# University of Alberta

Resistance and Membrane Fluidity of Endospores of *Clostridium* spp. During Pressure-Assisted Thermal Processing in the Presence of Antimicrobials

by

Simmon Christian Hofstetter

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

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science



Spring 2012
Edmonton, Alberta

## Acknowledgements

During the course of this degree program, various members of the scientific community have contributed valuable insights. From the international crowd, I would like to thank Dr. Roland Winter for his hospitality, Norbert Opitz and Christian Denter for their technical assistance and expertise with microscopy, as well as Jong Zhai and Shobhna Kapoor for their technical assistance. A heartfelt thank you goes to David Gebhardt for his humor, patience, and work ethic- may you never encounter endospores in a lab ever again.

From the University of Alberta, I thank Ken Fahner for his technical support, Kamila Moquin for her insights into bacterial membrane biochemistry, and Elena Dlusskaya for her encyclopedic knowledge of bacteria. As well, I thank Heather Vandertol-Vanier for her advice, Lynn Elmes for her honesty, Jody Forslund for her tremendous patience, Richard Smith for lively insight, and Max Amerongen for keeping the lights bright and blinking- and commenting on those which will be dim forever. I also thank Ming Chen for his electron microscopy expertise and willingness to teach- may retirement treat you well.

I thank the Natural Sciences and Engineering Research Council of Canada, the Alberta Livestock and Meat Agency, and the Canada Research Chair Program for having given me this opportunity. I also thank the Technische Universität Dortmund, Germany, for the opportunity to collaborate.

Thank you to Januana Teixiera, Linda Ho, Clarissa Schwab, Emefa Monu, Melissa Haveroen, Marcia Su, Brenna Black, Aaron Pleitner, Simona Palomba, Christine Liu, Petr Miller, Xiaoxi Lin, and Sandra Galle for all your conversation, humor, insights, and support. A special thank you goes to Christina Engels- a lab partner of solid gold. I also thank the Taylor family (that includes you, Dave), for having been a second home. A very special thank you also goes to Gabi Wojtanowska, and her son and daughter, for taking me in during my stay in Germany- I will not forget your kindness.

I also thank my committee members Dr. L.M. McMullen, Dr. M.G. Gänzle, Dr. D. Barreda, Dr. D. Sauvageau, and Dr. P. Setlow. Dan, you speak with choice words, and though you may not think it, these seed moments of reflection. For that, I thank you. Lynn and Michael, you have both been patient and knowledgeable mentors, with a

willingness to hear a rant now and then (and pass along one or two of your own). I could not ask for better guidance. Thank you.

Finally, I thank my family, especially my mother and father. Thank you for having taken the time to raise me with good food, books, and a warm house. Thank you for having shown me the value of a backbone, a sound mind, and a willingness to try. Thank you for all that you have done.

## Abstract

Endospores of *Clostridium* spp. pose an economic threat to the food industry and put consumers at risk of foodborne illness. An increase in consumer demand for minimally-processed, ready-to-eat products has driven research to examine ways to mitigate current thermal processing severity used to inactivate endospores, while maintaining food safety. Pressure-assisted thermal sterilization (PATS) in combination with antimicrobial compounds is a promising technology. The first objective of this study was to determine the resistance of endospores of *Clostridium* spp. to the antimicrobials nisin and reutericyclin during PATS. Nisin and reutericyclin target cell membranes, are effective against endospores, and have a unique mode of action. The presence of nisin enhanced inactivation of endospores during thermal treatments, whereas reutericyclin had no effect. The presence of nisin and reutericyclin enhanced inactivation of endospores during early stages of PATS. However, the presence of reutericyclin appeared to facilitate survival of endospores. Resistance to nisin and reutericyclin during thermal treatment and PATS is species-dependent.

The second objective of this study was to determine the composition of the inner membrane of endospores of *Clostridium* spp., and to assess membrane fluidity *in situ* and *ex situ* of thermal and high pressure / thermal treatment. Measurements of membrane fluidity may provide information to help explain species-dependent resistance of endospores to nisin and reutericyclin. The inner membrane of endospores was chosen as a target because of its barrier properties. Inner membranes of endospores of *Clostridium* spp. differed significantly in short chain:long chain fatty acid ratios, and saturated:unsaturated fatty acid ratios between species. The fluorescent probe LAURDAN was used to label and measure fluidity of the inner membranes of endospore populations. Labeling inner membranes of endospores with LAURDAN did not affect the viability or structure. Fluorescence measurements were done *in situ* and *ex situ* of thermal

and high pressure / thermal treatment. At ambient conditions, the inner membranes of endospores of *Clostridium* spp. were in an ordered state indicative of a gel-phase membrane. The presence of reutericyclin during thermal treatments disrupted the gel-phase membranes. Conversely, the presence of nisin during high pressure/thermal treatments stabilized the ordered state of inner membranes of endospores.

## Table of Contents

## List of Tables



## List of Figures

## List of Symbols and Abbreviations

| | |
|---|---|
| ATP | Adenosine triphosphate |
| *B.* | *Bacillus* |
| *C.* | *Clostridium* |
| Ca | Calcium |
| Ca-DPA | Calcium-dipicolinic acid |
| CLE | Cortex lytic enzyme |
| CLSM | Confocal laser scanning microscopy |
| DNA | Deoxyribonucleic acid |
| DPA | Dipicolinic acid |
| DPPC | Dipalmitoylphosphatidylcholine |
| FAME | Fatty acid methyl ester |
| FRAP | Fluorescence recovery after |
| FT-IR | photobleaching |
| GP | Fourier transform infrared |
| H | Generalized polarization |
| HHP | Hydrogen |
| K | High-hydrostatic pressure |
| LAURDAN | Potassium |
| LCFA | 6-dodecanoyl-2- |
| MAL | dimethylaminonaphthalene |
| MIC | Long chain fatty acid |
| MPa | Muramic acid lactam |
| OM | Minimal inhibitory concentration |

| | |
|---|---|
| Megapascal | UV |
| Outer membrane | Pressure-assisted thermal sterilization |
| PATS | Peptidoglycan |
| PG | Reinforced clostridial media |
| RCM | Small acid-soluble protein |
| SASP | Short chain fatty acid |
| SCFA | Saturated fatty acid |
| SFA | Species |
| spp. | Transmission electron microscopy |
| TEM | transfer-Ribonucleic acid |
| tRNA | Unsaturated fatty acid |
| USFA | Ultraviolet |

# 1. Introduction and Literature Review

## 1.1 Bacterial Endospores

Bacterial endospores formed by Gram-positive genera *Clostridium* and *Bacillus* are distributed throughout the environment (21, 116). Endospores are highly resistant and metabolically dormant (43, 130). Endospore formation, sporulation, is initiated by vegetative cells when nutrient levels are depleted (42, 43, 130). Sporulation is discussed in detail by Henriques and Moran (68). Endospores differ from vegetative cells in a variety of ways.

