulation. Another molecule in the core that is important for endospore resistance is a group of small, acid-soluble endospore proteins (SASP) which are responsible for saturating and protecting endospore DNA. These proteins make up 3 to 6% of the total endospore protein (Setlow, 2006). SASPs are unique to endospore formers because they do not share homology to any structural motifs identified in other proteins found in non-endospore formers (Setlow, 2006). There are different groups of SASPs, but α/β-type SASPs are the most abundant. The other function of the other types of SASPs is not known but may confer some protection to DNA (Setlow, 2007). α/β-type SASPs protect the DNA from wet and dry heat, UV radiation, desiccation and genotoxic chemicals by tightly binding and saturating the bacterial DNA . This causes conformational changes which protect the DNA from a variety of different treatments.

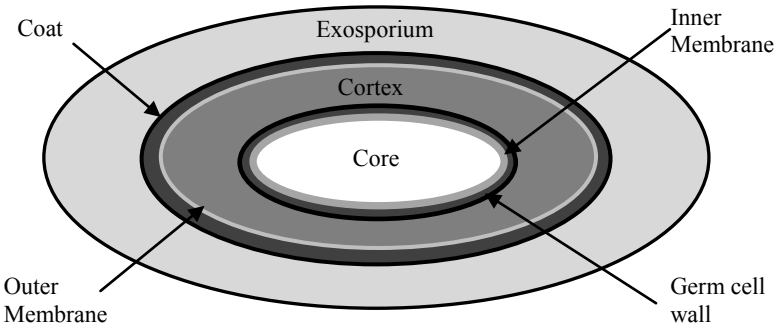

Figure 1.1: Structure of a bacterial endospore (Setlow, 2007).

1.4 Sporulation and Germination of Endospores

Sporulation is an energy intensive process. Starvation for carbon and/or nitrogen sources will generally induce sporulation. Phosphorylated SpoA protein, which acts as both an activator and repressor of gene expression, initiates the sporulation process. SpoA is required to activate expression of genes encoding two sigma (σ) factors. RNA polymerase will associate with the σ factors to determine specificity of the SpoA protein (Setlow, 2007). During the sporulation process, there is a generation of a large mother cell and a small forespore. The forespore is engulfed by the plasma membrane of the mother endospore. Both compartments participate in extensive "cross-talk" to synchronise events, especially during critical developmental phases (Setlow, 2007). Production of a thick peptidoglycan cortex between the inner and outer forespore membranes occurs in tandem with the decrease of water content and pH in the forespore protoplast. The mother cell produces a large amount of DPA that is taken up by the forespore. The uptake of DPA will further decrease the water content in the forespore. Using proteins produced by the mother cells, protein layers form a coat around the endospore. This even occurs during the later stages of sporulation. In some species, an exosporium is added. The final stage involves lyses of the mother cell and the release of the endospore into the environment (Setlow, 2007).

Endospores can germinate after several years of dormancy if specific nutrients are present. The nutrients required for germination is specific for the species of endospore former. Early research identified L-alanine and a variety of other

amino acids (ie: L-aminobutyric acid, L-cysteine, L-valine and L-leucine) as chemicals that induced germination (Hills, 1949). Other common nutrients that can trigger germination include L-amino acids, D-sugars and purine nucleosides (Setlow, 2007). In *Clostridium* spp., bicarbonate anions stimulate germination and lactate is an effective co-germinant (Gould, 2006). Germination that is triggered by nutrients begins with nutrients binding to proteins (germinant receptors) found in the endospore inner membrane. Release of monovalent cations (ie: H^+, K^+) and DPA with divalent cations is the result of the nutrient binding to the receptors. The increase in ions is followed by water uptake. The amount of water is not enough to allow for protein movement in the core or to end endospore dormancy (Cowan *et al.*, 2003). Resistance to moist heat is decreased with the uptake of water which initiates a cascade of events that will result in the germination of the endospore. Hydrolysis of the cortex peptidoglycan occurs when water is taken up into the endospore. This even leads to expansion of the endospore core, without membrane synthesis. Completion of germination occurs when water content in the core is equal to that of a growing cell. This whole process occurs without ATP production (Setlow, 2007). Proteins are mobile with the increased levels of water which leads to enzyme activity in the core. Enzyme activity results in endospore outgrowth with the following key events: SASP hydrolysis, metabolism of exogenous and endogenous compounds, macromolecular synthesis, and concluding with DNA replication (Setlow, 2007).

Germination can also be induced by "non-nutrient" means. Despite containing large amounts of DPA in the endospore core, exogenous calcium DPA can result in germination (Riemann and Ordal, 1961). Surfactants, such as *n*-dodecylamine, can be used a germinant. However, surfactant induced germination can result in death of germinated endospore (Rode and Foster, 1960) due to change in permeability of the membrane. Treatment with disulfide bond-breaking reagents will permeabilise the endospore coat. Lysozyme can then be used to initiate germination (Gould and Hitchins, 1963). Lastly, high hydrostatic pressure has been known to stimulate germination (Clouston and Wills, 1969). Germination, combined with antimicrobial agents, has been researched as a method to control endospores. However, germination occurs in a non-log linear fashion meaning there are long tails or "skips" during long-term storage of endospores (Gould *et al*., 1968). An example of this is *Cl. botulinum* endospores germinating after several months of incubation (Esty and Meyer, 1922).

Early endospore research revealed that endospores could be activated with mild heating (Hills, 1949). Activation would lead to rapid germination if the specific germinants were present. It has been elucidated that the endospore protoplast may be in a glassy state and may contribute to resistance (Sapru and Labuza, 1993a,b). Pre-heating endospores is thought to cause a temperature-induced glass transition (Ablett *et al.*, 1999) and lead to activation. However, there is not enough data that has completely validated this mechanism.

1.5 Endospore Resistance

Endospores are extremely resistant to three major treatments: heat (both wet and dry), chemicals, and radiation. All three methods have been used to try to control the outgrowth of endospores.

1.5.1 Resistance to Wet Heat

Resistance to wet heat is defined as resistance to heat when suspended in an aqueous environment. In aqueous environments, endospores can withstand temperatures approximately 40 ºC higher than that of vegetative cells of the same species (Gerhardt and Marquis, 1989; Nicholson *et al.*, 2000). The lower the core water content, the higher the resistance to wet heat. The core water content can be manipulated by temperature. Endospores formed at high temperatures exhibit a lower core water content than endospores produced at lower temperatures (Setlow, 2006). Thermophiles are more resistant to wet heat than mesophiles. Particular proteins present in thermophiles may confer resistance that would not be present in mesophiles. Inability to produce DPA renders the endospore more sensitive to wet heat as a lack of DPA results in higher water content in the core. Ions will mineralise the endospore core but not all will increase the resistance to wet heat. Endospores saturated with Ca^{2+} are most resistant to wet heat, followed by saturation with Mg^{2+} or Mn^{2+}; endospores saturated with K^{+} or Na^{+} ions instead of Ca^{2+} are the least resistant (Marquis and Bender, 1985; Gerhardt and Marquis, 1989). The mechanism by which low water content protects the core from wet heat is unknown. Low water content means that there is an extremely

low level of free water present which results in restricted macromolecular movement. Restricted movement could also result in enzymatic dormancy, thereby resulting in endospore resistance to wet heat (Gerhardt and Marquis, 1989). The initial step in endospore killing via wet heat is unknown; however, endospore killing by wet heat is usually accompanied by inactivation of core enzymes and rupture of the endospore inner membrane. Any deficiency in the endospore inner membrane means a deficiency in the permeability barrier (Warth, 1980; Setlow *et al.*, 2002). Endospore DNA saturated with α/β-type SASPs are resistant to wet heat. Wet heat does not kill endospores via DNA damage (Setlow, 2006). Endospores deficient in α/β-type SASPs are killed by wet heat due to depurination. Therefore, α/β-type SASPs are extremely effective in protecting endospore DNA.

1.5.2 Resistance to Dry Heat

Under dry heat conditions, endospores can withstand temperatures approximately 30 ºC higher than that of vegetative cells of the same species (Nicholson *et al.*, 2000). In contrast to wet heat, endospores of mesophiles and thermophiles are equally resistant to dry heat (Alderton and Snell, 1969). Resistance to dry heat also infers resistance to desiccation. Protection of DNA is vital to resistance to dry heat and desiccation. Dry heat kills endospores by means of DNA damage. Endospores lacking in DNA protection and DNA repair mechanisms are sensitive to dry heat (Setlow, 2006).

1.5.3 Resistance to Chemicals

A variety of chemicals have been used to control endospore outgrowth. Resistance to acids, bases, oxidising agents, alkylating agents, aldehydes and organic solvents has been observed. Some genotoxic chemicals, such as formaldehyde, nitrous acid, and some alkylating agents, have the ability to kill endospores via DNA damage. Hydrogen peroxide, a well known genotoxic chemical, has little to no effect on endospores (Setlow, 2006) because of the protective nature of the endospore coat. Mutations in the DNA will accumulate until eventually a mutation in the repair mechanism will render the endospores sensitive to these chemicals (Setlow *et al.*, 1998; Loshon *et al.*, 1998; Tennen *et al.*, 2000). Inner membrane damage caused by some oxidizing agents will sensitize endospores to other treatments (Cortezzo *et al.*, 2004). Oxidizing agents combined with wet heat can lead to cell death. There are some chemical agents that a mechanism of action has not been elucidated. Chemicals, such as glutaraldehyde and *ortho*-phthalaldehyde, kill endospores but the mechanism is not known (Tennen *et al.*, 2000; Cabrera-Martinez *et al.*, 2002). Strong acids kill endospores by indirectly rupturing the endospore inner membrane (Setlow *et al.*, 2002). The first major barrier to chemicals is the endospore coat. Proteins responsible for resistant nature of the endospore coat have not been determined, but the endospore coat is thought to detoxify the chemicals before they can reach the endospore core (Setlow, 2006). Superoxide dismutase, which renders hydrogen peroxide less toxic, has been coupled with the exosporium and/or endospore coats of some species (Henriques *et al.*, 1998; Lai *et al.*, 2003;

Redmond *et al.*, 2004). Another major barrier to chemicals is the inner membrane owing to its exceptionally low permeability. Mild heat treatment or sporulation at low temperatures can increase the permeability of the inner membrane. These treatments can be combined with genotoxic chemicals to kill endospores. The immobility of the inner membrane lipids may be responsible for the resistant nature (Setlow, 2006).

1.5.4 Resistance to Radiation

Endospores can withstand 10 to 50 fold more UV radiation at 254 nm than growing cells (Nicholson *et al.*, 2000). Even at longer wavelengths, endospores are more resistant than growing cells. The resistance of endospores to UV radiation depends on the efficient and rapid repair of endospore photoproducts. Endospore photoproducts are generated by 254 nm of radiation. The most common endospore photoproduct is thymidyl-thymidine adduct (Setlow, 2006). Endospore photoproducts are much less lethal than photoproducts generated in growing cells (Setlow, 2006). These photoproducts are repaired within minutes of endospore outgrowth by at least three repair mechanisms. Endospore photoproduct lyase (Spl), which is unique to endospores, is responsible for some of the repair process. Endospore photoproducts are also repaired by recombination and excision. These two pathways are independent of the Spl pathway. Protection of the DNA is also important to radiation resistance. α/β-type SASPs protect the DNA by binding to the DNA and changing the conformation. The conformational changes make the formation of easily repaired

endospore photoproducts more favourable (Setlow, 1992; Setlow, 1995 Frenkiel-Krispin *et al.*, 2004). There have been some studies which indicate that ionising radiation can sterilise food. The United States Army research laboratories at Natick, MA studied irradiation sterilisation of food during the 1950s and 1960s. Packages of food inoculated with *C. botulinum* were sterilised with 25 to 40 kGy of radiation (Ross, 1974; Anellis *et al.*, 1979).

1.6 Interventions

Contamination of raw meat during processing is unavoidable. Endospores can particularly be a problem because of their resistance to steam and acid washes used to clean the carcasses. Treatments used to kill or control endospores are generally harsher treatments; especially because of their resistance to several types of treatments (as outlined above). Coupled with consumer demands retention of organoleptic properties of fresh meat products without compromising shelf life, control of endospores in fresh meat has been a challenging.

Some interventions have been designed to delay the onset of sporulation. A carcass rinse with peroxyacetic acid (POAA) and its effects on "blown-pack" spoilage was investigated by Boerema *et al.* (2007). Fresh beef was treated with a POAA rinse and inoculated with three levels of *Cl. estertheticum* endospores (0, 4 and 40 endospores/cm^2). The packages were vacuum packaged and stored at refrigeration temperatures (-1.5, 0 and 2 ºC). The POAA rinse alone did not prevent the outgrowth of endospores in vacuum packaged chilled meats.

Inactivation of approximately 4 to 5 log CFU/mL of *Cl. estertheticum* endospores was obtained using POAA-based agents (Broda *et al*., 2007); this was done in vitro. Treatment with POAA-based rinses on carcass had no effect on the onset of gas production. The amount of bacteria present on the meat (ex. Initial level of contamination) did affect "blown-pack" spoilage. Onset of gas production occurred earliest for the package that was inoculated with 40 endospores/cm^2. Although meat inoculated with the highest concentration of endospores showed signs of gas production the quickest, a lower inoculum spoiled the meat well before the best before date. The uninoculated meat did not exhibit any gas production. Most of the contamination of carcasses occurs when the hide is removed and when the carcass is opened (Boerema *et al.*, 2002; Boerema *et al.*, 2007). In the same study, temperature was also an important factor in control of "blown-pack" spoilage. At -1.5 ºC, gas production was considerably delayed. Storage temperature was deemed to be the most important factor in controlling and influencing the growth of *Cl. estertheticum* (Boerema *et al.*, 2007). Onset of blowing occurred approximately 10 d later when the product was stored at -1.5ºC as compared to product stored at 1.5 ºC and 3.5 ºC increase was not significant. Packages that were stored at 0 and 2 ºC observed "blown-pack" spoilage at approximately the same time. The authors adamantly maintain that fresh meat should be stored at temperatures as close to -1.5 ºC in order to prevent "blown-pack" spoilage due to *Clostridium* spp. Attachment of *Clostridium* endospores to connective tissue or the fat tissue may be the reason for the inability of POAA to inactivate endospores in vivo. Due to the hydrophobicity of endospores (Rönner

et al., 1990), attachment to food surfaces is greater than that of vegetative cells (Husmark and Rönner, 1992). Effectiveness of sanitizers is affected by bacterial attachment to surfaces (Bower *et al.*, 1996). Because the surfaces of the carcasses are not even, sanitizers may not be able to reach certain areas of the carcass and therefore, will be ineffective. In an aqueous medium, there is more contact between the endospores and the sanitize resulting in endospores being more susceptible to sanitizer. Attachment of bacterial cells to surfaces can be affected by size of the inoculums (Iturriaga *et al.*, 2003; Takeuchi and Frank, 2000). Overall, POAA treatment is not effective in preventing outgrowth of low number of endospores on meat carcasses. However, in cases of high numbers of contamination, it can be used to slightly extend the shelf life of vacuum packed meats. Other interventions may be applied to prevent the outgrowth of endospores.

1.7 Bacteriocins

Fuelled by consumer demands for a "natural product", bacteriocins have been studied as a possible biopreservative in foods. Produced by bacteria, bacteriocins are defined as antimicrobial peptides (or proteins) and generally have a narrow spectrum of antibacterial activity against closely related species (Tagg *et al.,* 1976; Klaenhammer, 1993). Low concentrations of bacteriocins have the ability to exert strong antimicrobial affects. Bacteriocins can differ in spectrum of activity, how they exert their antimicrobial affects, their size and weight, genetic origins and their biochemical properties. Most of the current research is focused

on bacteriocins produced by LAB. Nisin, produced by *Lactococcus lactis* subsp. *lactis*, is one of the better known bacteriocins and it has been approved for use in foods in some countries.

Bacteriocins prevent the growth of Gram positive microorganisms but tend not to have an effect on Gram negative microorganisms. Gram negative microorganisms can be sensitised to bacteriocins with treatments of chelating agents, sub lethal temperatures (heating or freezing) or use of high pressure. This work is still quite preliminary and it is still not known if these methods could be feasible in a meat system (Lucke, 2000). Bacteriocins not only prevent the growth of certain spoilage microorganisms but can have antimicrobial affects against various pathogens. Spoilage and some pathogenic microorganisms are usually closely related to the producer (Schillinger *et al.*, 1996). There is a great number of bacteriocins that have inhibitory effects on *Listeria monocytogenes*. Some bacteriocins will affect the growth of other pathogens such as, *Staphylococcus aureus* and *Cl. botulinum*; however, this occurs at lesser frequency (Schillinger *et al.*, 1996). Not all bacteriocins will have the same spectrum of activity. The mode of action of bacteriocins is via the cell membrane. There is speculation that bacteriocins have the ability to form pores within the membrane and thus disrupt the proton motive force (Hechard and Sahl, 2002). Different bacteriocins will cause bactericidal or bacteriostatic effects against different microorganisms.

Bacteriocins produced by LAB can be classified into three groups based on genetic and biochemical properties (Eijsink *et al.*, 1998; Nes *et al.*, 1996). Class I and class II bacteriocins are more heavily studied due their smaller size. The class I bacteriocins are known as the lantibiotics. Produced ribosomally, lantibiotics undergo post-translation modification before they are released as active peptides. Nisin is a lantibiotic. Class I bacteriocins tend to be broad spectrum antimicrobial compounds and can have the ability to prevent the outgrowth of endospores. However, there are limitations to the use of class I bacteriocins in food because of interactions within the food complex. For example, nisin is inactivated in raw meats (Rose *et al*., 2002). This is due to the reaction of nisin with of glutathione in raw meats. Many of the bacteriocins produced by LAB are Class II bacteriocins. These bacteriocins are unmodified bacteriocins and are divided further into three subclasses. Class IIa are pediocin-like bacteriocins; class IIb are two-peptide bacteriocins; and Class IIc are non-pediocin-like, one-peptide bacteriocins (Drider *et al.*, 2006). The Class III bacteriocins are thermosensitive peptides (Klaenhammer, 1993). The last group of bacteriocins has the unique characteristic of being circular. These bacteriocins have not yet been classified although some researchers have suggested they be grouped into a separate class, Class IV (Maqueda *et al.*, 2007). Class IV also includes organisms that are not considered LAB. Several circular bacteriocins have been described including enterocin AS-48 from *Enterococcus faecalis* S-48, gassericin A from *Lactobacillus gasseri* LA39, reutericin 6 from *Lactobacillus reuteri* LA6, butyrivibriocin AR10 from *Butyrivibrio fibrisolvens* AR10, uberolysin from

Streptococcus uberis, circularin A from *Clostridium beijerinckii* ATCC 25752, subtilosin A from *Bacillus subtilis* (Maqueda *et al.*, 2007) *and* carnocyclin A from *Carnobacterium maltaromaticum* UAL307 (Martin-Visscher *et al.*, 2008). The circular bacteriocins are large and posttranslationally modified (Martin-Visscher *et al*., 2008).