Endospores exhibit significant size variance relative to vegetative cells and endospores of different strains (22). *Bacillus* spp. endospores differ significantly in mean length between species, and are roughly half the size of vegetative cells (22). Dormancy of an endospore is primarily due to a core with low water activity (35, 152, 155). Little to no energy-rich compounds, such as adenosine triphosphate (ATP), are found in endospores (158). Endospores do not metabolize endogenous or exogenous compounds (35, 158, 159), and metabolic inactivity can be maintained virtually indefinitely. The pH within the core is 1.0 to 1.5 units lower than vegetative cells, which have a cytoplasmic pH between 7.5 and 8.0 (120, 158). The core of an endospore contains enzymes, DNA, ribosomes, and tRNA in proportions identical to vegetative cells (158, 159). Endospores can persist for millions of years under suitable conditions (20, 82, 179). The primary function of an endospore appears to be survival until a favorable change in the environment occurs.

## 1.2 Germination

Endospores germinate and resume vegetative growth upon exposure to suitable growth conditions (120, 121). Muropeptide fragments of the cell wall, released by growing vegetative cells, can induce germination (160). A eukaryotic-like Ser/Thr kinase appears to act as a receptor to these fragments (160). Germination is also initiated by nutrients in the environment. The types of nutrients are species-dependent (156). Examples of such nutrients include L-amino acids, D-sugars, and purine nucleosides (156). Nutrient-induced germination is initiated without enzymatic action, which makes it a non-metabolic event (156). Nutrients simply bind to stereospecific receptors located in the endospore inner membrane, and a cascade of events is triggered (156). The number of germinant receptor operons tends to be lower in *Clostridium* spp. than in *Bacillus* spp. (188). The number of total germinant receptors within an individual endospore's inner membrane is estimated to be between 10 to 100 molecules per endospore (77, 156). Presumably, low numbers of germinant receptors ensure endospores initiate germination in conditions where nutrient levels are high enough to sustain growth. Muropeptide fragment-induced germination supports this line of reasoning, as vegetative cells produce these during conditions capable of sustaining growth.

At the onset of germination, an unknown mechanism releases ions ($K^+$, $H^+$, $Ca^{2+}$) and pyridine-2,6-dicarboxylic acid (DPA) from the core of endospores. The release of DPA from the cores is often used as an indicator of germination or damage during growth and inactivation studies (78, 104, 121, 187). The cortex lytic enzyme (CLE) CwlJ is activated during release of DPA (98). SleB is another CLE, but the method of activation of this enzyme is unknown. Following ion release both CwIJ and SleB hydrolyze the endospore cortex. The endospore cortex is a specialized layer of peptidoglycan (PG) that helps maintain dormancy (120, 131, 156). Cortex hydrolysis facilitates core rehydration, cell expansion, and an associated increase in wet weight. Proteins such as small acid-

soluble protein (SASP) proteases activate as rehydration occurs. These proteases are critical in generating energy for the germinating endospore from deoxyribonucleic acid (DNA)-bound SASPs (10). Cellular metabolism is initiated once rehydration completes, and macromolecular synthesis can begin (35, 120). Germination is followed by a phase called outgrowth. Outgrowth converts germinated endospores into growing cells (10). Outgrowth also utilizes high-energy amino acids, derived from degradation of SASPs via proteases, for metabolic functions until exogenous nutrients are taken up (10). Germination in conditions capable of sustaining growth is crucial because the process is irreversible, and its completion restores cell sensitivity to environmental factors.

A small proportion of superdormant endospores exist within every population of endospores (10). Superdormant endospores germinate extremely slowly. Slowed germination preserves endospores' resistance (11). The dehydrated core, SASPs, and inner membrane integrity all appear to be retained in superdormant endospores (10). The exact reason for this phenomenon is unknown, and little research into superdormant endospores has been done.

### 1.3 Endospore Adaptations

Endospores have physicochemical adaptations that make them well adapted to endure adverse environmental conditions. Solar ultraviolet (UV) radiation, extremes of pressure, dry heat, wet heat, desiccation, and even microbial-enzyme attacks are all repelled (117, 155). Endospores also resist chemicals such as acids, bases, oxidizers, alkylating agents, aldehydes, and organic solvents (155). Resistance of endospores is a result of their cellular architecture (Figure 1-1).

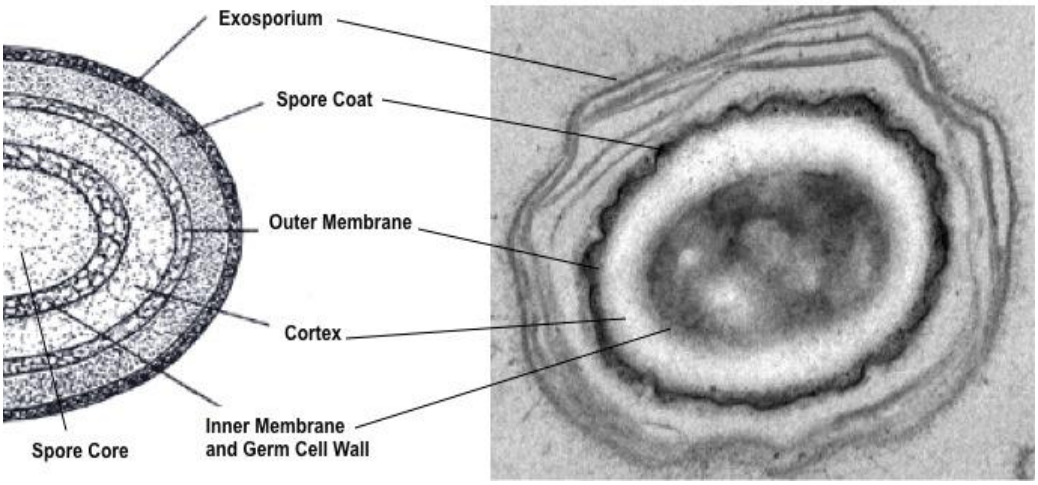


**Figure 1-1:** Diagram of the structure of a bacterial endospore (A), and a transmission electron microscopy image of an endospore of *C. sporogenes* ATCC 7955 (B). See section 3.2.6 of this thesis for the protocol used to take the image.