Bacteriocins are added to the food product as a pure or partially pure substance or a bacteriocin-producing culture is used to produce the bacteriocin in the food. Being peptides (or proteins) bacteriocins are digested when consumed and therefore do not generally disrupt the commensal microflora. In the case of nisin, a partially purified bacteriocin is used. An advantage with the use of purified bacteriocin is that one can control the amount added; however the high cost of purification may not be justifiable and some can be difficult to purify. With the addition of a bacteriocin-producing culture, there is growth of the microorganism on the food and the bacteriocin will be produced in situ. To ensure that the food is safe, the strain chosen must produce an adequate amount of bacteriocin in vivo. At the same time, the strain must not produce excess amounts of unwanted metabolites (Schillinger *et al.*, 1996). These metabolites can include organic acids, peroxides, or any substance that will cause a sensory change in the food product. Although it is more economically feasible to inoculate food with a bacteriocin-producing culture, one has to take into consideration that some bacteriocins are encoded via plasmids that can be unstable (Schillinger *et al.*, 1996). Also, some bacteriocin-producing microorganisms will only produce

bacteriocins a specific temperature. These are factors that should be taken to consideration when choosing a bacteriocin-producing culture to preserve food. Despite all of the disadvantages, there is still a considerable amount of research on the use of bacteriocin-producing LAB as biopreservatives in food.

Incorporation of bacteriocin producing cultures, especially LAB, has been studied in a variety of food products. Bacteriocin producing cultures have been added to fermented products such as yogurt, cheese, sausages as well as vacuum packaged meat products. Most of the work with vacuum packaged meat products has examined the effects of bacteriocin-producing bacteria on *L. monocytogenes*. There has been little work done on the effects of bacteriocin-producing LAB and the prevention of outgrowth of *Clostridium* spp. A strain of *Lactobacillus helveticus* has strong activity against *Clostridium sporogenes*. Unfortunately, bacteriocin production in *Lb. helveticus* has not been determined by standard methods (Topisirovic *et al.*, 2006). Nisin-producing strains have been co-incubated with *Cl. botulinum* in vivo but not in a meat a product. At temperatures of 10 ºC, *Cl. botulinum* was not detected after 10 days of co-incubation with a protective culture (*Lactococcus lactis* and *Pediococcus pentosaceus*) (Rodgers *et al.*, 2004). However, at 15 ºC, *Cl. botulinum* was not inhibited by the protective cultures. When samples were stored at 10 ºC, reduction of *Cl. botulinum* coincided with the production of nisin by *Lc. lactis* (Rodgers *et al.*, 2004); but production of nisin was not rapid enough to prevent the detection of botulinal

toxin. Refer to Table 1.2 summarising other studies involving bacteriocin-producing cultures and *Clostridium* spp.

Table 1.2 Application of bacteriocin-producing culture in non-fermented refrigerated foods

Bacteriocin-producing cultures	**Target microorganism**	**Food**	**Reference**
Enterococcus faecium	*Cl. botulinum*	Sous vide fish	Smith (1975)
Lactococcus lactis	*Cl. botulinum*	Chicken a la king	Saleh and Ordal (1955)
Lactobacillus plantarum ATCC 8014	*Cl. botulinum* type A, B, and E	Refrigerated can pea soup	Skinner *et al.* (1999)
Lactobacillus plantarum	*Cl. botulinum*	Cured meat	Tanaka *et al.* (1980)
Pediococcus acidilactici	*Cl. botulinum*	Chicken salad	Hutton *et al.* (1991)

Adapated from Rodgers (2001)

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 of *Clostridium* spp. More research needs to be done with co-inoculation of bacteriocin producing cultures and *Clostridium* spp. on meat products. As well, a range of bacteriocins producing cultures should be examined. Little or no work has been done to examine the effect of class II and the new class of circular bacteriocins on *Clostridium* spp. in meat products; and no work has been completed in vacuum packaged fresh beef.

1.8 Lactic Acid Bacteria and Biological Preservation

The addition of desirable microorganisms, particularly LAB, to cooked meat products has been studied to provide a novel method of preservation. Lactic acid

is produced as the major fermentation end product. LAB have a long history of safe use in foods. They are essential to the dairy industry because they are used to produce cheeses and yogurts. LAB are also utilised in baking, wine making, curing meat products and in pickling of vegetables. Biological preservation with bacteriocin-producing LAB is a relatively new approach to improve the safety of foods as well as prolong the shelf life of foods. LAB will naturally dominate the microflora of raw meats and meat products that are stored under vacuum or modified carbon dioxide atmosphere. Therefore, under these condition and with the ability to produce bacteriocins, LAB can be used a protective culture.

Carnobacterium maltaromaticum (one of the LAB) is isolated from both cold and temperate environments and can predominate in food. They are frequently isolated from fish, meat and some dairy products (Leisner, 2007). *C. maltaromaticum* is tolerant to freezing, thawing and high pressure. These microorganisms have the ability to grow at low temperatures, under anaerobic conditions, and in environments with increased carbon dioxide concentrations (Leisner, 2007).

Several *Carnobacterium* spp. produce bacteriocins and these microorganisms are frequently isolated from food. Bacteriocins isolated from *Carnobacterium* spp. have been characterised as Class I and II; all but one belonging to Class II (Stoffels *et al.*, 1992a,b). Most of the Class II bacteriocins have been identified as being small pediocin-like bacteriocins. These bacteriocins are ribosomally

synthesised and modified posttranslationally to activate the prepeptides. The mechanism of action of these bacteriocins is via the formation of pores which causes leakage of internal low molecular weight substances and dissipates the membrane potential (Suzuki *et al.*, 2005; Drider *et al.*, 2006). Production of bacteriocins by *Carnobacterium* spp. is affected by external parameters. NaCl concentration, temperature, presence of acetate and pH are all external factors that can modify bacteriocin production. Presence of bacteriocin-sensitive microorganisms will induce bacteriocin production (Sip *et al.*, 1998). External factors should be taken into consideration when using a bacteriocin producing *Carnobacterium* as a biopreservation method in foods.

Carnobacterium spp. are applied in foods to inhibit *L. monocytogenes* and spoilage microorganisms and to extend the shelf life. There are several concerns associated with the use of *Carnobacterium* spp. for biopreservation these include resistance, production of unwanted metabolites, and safety. Resistance of *L. monocytogenes* to divergicin M35, a Class IIa bacteriocin, has been documented (Naghmouchi *et al.*, 2006). Modification of the cell wall fatty acid composition of *L. monocytogenes* may be responsible for resistance. However, if the bacteriocin-producing culture has the ability to produce several bacteriocins, development of resistance may be deferred or prevented. Combinations of bacteriocins with different mechanisms of action have a better chance of preventing resistance than those with similar mechanism of action. Some *Carnobacterium* spp. have the ability to produce a variety of bacteriocins.

Carnobacterium maltaromaticum UAL307 produces carnobacteriocin BM1, piscicolin 126 and carnocyclin A. Carnobacteriocin BM1 and piscicolin 126 are Class IIa bacteriocins (Gursky *et al*., 2006; Jack *et al.*, 1996; Quadri *et al.*, 1994). Carnocyclin A is a novel circular bacteriocin and is a first of its kind to be isolated from *Carnobacterium* spp. (Martin-Visscher *et al.*, 2008). Other circular bacteriocins are cationic, possess a high isoelectric point and have a high content of hydrophobic residues; all characteristics which carnocyclin A shares. Circular bacteriocins, carnocyclin A included, has potent bioactivity, stability to variation in temperature and pH and resistance to proteolysis (Kawai *et al.*, 2004; Ma *et al.*, 2003). The circular structure of the bacteriocin may confer these properties. The ability to produce several bacteriocins is beneficial because it increases the survival of the bacteria in a competitive environment (Deegan, 2006; Jeziorowski and Gordon, 2007; Nes *et al.*, 2007).

The presence of any microflora may affect sensory properties of food because of metabolites that may be produced. In naturally contaminated food products, presence of non LAB generally has a greater impact on quality and spoilage (Leisner *et al.*, 2007). However, some *C. maltaromaticum* strains are considered moderate spoilage microorganisms. Combinations of *C. maltaromaticum*, *B. thermosphacta* will produce a synergistic spoilage effect in modified atmosphere packaged shrimp (Mejlholm *et al.*, 2005; Laursen *et al.*, 2006). *C. maltaromaticum* is generally considered to be homofermentative, utilizing glucose to produce lactic acid (Leisner *et al.*, 2007). Some other metabolites that are

produced by *C. maltaromaticum* include acetic acid, ethanol, acetoin, diacetyl, volatile alcohols, and ketones. Carbohydrate catabolism results in a diverse range of metabolites. Although some metabolites are potent flavour compounds, the concentrations produced have a minimal effect on the sensory properties of food. Other microorganisms that are present in naturally contaminated meat products generally have a greater effect on sensory and spoilage. Production of tyramine from tyrosine is of concern for food safety. This is the only known metabolic pathway in which *Carnobacterium* spp. that causes a food safety concern (Leisner, 2007). Various strains of *C. maltaromaticum* will produce different amounts of tyramine (Leisner *et al*., 1994; Masson *et al.*, 1996; Bover-Cid and Holzapfel, 1999; Laursen *et al.*, 2006). Tyramine production by *C. maltaromaticum* has been described in a variety of meat products. These include meat (up to 28 mg/kg, Edwards *et al.*, 1987), a meat-fat mixture (up to 121 mg/kg, Masson *et al.*, 1999), cold-smoked salmon (up to *c.* 370 mg/kg, Duffes *et al.*, 1999b; Jørgensen *et al.*, 2000; Connil *et al.*, 2002; Brillet *et al.*, 2005), frozen and thawed salmon (up to 40 to 60 mg kg^{-1}, Emborg *et al.*, 2002), and shrimp (up to 20 to 60 mg kg^{-1}, Laursen *et al.*, 2006). The concentration of tyramine found in these products has no adverse effect on most people. However, those with reduced monoamine oxidase activity may have some adverse symptoms because their diet is limited to no more than 5 mg of tyramine per meal (McCabe, 1986). Hereditary deficiency and reduced monoamine oxidase due to oral medications may cause migraine headaches with high consumption of tyramine. Therefore, production of tyramine by *Carnobacterium* spp. may be an issue for those who

are sensitive. Those that are sensitive to tyramine are advised to consume fish and meat products that products those are newly processed and not close to their best before dates to minimise sensitivity (Leisner, 2007). Environmental parameters will affect the production of metabolites. The addition of *C. maltaromaticum* will rapidly spoil meat stored under aerobic conditions at 7 ºC but sensory properties of beef will be unaffected during long storage in vacuum packs at 2 ºC (Leisner *et al.*, 1995). Therefore, product storage time and temperature will play an important role in spoilage and sensory effects with the addition of *Carnobacterium* spp. as a protective culture.

1.9 Objectives

The objectives of this study were to 1) isolate and identify "blown-pack" spoilage microorganism(s); 2) confirm the ability of the microorganism to cause spoilage and understand the role of the background microflora in causing "blown-pack" spoilage; and 3) use bacteriocin-producing microorganisms as a method of biopreservation.

2. Materials and Methods

2.1 Raw Vacuum packed Meat

Packages of grossly distended, vacuum packaged, chilled, raw beef were obtained from an Alberta processor for investigation. The meat package was inspected for leakage and stored at 4 ºC until a sample could be taken.

Prior to sampling, a silicone plug was placed on the surface of the package. A needle connected to an aspirator was inserted into the silicon plug and the gas was removed without disturbing the anaerobic environment. The whole package was transferred to an anaerobic hood (Forma 1025/1029 Anaerobic Chamber, Thermo Scientific, Asheville, NC) operating at 21 ºC. The packaged sample was opened in the hood and using a sterile pipette, two 1 mL samples of purge (liquid that accumulates in the bottom of the meat package during storage) were obtained. Surface samples (5 x 2 cm) of meat were also collected using aseptic techniques.

A smear of purge was prepared on a microscope slide and viewed under a phase contrast microscope to observe the presence of endospores. Endospores were observed as refractive bodies under contrast microscopy.

2.2 Profiling of Organic Acids and Alcohols

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.

To remove particulates, 1.0 mL of purge was incubated at overnight at 4 ºC with 1.0 mL of 70% perchloric acid. The precipitate was removed by centrifugation (6000 x *g* for 5 min). The supernatant (1 mL) was transferred to a high performance liquid chromatograph (HPLC) vial and stored at -20 ºC 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 (Broda *et al.*, 1996). Solutions of lactic acid (Fisher Scientific), valeric acid (Sigma Aldrich), propionic acid (Sigma Aldrich) were also used to determine unknown peaks. Standard solutions were made from analytical grade acids and alcohols. Standard solutions (1 mL) were transferred to a HPLC vial and stored at -20 ºC 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 were monitored 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 and analyse chromatographs.

2.3 Microbiological Examination

2.3.1 Bacteria

Clostridium perfringens ATCC 13124, *Clostridium frigidicarnis* BAA155, *Clostridium frigidicarnis* BAA154, and *Clostridium putrefaciens* ATCC 25786 were used as control in this study. *Clostridium estertheticum* (Yang *et al*., 2009), which was recently isolated from a sample of purge from a "blown" package of beef, was obtained from Agriculture and Agri-Food Canada (AAFC) Meat Research Centre, Lacombe. Strains were grown in 10 mL of cooked meat medium (Becton, Dickinson and Company, Sparks, MD) and incubated at 21 ºC under anaerobic conditions. The gas mixture used in the anaerobic hood consisted of 85% N_2, 10% H_2 and 5% CO_2 (Praxair, Edmonton, Alberta). Strains were maintained in peptone-yeast-glucose-starch agar medium (PYGS, Lund *et al.*, 1990) and 55% glycerol (Fisher Scientific) at -80 °C. The peptone, yeast extract and agar were obtained from Becton, Dickinson and Company (Difco, Sparks, MD). Glucose and starch were obtained from Fisher Scientific.

2.3.2 Sampling Procedure

All media (both broth and plates) used during sampling was pre-reduced by leaving the plates in the anaerobic hood for 24 h prior to use. One sample of purge was used for determination of the general spoilage microflora and was transferred into a test tube containing 9 mL of dilution fluid (0.1 % peptone, 0.85% NaCl) (Broda *et al*., 1996). The other sample of purge was transferred to a test tube containing 9 mL of pre-reduced Reinforced Clostridial Medium (RCM; Difco; Becton, Dickinson and Company) (Broda *et al*., 1996) to stimulate growth of endospores that may be present in the purge.

A sample of meat was also obtained and used for microbial analysis. A sterile scalpel was used to cut a 2 x 5 cm surface section of the meat and the meat sample was placed into a sterile stomacher bag. This sample was massaged with 90 mL of dilution fluid. An aliquot (1 mL) of the homogenate was transferred to 9 mL of dilution fluid for enumeration of the microbial population and 1 mL was transferred to 9 mL of pre-reduced RCM for enumeration of anaerobic species.

Total aerobic bacteria, *Enterobacteriaceae*, lactic acid bacteria (LAB), and *Brochothrix thermosphacta* were enumerated using serial dilutions of the purge and the meat homogenate. Dilutions were made and 0.1 mL of each dilution were spread onto Plate Count Agar (PCA; Becton, Dickinson and Company), Violet Red Bile Glucose Agar (VRBG; Becton, Dickinson and Company), de Man,

Rogosa Sharpe agar (MRS; Becton, Dickinson and Company) and Streptomycin Sulphate-Thallous Acetate-Acitidone Agar (STAA; Gardner 1966). Plates of PCA, APT, and STAA were incubated at room temperature (approximately 20 °C) and VRBG plates were incubated at 37 °C. Dilutions prepared from purge and meat were incubation at 23 °C for 24 h and plated onto VRBG. All plating was completed in duplicate.

For analysis of the anaerobic microflora, sample preparation and plating were done in an anaerobic hood. Appropriate dilutions of the purge were prepared in pre-reduced RCM and 0.1 mL volumes were spread onto modified RCM Agar (Broda *et al.*, 1996). The modified RCM contained 5% sterile defibrinated sheep blood (Oxoid SR51; Ryegate, MT), 0.5% glucose (Fisher Scientific) and 1.5% agar (Becton, Dickinson and Company). Plastic Petri plates were placed into an anaerobic jar with a BBL GasPakPlus Anaerobic System Envelopes with palladium catalyst (Becton, Dickinson and Company). An anaerobic indicator strip (Becton, Dickinson and Company) was also added to the jar to monitor anaerobic conditions. The anaerobic jars were removed from the anaerobic chamber and incubated at 4 °C for 3 weeks before colonies were enumerated.

To determine counts of anaerobic endospores, two treatments were applied to destroy vegetative cells. To destroy viable cells with ethanol, a portion of the sample to be plated was mixed with an equal volume of ethanol and incubated at room temperature for 30 min. To destroy viable cells and any heat susceptible

endospores, samples of the purge were heated at 80 ºC for 10 min before further dilutions were prepared. The dilutions were spread onto Shahidi Ferguson Perfringens Agar (SFP; Becton, Dickinson and Company) containing 10% egg yolk solution (Remel; Lenexa, KS) and 10% glucose. All plating was completed in duplicate. Plates of SFP were incubated anaerobically at 30 °C for 7 d.