Endospores of *Bacillus* species are structurally complex as demonstrated by electron microscopy studies (85). Multiple layers surround a dehydrated core of an endospore (68). Each of these layers is chemically distinct and some contribute to resistance. The outermost of these layers, and not present in all species, is called the exosporium. The exosporium is commonly found on endospores of *Bacillus cereus* (43, 88, 142, 180). An exosporium has no known function and consists of proteins, including species-specific glycoproteins (38, 88, 142, 175). Beneath the exosporium is an arrangement of species-specific proteins called the coat.

In *Bacillus* species the coat consists of more than 50 different individual proteins, many of which are cross-linked (43, 44, 88, 68). The CLEs SleB and CwlJ reside within the coat protein layer, although some SleB is located within or adjacent to the inner membrane (5, 26). Coats play little to no role in thermal and radiation resistance (44, 117, 153). However, a coat can help endospores resist bactericidal chemicals (155). The mechanism by which a coat resists chemicals is not known. Coat proteins may react with, and detoxify chemicals before they access inner endospore layers (60). Coats are also semipermeable, allowing the passage of molecules less than 5 kDa in size (44, 68, 156).

Large exogenous lytic enzymes, such as lysozyme, cannot penetrate and damage the layers beneath a coat (44, 68, 84). Below an endospore's protein coat lays the outer membrane.

The presence of an outer membrane is determined by sporulation (130). The outer membrane was originally part of the mother cell that gave rise to the respective endospore, and is involved in cortex synthesis. Removal of an outer membrane has no effect on endospore resistance to heat, radiation, and chemicals (117, 153). Outer membranes may passively sequester hydrophobic molecules, thus passively protecting against some antimicrobial compounds.

A layer of peptidoglycan (PG) called the cortex lies beneath the outer membrane (131). An endospore's cortex is essential for proper sporulation, as well as the reduction and maintenance of low core water content (155). The mechanism by which a cortex assists with maintaining low core water content is likely similar to maintenance of turgor pressure in vegetative cells facilitated by the PG cell wall. The lower water activity within endospore cores results in higher mechanical stress via osmotic pressure than in vegetative cells; the cortex is thicker than that of cell walls in vegetative cells in order to compensate. The cortex also indirectly contributes to thermal resistance by helping to maintain a core's low water activity. Cortex PG is chemically distinct from the germ cell wall PG underneath it, although both can be recognized and degraded by lysozyme (124, 138). Cortex PG contains diaminopimelic acid, and much of the muramic acid present is muramic acid-lactam (MAL) (131). The muramic acid moieties act as recognition sites for CLEs during germination. Cortex degradation is essential for core expansion and subsequent growth (120, 156). The germ cell wall beneath a cortex is composed of PG similar to that of vegetative cells, and becomes the cell wall of a germinated, outgrowing endospore (131). Germ cell walls have not been identified as contributing to endospore resistance. Underneath a germ cell wall rests the inner membrane.

The inner membrane acts as a permeability barrier, preventing the passage of chemicals and water, to the endospore's core (30, 117, 157). Impermeability of the inner membrane may be due to its compressed state (34). The inner membranes of endospores of *Bacillus* spp. have a chemical composition similar to membranes of respective vegetative cells (30, 31), although the actual structural arrangement is unknown (10). As a permeability barrier, the inner membrane contributes to thermal resistance by preventing rehydration of the core.

The center of an endospore is called its core. Within a core, α/β-type SASPs are bound to cellular DNA to the point of saturation. Bound DNA has an altered conformation, resisting thermal denaturation and cross-linking during UV irradiation (59). The majority of an endospore's core material is a calcium-DPA (Ca-DPA) matrix (59, 155). The DPA in the core is likely in the form of a precipitate, but the exact state is unclear (155). Presence of DPA within a core prevents spontaneous germination (98). The Ca-DPA matrix also excludes water (121, 152). Water exclusion keeps core water content between 25-50% of an endospore's wet weight, and also helps protect DNA and proteins (123, 152). The water content of bacterial endospores is inversely related to wet-heat resistance (59). As such, Ca-DPA's exclusion of water is a major contributor to endospore resistance to heat.

Macromolecular movement is restricted due to low levels of free water in endospore cores (35). Lack of molecular movement contributes to wet heat resistance by preventing protein denaturation (59). The release of DPA from endospores of *Bacillus* correlates to inactivation during thermal treatments (129). In addition, removing DPA from endospores, by creating DPA-deficient mutants, correlates with thermal and high-pressure sensitivity (102). Sporulation conditions also significantly impact endospore resistance. Prevalence of different divalent and monovalent cations in sporulation media has varying effects on thermal resistance within strains, and media made from soil extract

significantly increases thermal and high-pressure resistance (102, 103, 104). Endospores formed in the higher range of growth temperatures have lower water content than those formed at lower temperatures, with the latter also exhibiting a lowered heat resistance (155). Thus, a loss of thermal resistance occurs when DPA is released during germination, breach of endospore integrity, or compromising DPA reserves (87). Maintenance of an endospore's dehydrated core is ultimately responsible for the majority of resistance (155).

Endospores are a cellular response to adverse environmental conditions that effectively puts bacteria into stasis until more favorable conditions arise. Although other examples of dormant metabolic states exist, none are as hardy or widely distributed endospores. Endospores are present in virtually all of the Earth's environments- from the deepest points of the ocean floor to the soil beneath our feet. Such a pervasive form of life is bound to interfere with some aspect of human activity.