To select psychrotrophic and cold tolerant *Clostridium* spp., six colonies were initially chosen from the modified RCM based on morphology and streaked onto Columbia Blood Agar (CBA; incubated at 15 °C) under anaerobic conditions. Colonies were also chosen from RCM blood agar and SFP agar. However, only isolates from RCM blood agar produced strict anaerobic microorganisms. Isolation of colonies from RCM blood agar yielded a total of 66 isolates. These colonies were incubated on RCM blood agar plates in anaerobic conditions at 15 ºC until colonies became visible (usually within 3 d). The 66 colonies were streaked for purity on RCM blood agar and PYGS, both incubated anaerobically at 15 ºC. The isolates were viewed under a microscope for morphology, Gram reaction and for the presence of endospores (Broda *et al.*, 1996). Once isolates were chosen, they were subjected to screening tests including growth in the presence of oxygen, cell morphology, presence of catalase, and presence of oxidase (Weenk *et al.*, 1991).

2.4 Molecular Analysis

2.4.1 Confirmation of the Presence of *Clostridium* spp.

To confirm the presence of *Clostridium* spp., species-specific polymerase chain reaction (PCR) was used. To destroy viable cells, one 5-mL sample of purge was subjected to heat treatment and another 1-mL sample was subjected to ethanol treatment. A1-mL sample of the purge, as well as a purge sample from the AAFC Meat Research Centre Lacombe did not undergo any treatment. The samples were grown in PYGS broth. There was also one purge sample that did not undergo any treatment. The isolates from RCM, egg yolk agar, and the reference strains *Clostridium* spp. were also subjected to species-specific PCR. Genomic DNA was isolated from each sample using DNeasy Blood and Tissue Kit (Qiagen; Mississauga, ON). The manufacturer's recommended protocol for isolation of nucleic acid from Gram positive bacteria was followed, with modification to the lysis procedure. To improve lysis, the cells were resuspended in enzymatic lysis buffer (50 mg/mL lysozyme; Sigma Aldrich) and incubated at 37 ºC for 90 min. From this step the recommended protocol was followed. A sample 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) was used. The eluted DNA was stored at -20 ºC pending PCR amplification.

PCR amplification was performed using three primers used by Broda *et al.* (2003), one primer used by Boerema *et al.* (2002) and primers 16CFF/CFR,

16FRIF/FRIR were designed using IDT Primer Quest (see Table 2.1). The primers were based on the 16S rDNA sequence obtained from GenBank.

Table 2.1: Species-specific primers used to detect "blown-pack" spoilage microorganisms in the meat purge obtained from a meat package

Primer Name	Sequence	Expected size	Species Detected	Source
16 SEF/SER	F: 5′- TCG GAA TTT CAC TTT GAG -3′ R: 5′- AAG GAC TTC ACT CAT CTC TG -3′	790 bp	*Cl. estertheticum*	Broda *et al.* (2003)
16 SDBF/SDBR	F: 5′- GAG AGG AGT TCT TCG GAA CGA -3′ R: 5′- AAG CSA CTT CCC CAA TTA C -3′	935 bp	*Cl. gasigenes*	Broda *et al.* (2003)
16 EISRF/EISRR	F: 5′- GTA GAT GTA TTG ACT TCG G - 3′ R: 5′- ACC TAT AAC CAC TCT CGT -3′	230 bp	*Cl. estertheticum*	Broda *et al.* (2003)
16 CAF/CAR	F: 5′- AGT TAT TCC TTC GGG RA - 3′ R: 5′- ACG GAG GAT TGG TAT CC -3′	756 bp	*Cl. algidicarnis, Cl. putrefaciens*	Boerema *et al.* (2002)
16 CFF/CFR	F: 5′- TTT ACT GGG CGT AAA GGG TGC GTA - 3′ R: 5′- TGC GGG ACT TAA CCC AAC ATC TCA -3′	541 bp	*Cl. frigidicarnis*	GenBank Accession: AF06942
16 FRIF/FRIR	F: 5′- GTA GAT GTA TTG ACT TCG G - 3′ R: 5′- TGC GGG ACT TAA CCC AAC ATC TCA-3′	299 bp	*Cl. frigidicarnis*	GenBank Accession: AF06942

The PCR protocol obtained from Broda *et al.* (2002) was modified. Each PCR reaction mix consisted of: 10 µL of 10X PCR buffer, 3 µL of $MgCl_2$ (50 nM), 0.5 µL of each primer (forward and reverse), 0.3 µL of each deoxynucleoside triphosphate (dNTPs; Invitrogen), 0.5 µL of Taq polymerase (Invitrogen), 10 µL of template DNA, balance Milli-Q water for a total volume of 100 µL. Amplifications were performed in a thermal cycler (GeneAmp, Applied Biosystems, Foster City, CA). The initial denaturation was done at 93 ºC for 3 min. Each DNA amplification cycle consisted of: denaturation for 1 min at 92 ºC,

annealing for 1 min at 55 ºC, and extension for 2 min at 72 ºC. Target DNA was amplified in 30 cycles. The final extension was completed at 72 ºC for 3 min. PCR products were held at 4 ºC until further analysis.

PCR products were separated by gel electrophoresis in 2.0 % (w/v) agarose gel at 90 V for 1.5 h. A 1 Kb Plus DNA molecular weight marker was used as a size marker (Invitrogen). The gel was stained with ethidium bromide and was visualised using a UV transilluminator. Three replicates of DNA isolation and species specific PCR were done.

2.4.2 Characterising Pure Culture Isolates

Once confirmation of presence of *Clostridium* spp. was completed, analysis of 16S rDNA was done. The reference strains used for molecular comparison included *Cl. frigidicarnis* ATCC BAA154 and ATCC BAA155 and *Cl. putrefaciens* ATCC 25786.

Using the same methods described for species-specific PCR, DNA isolation from reference and meat isolates was done on exponentially growing cells harvested from PYGS broth incubated for 17 h at 30 ºC.

To analyse the 16S rDNA gene, PCR was performed using universal (eu) bacterial primers. 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'

(Hutson *et al*. 1993). These primers are complementary to the conserved region of the 16S rDNA gene. The genomic DNA isolated from each culture was used as the template. PCR was completed using the modified Broda *et al.* (2002) protocol as previously described.

For restriction length polymorphism analysis (RFLP), PCR-amplified 16S rDNA of the reference (type strains) and meat strains were digested with *Alu*I, *Hha*I, and *Hae*III endonucleases (Invitrogen). 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 (Broda *et al*., 2002). The mixtures were incubated overnight at 37 ºC.

Products of the restriction digest were separated by electrophoresis in 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 ethidium bromide and banding patterns were visualised using a UV transilluminator. Restriction of PCR products was replicated in triplicate.

Sequencing of the 16S rDNA of two clonal isolates, isolated from RCM blood agar, was done by to the Macrogen (Rockville, MD). Primers (pH* and pA) were diluted to a concentration of 1.6 pmol/µL. 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 (NCBI and Ribosome Project). Sequencing was done twice for each microorganism.

2.5 Inhibition of *Clostridium* spp. by Bacteriocin-producing Microorganisms

To determine if the isolate from "blown-pack" spoiled meat was susceptible to bacteriocin-producing cultures, spot-on-lawn and deferred inhibition assays (Gursky *et al.*, 2006) were done. Stock cultures of *Cl. frigidicarnis* ATCC BAA154 *Cl. frigidicarnis* ATCC BAA155, *Cl. putrefaciens* ATCC 25786 and the "blown-pack" meat isolate were grown in PYGS broth and stored at 21 ºC for 24 h in the anaerobic hood. Endospores were prepared by inoculation of cooked meat medium (Becton, Dickinson and Company), which was incubated at 21 ºC in an anaerobic hood until the medium turned black. To prepare endospores of the presumptive *Clostridium* spp. and control (ATCC) strains for use in deferred inhibition and for spot-on-lawn assays, 1 mL portions of inoculated cook meat medium were heated at 80 ºC for 10 min to destroy vegetative cells. Presence of endospores was verified using phase contrast microscopy.

Stock cultures of *Brochothrix campestris* ATCC 43754, *Carnobacterium maltaromaticum* UAL307, *Leuconostoc gelidum* UAL187, *Pediococcus acidilactici* PA1.0 were grown in All Purpose Tween broth (APT, Becton, Dickinson and Company, Sparks, MD) and incubated at 21 ºC for 24 h. All bacteriocin-producing cultures were maintained in APT broth and 80% glycerol and stored at -80 °C. *C. maltaromaticum* UAL26 was grown in APT broth and

incubated at 16 ºC for 48 h to stimulate bacteriocin production in a broth culture (Gursky *et al.*, 2006).

For deferred inhibition assays, producer strains were spotted (2 to 5 µL) onto APT agar and incubated aerobically at 21ºC for 24 h. The plates were moved into the anaerobic hood and left for at least 2 h prior to further experimentation. PYGS soft agar (5 mL) was seeded with the vegetative cells (10^4 CFU/cm^2) or endospores (10^4 CFU/cm^2) of the indicator strains. Endospores were obtained from cultures grown in cooked meat medium. APT plates with the producer strains were overlayered with the soft agar inoculated with either endospores or vegetative cells of *Clostridium* spp. The plates were incubated at 21 ºC for 24 h (vegetative cells) or 72 h (endospores). Each assay was done in duplicate and three replicates of the experiment were completed.

For spot-on-lawn assays, a 24-h culture of the bacteriocin-producing cultures were centrifuged at 1600 x *g* for 15 min at 4 ºC and the supernatant was transferred into a sterile test tube. The supernatant was heated in a water bath at 65 ºC for 30 min to kill vegetative cells. The supernatant was spotted (2 x 5 µL) onto APT plates and allowed to dry. Protease (5 µL of 1 mg/mL solution in 50 mM tris-HCl buffer) was spotted next to the supernatant and allowed to dry. The plates were moved into the anaerobic hood and left for at least 2 h prior to use in the experiment. PYGS soft agar (5 mL) was seeded with the vegetative cells (10^4 CFU/cm^2) or endospores (10^4 CFU/cm^2) of the indicator strains (50 µL). The

APT plates were overlayered with the seeded soft agar. The plates were incubated at 21 ºC for 24 h (vegetative cells) or 72 h (endospores). Each assay was done in duplicate and three replicates of the experiment were completed.

2.6 Confirmation and Control of "Blown-pack" Spoilage

To confirm the ability of the isolate from the commercially spoiled "blown-pack" meat to cause "blown-pack" spoilage, the isolate was added to meat and stored for a total of 84 d. To determine the effect of the background microflora on the growth of the BP isolate on the meat, the meat was prepared aseptically and non-aseptically. A biopreservative culture was added to aseptically prepared meat as a method of controlling "blown-pack" spoilage.

Boneless inside round of beef was obtained from a federally inspected meat processing facility. The inside rounds were stored at -20 ºC and thawed overnight at 4 ºC prior to use. To prepare aseptic meat, the meat was immersed in ethanol and flamed. The outside was trimmed and the remaining meat was immersed in ethanol and flamed a second time. The meat was transferred to a sterilised cutting board. The charred surfaces were trimmed using sterile instruments. The meat was cut into steaks, approximately 9 x 9 x 2 cm using a Berkel X13 meat slicer (Berkel, Louisville, KY). The meat slicer was sanitised with MATRIXX (Ecolab Food and Beverage, Mississauga, ON) and 70% ethanol. To prepare non-aseptic steaks the inside rounds were trimmed without any surface sterilisation, sliced, and the steaks were placed into a sterile bag. Purge and trim from the package

was added to the bag and the meat was massaged to ensure even distribution the background microflora. Steaks were placed individually into 3 mil nylon/poly coex vacuum bags (Unipac Packaging Products Ltd., Edmonton, Canada) with an oxygen transmission rate of 52 cc/m^2/24 h at 0 % r.h. For the aseptic samples, this was completed as the steaks were sliced. For the non-aseptic steaks, this was done after the massaging of the steaks. The bags were moved to a laminar flow room for inoculation of the steaks.

Cl. frigidicarnis ATCC BAA154 and the presumptive *Clostridium* isolate were grown as described previously to produce endospores. Prior to inoculation, endospores were heated at 80 ºC for 10 min and diluted to a concentration of log 5 CFU/mL. *C. maltaromaticum* UAL307 was grown in APT broth at 21 ºC for 24 h. Before inoculation, *C. maltaromaticum* UAL307 was diluted to a concentration of log 6 CFU/mL. There were eight treatments in total that included aseptically prepared meat inoculated with *Clostridium* isolate (this study); aseptically prepared meat inoculated with *Cl. frigidicarnis* ATCC BAA154; aseptically prepared meat inoculated with salinated peptone (0.1% peptone, 0.85% sodium chloride; control); aseptically prepared meat inoculated *Clostridium* isolate (this study) and *C. maltaromaticum* UAL307; aseptically prepared meat inoculated *C. maltaromaticum* UAL307; non-aseptically prepared meat inoculated with *Clostridium* isolate (this study); non-aseptically prepared meat inoculated *Cl. frigidicarnis* ATCC BAA154; and non-aseptically prepared sterilised salinated peptone (control).

The required volume of endospores, *C. maltaromaticum* or sterile 0.1% peptone with 0.85% NaCl added (150 µL) was pipetted onto each side of the steaks for a total of volume 300 µL. The *Clostridium* endospores were inoculated so that the concentration on the meat was approximately 10^4 CFU/cm^2 and the concentration of *C. maltaromaticum* was approximately 10^5 CFU/cm^2. The inoculum was evenly distributed by gently massaging the steaks in the package. The packages (total of 144 per replicate) were vacuum packaged on a Multivac C200 (Multivac, Kansas City, MO). Duplicates of each treatment were made. The steaks were stored at 2 ºC in a walk in cooler. Operating temperatures were monitored by TempLog Dataloggers (Oakton, Vernon Hills, IL). The storage trial was terminated after 84 days. Three replicates of the storage experiments were completed.

Packages were monitored for the first sign of "blown-pack" spoilage, indicated by the presence of gas bubbles in the purge (Broda *et al.*, 2002) and inspected for changes in colour. Samples were opened in the anaerobic hood operating at 21 ºC. Two packages of each treatment were sampled at day 0, 14, 28, 42, 56, 63, 70, 77 and 84. Purge (1 mL) was collected from each package and stored in a sterile eppendorf tube at -20 ºC, pending HPLC analysis. The remaining meat and purge were used for microbial analysis.

To determine counts of anaerobic bacteria, pre-reduced dilution fluid (90 mL) was added to the packages containing the steak and any purge that was left in the

package. The sample was massaged by hand in the anaerobic hood for 2 min prior to preparation of serial dilutions of the homogenate in dilution fluid. Appropriate dilutions were made and 0.1 mL of each dilution was spread onto pre-reduced RCM. These plates were incubated at 21 ºC for 7 d and colonies were enumerated.

After sampling for anaerobic bacteria, the packages were moved out of the anaerobic hood and serial dilutions of homogenate were prepared in dilution fluid to enumerate the aerobic microorganisms. Appropriate dilutions (0.1 mL in volume) were spread onto PCA and APT plates. The PCA and APT plates were incubated at 21 ºC and enumerated after 48 h. VRBG counts were done as pour plates to enumerate *Enterobacteriaceae*. The plates were incubated at 35 ºC for 24 h. All counts were done in duplicate. Bacterial counts were converted to log CFU/cm^2 and statistical analysis was completed on the counts using Statistical Analysis Software (SAS).

The BP isolate was isolated from packages and PCR-RFLP was completed as previously described to verify the presence of the microorganism in the meat.

The purge samples chosen for HPLC analysis included: the original purge sample and purge collected on 0, 42, and 84 d for each replicate. HPLC analyses were completed as previously described. SigmaPlot was used to obtain graphs from the refractive index and UV detection.

3. Results

3.1 Characterisation of Spoilage

The meat, obtained from a local processor, was approximately 6 weeks old on receipt and had not been subjected to temperature abuse. The meat package exhibited gross pack distension (Figure 3.1). The exterior surface of the lean tissue was dark and purple and when cut the interior was pink. When a sample of meat exposed to oxygen, the meat turned grey. A large amount of purge had collected in the "blown" vacuum package and this was collected for analysis. The odour of the meat and purge was extremely offensive. Odours of the spoiled meat were described as "cheesy", "dairy" and "putrid". Endospores were observed as refractive bodies under contrast microscope.

Figure 3.1: Commercial beef exhibiting "blown-pack" spoilage

3.2 Profiling of Organic Acids and Alcohols Using HPLC

Quantitative analysis of organic and alcohols showed that butyric acid, acetic acid, propanol and butanol were present in the purge collected from the distended vacuum package. Table 3.1 lists the concentration of volatiles found in the

commercial vacuum packaged beef. The most prevalent metabolite found was butyric acid, which was probably responsible for some of the putrid smells permeating from the "blown" packages. Acetic acid and butanol were also found in the purge. Propanol was detected but was deemed to be insignificant compared to the other metabolites present in the purge sample.

Table 3.1
Concentration of organic acids and alcohols in the purge obtained from spoiled vacuum packaged beef obtained from a commercial processor

Volatiles	**Concentration (mM)**
Acetic acid	21.6
Butyric acid	76.3
Butanol	24.3
Propanol	< 1

The retention times for acetic acid and butyric acid fell at 21 and 28 min, respectively (Figure 3.2). There are two substantial peaks at 22 and 24 min that may be due to the presence of a three-carbon organic acid or a hydroxyl-four-carbon organic acid. Butanol was observed at approximately 48 min and the propan-1-ol peak at 38 min (Figure 3.2). Propan-2-ol was not present in the purge. The propan-1-ol peak was smaller than that of butanol. Propan-1-ol was present in small quantities in the purge sample of "blown" vacuum packaged beef.