### 1.4 Relevance of Endospores to the Food Industry

Endospores' ability to germinate and widespread distribution presents a serious problem for the food industry (10). Germination within food can lead to spoilage and mild to severe foodborne illness (16, 32). Economic losses due to spoilage, product recalls, and consumer avoidance can be substantial. Endospores of both *Clostridium* and *Bacillus* spp are problematic. The most serious threat to consumers is posed by germinating endospores of *Clostridium botulinum*. *C. botulinum* is capable of producing a toxin during growth, and if the toxin is consumed it can cause botulism (97). Foodborne botulism is characterized by muscle paralysis leading to asphyxiation (161). Endospores of *C. botulinum* are distributed worldwide and capable of causing foodborne intoxication associated with meats, fish, vegetables and milk products (83). Many food industry regulations and processing methods are based around eliminating endospores of *C.*

*botulinum* (10). The production of *Clostridium perfringens* endospores is responsible for some foodborne illnesses associated with consumption of cooked meat products. *C. perfringens* intoxication exhibits symptoms of acute abdominal pain, diarrhea, and nausea (165). *Bacillus licheniformis* and *Bacillus subtilis* are both associated with outbreaks of foodborne illness (16, 150). *Bacillus cereus* is commonly associated with foods that interact heavily with soil, and is known as both a spoilage and toxin-producing organism (14). *Clostridium beijerinckii* endospores have been identified as causative agents in "blown pack" spoilage of chilled vacuum-packed red meats, mainly posing an economic threat (16). Thermo-acidophilic *Alicyclobacillus* spp that have germinated can also cause spoilage, mainly in concentrated fruit juices (25, 166, 169).

### 1.4.1 Thermal Processing

Endospores are highly resistant to thermal processing used to extend the storage life of foods with a pH greater than 4.5 (89, 140). Thermal resistance of endospores was identified in the early 1900's (99). Toxicity and thermal endurance of endospores of *C. botulinum* was well established at this time (47, 167). Since then, endospores of *C. botulinum* have taken priority as the most concerning cause of endospore-related foodborne illness. Food producers currently employ thermal processing to control endospores. Such treatment entails bringing a product to 121°C for a minimum of 3 minutes (10). Thermal treatment of this severity aims to achieve commercial sterility.

Thermal processing that eliminates endospores to such a high degree also compromises the nutrient and sensory properties of foods (107, 134). The food industry aims to produce shelf-stable, low acid foods that retain quality with minimal use of additives. Thermal processing has been used with additional measures to control endospores to help mitigate thermal severity. For instance, the addition of nitrite, liquid smoke, and lowered pH of foods below 4.5, has all been used. Unfortunately, the food

industry still encounters challenges in eliminating endospores without compromising product quality (68, 102). Non-thermal treatments that may be capable of producing minimally processed, safe, high quality food products are being examined (10).

### 1.5 High Hydrostatic Pressure (HHP) Technology

Processing foods by pressurization has become an attractive technology for ensuring safety and extending storage life (74, 162). In the late 19$^{th}$ century HHP was evaluated for exactly this purpose (72). Since then, we have come to understand that high HHP causes less deterioration of vitamins, phytochemicals, and aroma compounds compared to thermal treatments with comparable bactericidal effects (24). High hydrostatic pressure can inactivate most vegetative bacteria, including pathogens (46, 184). For example, HHP readily inactivates cells of *Escherichia coli* and *Listeria innocua* in juice (19). Various molds, yeast, and fungal spores can also be reduced by up to 6-log cycles at or below 690 MPa at ambient temperature (48, 136, 137). Acceptance of HHP as a processing technique has grown, and has been introduced in several countries, including Canada (86).

#### 1.5.1 High Hydrostatic Pressure (HHP) and Endospores

Endospores have proven to be a resistant target for HHP (71). In the 1960's it was established that pressure alone does not inactivate endospores, specifically those of *B. subtilis* and *Bacillus alvei* (173). Endospores can survive pressures above 1000 MPa, unless temperatures approaching 100 °C are simultaneously applied (24). Endospores of *C. botulinum* are among the most pressure-resistant (126). High hydrostatic pressure alone cannot inactivate endospores, and high hydrostatic pressure with temperature could not be done with commercial units until recently (111, 151). Consequently, commercial production of low-acid, shelf-stable foods using HHP has not yet been done (10).

Favorable side effects of HHP have led researchers to examine its efficacy in endospore inactivation when used prior to high temperatures or antibacterial agents (10).

The sequential application of HHP with other technology is based on the observation that pressure can induce germination. At moderately high pressure (50 to 300 MPa), nutrient receptors are activated in the absence of nutrients and germination proceeds as per the nutrient-induced pathway (13, 121, 186). Higher pressures (400 to 800 MPa) do not trigger germination via nutrient receptors, but cause a substantial DPA release. Released DPA activates the CLE CwIJ, which in turn forces a cascade of germination events (11, 121, 186). Other agents are also capable of inducing germination, including PG lytic enzymes, cationic surfactants (11, 156), and mechanical abrasion (79). Adequate mechanical abrasion damages the cortex, leading to activation of CLEs and the germination cascade (79). Germinated endospores are more sensitive to subsequent antimicrobial and thermal treatments (11). High hydrostatic pressure-induced germination is thought to be the primary mechanism responsible for enhancing thermal inactivation of endospores (11). The sequential application of antimicrobial treatments in order to achieve a synergistic effect has been termed "hurdle technology" (91).

Hurdle technology using HHP with a variety of non-thermal food processing methods has been evaluated against endospores. Ultraviolet irradiation, pulsed-electric fields, and ultrasound have all essentially proven ineffective for endospore inactivation in combination with HHP (148). Irradiation and HHP treatments have exhibited additive endospore inactivation, but not enough data exists to make it an accepted commercial technology (36, 61). Resistance to ultraviolet light can also persist in endospores, regardless of the increased water content and thermal sensitivity during germination (187). The application of a heating step following HHP has also been ruled out as an effective endospore control.

Rejection of sequential pressurization and heating is due to the existence of superdormant endospores within a given population. An HHP process that induces germination (50 to 400 MPa) and is followed with a pasteurization step will not inactivate superdormant endospores, and is therefore unlikely to be used in a commercial setting (11). As was mentioned, superdormant endospores germinate very slowly and retain thermal resistance. High pressures in combination with temperature are required to make acceptable reductions in endospore populations of *Bacillus* or *Clostridium* species (48, 136, 137). Simultaneous use of pressure and heat is called pressure-assisted thermal sterilization (PATS), and may be a more effective process (110, 183).

### 1.6 Pressure-Assisted Thermal Sterilization (PATS)

The food industry is interested in PATS due to growing consumer preference for safe, high-quality foods with minimal additives (33). Pressure-assisted thermal sterilization involves the simultaneous application of pressure, up to 700 MPa, and sub-retorting temperature (90-120°C) (2). The heat used in PATS can be lower than that of thermal processing due to synergistic protein denaturation, thereby maintaining high product quality and safety (107). Pressure-assisted thermal sterilization is less detrimental to flavor, texture, and nutritional quality compared to conventional thermal processing (4, 110). Pressure-assisted thermal sterilization is also more effective than thermal treatment for inactivating vegetative bacteria (127). These factors make PATS a leading candidate in alternatives to severe thermal processing. Pressure-assisted thermal sterilization is not, however, a straightforward technology.

Various factors influence the efficacy of PATS sporicidal effects. Initial temperature, target pressure, holding time, matrix compressibility, and rates of heat loss all influence the inactivation of endospores (7). Lack of adequate temperature control may contribute to tailing phenomena of endospores observed in thermal inactivation

kinetics (174). Heating associated with the compression of water, termed adiabatic heating, is a beneficial side-effect with regard to endospore inactivation. Adiabatic heating of water was first observed and recorded in 1912 (15), and is equally distributed within a sample almost instantaneously. All compressible substances exhibit an increase in temperature upon compression (174). Adiabatic heating of water occurs at a rate of approximately 3°C for every 100 MPa increase (6). In foods that are high in fat and alcohol, adiabatic heating approaches 9°C for every 100 MPa (134). Most foods have a sufficiently high water activity that adiabatic heating is comparable to that of water (11). Regardless of medium, adiabatic heating effects are homogeneously distributed during pressurization (11). This attribute of HHP aids in destruction of pathogenic bacteria, including endospores, while decreasing heating costs.

### 1.6.1 Inactivation of Endospores by Pressure-Assisted Thermal Sterilization (PATS)

Pressure-assisted thermal sterilization-mediated inactivation of endospores is a promising technology (4, 119, 148). In general, PATS accelerates inactivation of endospores when compared to thermal treatments (2). Increases in PATS temperatures, from ambient to just above 50°C, are needed to enhance inactivation of *Bacillus* and *Clostridium* endospores (112, 140). This applies to both buffer and meat systems (112, 141). For example, *B. subtilis* were reduced by 0.5-logs at 404 MPa and 25°C, but a more industrially acceptable 5-log reduction was observed in numbers of endospores when the temperature was increased to 70°C at the same pressure (168). Gao et al (57) observed similar results, showing that 579 MPa and 87°C, for 13 min, was optimal for a 6-log reduction of endospores of *B. subtilis*. Such results are promising because they show that PATS can achieve a 5-log inactivation of endospores below 121°C.

Pressure-assisted thermal sterilization has been assessed for destruction of highly resistant, food-relevant endospores. *Bacillus amyloliquefaciens* FAD 11/2 produces one

of the most resistant endospores known, and was originally isolated from ropy bread (1, 102, 133, 146). Endospores of *B. amyloliquefaciens* are inactivated by 4.5-logs at 700 MPa and 121°C (2). These parameters are rather severe. By comparison, a 7-8-log inactivation was observed for endospores of other species at identical processing parameters (2). Strains genetically proximal to *C. botulinum,* and *C. botulinum* itself, have also been subjected to PATS. Negligible endospore inactivation occurs in *Clostridium sporogenes* after treatment at 600 MPa for 30 min at 20°C (111). This highlights endospore resistance to pressure at ambient temperature. However, at 400 MPa, for 30 min at 60°C a 3-log reduction in *C. sporogenes* endospores occurred (111). These conditions constitute relatively moderate processing parameters. In contrast, treatment of 700 MPa and 121°C, for 1 min, was capable of inactivating 7-8-logs of endospores of *C. sporogenes* and *Clostridium tyrobutyricum* (2). The endospores of *C. botulinum* exhibit more concerning results.

Endospores of *C. botulinum* are among the most resistant to PATS, next to strains of *B. amyloliquefaciens*. Effective PATS parameters for the elimination of endospores of *C. botulinum* range from 60 to 121°C, and include pressures up to 800 MPa (67). *C. botulinum* strains also exhibit strain-specific sensitivity to PATS. Vegetative bacteria treated with PATS originally displayed this variability (128). It seems that strain-dependent resistance carries over to endospores (Table 1-1). Varying temperature or time appears to have the greatest effect on optimizing endospore inactivation. Endospores of *C. botulinum* can exhibit resistance ranging from no inactivation to a 5.5-log reduction at 600 MPa and 80°C (103).

**Table 1-1.** *C. botulinum* endospore strain resistance to pressure-assisted thermal processing.

| Strain | Pressure (MPa) | Temperature (°C) | Time | Inactivation (logs) | Reference |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| Type A BS-A<br>Type A 62-A | 827 | 75 | 20 min | 3 | (140) |
| Type B ATCC 25765<br>Type B TMW 2.518 | 600 | 80 | 1 s | >5.5 | (103) |
| Type E | 827 | 60 | 5 min | >5 | (141) |
| 2-B<br>KAP9-B | 827 | 75 | 20-30 min | 6 | (139) |
| 17-B | 620 | 100 | 10 min | 5.5 | |
| TMW 2.357 | 800 | 120 | 4 min | 6 | (102) |

Pressure resistance of endospores appears to be unrelated to thermal resistance (103, 104, 115). For example, endospores of *B. subtilis* IAM12118 are highly pressure-resistant, but exhibit higher heat sensitivity than endospores of *Bacillus stearothermophilus*, which has similar pressure resistance (115). Pressure stabilizes endospores to thermal effects in some cases (2, 102, 125, 133). Increasing temperature decreases D-values of endospores until 121°C, where the contribution of pressure to inactivation becomes less pronounced (133). Temperatures of 100°C can be more effective at inactivating endospores than a combination of 600-800 MPa and 120°C (102). Tailing effects in inactivation curves of endospores are common during PATS, and may be a consequence of pressure stabilization (2, 102). A consensus on the parameters for PATS that is adequate for industrial application cannot easily be distilled from the literature. A reliable way of enhancing inactivation of endospores by PATS may be through the use of highly specific antimicrobial compounds.