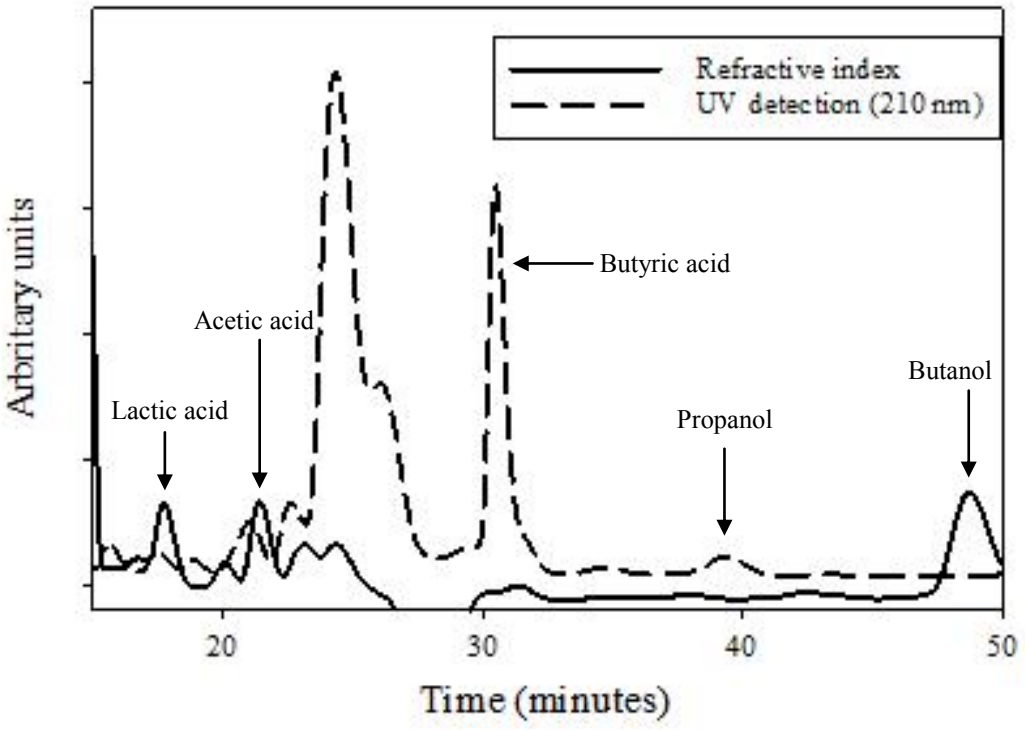

Figure 3.2: HPLC chromatographs of organic acids and alcohols present in the purge obtained from "blown" vacuum packed beef. Refractive index is used to monitor alcohols and UV detection at 210 nm was used to detect organic acids.

3.3 Enumeration of Aerobic Spoilage Microflora

The counts for aerobic bacteria in the meat and purge samples can be found in Table 3.2. Total aerobic counts were high but not as high as the total count for LAB meaning that LAB was the dominant microflora on the meat. *Enterobacteriaceae* were not detected by surface plating on VRBG, but after storage of the dilutions for 24 h at 23 °C and plating the stored dilutions onto VRBG, they were detected. The purge had slightly higher counts (1 log) than that found on the meat samples on STAA agar, which is selective for *Brochothrix thermosphacta*.

Table 3.2

Counts of the aerobic microflora (log CFU/mL) in the purge and meat samples obtained from "blown" vacuum packed beef.

	Incubation Temperature (°C)	**Meat (log CFU/mL)**	**Purge (log CFU/mL)**
Total aerobic bacteria	25	7.8	8.2
LAB	25	7.1	7.5
Enterobacteriaceae	37	TFTC	TFTC
Brochothrix thermosphacta	25	3.9	4.6

TFTC – too few to count

3.4 Enumeration of Anaerobic Bacteria

Anaerobic bacteria from meat and purge samples were enumerated on RCM agar (Table 3.3). Counts of anaerobic bacteria in the purge enumerated on RCM were slightly higher than those obtained from the meat samples. Total anaerobic counts were relatively high, reaching log 7.6 CFU/mL in the purge. When the sample of purge was either heated or treated with ethanol and plated on SFP agar, no growth of endospores was observed.

Colonies from the RCM plates were chosen based on colony morphology and characterisation of the colonies was done. The colonies were streaked onto CBA to ensure pure cultures were isolated. In total, 66 colonies were picked but only 26 isolates were strict anaerobes. Some of the remaining isolates were LAB. Colony morphology was noted and oxidase and catalase tests for 26 isolates were negative. Gram stains were prepared; however, due to the age of the cultures, most of the cells were Gram variable (results not shown). All cultures were able

to growth at both 15 and 30 ºC. Growth was observed on RCM and CBA medium.

Table 3.3
Counts of anaerobic bacteria in "blown" vacuum packed beef.

	Incubation Temperature (°C)	**Meat (log CFU/mL)**	**Purge (log CFU/mL)**
Total anaerobic bacteria (enumerated on RCM)	25	6.8	7.6
Endosporeformers – heat treated (enumerated on SFP)	30	ND	ND
Endosporeformers – ethanol treated (enumerated on SFP)	30	ND	ND

ND=not detected (with a detection limit of 2.0 log CFU/mL)

On SFP medium, there was some growth and the colonies were white indicating that no hydrogen sulphide was produced. Some colonies had clear zones indicating some of the cultures had lecithinase activity. Under a phase contrast microscope, the 26 isolates were observed as rods with terminal endospores. On the RCM agar, the colonies were slightly raised, circular white with an irregular grey halo. When the isolates that formed endospores and *Cl. frigidicarnis* ATCC BAA154, *Cl. frigidicarnis* ATCC BAA155, and *Cl. estertheticum* ATCC 51254 were grown on the cooked meat medium, the amount of gas produced was substantial enough to lift the cooked meat medium from the bottom of the test tube. There was also an abundance of gas production observed when these microorganisms were grown in PYGS medium.

3.5 Detection of *Clostridium* spp. in the Purge

Species-specific PCR was used to verify the presence of *Clostridium* spp. in the purge from the commercially spoiled meat. The purge was enriched to increase the numbers of *Clostridium* spp. and to increase DNA isolation efficiency. A total of six primers were used to detect the presence of *Clostridium* spp. Amplification of the DNA extracted from the isolates that formed endospores, enriched purge present in "blown-pack" spoiled meat, and ethanol or heated purge samples using 16SEF/16SER, 16SDBF/16 SDBR, and 16EISRF/EISRR primers was completed. These primers were used to detect *Cl. estertheticum* and *Cl. gasigenes*. Two primer pairs were used to detect *Cl. estertheticum*: 16SEF/16SER and 16EISRF/16EISRR. The expected sizes of the PCR products were 790 bp and 230 bp, respectively.

Purge samples that were enriched, heat treated and treated with ethanol gave the expected size of PCR products for *Cl. estertheticum* (Figure 3.3). The ATCC cultures and the isolates that formed endospores, as well as isolates obtained from SFP medium did not produce any bands using the *Cl. estertheticum* primer sets. There was no *Cl. gasigenes* detected in any of the samples. Amplifications with DNA extracted from the isolates that formed endospores, purge that had been enriched, and ethanol or heated purge samples using 16 CAF/CAR, 16 CFF/CFR, and 16FRIF/FRIR primer pairs was also completed. The expected sizes for the PCR products were 756 bp, 541 bp and 299 bp, respectively.

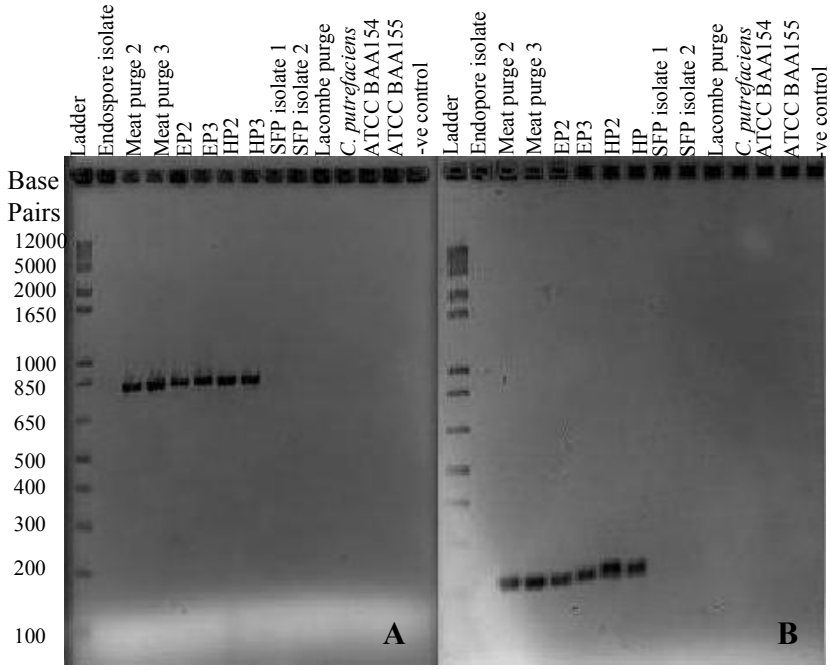

Figure 3.3: Banding patterns using species-specific primers for *Cl. estertheticum.* Panel A and B: primers (16SE and16EISR) used to detect *Cl. estertheticum*. The endospore isolate is the isolate that was obtained from RCM blood agar. The designation meat purge indicates that the purge had been incubated overnight at 30°C anaerobically in PYGS. EP was the ethanol treated purge sample. HP was a heated purge sample. Lacombe purge was a purge sample that was obtained from the AAFC Meat Research Centre. SFP isolates were samples of DNA isolated from strains obtained from SFP agar. *Cl. frigidicarnis* ATCC BAA154 and BAA155 and *Cl. putrefaciens* ATCC 25786 were used as reference strains. The negative control was a sample with PCR reaction mixture and water.

Cl. putrefaciens and *Cl. algidicarnis* were detected using 16CAF/CAR. These two microorganisms are genetically closely related. There were no bands present at 756 bp for the reference strains *Cl. frigidicarnis* BAA154, *Cl. frigidicarnis* ATCC BAA155, *Cl. estertheticum* ATCC 51254 (Figure 3.4). There was a band for *Cl. putrefaciens* ATCC 25786. Purge samples that were enriched, heated or treated with ethanol gave the expected size of PCR products for *Cl. putrefaciens* and *Cl. algidicarnis*. *Cl. frigidicarnis* was detected using 16CFF/CFR and 16FRIF/16FRIR primer sets. All purge samples produced the expected PCR

products. The BP isolate also gave the PCR products expected for all three microorganisms. The reference strains produced expected PCR products for primers CFF/R and FRIF/R. Even *Cl. estertheticum* ATCC 51254 produced bands at 541 bp and 299 bp.

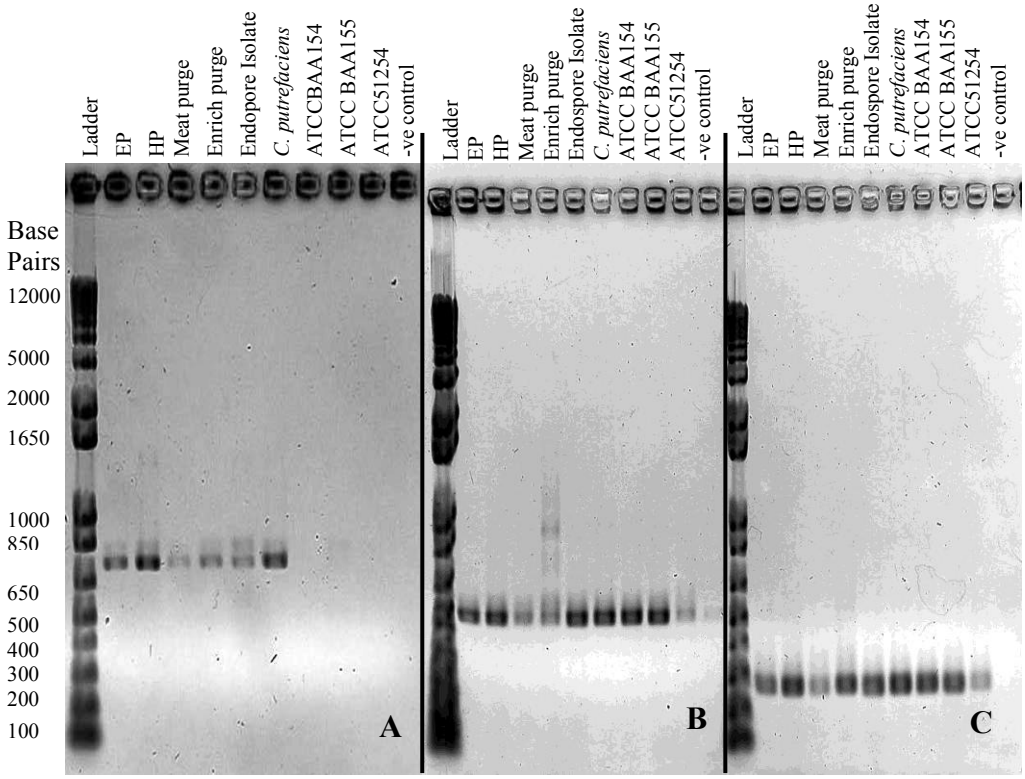

Figure 3.4: Banding patterns using species-specific primers for *Cl. algidicarnis*, *Cl. putrefaciens*, and *Cl. frigidicarnis*. Panel A: primers (16CA) used to detect *Cl. algidicarnis* and *Cl. putrefaciens*. Panel B and C: primers (16CF and 16FRI) used to detect *Cl. frigidicarnis*. Meat purge was purge collected that did not undergo any treatment. Enrich purge is purge that had been enriched using PYGS. EP was the ethanol treated purge sample. HP was a heated purge sample. *Cl. putrefaciens* ATCC 25786, *Cl. frigidicarnis* ATCC BAA154 and BAA155, and *Cl. estertheticum* ATCC 51254 were used as reference strains. The negative control was a sample with PCR reaction mixture and water.

3.6 RFLP Analysis of 16S rDNA Isolated from "Blown-pack" Meat

To identify clonal isolates within the 26 anaerobic isolates, RFLP analysis was completed. DNA was successfully isolated from the 26 strict anaerobes and the 16S rDNA was amplified using PCR. The PCR product for all the isolates and cultures was approximately 1650 bp (results not shown). Initial examination of banding patterns indicated that all 26 isolates were clonal isolates (results not shown). The isolate was referred to as "BP isolate" to indicate its origin from "blown-pack" spoiled meat.

RFLP analysis of the 16S rDNA produced complex banding patterns (Figure 3.5). Three restriction endonucleases were used: *Alu*I, *Hae*III and *Hha*I (Broda *et al.*, 2000a). The BP isolate and *Cl. putrefaciens* ATCC 25786 had the same banding pattern when digested with *Alu*I. Both strains of *Cl. frigidicarnis* had similar banding patterns when digested with *Alu*I. The banding pattern for the BP isolate did not match that obtained with *Cl. frigidicarnis* regardless of the restriction enzyme used. The banding pattern obtained when *Hae*III and *Hha*I were used produced slightly different results. With the *Hae*III restriction enzyme, there was a light band at 230 bp with *Cl. putrefaciens* ATCC 25786 but this band was not present in the digest for the BP isolate. The banding pattern was also different when *Hha*I was used as there was one band present in the restriction digest of the BP isolate that was not present in the restriction digest of *Cl. putrefaciens* ATCC 25786.

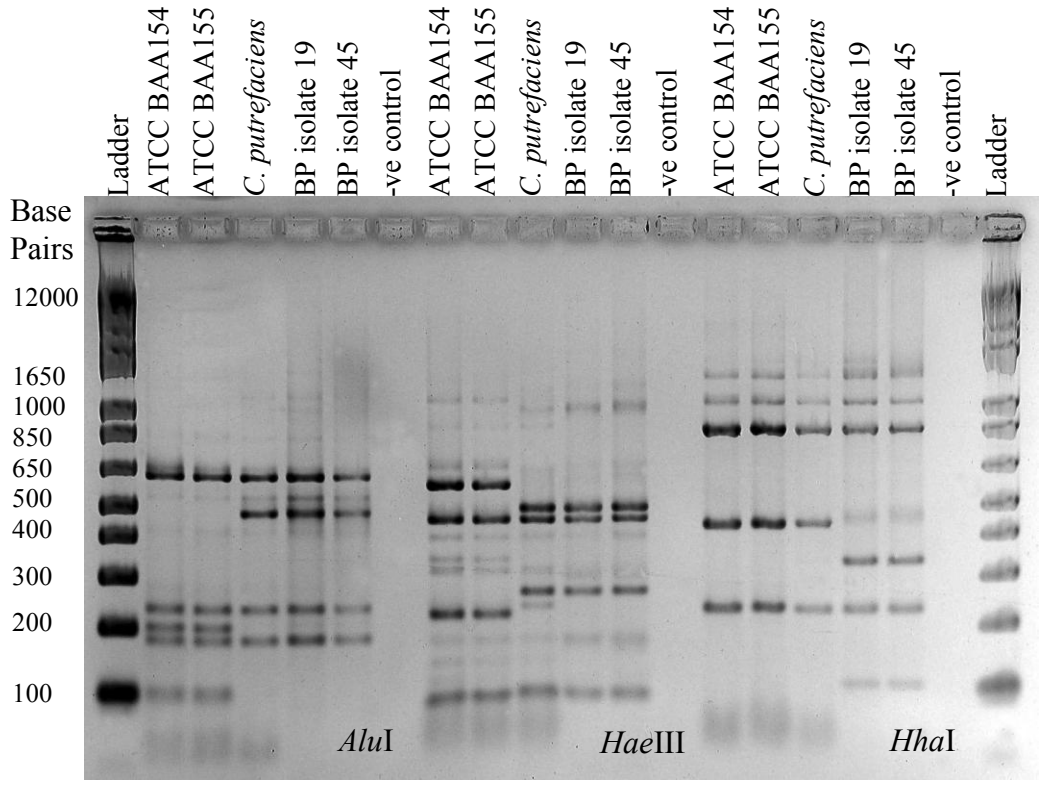

Figure 3.5: Restriction patterns of anaerobic isolates using *Alu*I, *Hae*III, and *Hha*I restriction endonucleases. *Cl. frigidicarnis* ATCC BAA 154 and BAA 155, *Cl. putrefaciens* ATCC 25786 were used as reference strains. BP isolates are the isolates that formed endospores obtained from the "blown-pack" spoiled meat. The negative control was a sample with PCR reaction mixture and water.

To determine the sequence obtained from the PCR product of the 16S rDNA from the BP isolate, the sequence was compared to type strains using gene databases (NCBI and Ribosome Project). Comparison of the sequence obtained from the PCR product identified the isolate to be *Clostridium putrefaciens* DSM1291 (accession number of NR_024995.1). The BP isolate 16S rDNA sequence, with a query length of 1050 base pairs, matched with an identity of 97%. Sequencing of the aerobic organisms isolated on the RCM were identified as LAB. Some of the LAB were identified to be *Carnobacterium* spp.