Pressure-assisted thermal sterilization in combination with low pH was briefly assessed against endospores. Acidic environments can enhance HHP inactivation of vegetative cells (3, 164). With the exception of *Alicyclobacillus acidoterrestris*, sporeforming bacteria cannot germinate in low pH foods, thus making pH an attractive candidate for PATS hurdle technology (166). *Clostridium* endospores, reduced by less than 0.5-logs at neutral pH, 404 MPa at 25°C, for 30 min, are reduced by 2.5-logs

following treatment at the same conditions in a medium at pH 4.0 (168). The use of citric acid (pH of 4.0) and PATS to eliminate endospores of *Bacillus* spp achieved a 6-log reduction using 400 MPa and 70°C, for 30 min (145). In contrast, Wuytack and Michiels (185) found that endospores of *B. subtilis* resist inactivation over a pH range of 3.0 to 8.0 at pressures of 100 to 600 MPa at 40°C. Reasons for this variability remain unknown. However, nutrient and pressure-induced germination are favored under neutral pH (185), and acidity may counteract the inactivating effects of HHP by inhibiting germination. Endospores have shown either enhanced inactivation or complete resistance to acidic conditions and PATS (145, 185). The manipulation of pH to enhance endospore inactivation during PATS is not a favorable technique because it is unreliable and ultimately alters product quality.

### 1.7 Enhancing Pressure-Assisted Thermal Sterilization (PATS)

Understanding the physicochemical effects of HHP on bacterial cells is crucial to selecting antimicrobial compounds that may enhance endospore inactivation during PATS. Pressure-mediated cell inactivation is attributable to structural and physiological damage (18, 143). Application of HHP forces a decrease in void volumes that require potentially irreversible molecular reconfigurations (149). High hydrostatic pressure acts on the spatial structures of biomacromolecules in an isostatic fashion (149, 184), which ultimately leads to pressure-induced denaturation of proteins and cellular inactivation as vital functions are compromised. Molecular rearrangement eliminates void volumes and is the primary contributor to protein denaturation in aqueous solutions (69, 76). Reconfigurations are capable of adversely affecting cellular physiology, including metabolism, membrane function, compound transport, transcription, and translation (8, 149). High hydrostatic pressure also favors ionic dissociation, as these favorably decrease volume through water interaction (149). High hydrostatic pressure can strengthen

hydrogen bonds, but does not disrupt covalent bonds unless extremely high pressures are applied (149). Winter and Jeworrek (184) have postulated that pressure can also affect structural properties of the surrounding solvent (*ie*. water), which may further induce structural and dynamical changes. Bacterial cell membranes are particularly susceptible to HHP.

Bacterial cell membranes are relatively large, dynamic aggregates of lipids, proteins, and various carbohydrate derivatives. They are highly sensitive to HHP due to this heterogeneous composition (184). Application of HHP to membrane systems increases the packing order of membranes, mimicking the effects of cooling, and can form additional phases not observed at ambient pressures (184). Ordering effects can protect membranes, as in the case where water freezes and lamellar membrane structures are not destroyed by crystallization (37). However, HHP does not necessarily result in favorable stabilization of molecular aggregates within membranes. Pressure can lead to unfolding of proteins and exposure of chemical moieties, which may interact and form structures that do not revert to their original structure upon depressurization (149). Pressure-induced germination is likely caused by similar conformational disruptions of germinant receptors within inner membranes (11, 121, 186). Pressure-induced germination has yielded information on why PATS is more effective than thermal processing for inactivation of endospores.

Endospore inactivation using HHP was proposed as a two-step process, beginning with induction of germination followed by inactivation of sensitive cells (10, 11). Pressurization during PATS involves a "come-up" time to reach the specified pressure, during which considerable inactivation of endospores can occur at appropriate temperatures (10). Once pressurization is achieved, heat and pressure act synergistically to enhance protein denaturation and enzyme inactivation. Additional protein denaturation is considered the main reason for PATS' enhanced inactivation of endospores (147, 149).

High temperatures are thought to disrupt inner membrane stability, which results in the release of DPA (29). During PATS there is also an associated release of intracellular substances such as metallic ions and SASPs, irreversible hydrolysis of the cortex, and a decrease in intracellular pH (65, 90, 151, 164). The evidence indicates that compromising the inner membrane makes an endospore vulnerable to inactivation by heat and pressure similar to vegetative cells. Therefore, antimicrobial compounds capable of compromising inner membrane integrity should be capable of enhancing PATS.

### 1.8 Targeting the Inner Membrane of Endospores

The inner membrane of endospores is a good target for an inactivation strategy combining PATS with antimicrobials. High hydrostatic pressure in combination with antimicrobial peptides has been examined to extend storage life and ensure food safety against vegetative cells (105, 113, 144). This strategy is gaining interest for use against endospores due to the inner membrane's role as a permeability barrier, its hydrophobicity, and the endospores' metabolic dormancy (63). These three factors highlight the inner membrane's role in maintaining endospore resistance, accessibility by hydrophobic compounds, and the inability of endospores to mount adaptive resistance. The cellular membrane is known as one of the most pressure-sensitive components of bacteria (184). The inner membranes of endospores have not been assessed in this respect. However, they are susceptible regions for HHP effects because they exhibit a high propensity for morphological and physiological change (45, 190). In addition, germinant receptors are located within the inner membrane (77, 156), and DPA crosses this barrier during germination and inactivation (78, 104, 121, 187). These factors suggest the inner membrane plays a significant role in inactivation of endospores.

#### 1.8.1 Inner Membrane Impermeability

Intact inner membranes are barriers to chemicals that would otherwise enter the cores of endospores (155). For instance, the endospore inner membrane is impermeable to the small hydrophilic molecule methylamine, which rapidly crosses the membranes of germinated and vegetative cells (31, 154, 170). This membrane is also a barrier to water, and helps maintain low water activity in conjunction with the cortex. When the inner membrane integrity is compromised, the core rehydrates and this indirectly facilitates inactivation of endospores by heat and antimicrobials (58, 95, 109, 156). Mutant endospores with an altered inner membrane composition have been shown to have higher core water content (63). Although the mechanism of endospore dehydration is unknown, these modified endospores exhibit up to a ten-fold increase in sensitivity to wet heat (63). Griffiths and Setlow (63) provide compelling evidence that compromising inner membranes leads to loss of wet heat resistance that correlates with increased core water content. The mechanism for inner membrane impermeability has not been explained (31, 63, 154, 170). However, the evidence suggests that impermeability is a consequence of unique structural cohesion. Compromising inner membrane structural integrity should be a priority in strategies for inactivation of endospores.

### 1.8.2 Hydrophobic Character

Endospores are hydrophobic which allows them to aggregate and leads to increased resistance to high temperature and pressure (92, 182). Aggregation likely shields the innermost endospores from disruptive thermodynamic effects. Endospore aggregation is not attributed to highly specific molecular interactions, and is thought to be the result of simple hydrophobic interactions. Inner membranes are unlikely to be involved, as endospore architecture makes direct interaction improbable. However, the observation of aggregation highlights the hydrophobic properties of endospores. The hydrophobicity of endospores does not change appreciably during PATS (1).

These two observations about endospores emphasize the potential for hydrophobic antimicrobial compounds to be used in combination with PATS. Hydrophobic antimicrobial compounds should be attracted to endospores from different species. Such attraction may facilitate accurate localization of compounds to the inner membrane of the endospore. Resilience of hydrophobicity during PATS suggests that hydrophobic antimicrobials may be effectively retained once properly situated. It remains to be seen if such antimicrobials can exert activity to compromise the inner membrane. Selection of antimicrobials must take into account inner membrane dynamics.

### 1.8.3 Metabolic Dormancy of Endospores

Lipids of the inner membrane are largely immobile, with mobility returning upon germination (34). The inner membrane exists in a compressed state, with surface area expanding 1.5-fold upon germination in the absence of lipid synthesis (34). It is important to note that the membrane lipids of endospores of *Bacillus* spp are also similar, in both quality and quantity, to those of vegetative cells (9, 106, 118, 132). Similar findings have not been confirmed for *Clostridium* spp. The evidence suggests that the inner membranes of endospores are dormant, with little molecular movement, and that the composition may allow entry by hydrophobic antimicrobial compounds.

Proteins involved in germination are located within inner membranes. This includes nutrient germinant receptors and SpoVA proteins (75, 122, 177, 178). Modifying the lipid content of inner membranes has marginal effects on germination (63). Germination proteins facilitate the release of Ca-DPA and a concomitant entry of water. Membrane proteins can function within the inner membrane, suggesting that proteins with membrane activity, such as nisin, may also retain activity. Activity of membrane-targeting antimicrobials is supported by the use of nisin to control the outgrowth of endospores of *C. botulinum* (28, 39, 66, 81, 172). The effects of PATS on this activity

have not been characterized. It is unknown whether non-protein, hydrophobic antimicrobials will retain activity within the inner membrane during PATS.

Oligomeric proteins dissociate at low pressures (<200 MPa) (69). Proteins dissolved in membranes appear to be highly sensitive to pressure changes (176, 191). Pressure-assisted thermal sterilization may inhibit the activity of hydrophobic antimicrobials, be they protein or otherwise. Proteins may be the most susceptible to these effects, as the activity and inactivation of many membrane-bound enzymes depends on consistent protein structure, a certain degree of membrane fluidity, or both (27, 40). Membrane proteins and lipids can influence one another simply as a result of their biochemical nature, making for unpredictable effects when PATS is also applied (184). Regardless, the prior success in controlling endospores using nisin supports a more in-depth examination of antimicrobial effects upon endospores during PATS.

Selection of hydrophobic antimicrobials for endospore control during pressure-assisted thermal sterilization (PATS) should be based on a generic affinity for cellular membranes. This will ensure targeting of inner membranes of endospores. In other words, hydrophobic antimicrobials that execute membrane insertion via hydrophobic interaction, and then compromise membrane barrier properties, are favorable. Nisin and reutericyclin are two such molecules.

## 1.9 Nisin and Reutericyclin

### 1.9.1 Origin of Nisin and Use in Food Processing

Many lactic acid bacteria (LAB) associated with foods produce small, narrow-spectrum antimicrobial peptides known as bacteriocins (33, 171). Gratia (62) was the first to observe pathogen inhibition by bacteriocins over 80 years ago. In 1947, a proteinaceous bacteriocin, originally identified in 1933, was named nisin for “group N inhibitory substance” (108, 181). It wasn’t long before the deliberate use of bacteriocins

as food preservatives was proposed (70). Nisin was at the forefront of bacteriocins considered. First marketed in 1953, nisin has since achieved GRAS status ("generally regarded as safe") in the USA (49), and as of 2005 was approved for use in food in over 48 countries (33). In the USA specifically, nisin is confirmed for use against *C. botulinum* endospores in pasteurized cheese spreads, as it readily prevents outgrowth and toxin formation (49, 172). In hard cheese, nisin prevents "late gas blowing" brought about by germination and outgrowth of endospores of *Clostridium* spp (39). Nisin is used in high- and low-acid canned foods to prevent *Clostridium* and *Bacillus* outgrowth (28). Using bacteriocins produced by lactic acid bacteria (LAB) from food fermentations represents an unprecedented opportunity to manipulate food systems with naturally occurring compounds, for the express purpose of enhancing food safety (33). Cotter et al (33) make a sobering assessment, noting that before any naturally occurring compound can be used in food applications it must first be thoroughly understood on a biological level.

### 1.9.2 Nisin Mode of Action and Endospore Control During PATS

Nisin belongs to a class of bacteriocins called lantibiotics. Nisin is a polycyclic peptide with binding specificity for lipid II molecules, and lyses cells via formation of pores (64). Lantibiotic peptides are posttranslationally modified to form lanthionine rings (23, 93, 189). Nisin is positively charged, composed of 34 amino acids, and is produced by the LAB *Lactococcus lactis* subspecies *lactis* (41). Nisin is capable of effectively inactivating bacteria through two mechanisms. First, nisin binds the lipid II molecule in bacterial cell membranes, preventing completion of new cell septa and preventing cell wall synthesis (64). This activity is bactericidal in a growing cell culture. The second mechanism of bacterial inactivation is via trans-membrane pore formation (41). Pore-formation leads to a loss of intracellular molecules and dissipation of proton motive force (41). Nisin pores can dissipate a variety of ion gradients, including potassium (53).

Nisin, among other bacteriocins, has been proven to have an inhibitory effect on endospores (94, 114). The combined effects of nisin and HHP against bacteria have been examined (66, 81). Lopez-Pedemonte et al (94) examined the effects of nisin and lysozyme on endospores during HHP alone. Nisin facilitated an inactivation of approximately 2.5-log after moderate pressure treatment (400 MPa), whereas lysozyme had little to no effect (94). The effects of HHP and nisin in combination with heat on Gram-positive endospores, including those of *C. botulinum,* have also been examined (56, 168). The use of HHP and 60°C with either nisin or pediocin extended the storage life of cooked roast beef slices, based on inhibition of bacterial growth, when held at 4°C to 84 days (80). In a separate example, pressures between 300 and 700 MPa were used in concert with temperatures between 30°C and 70°C (56); the application of nisin during PATS reduced the temperature, pressure, or time parameters required to inactivate food-relevant endospores, as in the case of *C. botulinum* 33A (56). Other bacteriocins in combination with HHP have also shown potential in this respect (12, 81). Nisin is an ideal antimicrobial for studying the effects of hydrophobic antimicrobials on endospores. Nisin's ability to enhance inactivation of endospores by PATS, its targeting of cell membranes, and well-characterized biochemistry makes it ideal for the study of physicochemical effects of hydrophobic antimicrobials on the inner membranes of endospores during PATS.