3.7 Control of "Blown-pack" Spoilage with a Bacteriocin-producing LAB

Most of the bacteriocin- producing cultures tested had an inhibitory effect against the *Clostridium* spp. The zone of inhibition for *C. maltaromaticum* UAL26 was very small compared to that obtained with *C. maltaromaticum* UAL307 (Figure 3.6). *L. gelidum* UAL187 had more activity against the vegetative cells of *Cl. frigidicarnis* ATCC BAA154 compared to *P. acidilactici* PA1.0, *B. campestris* ATCC 43754 and *C. maltaromaticum* UAL26. However, there was no activity against the endospores of the same microorganism. Only *C. maltaromaticum* UAL307 had defined zones of inhibitions against both vegetative cells and endospores of the *Clostridium* spp. tested.

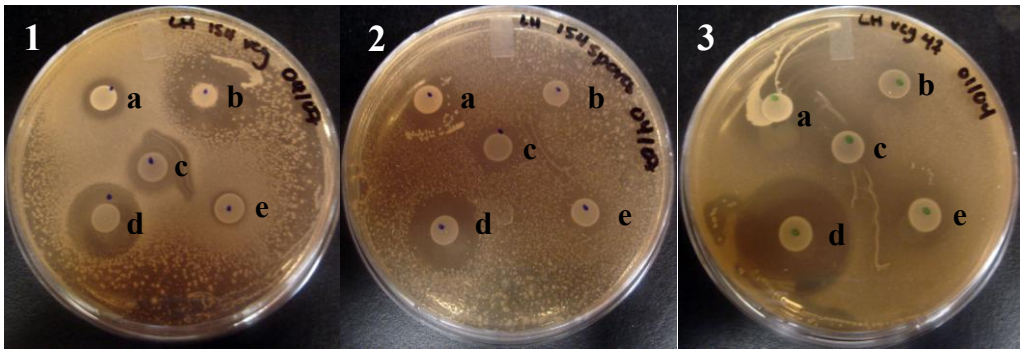

Figure 3.6: Antimicrobial activity of bacteriocin-producing cultures (a) *P. acidilactici* PA1.0, (b) *L. gelidum* UAL187, (c) *B. campestris* ATCC 43754. (d) *C. maltaromaticum* UAL307, (e) *C. maltaromaticum* UAL26 used in a deferred inhibition assay against (1) vegetative cells of *Cl. frigidicarnis* ATCC BAA154, (2) endospores of *Cl. frigidicarnis* ATCC BAA154, (3) vegetative cells of BP isolate.

Results of deferred inhibition assays with vegetative cells and endospores are shown in Table 3.4. *B. campestris* ATCC 43754 only inhibited the growth of vegetative cells of *Cl. frigidicarnis* ATCC BAA154, *Cl. putrefaciens* ATCC 25786 and the BP isolate obtained in this study. *C. maltaromaticum* UAL307 and UAL26 inhibited the growth of all of the vegetative cells of *Clostridium* spp. used in this study. *L. gelidum* UAL187 and *P. acidilactici* PA 1.0 did not inhibit the growth of the vegetative cells of *Cl. frigidicarnis* ATCC BAA155. All of the LAB used as bacteriocin producers inhibited the growth of the endospores of *Cl. frigidicarnis* ATCC BAA154 and the BP isolate. Unfortunately, endospores of *Cl. frigidicarnis* ATCC BAA155 and *Cl. putrefaciens* ATCC 25786 did not produce a confluent lawn were inhibition zones could be observed.

When spot-on-lawn assays with each of the producer strains were done, *Clostridium* spp. were not inhibited with the exception of the BP isolate. The BP isolate was inhibited by the supernatant obtained from *C. maltaromaticum* UAL307. Based on the results of spot-on-lawn and deferred inhibition assays, *C. maltaromaticum* UAL307 was chosen to determine if it would control "blown-pack" spoilage in meat.

Table 3.4
Results of deferred inhibition assays with vegetative cells and endospores of *Clostridium* spp. *B. campestris* ATCC 43754, *C. maltaromaticum* UAL307 and UAL26, *L. gelidum* UAL187 and *P. acidilactici* PA1.0 were used as bacteriocin-producing strains.

Strain of clostridia	Bacteriocin producers				
	B. campestris ATCC 43754	*C. maltaromaticum* UAL 307	*C. maltaromaticum* UAL26	*L. gelidum* UAL 187	*P. acidilactici* PA 1.0
Vegetative cells:					
Cl. frigidicarnis ATCC BAA154	+++	+++	+++	NI	+++
Cl. frigidicarnis ATCC BAA155	NI	+++	+++	NI	NI
Cl. putrefaciens ATCC 25786	+++	+++	+++	+++	+++
BP isolate (this study)	+++	+++	+++	NI	+++
Endospores:					
Cl. frigidicarnis ATCC BAA154	NI	+++	+++	+++	+++
Cl. frigidicarnis ATCC BAA155	NG	NG	NG	NG	NG
Cl. putrefaciens ATCC 25786	NG	NG	NG	NG	NG
BP isolate (this study)	NI	+++	+++	+++	+++

NI – no inhibition; NG – no growth; +++ – inhibition

3.8 Confirmation of "Blown-pack" Spoilage and use of a Biopreservative Culture

To confirm that the "BP isolate" obtained from spoiled vacuum packaged beef was capable of causing "blown-pack" spoilage, the endospores were inoculated onto aseptically and non-aseptically prepared beef. Samples were stored at 2 °C for 84 d. During storage the packages were examined for changes in colour and for gas production.

Initially, the colour was bright red and very little purge was observed. There was no gas present at day 0. During storage of the meat, the colour changed from bright red to pale pink to brown. There was also accumulation of purge in the packages as the storage time continued. The gas production was first observed on day 42 in the packages containing non-aseptically prepared meat that were inoculated with *Cl. frigidicarnis* ATCC BAA154. By the end of the study, the only packages in which gas production was observed were samples inoculated with either the ATCC strain or the BP isolate. The production of gas was not enough to cause the distension that was observed in the commercially spoiled vacuum packaged beef. Packages containing aseptically prepared meat inoculated with endospores also showed signs of gas production but it was observed only after 56 d of storage. Control samples and packages with meat inoculated with *C. maltaromaticum* UAL307 did not show any signs of gas production in the packages.

The total aerobic counts enumerated on PCA were different between the aseptically and non-aseptically prepared meat samples (Figure 3.7). After 28 d of storage, there was a 5 to 7 log CFU/cm^2 difference between the total aerobic plate counts obtained from aseptic and non-aseptically treated samples. After 56 d of storage, samples inoculated with *C. maltaromaticum* UAL307 had total aerobic plate counts of 8 log CFU/cm^2. The prevalent microorganisms that grew on the non-aseptically treated meat were LAB with counts equalling the counts of total aerobic bacteria. There was some increase in numbers of total aerobic and LAB for the aseptic control; however, most of the aseptically treated samples had total aerobic counts of 2 log CFU/cm^2. *Enterobacteriaceae* counts were higher in non-aseptically treated samples compared to aseptically treated samples. The samples inoculated with *C. maltaromaticum* UAL307 had counts of *Enterobacteriaceae* similar to those of the aseptically treated samples.

After 56 d of storage, the anaerobic counts determined on RCM were higher for the non-aseptically treated samples compared to the aseptically treated samples. The anaerobic counts from non-aseptically prepared control samples and those with *Cl. frigidicarnis* ATCC BAA154 or the BP isolate remained below 10^5 CFU/cm^2 throughout storage; however, anaerobic counts of aseptic samples inoculated with *Cl. frigidicarnis* ATCC BAA154 and the BP isolate and both samples inoculated with *C. maltaromaticum* UAL307 reached log 8 to 9 CFU/cm^2 by 63 d of storage. The counts of anaerobic bacteria of the aseptically prepared samples were slightly higher than the aerobic counts.

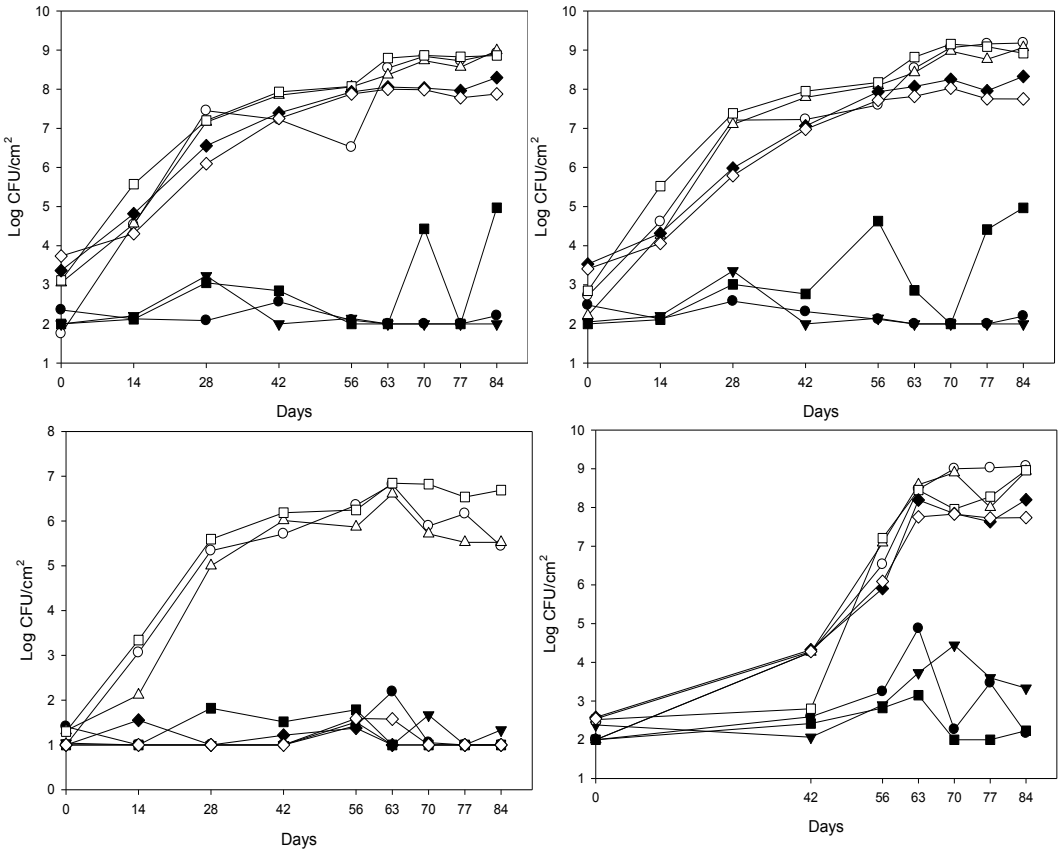

Figure 3.7: Counts of total aerobic bacteria (A), LAB (B), *Enterobacteriaceae* (C) and total anaerobic counts, (D) on aseptically prepared (solid symbols) and non-aseptically prepared (open symbols) beef inoculated with *C. frigidicarnis* ATCC BAA154 (circles), BP isolate (triangles), and uninoculated control samples (squares). Bacterial counts on samples inoculated with *C. maltaromaticum* UAL307 (open diamond) and *C. maltaromaticum* UAL307 and the BP isolate (solid diamond) were also included. n=3 with a standard error of 0.22.

PCR-RFLP analysis of microorganisms isolated at the end of the experiment verified the presence of original microorganism inoculated onto the meat (results not shown). Some of the isolates from the samples inoculated with *Cl. frigidicarnis* ATCC BAA154 and the BP isolate had banding patterns that were identical to those obtained from the pure culture strains used for inoculation. Some of the isolates did have unique banding patterns.

Metabolites in the meat purge were determined using HPLC. The purge sample from commercially packaged beef that had been stored for 24 months had a variety of metabolites that were different from the purge detected from fresh meat (Figure 3.8A). The major metabolites detected in commercially spoiled "blown-pack" meat included: acetic, propionic, butyric, isovaleric and valeric acids. Lactic acid and an unidentified metabolite were detected in the purge from a sample of fresh meat. Lactic acid was also the main metabolite detected in the purge of meat inoculated with *C. maltaromaticum* UAL307 (Figure 3.8B). Propionic, valeric, and acetic acids were also detected in meat inoculated with *C. maltaromaticum* UAL307. Higher concentrations of valeric acid were detected in meat inoculated with *C. maltaromaticum* UAL307 and stored for 70 d as compared to that detected in the purge from meat inoculated with the BP isolate and *C. maltaromaticum* UAL307.

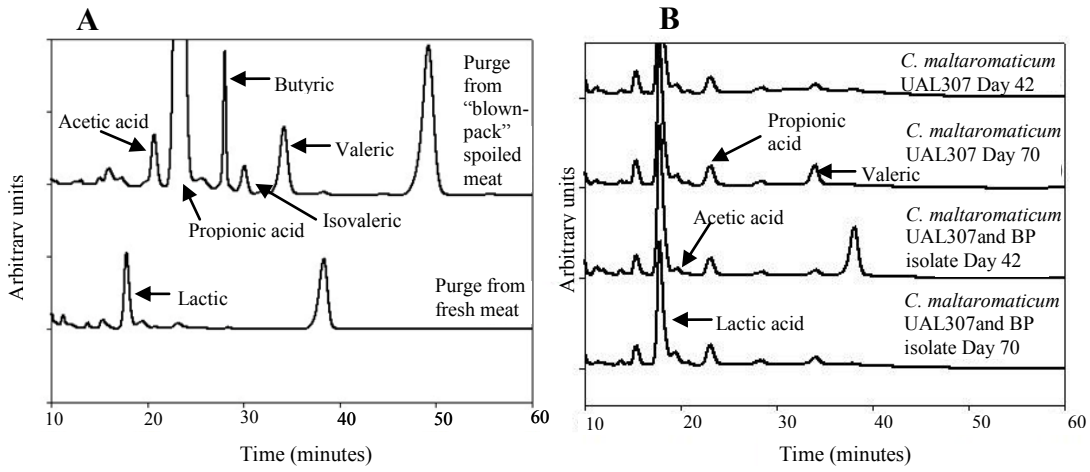

Figure 3.8: HPLC profile using UV detection of organic acids dissolved in purge obtained from (A) "blown" vacuum packaged beef and purge from fresh meat; and (B) from vacuum packaged meat inoculated with *C. maltaromaticum* UAL307 and *C. maltaromaticum* UAL307 and the BP isolate and stored for 42 or 70 d at 2 °C.

The major metabolites present in the meat purge obtained from aseptically prepared meat were lactic, acetic, propionic acid and butyric acids (Figure 3.9). There was no major difference in metabolites detected in samples stored for 42 (Figure 3.9A) or 70 d (Figure 3.9B). When comparing the different treatments within the group of samples made with aseptically prepared meat, the metabolites were the same but the relative amounts of each organic acid differed. Purge from samples inoculated with *Cl. frigidicarnis* ATCC BAA154 had larger peaks for acetic and propionic acids compared to those obtained from purge of samples inoculated with the BP isolate and the uninoculated control samples. The purge samples from meat inoculated with the BP isolate had slightly larger peaks than that of the control samples. When compared to the analysis of the purge from the commercially spoiled "blown-pack" meat sample, there are a few metabolites that were not detected in the inoculated samples. Isovaleric and valeric acid were not present in the aseptically treated samples. A much higher concentration of the butyric acid was present in the commercially spoiled "blown-pack" meat sample compared to that detected in the purge from aseptically treated samples inoculated with the BP isolate and/or *Cl. frigidicarnis* ATCC BAA154. Lactic acid was detected in all of the aseptically treated samples but was not detected in the purge from commercially spoiled "blown-pack" meat sample.

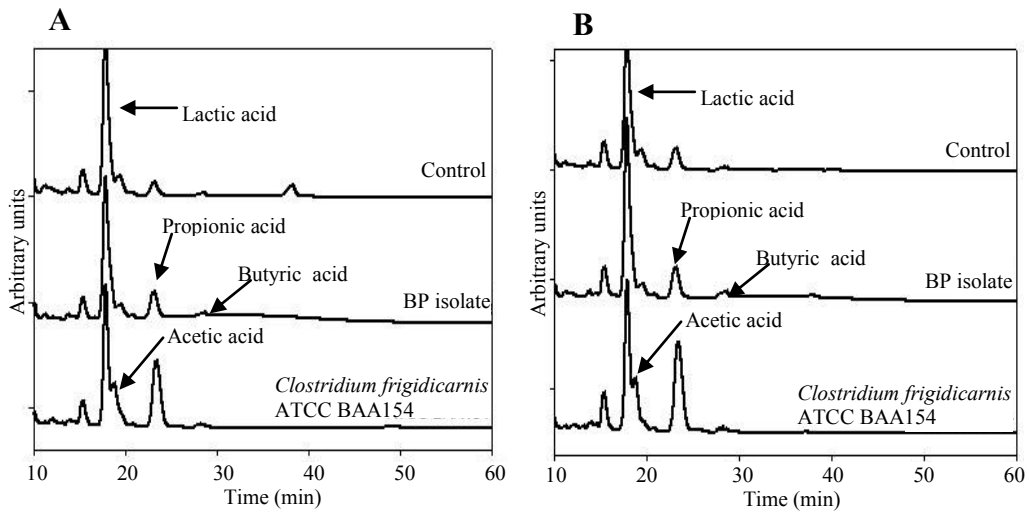

Figure 3.9: HPLC chromatographs of organic acids present in the purge obtained from aseptically prepared vacuum packaged meat stored for 42 (A) or 70 d (B). Samples included an uninoculated control, meat inoculated with the BP isolate and samples inoculated with *Cl. frigidicarnis* ATCC BAA154.

After 42 d of storage, the metabolites present in the purge from non-aseptically prepared meat included lactic, acetic, propionic, and butyric acids (Figure 3.10). After 42 d of storage, lactic, propionic and butyric acids were detected in the purge from the samples inoculated with the endospores of *Cl. frigidicarnis* ATCC BAA154 (Figure 3.10A). Acetic acid was only detected in samples inoculated with *Cl. frigidicarnis* ATCC BAA154. Purge from samples inoculated with *Cl. frigidicarnis* ATCC BAA154 had larger peaks for propionic acids compared to those obtained from purge of samples inoculated with the BP isolate and the uninoculated control samples. After 70 d of storage, isovaleric and valeric acid were present in non-aseptic meat inoculated with endospores of *Cl. frigidicarnis* ATCC BAA154. Valeric acid was also present in the purge from samples inoculated with the BP isolate. Butyric acid was present in day 42 and day 70 in all treatments. However, the amount of butyric acid was not present at

concentrations that were found in purge from commercial "blown-pack" spoiled beef. Also, lactic acid was present in the purge from non-aseptic samples where it was not present in the purge from the commercial "blown-pack" spoiled beef.

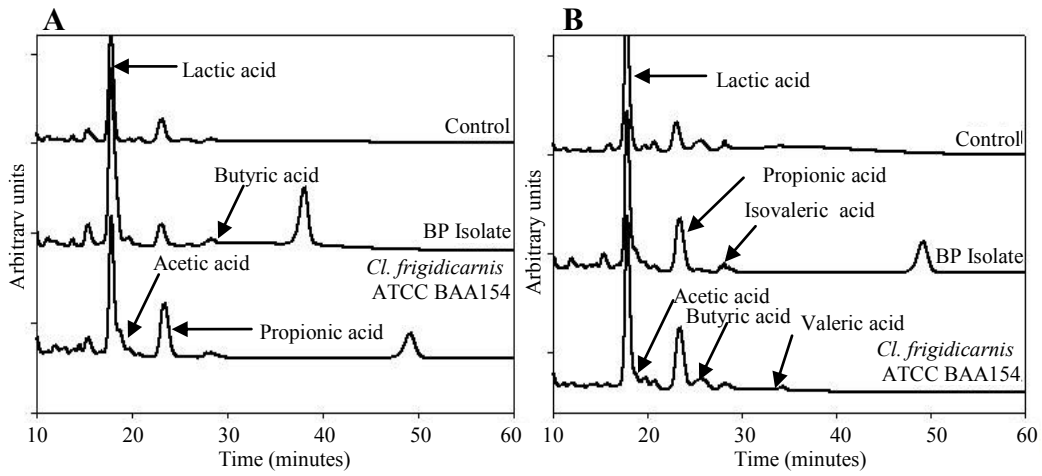

Figure 3.10: HPLC chromatographs of organic acids present in the purge obtained from non-aseptically prepared vacuum packaged meat stored for 42 (A) or 70 days (B). Samples included an uninoculated control, meat inoculated with the BP isolate and samples inoculated with *Cl. frigidicarnis* ATCC BAA154.

4. Discussion

Traditionally, *Clostridium* spp. have not been implicated as major cause of meat spoilage. Mesophilic *Clostridium* spp. have been implicated as the causative agent in deep tissue spoilage (also known as "bone taint") of beef and cured hams (Ingram, 1952; Callow and Ingram, 1955; Ross, 1965). More recently, the causative agent of "blown-pack" spoilage of vacuum packaged meats has been identified as being *Clostridium* spp. (Dainty *et al*., 1989; Kalchayanand *et al*., 1989; Broda *et al*., 1996). Compared to deep tissue spoilage, "blown-pack" spoilage occurs during chilled storage and in the absence of temperature abuse. *Cl. estertheticum* has been implicated in "blown-pack" spoilage of beef from southern Africa, northern Europe and North America (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Helps *et al.*, 1999; Yang *et al.*, 2009) and in vacuum packaged lamb from New Zealand (Broda *et al.*, 1996). *Cl. gasigenes*, a psychrotrophic microorganism, has only been implicated in "blown-pack" spoilage in vacuum packaged lamb from New Zealand (Broda *et al.*, 2002). The incidence of "blown-pack" spoilage of vacuum packaged beef is described as sporadic because spoilage only occurs in a fraction of the product packaged at the same time (Dainty *et al.*, 1989). Variability in contamination of the meat product may be the cause of the sporadic nature of "blown-pack" spoilage (Boerema *et al.*, 2002).

The commercial meat sample obtained from a local processor displayed characteristics typical of "blown-pack" spoilage. The vacuum packaged meat

exhibiting gross package distension, in the absence of temperature abuse is indicative of spoilage by psychotropic *Clostridium* spp. The production of offensive odours in spoiled vacuum packaged meat is not restricted to a single bacterial species. In the absence of oxygen and the presence of carbon dioxide and hydrogen, it is very likely that a fermentative metabolism occurs with butyric acid being the major metabolite (Jones and Woods, 1986). HPLC analysis of the purge from the commercial product revealed that one of the major products was butyric acid. The other major products were butanol and acetic acid. The presence of butyric and acetic acid is characteristic of "blown-pack" spoiled meat (Broda *et al.*, 1996). Butanol is also known to be produced by some *Clostridium* spp. (Broda *et al.*, 1996). Further analysis of the products in the purge, and identification of the gases in the headspace of the distended package could identify more of the metabolites produced by the spoilage microorganisms. The compounds associated with "blown-pack" spoilage include esters (especially butyl derivatives), sulphur-containing compounds, 1-butanol and butanoic acid (Dainty *et al.*, 1989). These compounds were not detected, with the exception of butanol, in the current study because the column used for HPLC analysis was for separation of organic acids.

Enterobacteriaceae were not detected on the commercially produced product unless the dilutions were incubated overnight prior to plating on selective agars, indicating that low numbers of these microorganisms were present. The low numbers of *Enterobacteriaceae* in the purge indicated that this group of microorganisms is not responsible for gas production and spoilage of the vacuum

packaged meat. The low numbers of *Enterobacteriaceae* suggests that the meat package was most likely not subjected to temperature abuse during its transport from the processing plan to the retailer (Broda *et al*., 1996). Commercial incidences of "blown-pack" spoilage of vacuum packaged meats have generally been due to temperature abuse which leads to the growth of gas-producing *Enterobacteriaceae*. *Serratia liquefaciens*, *Enterbacter aerogenes* and *Hafnia alvei* have been isolated in significant numbers and confirmed as gas producers in "blown-pack" spoiled vacuum packaged beef that have been temperature abused (Hanna *et al*., 1979). The low numbers of *Enterobacteriaceae* and the absence of temperature abuse indicated that the gas production was most likely the cause of a different microorganism. In contrast, Broda *et al.* (1996) found approximately log 3 CFU/mL *Enterobacteriaceae* on "blown-pack" spoiled vacuum packaged lamb, venison and beef.

Enumeration of aerobic microorganisms revealed high numbers of LAB. When grown on anaerobically stored products, LAB will become the prevalent microorganisms and thus anaerobic counts may not be representative of *Clostridium* spp. in the spoilage microflora (Broda *et al*., 1996). The numbers of LAB detected on the commercially packaged beef with "blown-pack" spoilage were similar to that found on "blown-pack" spoiled vacuum packaged beef (Broda *et al.*, 1996). The presence of *Brochothrix thermosphacta* is typical of spoiled meat. The numbers detected in the current study were not as high as the LAB numbers and thus these microorganisms were not part of the prevalent microflora.

B. thermosphacta counts were consistent with those found in "blown-pack" spoiled vacuum packaged lamb (Broda *et al.*, 1996).

Psychrotrophic *Clostridium* spp. are fastidious microorganisms and there has been previous reports that they can be difficult to recover from solid media (Dainty *et al*., 1989). There has also been some speculation that bacteriocin producing LAB may play a role in the poor recovery of psychrotrophic *Clostridium* spp. on solid media (Crandall and Montville, 1993). Of the 66 isolates, only 26 of the isolates recovered were strict anaerobes, which is a relatively low recovery rate of strict anaerobes from "blown-pack" spoiled meats. The high numbers of microorganisms on the samples used in the current study may have contributed to the difficulty in isolation of *Clostridium* spp. Crandall and Montville (1993) indicated that the LAB that are naturally present in meat produced bacteriocins. The bacteriocins were found to have an inhibitory effect on *Clostridium* spp. Other factors that have been implicated in the poor recovery of psychrotrophic *Clostridium* spp. include increased sensitivity to metabolites by some *Clostridium* spp. Butanol toxicity may suppress the recovery of microorganisms; especially when carbon sources become limiting after prolonged storage of meat (Cortinas *et al.*, 1994). Some *Clostridium* spp. will be sensitive to low concentrations of butanol, which are generally considered to be non-toxic. Cell injury can also to increased sensitivity of the selective agents present in a solid culture medium (Mossel and Van Netten, 1984). When enumerating the bacteria in the purge and on meat samples, the purge samples consistently had higher

counts than the meat samples. It is possible that the crevices of the muscle structure could have harboured the bacteria or endospores, making their recovery more difficult. Disruption of the structure of the meat is required to release the bacteria and endospores for enumeration. If psychrotrophic *Clostridium* spp. are fragile microorganisms, cell injury could play a role in recovery rates. Endospore enumeration media, such as SFP, can be used to give an approximate indication of the number of psychrotrophic *Clostridium* spp. that may be present on the meat and in the purge. However, the use of SFP has limitations, because *Bacillus* spp. may also grow and counts not be representative of the population of *Clostridium* spp. in the spoiled meat. Addition of ethanol or heat treatment before enumeration is not suitable because *Bacillus* endospores, like *Clostridium* endospores, will survive these treatments. In the current study, no microorganisms were isolated on SFP medium.

Species-specific PCR was used to detect the presence of *Clostridium* spp. in the "blown-pack" spoiled meat obtained from an Alberta processing plant. Results indicated the presence of *Cl. estertheticum*, *Cl. frigidicarnis*, *Cl. putrefaciens* and *Cl. algidicarnis* in the purge. *Cl. gasigenes* was not detected in the purge. *Cl. gasigenes* has the ability to produce enough gas to cause package distension in vacuum packaged lamb (Broda *et al.*, 2000b) and also produces the metabolites that are consistent with metabolites found in the gas and purge of "blown-pack" spoiled meats. The primer pair used to detect *Cl. frigidicarnis* was not specific enough because all ATCC reference cultures used produced bands with these

primers. Although *Cl. estertheticum* was detected using PCR, the microorganism could not be cultured from the purge or meat samples. This microorganism was recently identified and isolated from a vacuum pack of Canadian beef that had undergone "blown-pack" spoilage (Yang *et al.*, 2009). PCR results indicated that the microorganism isolated from the purge in the current study could be *Cl. putrefaciens* or *Cl. algidicarnis*. *Cl. algidicarnis*, a psychrotrophic cold-tolerant species that has been associated with production of sickly offensive odours during chilled storage of cooked vacuum packaged chilled pork (Lawson *et al.*, 1994). *Cl. putrefaciens* is proteolytic, causing slight disintegration of meat particles and is accompanied by a strong characteristic, sour, putrefactive odour (Sturges and Drake, 1927). However, differentiation between *Cl. putrefaciens* and *Cl. algidicarnis* cannot be completed using the 16CAF/CAR primers because the 16S rDNA gene sequences are highly homologous, with 99.5% of the nucleotides being identical (Broda *et al.*, 2002). Species-specific PCR could not be used to identify the microorganism because there is no differentiation between *Cl. putrefaciens* and *Cl. algidicarnis* (Broda *et al*., 2000a). *Cl. putrefaciens* and *Cl. algidicarnis* are known meat spoilage microorganisms but are not known to cause "blown-pack" spoilage. These microorganisms have been implicated in commercial deep tissue spoilage incidents (Boerema *et al.*, 2002). PCR was used to detect both of these *Clostridium* spp. in the "blown-pack" lamb (Boerema *et al.*, 2002). Both of these microorganisms are generally found in high numbers in the stifle joint but were not found in the drip or the external surfaces of the lamb leg (Boerema *et al.*, 2002). The absence of *Cl. putrefaciens* and *Cl. algidicarnis*

in the drip or on the surface indicates these microorganisms were not introduced to the surface during dressing or slaughter but instead were present in the joint during slaughter. Since the BP isolate recovered in this study was obtained from a commercial package of meat, contamination occurred during the slaughter or cutting process. The BP isolate could have been introduced onto the meat via the hide during dressing or from internal sources (ie. the joints) during cutting and deboning. Generally with "blown-pack" spoilage, the point of contamination is from the surface of the meat or the hide. Point of contamination will be important when designing effective intervention strategies.

RFLP was used as a molecular method to differentiate the microorganisms isolated from the spoiled packages of meat. Other methods, such as Random Amplified Polymorphic DNA analysis (RAPD) and repetitive element sequence-based PCR (rep-PCR), have also been used to characterise *Clostridium* spp. (Hyytiä *et al.*, 1999). However, most of the identification of psychrotrophic *Clostridium* spp. has been done through a combination of RFLP and 16S rDNA sequencing. RAPD and rep-PCR procedures can be more difficult in terms of replication because it may be difficult to obtain similar banding profiles each time. RAPD could be useful in identification of clonal isolates because most of the psychrotrophic microorganisms have yet to be sequenced and RAPD does not require knowledge of the DNA sequence. RFLP was used to differentiate the 26 strict anaerobic isolates. The isolates were determined to be clonal isolates because of the identical restriction patterns for all three restriction endonucleases.

It was not surprising that the 26 strict anaerobic isolates only yielded one isolate. In a study done by Broda *et al.* (2000a), 22 strains isolated from meat produced 8 different genotypes. Of the 8 genotypes, 7 strains were different from reference strains and only 4 strains were thought to represent undescribed strains. In the current study, although species-specific PCR presumptively identified the BP isolate as being *Cl. putrefaciens* or *Cl. algidicarnis*, the restriction pattern indicated otherwise. Comparison of the patterns of the *Cl. putrefaciens* ATCC 25786 and the BP isolate indicated that the microorganisms were not likely the same. Differing restriction patterns using *Hae*III could be caused by intraspecific nucleotide differences as genotypes of two meat strains differed only by *Hae*III restriction patterns but exhibited 99.8 % homology in their 16S rDNA sequence (Broda *et al.*, 2000a). In this study, there was a more obvious difference in the *Hha*I digest. The reference strains *Cl. frigidicarnis* ATCC BAA154 and *Cl. frigidicarnis* ATCC BAA155 could not be differentiated with the three restriction digests. The reference strains *Cl. frigidicarnis* ATCC BAA154 and BAA155 were differentiated from the *Cl. putrefaciens* ATCC 25786 with the restriction endonucleases *Alu*I and *Hae*III. Comparison of the restriction pattern of *Cl. frigidicarnis* (both ATCC BAA154 and ATCC BAA155) with the BP isolate confirmed that the BP isolate was not *Cl. frigidicarnis*. Broda *et al.* (2000a) indicated that one restriction enzyme, *Alu*I was sufficient to differentiate six genotypic species among 22 meat strains of psychrotrophic *Clostridium* spp. In this particular study, the restriction enzyme *Alu*I did not differentiate between the BP isolate and *Cl. putrefaciens* ATCC 25786 but the banding patterns obtained

from using the restriction enzymes *Hae*III and *Hha*I differentiated between the BP isolate and *Cl. putrefaciens* ATCC 25786.

Sequencing of the 16S rDNA identified the BP isolate as a *Clostridium* spp. and identified it as *Clostridium putrefaciens*. Although there was a match to a type strain, the RFLP patterns of the *Cl. putrefaciens* ATCC 25786 and the BP isolate did not match for two restriction enzymes. There are some differences in phenotypic traits between *Cl. putrefaciens* and the BP isolate. *Cl. putrefaciens* is not known to grow at refrigeration temperatures (Cato *et al*., 1986). The BP isolate grew well at refrigeration temperatures and thus is described as psychrotrophic. The BP isolate did not produce hydrogen sulphide on SFP agar and does not exhibit the same amount of proteolysis compared to *Cl. putrefaciens*. Slight production of hydrogen sulphide and digestion of meat until disintegration in minced pork is characteristic of *Cl. putrefaciens* (Sturges and Drake, 1927). Although there was a match with the species-specific PCR, based on phenotypic characterisation, RFLP analysis and DNA sequencing it was determined that more sequencing needed to be done to identify the BP isolate.

Use of bacteriocin-producing cultures was explored as an intervention that could prevent the growth of *Clostridium* spp., especially those thought to be responsible for "blown-pack" spoilage. *C. maltaromaticum* UAL307 had the largest and most defined zones of inhibition against *Clostridium* spp. compared to the other bacteriocin-producing cultures. *C. maltaromaticum* UAL26 had the second

greatest inhibitory effect on the *Clostridium* spp. Both of these microorganisms produce multiple bacteriocins including class IIa bacteriocins, piscicolin 126 and carnobacteriocin BM1. Production of multiple bacteriocins can be beneficial to the microorganisms, particularly in competitive environments, because it provides another mechanism to facilitate survival (Deegan *et al.*, 2006; Nes *et al.*, 2007; Martin-Visscher *et al.*, 2008). *C. maltaromaticum* UAL307 produces an additional bacteriocin named carnocyclin A (Martin-Visscher *et al*., 2008). Carnocyclin A is a novel circular bacteriocin. The other bacteriocin-producing cultures used in this study are not known to produce any circular bacteriocins. The presence of a circular bacteriocin could explain the greater antimicrobial effect of *C. maltaromaticum* UAL307 against the vegetative cells of *Clostridium* spp. Different classes of bacteriocins can have different mechanisms of action. This may be advantageous because there is decreased probability of the target microorganisms developing resistance. The mechanism of carnocyclin A has not been elucidated but the structure is similar to that of another circular bacteriocin, enterocin AS-48 (Martin-Visscher *et al.*, 2008). Structural similarity may mean similar mechanism of action. Insertion of the enterocin AS-48 into the cell membrane causes ion permeation and collapse of the membrane potential (Maqueda *et al.*, 2007). It is unknown how exactly enterocin AS-48 interacts with the membrane (such as pore formation) to disrupt the membrane potential. Because of its good inhibition of both the endospores and viable cells of the clostridia isolate, *C. maltaromaticum* UAL307 was chosen as the bacteriocin-producing microorganism in further studies.

Some work has been completed with LAB with inhibitory activity against "blown-pack" spoilage microorganisms. The growth of *Cl. estertheticum* endospores was inhibited by *Lactococcus lactis* but not by the supernatant (Jones *et al.*, 2008). In some cases, the supernatant is not concentrated enough to inhibit the growth of target microorganism. Antimicrobial activity may also be dependent on cell-bound molecules (Jones *et al.*, 2008). Bacteriocins that are cell bound will not be found in the supernatant. A variety of factors such as substrate composition, cell density and population kinetics can affect production and release of bacteriocins by LAB (Delgado *et al.*, 2007; Holck *et al*., 1992; Stoyanova and Levina, 2006; Tagg and Wannamaker, 1978).

To determine if the BP isolate obtained from "blown-pack" spoiled meat were the cause of with this particular type of spoilage, they were added back to meat, vacuum packaged and stored at 2 °C. The interaction of the background microflora and the use of a bacteriocin-producing microorganism as a protective culture were also investigated. Presence of background microflora did not delay the growth of *Clostridium* spp. based on the observation of gas production in non-aseptically prepared beef. Both *Cl. frigidicarnis* ATCC BAA154 and the BP isolate caused gas production after 42 d of storage when inoculated onto non-aseptically prepared meat. Compared to other studies that have investigated the ability of *Clostridium* spp. to cause "blown-pack" spoilage, the storage time required for gas production was relatively long. Vacuum packaged chilled lamb

showed signs of gas production and pack distension when inoculated with *Cl. estertheticum* and *Cl. estertheticum* subsp. Laramiense within 14 d of storage at 1 to 3 ºC (Kalchayanand *et al.*,1989; Broda *et al.*, 2000b). *Cl. gasigenes* sp. nov., another psychrophile that causes "blown-pack" spoilage, was observed to produce enough gas for package distension within 14 d at 2 ºC (Broda *et al.*, 2000b). Production of gas was slower for *Cl. gasigenes* sp. nov. compared to *Cl. estertheticum* and *Cl. estertheticum* subsp. Laramiense (Broda *et al.*, 2000b). *Cl. frigidicarnis* ATCC BAA154 produces an abundant amount of gas when grown in PYGS broth and cooked meat medium. The amount of gas production exhibited by both microorganisms was substantial enough to lift the cooked meat medium from the bottom of the test tube. The gas production by the BP isolate from this study resulted in similar observations. Broda *et al.* (2000b) found that *Cl. gasigenes* sp. nov., *Cl. estertheticum* and *Cl. estertheticum* subsp. Laramiense produced sufficient amounts of gas to produce distension of the package and noted that a consortium of *Clostridium* spp. may be the cause of "blown-pack" spoilage. Differences in inoculation numbers, size of meat samples and packages could account for the different results observed in the current study.

To determine the importance of the presence of a background microflora to the growth of *Clostridium* spp., meat was prepared under aseptic and non-aseptic conditions. As expected, high aerobic and LAB counts were obtained on the non-aseptically prepared samples and they were low on the aseptically prepared samples. The total aerobic counts were equal to the counts for LAB.

Enterobacteriaceae counts were completed because there have been reported incidences where psychrotolerant *Enterobacteriaceae* have cause "blown-pack" spoilage. Some *Enterobacteriaceae* spp. that can cause "blown-pack" spoilage include *Hafnia*, *Enterobacter*, *Serratia*, *Rahnella* and *Ewingella* spp. (Brightwell *et al.*, 2007). Typical "blown-pack" spoilage due to high numbers (10^5 CFU/cm^2) of *Enterobacteriacae* exhibits gross gas production with pack distension, putrid off odours and greening of the meat (Brightwell *et al.*, 2007). Generally "blown-pack" caused by *Enterobacteriaceae* occurs at sub-optimal temperatures. In this confirmation study, the number of *Enterobacteriaceae* did reach 10^5 CFU/cm^2 by 42 days of storage but it was not identified as the cause of gas production because the aseptically prepared meat, which had low counts of *Enterobacteriacae*, also had presence of gas. Also, the discolouration associated (greening) with *Enterobacteriaceae* was not observed.

Gas production was initially observed in packages that were inoculated with endospores and prepared non-aseptically (presence of background microflora). Gas production was observed in the aseptically treated packages inoculated with endospores; however, this occurred about 1 to 2 weeks later. It is possible that presence of background microflora may stimulate the growth of *Clostridium* spp. There was no gas production observed in the absence of endospores.

The presence of *C. maltaromaticum* UAL307 inhibited the gas production that was observed when the BP isolate and with *Cl. frigidicarnis* ATCC BAA154 were

inoculated onto beef. The microbial profiles of samples inoculated with *C. maltaromaticum* UAL307 with or without the BP isolate were very similar and with the methods used, inhibition of the growth of the BP isolate could not be detected. However the inhibition of gas production indicates that its growth was likely inhibited. There has been no work reported with "blown-pack" spoilage microorganisms and bacteriocin-producing cultures in fresh meat products. The only work that has been completed with "blown-pack" spoilage *Clostridium* spp. and inhibitory LAB was with *Cl. estertheticum* (Jones *et al.*, 2008). The study investigated the antimicrobial activity of inhibitory lactic acid bacteria in whole broth culture, supernatant and supernatant heated to 80ºC. Seven different inhibitory LAB were used including *Leuconostoc carnosum* 15, *Lactobacillus sakei* 27, *Lactobacillus sakei* 44, *Lactobacillus sakei* 63, *Lactococcus garvieae* 69, *Lactococcus lactis* 75, and *Lactobacillus sakei* Lb706. *Cl. estertheticum* endospores were only inhibited by the whole broth culture of *Lc. lactis* 75. The cell free supernatant and the heated supernatant did not have any inhibitory effects. Strains of *Lc. lactis* have the ability to produce nisin-like bacteriocins (Schillinger *et al.*, 1996) which may account for the inhibition observed. However, *Lc. lactis* does not grow on meat stored at refrigeration temperatures and nisin is not active on raw meats (Rose et al., 1999). No work was done in the study by Jones *et al.* (2008) with the inhibitory LAB and *Cl. estertheticum* in a meat system.

PCR-RFLP analysis verified the presence of the original microorganisms inoculated onto the meat. There were banding patterns that did not match the banding patterns of the original microorganisms. It is possible that these microorganisms could be LAB because of the high counts of LAB in the confirmation study.

The major components of meat are protein, lipids and some carbohydrates (glycogen). The availability of meat substrates will determine the growth of the *Clostridium* spp. Microorganisms present in the background microflora may accelerate the breakdown of these larger substrates to produce usable metabolites for the "blown-pack" spoilage *Clostridium* spp. The aqueous phase of the meat is the phase that provides the nutrients. Glucose, lactic acid, certain amino acids, nucleotides, urea and water soluble proteins are present in this phase (Gill, 1976; Nychas *et al.,* 1988; Drosinos, 1994) and are available as energy and nutrient sources for meat microflora. The availability of low molecular weight substrates in meat is known to affect the growth and spoilage activities of other meat spoilage bacteria (Nychas et al., 1998). *Cl. estertheticum*, when incubated in meat juice medium, will rapidly consume glucose first (Yang *et al*., 2009). Glucose is consumed by *Cl. estertheticum* ATCC 51877 within 4 d of incubation in meat juice medium (Gill, 1976) at 10 ºC anaerobically. Glycogen, another major component of meat, was utilised rapidly between d 2 and 4 of incubation (Yang *et al.*, 2009). After the 4 d of incubation, there was very little utilisation of glycogen (Yang *et al.*, 2009). Lactate can also be utilised by *Clostridium* spp. in meat.

Lactate can be present intrinsically or as a metabolite produced by LAB that are generally present in the background microflora. Lactate was not consumed by *Cl. estertheticum* within the first 4 d of incubation in meat juice medium; however, the concentration of the lactate began to decrease after 4 d and continued until 8 d of storage (Yang *et al.*, 2009). There was no change in free amino acids. Yang *et al.* (2009) infers that the succession of substrate usage by *Cl. estertheticum* ATCC 51877 is first glucose, glycogen and is followed by lactate. In the current experiment, there was a high concentration of lactate present in the meat with or without a background microflora and with most of the packages that were inoculated with the *Clostridium* spp. or *C. maltaromaticum* UAL307 and stored at 2 °C. The high concentrations of lactate can be attributed to both the lactate that is already present naturally in the meat as well as the growth of LAB, which most likely consumed glucose first, leaving lactate as a substrate for the *Clostridium* spp. to utilise. Since the non-aseptically prepared samples had much higher numbers of microorganisms as a background microflora, it is possible that the inoculated *Clostridium* endospores utilised lactate as the substrate. There was no lactate present in the commercial "blown-pack" spoiled meat but high concentrations of butyric acid, butanol and acetic acid were detected. Acetic and butyric acids are products of glucose metabolism in *Clostridium* spp. (Yang *et al.*, 2009). In meat juice medium inoculated with *Cl. estertheticum*, acetate was detected and reached its maximum concentration by 2 d of storage at 4 °C (Yang *et al.*, 2009). Butyric acid and butanol were detected after 2 d of storage. Production of butyric acid and butanol is most likely due to the concurrent

catabolism of lactate. Although meat generally has a high concentration of glycogen, not all of the glycogen is utilised. Glycogen is only metabolised by *Cl. estertheticum* ATCC 51877 when glucose is present (Yang *et al.*, 2009). Although *Cl. estertheticum* ATCC 51877 can utilise glucose initially, there is generally not enough glucose in the meat to sustain the growth required for "blown-pack" spoilage to occur. It was noted that lactate plays an important role in the growth of "blown-pack" spoilage microorganisms and when glucose is depleted, maximum numbers of *Cl. estertheticum* ATCC51877 was not reached (Yang *et al.*, 2009). Utilisation of lactate may be limited by other factors. Other *Clostridium* spp. that can ferment lactate require co-utilisation of acetate and pyruvate (Ingham *et al.*, 1998). This requirement has not been determined for "blown-pack" spoilage *Clostridium* spp.

The most common metabolites detected in packages inoculated with endospores were lactic acid, propionic acid, butyric, valeric and isovaleric acid. All of these metabolites, with the exception of lactic acid, were also detected in the purge obtained from the commercial "blown-pack" spoiled beef in higher proportions. The only major metabolite present in fresh meat is lactic acid. The lactic acid may have been used as a substrate by *Clostridium* spp., hence the lack of lactic acid peak in the purge obtained from the commercial "blown-pack" spoiled beef. The packages inoculated with the BP isolate and *Cl. frigidicarnis* ATCC BAA154 did have some butyric acid but not as much as the commercial sample of "blown-pack" spoilage. Propionic acid, valeric acid and isovaleric acid have not been

identified in as typical metabolites from "blown-pack" spoiled vacuum packaged beef (Broda *et al.*, 1996). However, most analysis of volatiles of "blown-pack" vacuum packaged spoiled meat has been done with gas chromatography and HPLC may not be able to detect the same compounds in significant concentrations. The presence of butyric acid indicates that there may be some growth of the *Clostridium* spp. but not to the extent required to produce enough gas to cause gross distension of the package. The presence and initial activity of the *Clostridium* spp. could explain the gas production that occurred only in packages inoculated with *Clostridium* spp. In samples inoculated with *Cl. frigidicarnis* ATCC BAA154, there was a difference between non-aseptic and aseptically prepared samples in terms of metabolites. The metabolites present were the same, but the concentration of propionic and acetic acid was higher in aseptically prepared samples. Unidentified metabolites were present in the non-aseptically prepared samples. These peaks are not present in the aseptically treated packages. These metabolites were not likely due to the growth of the from background microflora because these peaks were not present in the control samples. The BP isolate and the *Cl. frigidicarnis* ATCC BAA154 may not have been totally responsible for "blown-pack" spoilage observed because their volatile acid profiles did not match the volatile acid profiles of the commercial "blown-pack" spoiled sample. "Blown-pack" spoilage is most probably due to a number of gas producing, psychrotrophic *Clostridium* spp.

5. Conclusions

In this study, spoiled vacuum packaged beef observed to be typical of "blown-pack" spoilage, was obtained from a local processor. To characterise the spoilage, the metabolite profile was determined using HPLC and the ecology of the spoilage microflora was determined using culture techniques. The metabolite profile of the commercially spoiled beef was similar to that of a psychrotrophic *Clostridium* spp. previously identified in "blown-pack" spoiled vacuum packaged beef. Spoilage microorganisms that were detected in the "blown-pack" meat purge included LAB, *Enterobacteriaceae*, *Brochothrix thermosphacta* and unidentified anaerobic spoilage microorganisms. Detection and isolation of the anaerobic isolates were completed using molecular methods such as PCR-RFLP and sequencing. A total of 66 isolates were cultured, of which 26 isolates were strict anaerobes. PCR-RFLP was used to separate these 26 isolates into groups; however, analysis indicated that these 26 microorganisms were clonal isolates. Sequencing of the 16S rDNA was completed and the BP isolate was identified as *Cl. putrefaciens*. There were differences in the phenotypic traits between *Cl. putrefaciens* ATCC 25786 and the BP isolate (from this study), as well as in the RFLP patterns. Although the sequencing results identified the BP isolate as *Cl. putrefaciens*, differences in the phenotype and in the RFLP banding pattern suggests the possibility of a new strain of *Clostridium*.

A confirmation study was completed to verify the ability of the BP isolate to cause similar spoilage observed in the commercially spoiled beef. The BP isolate

was added to aseptically prepared meat and stored at 2 °C for 84 d. The BP isolate produced gas. Compared to commercially spoiled meat, the amount of gas was less. Endospores were also added to non-aseptically prepared meat to observe the effect of a background microflora. When the background microflora was added, gas production occurred earlier than when there was no background microflora present. Biopreservation was also explored in this study. A biopreservative culture was added to aseptically-prepared, endospore-inoculated meat to determine whether these cultures could prevent "blown-pack" spoilage. *C. maltaromaticum* UAL307 was chosen based on preliminary studies done with different *Clostridium* endospores. The use of *C. maltaromaticum* UAL307 as a biopreservative was successful in the meat model because there was no gas production observed after storage at 2 °C for 84 d. Metabolite profiling was also completed during the confirmation and biopreservative studies. The metabolite profile did not match what was found in the commercially spoiled vacuum packaged beef. Therefore, it is likely that the BP isolate was not the only microorganism responsible for the spoilage observed from the commercially spoiled beef.

From these results and the results of other studies (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Broda *et al.*, 1996), "blown-pack" spoilage was likely a result of a consortium of psychrotrophic *Clostridium* spp. The BP isolate was not the only cause of the "blown-pack" spoilage because the amount of gas produced was not equal to that observed in the commercially spoiled beef.

Although the BP isolate was not the sole cause of spoilage, it was associated with "blown-pack" spoilage producing gas in PYGS medium and in the confirmation study. The source of the *Clostridium* spp. has not been established; however, the use of *C. maltaromaticum* UAL307 has shown promise in preventing these microorganisms from producing gas in vacuum packaged fresh beef.

Further research could include investigation of the effect of different amounts of inoculum of the BP isolate on meat to determine total gas production and identification of the gas produced. The addition of a cocktail of psychrotrophic *Clostridium* spp. to observe effect on total gas production in vacuum packaged meats would also be advantageous to determine if a biopreservatives culture could control spoilage caused by a consortium of microorganisms. More phenotypic typing and molecular characterisation needs to be completed to verify the isolation of a new species.

6. References

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

Alderton G, Snell N. 1969. Chemical states of bacterial spores – dry heat resistance. Appl Microbiol 17:745-9.

Anellis A, Rowley DB, Ross EW. 1979. Microbiological safety of radappertized beef. J Food Prot 42:927-32.

Bassam BJ, Caetano-Anolles G, Gresshoff PM. 1992. DNA amplification fingerprinting of bacteria. Appl Microbiol Biotechnol 38:70-6.

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

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

Boerema JA, Broda DM, Penney N, Brightwell G. 2007. Influence of peroxyacetic acid-based carcass rinse on the onset of "blown pack" spoilage in artificially inoculated vacuum packed chilled beef. J Food Prot 70:1434-9.

Bover-Cid S, Holzapfel WH. 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. Int J Food Microbiol 53:33-41.

Bower CK, McGuire J, Daeschel MA. 1996. The adhesion and detachment of bacteria and spores on food-contact surfaces. Trends Food Sci Technol 7:152-7.

Brightwell G, Clemens R, Urlich S, Boerema J. 2007. Possible involvement of psychrotolerant *Enterobacteriaceae* in “blown-pack” spoilage of vacuum packaged raw meats. Int J Food Microbiol 119:334-9.

Brillet A, Pilet MF, Prevost H, Cardinal M, Leroi F. 2005. Effect of inoculation of *Carnobacterium divergens* V41, a biopreservative strain against *Listeria monocytogenes* risk, on the microbiological, chemical and sensory quality of cold-smoked salmon. Int J Food Microbiol 104:309-24.

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

Broda DM, Boerema JA, Bell RG. 1998. A PCR survey of psychrotrophic *Clostridium botulinum*-like isolates for the presence of BoNT genes. Lett Appl Microbiol 27:219-23.

Broda DM, Lawson PA, Bell RG, Musgrave DR. 1999. *Clostridium frigidicarnis* sp nov., a psychrotolerant bacterium associated with "blown-pack" spoilage of vacuum packed meats. Int J Syst Bacteriol 49:1539-50.

Broda DM, Musgrave DR, Bell RG. 2000a. 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 88:107-18.

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

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

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

Broda DM, Musgrave DR, Bell RG. 2003. Molecular differentiation of clostridia associated with "blown-pack" spoilage of vacuum packed meats using internal transcribed spacer polymorphism analysis. Int J Food Microbiol 84:71-7.

Broda DM. 2007. The effect of peroxyacetic acid-based sanitizer, heat and ultrasonic waves on the survival of *Clostridium estertheticum* spores in vitro. Lett Appl Microbiol 45:336-41.

Cabrera-Martinez RM, Setlow B, Setlow P. 2002. Studies on the mechanisms of the sporicidal action of ortho-phthalaldehyde. J Appl Microbiol 92:675-80.

Callow E H, Ingram M. 1955. Bone taint in beef. Food 24:52-5.

Cano RJ, Borucki MK. 1995. Revival and identification of bacterial spores in 25 million year old to 40 million year old Dominican amber. Science 268:1060-4.

Cato EP, George WL, Finegold SM. 1986. Genus *Clostridium* Prazmowski, 1880. p. 1141-2000. In: P. H. A. Sneath, N. S. Mair, M. E. Sharpe, J. G. Holt, editors. Bergey's Manual of Systematic Bacteriology. The Williams & Wilkins Co., Baltimore.

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

Cohn F. 1876. Untersuchungen über Bakterien. Beitr Biol Pflanz 2:249-76.

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

Connil N, Prevost H, Dousset X. 2002. Production of biogenic amines and divercin V41 in cold smoked salmon inoculated with *Carnobacterium divergens* V41, and specific detection of this strain by multiplex-PCR. J Appl Microbiol 92:611-7.

Cortezzo DE, Koziol-Dube K, Setlow B, Setlow P. 2004. Treatment with oxidizing agents damages the inner membrane of spores of *Bacillus subtilis* and sensitizes spores to subsequent stress. J Appl Microbiol 97:838-52.

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

Cortinas TI, Micalizzi B, deGuzman AMS. 1994. Influence of culture conditions on growth and protective antigenicty of *Clostridium chauvoei*. J Appl Bacteriol 77:382-7.

Cowan AE, Koppel DE, Setlow B, Setlow P. 2003. 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 100:4209-14.

Cowan AE, Olivastro EM, Koppel DE, Loshon CA, Setlow B, Setlow P. 2004. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. Proc Natl Acad Sci USA 101:7733-8.

Crandall AD, Montville TJ. 1993. Inhibition of *Clostridium botulinum* growth and toxigenesis in a model gravy system by coinoculation with bacteriocin-producing lactic acid bacteria. J Food Prot 56:485-8.

Crook PG. 1952. The effect of heat and glucose on endogenous endospore respiration utilizing a modified Scholander Microrespirometer. J Bacteriol 63:193-8.

Dainty RH, Edwards RA, Hibbard CM. 1989. Spoilage of vacuum packed beef by a *Clostridium* sp. J Sci Food Agric 49:473-86.

Deegan LH, Cotter PD, Hill C, Ross P. 2006. Bacteriocins: Biological tools for bio-preservation and shelf-life extension. Int Dairy J 16:1058-71.

Delgado A, Arroyo Lopez FN, Brito D, Peres C, Fevereiro P, Garrido-Fernandez A. 2007. Optimum bacteriocin production by *Lactobacillus plantarum* 17.2b requires absence of NaCl and apparently follows a mixed metabolite kinetics. J Biotechnol 130:193-201.

Desser H, Broda E. 1969. Radiochemical investigation of respiration of spores of *Bacillus cereus*. Arch Mikrobiol 65:76-86.

Drider D, Fimland G, Hechard Y, McMullen LM, Prevost H. 2006. The continuing story of class IIa bacteriocins. Microbiol and Mol Biol R 70:564-82.

Drosinos EH, Board RG. 1994. Metabolic activities of pseudomonads in batch cultures in extract of minced lamb. J Appl Bacteriol 77:613-20.

Duffes F, Corre C, Leroi F, Dousset X, Boyaval P. 1999. Inhibition of *Listeria monocytogenes* by in situ produced and semipurified bacteriocins of *Carnobacterium* spp. on vacuum packed, refrigerated cold-smoked salmon. J Food Prot 62:1394-403.

Edwards RA, Dainty RH, Hibbard CM, Ramantanis SV. 1987. Amines in fresh beef of normal pH and the role of bacteria in changes in concentration observed during storage in vacuum packs at chill temperatures. J Appl Bacteriol 63(5):427-34.

Eijsink VGH, Skeie M, Middelhoven PH, Brurberg MB, Nes IF. 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl Environ Microbiol 64:3275-81.

Emborg J, Laursen BG, Rathjen T, Dalgaard P. 2002. Microbial spoilage and formation of biogenic amines in fresh and thawed modified atmosphere-packed salmon (*Salmo salar*) at 2 °C. J Appl Microbiol 92:790-9.

Esty JR, Meyer KF. 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes XI. J Infect Dis 31:650-63.

Frenkiel-Krispin D, Ben-Avraham I, Englander J, Shimoni E, Wolf SG, Minsky A. 2004. Nucleoid restructuring in stationary-state bacteria. Mol Microbiol 51:395-405.

Gardner GA. 1966. A selective medium for the enumeration of *Microbacterium thermosphacta* in meat and meat products. J Appl Bacteriol 29:455-60.

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

Gill CO. 1976. Substrate limitation of bacterial growth at meat surfaces. J Appl Bacteriol 41:401-10.

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

Gould GW, Hitchins AD. 1963. Sensitization of bacterial spores to lysozyme and to hydrogen peroxide with agents which rupture disulphide bonds. J Gen Microbiol 33(3):413-23.

Gould GW, Jones A, Wrighton C. 1968. Limitations of initiation of germination of bacterial spores as a spore control procedure. J Appl Bacteriol 31:357-66.

Gursky LJ, Martin NI, Derksen DJ, van Belkum MJ, Kaur K, Vederas JC, Stiles ME, McMullen LM. 2006. Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. Arch Microbiol 186:317-25.

Hanna MO, Smith GC, Hall LC, Vanderzant C. 1979. Role of *Hafnia alvei* and a *Lactobacillus* species in the spoilage of vacuum packaged strip loin steaks. J Food Prot 42:569-71.

Hechard Y, Sahl HG. 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie 84:545-57.

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

Henriques AO, Melsen LR, Moran CP. 1998. Involvement of superoxide dismutase in spore coat assembly in *Bacillus subtilis*. J Bacteriol 180:2285-91.

Hills GM. 1949. 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 45:363-70.

Holck A, Axelsson L, Birkeland SE, Aukrust T, Blom H. 1992. Purification and amino acid sequence of Sakacin-A, a bacteriocin from *Lactobacillus sake* Lb706. J Gen Microbiol 138:2715-20.

Husmark U, Ronner U. 1992. The influence of hydrophobic electrostatic and morphologic properties on the adhesion of *Bacillus* spores. Biofouling 5:335-44.

Hutson RA, Thompson DE, Collins MD. 1993. Genetic interrelationships of saccharolytic *Clostridium botulinum* types B, E and F and related clostridia as revealed by small-subunit rRNA sequences. FEMS Microbiol Lett 108:103-10.

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

Hyytia E, Bjorkroth J, Hielm S, Korkeala H. 1999. Characterisation of *Clostridium botulinum* groups I and II by randomly amplified polymorphic DNA analysis and repetitive element sequence-based PCR. Int J Food Microbiol 48:179-89.

Ingram M. 1952. Internal bacterial taints (bone taint or souring) of cured pork legs. J Hyg 50:165-81.

Ingham SC, Hassler JR, Tsai Y-, Ingham BH. 1998. Differentiation of lactate-fermenting, gas producing *Clostridium* spp. isolated from milk. Int J Food Microbiol 43:173-83.

Iturriaga MH, Escartin EF, Beuchat LR, Martinez-Peniche R. 2003. Effect of inoculum size, relative humidity, storage temperature, and ripening stage on the attachment of *Salmonella montevideo* to tomatoes and tomatillos. J Food Prot 66:1756-61.

Jack RW, Wan J, Gordon J, Harmark K, Davidson BE, Hillier AJ, Wettenhall REH, Hickey MW, Coventry MJ. 1996. Characterisation of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Appl Environ Microbiol 62:2897-903.

Jeziorowski A, Gordon DM. 2007. Evolution of microcin V and colicin Ia plasmids in *Escherichia coli* . J Bacteriol 189:7045-52.

Jones DT, Woods DR. 1986. Acetone-butanol fermentation revisted. Microbiol Rev 50:484-524.

Jones RJ, Hussein HM, Zagorec M, Brightwell G, Tagg JR. 2008. Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. Food Microbiol 25:228-34.

Jorgensen LV, Huss HH, Dalgaard P. 2000. The effect of biogenic amine production by single bacterial cultures and metabiosis on cold-smoked salmon. J Appl Microbiol 89:920-34.

Kalchayanand N, Ray B, Field RA. 1993. Characteristics of psychotrophic *Clostridium laramie* causing spoilage of vacuum packaged refrigerated fresh and roasted beef. J Food Protein 56:13-7.

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

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

Kennedy MJ, Reader SL, Swierczynski LM. 1994. Preservation records of microorganisms - evidence of the tenacity of life. Microbiology-Sgm 14:2513-29.

Kim H, Hahn M, Grabowski P, McPherson DC, Otte MM, Wang R, Ferguson CC, Eichenberger P, Driks A. 2006. The *Bacillus subtilis* spore coat protein interaction network. Mol Microbiol 59:487-502.

Klaenhammer TR. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12:39-86.

Klobutcher LA, Ragkousi K, Setlow P. 2006. The *Bacillus subtilis* spore coat provides "eat resistance" during phagocytic predation by the protozoan *Tetrahymena thermophila*. Proc Natl Acad Sci U S A 103:165-70.

Lai EM, Phadke ND, Kachman MT, Giorno R, Vazquez S, Vazquez JA, Maddock JR, Driks A. 2003. Proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*. J Bacteriol 185:1443-54.

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

Lawson P, Dainty RH, Kristiansen N, Berg J, Collins MD. 1994. Characterisation of a psychrotrophic *Clostridium* causing spoilage in vacuum packed cooked pork: description of *Clostridium algidicarnis* sp. nov. Lett Appl Microbiol 19:53-7.

Leisner JJ, Greer GG, Dilts BD, Stiles ME. 1995. Effect of growth of selected lactic acid bacteria on storage life of beef stored under vacuum and in air. Int J Food Microbiol 26:231-43.

Leisner JJ, Millan JC, Huss HH, Larsen LM. 1994. Production of histamine and tyramine by lactic acid bacteria isolated from vacuum packed sugar salted fish. J Appl Bacteriol 76:417-23.

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

Loshon CA, Beary KE, Gouveia K, Grey EZ, Santiago-Lara LM, Setlow P. 1998. Nucleotide sequence of the *ssp*E genes coding for gamma-type small, acid-soluble spore proteins from the round-spore-forming bacteria *Bacillus aminovorans*, *Sporosarcina halophila* and S-ureae. BBA-Gene Stru Expr 1396:148-52.

Lucke FK. 2000. Utilization of microbes to process and preserve meat. Meat Sci 56:105-15.

Lund BM. 1986. Anaerobes in relation to foods of plant origin. p 351-72. In: E. M. Barnes, G. C. Mead, editors. Anaerobic Bacteria in Habitats Other Than Man. 13th ed., Blackwell Scientific, Oxford.

Lund BM, Graham AF, Georges SM, Brown D. 1990. The combined effect of incubation temperature, pH and sorbic acid on the probability of growth of non-proteolytic type B *Clostridium botulinum*. J Appl Bacteriol 69:481-92.

Ma B, Zhang KZ, Hendrie C, Liang CZ, Li M, Doherty-Kirby A, Lajoie G. 2003. PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. Rapid Commun Mass Spec 17:2337-42.

Maqueda M, Sanchez-Hidalgo M, Fernandez M, Montalban-Lopez M, Valdivia E, Martinez-Bueno M. 2008. Genetic features of circular bacteriocins produced by Gram-positive bacteria. FEMS Microbiol Rev 32:2-22.

Marquis RE, Bender GR. 1985. Mineralization and heat-resistance of bacterial spores. J Bacteriol 161:789-91.

Martin-Visscher LA, van Belkum MJ, Garneau-Tsodikova S, Whittal RM, Zheng J, McMullen LM, Vederas JC. 2008. Isolation and characterisation of carnocyclin A, a novel circular bacteriocin produced by *Carnobacterium maltaromaticum* UAL307. Appl Environ Microbiol 74:4756-63.

Masson F, Johansson G, Montel MC. 1999. Tyramine production by a strain of *Carnobacterium divergens* inoculated in meat-fat mixture. Meat Sci 52:65-9.

Masson F, Talon R, Montel MC. 1996. Histamine and tyramine production by bacteria from meat products. Int J Food Microbiol 32:199-207.

Mccabe BJ. 1986. Dietary tyramine and other pressor amines in Maoi regimens - a Review. J Am Diet Assoc 86:1059-64.

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

Mossel, D.A.A. and Van Netten, P. 1984. Harmful effects of selective media on stressed micro-organisms: nature and remedies. p 329-69. In: Andrew, M.H.E. and Russel, A.D., editor. The Revival of Injured Microbes. 1st ed. Academic Press, London.

Naghmouchi K, Drider D, Kheadr E, Lacroix C, Prevost H, Fliss I. 2006. Multiple characterisations of *Listeria monocytogenes* sensitive and insensitive variants to divergicin M35, a new pediocin-like bacteriocin. J Appl Microbiol 100:29-39.

Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Anton Leeuw Int J G 70:113-28.

Nes I, Yoon S, Diep WB. 2007. Ribosomally synthesised antimicrobial peptides (bacteriocins) in lactic acid bacteria: A review. Food Sci Biotech 16:675-90.

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

Nychas GJ, Dillon VM, Board RG. 1988. Glucose, the key substrate in the microbiological changes occurring in meat and certain meat products. Biotechnol Appl Biochem 10:203-31.

Popham DL. 2002. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. Cell Mol Life Sci 59:426-33.

Purnell G, Loeffen M. 2006. Automation for the modern slaughterhouse. In: L. M. L. Nollet, F. Toldra, editors. Advanced Technologies for Meat Processing. p 43-73.

Quadri LEN, Sailer M, Roy KL, Vederas JC, Stiles ME. 1994. Chemical and genetic characterisation of bacteriocins produced by *Carnobacterium piscicola* Lv17b. J Biol Chem 269:12204-11.

Redmond C, Baillie LWJ, Hibbs S, Moir AJG, Moir A. 2004. Identification of proteins in the exosporium of *Bacillus anthracis*. Microbiology-Sgm 150:355-63.

Riemann H, Ordal ZJ. 1961. Germination of bacterial endospores with calcium and dipicolinic acid. Science 133:1703-4.

Rode LJ, Foster JW. 1960. The action of surfactants on bacterial spores. Arch Mikrobiol 36:67-94.

Rodgers S. 2001. Preserving non-fermented refrigerated foods with microbial cultures - a review. Trends Food Sci Technol 12:276-84.

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

Ronner U, Husmark U, Henriksson A. 1990. Adhesion of *Bacillus* spores in relation to hydrophobicity. J Appl Bacteriol 69:550-6.

Rose NL, Sporns P, Stiles ME, McMullen LM. 1999. Inactivation of nisin by glutathione in fresh meat. J Food Sci. 64:759-762.

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

Ross EW. 1974. Statistical estimation of 12d for radappertized foods. J Food Sci 39:800-6.

Ross HE. 1965. *Clostridium putrefaciens* - a neglected anaerobe. J Appl Bacteriol 28:49-51.

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

Sapru V, Labuza TP. 1993a. Glassy state in bacterial spores predicted by polymer glass transition theory. J Food Sci 58:445-8.

Sapru V, Labuza TP. 1993b. Temperature dependence of thermal inactivation rate constants of bacterial spores in a glassy state. J Ind Microbiol 12:247-50.

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

Setlow P. 1983. Germination and outgrowth. p 211-54. In: A. Hurst, G. W. Gould, editors. The Bacterial Spore. Vol. II ed. Academic Press, London.

Setlow P. 1992. I will survive - protecting and repairing spore DNA. J Bacteriol 174:2737-41.

Setlow P. 1994. Mechanisms which contribute to the long term survival of spores of *Bacillus* species. J Appl Bacteriol 76:S49-60.

Setlow P. 1995. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. Annu Rev Microbiol 49:29-54.

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

Setlow P. 2007. I will survive: DNA protection in bacterial spores. Trends Microbiol 15:172-80.

Setlow B, Loshon CA, Genest PC, Cowan AE, Setlow C, Setlow P. 2002. Mechanisms of killing spores of *Bacillus subtilis* by acid, alkali and ethanol. J Appl Microbiol 92:362-75.

Setlow B, Setlow P. 1996. Role of DNA repair in *Bacillus subtilis* spore resistance. J Bacteriol 178:3486-95.

Setlow B, Tautvydas KJ, Setlow P. 1998. Small, acid-soluble spore proteins of the alpha beta type do not protect the DNA in *Bacillus subtilis* spores against base alkylation. Appl Environ Microbiol 64:1958-62.

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

Smith LD. 1975. Inhibition of *Clostridium botulinum* by strains of *Clostridium perfringens* isolated from soil. Appl Microbiol 30:319-23.

Spring S, Merkhoffer B, Weiss N, Kroppenstedt RM, Hippe H, Stackebrandt E. 2003. Characterisation of novel psychrophilic clostridia from an Antarctic microbial mat: description of *Clostridium frigoris* sp nov., *Clostridium lacusfryxellense* sp nov., *Clostridium bowmanii* sp nov and *Clostridium psychrophilum* sp nov and reclassification of *Clostridium laramiense* as *Clostridium estertheticum* subsp *laramiense* subsp nov. Int J Syst Evol Microbiol 53:1019-29.

Stoffels G, Nes IF, Guomundsdottir A. 1992a. Isolation and properties of a bacteriocin producing *Carnobacterium piscicola* isolated from fish. J Appl Bacteriol 73:309-16.

Stoffels G, Nissenmeyer J, Gudmundsdottir A, Sletten K, Holo H, Nes IF. 1992b. Purification and characterisation of a new bacteriocin isolated from a *Carnobacterium* sp. Appl Environ Microbiol 58:1417-22.

Stoyanova LG, Levina NA. 2006. Components of fermentation medium regulate bacteriocin synthesis by the recombinant strain *Lactococcus lactis* subsp lactis F-116. Microbiology 75:286-91.

Sturges WS, Drake ET. 1927. A complete description of *Clostridium putrefaciens* (McBryde). J Bacteriol 14:175-9.

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

Tagg JR, Wannamaker LW. 1978. Streptococcin AFf22 nisin like antibiotic substance produced by a Group-A *Streptococcus*. Antimicrobial Agents Chemother 14:31-9.

Takeuchi K, Frank JF. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. J Food Prot 63:434-40.

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

Tennen R, Setlow B, Davis KL, Loshon CA, Setlow P. 2000. Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. J Appl Microbiol 89:330-8.

Topisirovic L, Kojic M, Fira D, Golic N, Strahinic I, Low J. 2006. Potential of lactic acid bacteria isolated from specific natural niches in food production and preservation. Int J Food Microbiol 112:230-5.

Vreeland RH, Rosenzweig WD, Powers DW. 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature 407:897-900.

Warth AD. 1980. Heat stability of *Bacillus cereus* enzymes within spores and in extracts. J Bacteriol 143:27-34.

Weenk G, Fitzmaurice E, Mossel DAA. 1991. Selective enumeration of spores of *Clostridium* species in dried foods. J Appl Bacteriol 70:135-43.

Woese CR. 1987. Bacterial evolution. Microbiol Rev 51:221-71.

Yang X, Balamurugan S, Gill CO. 2009. Substrate utilization by *Clostridium estertheticum* cultivated in meat juice medium. Int J Food Microbiol 128:501-5.