### 1.9.3 Origin, Mode of Action, and Use of Reutericyclin in Food Processing

Reutericyclin was the first low molecular weight antibiotic isolated from a lactic acid bacteria fermentation (73). *Lactobacillus reuteri*, a sourdough starter culture, was identified as producing reutericyclin (54). Reutericyclin has undergone substantial study to elucidate its biochemistry, and it is now known that this compound is a unique tetramic acid (51, 54, 73). Reutericyclin is highly hydrophobic, negatively charged, and partitions

favorably into cytoplasmic membranes (53, 54). Reutericyclin also exhibits bactericidal activity.

Reutericyclin inactivates Gram-positive organisms via proton-ionophore activity (51, 54). The outer membrane of Gram-negative organisms affords them protection. Once inserted into a cell membrane, reutericyclin effectively dissipates proton gradients and nullifies any associated proton motive force (51, 54). Reutericyclin can inhibit lactic acid bacteria, as observed in *Lactobacillus* spp (53). Reutericyclin can inhibit germination of endospores and cell growth in *Bacillus* spp (51). It is not presently added intentionally to food products to improve food safety, but reutericyclin can enhance the persistence of sourdough starter cultures (52). Reutericyclin can be added indirectly via appropriate starter culture selection (50). Reutericyclin has potential as an additive for extending product shelf life and may achieve acceptance for this purpose quickly, given its origin (33).

No studies using PATS in combination with reutericyclin for control of endospores have been reported. Reutericyclin's hydrophobic nature, and proven ability to prevent germination of endospores of *Bacillus* spp make it a suitable candidate for this study. It is important to note that divalent cations in millimolar concentrations can counteract the activity of reutericyclin (55). It remains to be seen if calcium ion levels released from the cores of endospores during PATS could result in a protective effect. This consideration is relevant due to the use of calcium in canning of foods (163).

## 1.10 Research Objectives

Consumer demand is increasing for low-acid, shelf-stable, minimally processed foods that retain a high degree of quality. Conventional thermal processing cannot meet these demands. Pressure-assisted thermal sterilization can minimize the processing parameters needed, but bacterial endospores exhibit variable resistance, making industrial implementation of this technology problematic. It may be possible to enhance inactivation of endospores by PATS by simultaneously using membrane-targeting antimicrobial compounds. Research into the efficacy of this hurdle approach is in its infancy, and a comprehensive understanding of the physicochemistry does not yet exist. Expanding current knowledge about PATS hurdle technology will help facilitate its acceptance by the food industry and regulators for control of endospores.

This project examined the hypothesis that antimicrobial compounds can be used to target inner membranes of endospores and thereby enhance inactivation by PATS. The objectives of this project were as follows:

1. Determine the resistance of endospores of *Clostridium* spp to nisin and reutericyclin during PATS.
2. Determine the composition of the inner membrane of endospores of *Clostridium* spp, and assess membrane fluidity *in situ* and *ex situ* of PATS treated endospores to provide a physicochemical explanation for variability in resistance.

The thesis project aimed to undertake two stages of research in order to assess a PATS hurdle approach that targets inner membranes of endospores. Efficacy of membrane-targeting compounds must first be assessed. In doing so, endospore inactivation profiles must be established in combination with commercially relevant PATS parameters and bacterial strains. Use of species of *Clostridium* is preferable, due to their problematic association with food and genetic relation to *C. botulinum*. Using endospores other than *C. botulinum* as surrogates is an accepted method within the

literature (2). Endospores of *Bacillus* spp have been well studied, due to ease of genetic and microbiological manipulation. Endospores of *Clostridium* spp, in contrast, remain shrouded in mystery (155).

The second stage of research should attempt to explain differences observed in endospore inactivation kinetics. The chemistry of antimicrobials must be well characterized, and modes of activity known, as this information is critical in complementing gaps in knowledge of endospore physiology. These characteristics may also help explain novel observations. Nisin and reutericyclin are well suited to this study because both are well characterized, of lactic acid bacteria origin, and target bacterial cell membranes (51, 96). Nisin and reutericyclin also have different mechanisms of antimicrobial activity that will allow for comparison. It may be necessary to use unconventional approaches to explain effects on the inner membrane, but it is not uncommon in HHP biophysical studies to use unique methods (184).

## 1.11 References


1. **Ahn, J., and Balasubramaniam, V.M.** 2007. Physiological responses of *Bacillus amyloliquefaciens* spores to high pressure. J. of Microbiol. and Biotechnol. **17:** 524-529.
2. **Ahn J., Balasubramaniam V.M., and Yousef A.E.** 2007. Inactivation kinetics of selected aerobic and anaerobic bacterial spores by pressure-assisted thermal processing. Int. J. Food Microbiol. **113:** 321-329.
3. **Alpas, H., Kalchayanand, N., Bozoglu, F., and Ray, B.** 2000. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. Intl. J. Food Microbiol. **60:** 33-42.
4. **Ananta, E., Heinz, V., Schluter, O., and Knorr, D.** 2001. Kinetic studies on high-pressure inactivation of *Bacillus stearothermophilus* spores suspended in food matrices. Innov. Food Sci. and Emerging Tech. **2:** 261-272.
5. **Bagyan, I., and Setlow, P.** 2002. Localization of the cortex lytic enzyme CwlJ in spores of *Bacillus subtilis*. J. Bacteriol. **184:** 1289-1294.
6. **Balasubramaniam, V.M., Ting, E.Y., Stewart, C.M., and Robbins, J.A.** 2004. Recommended laboratory practices for conducting high-pressure microbial inactivation experiments. Innov. Food Science and Emerging Tech. **5:** 299-306.
7. **Balasubramanian, S., and Balasubramaniam, V.M.** 2003. Compression heating influence of pressure-transmitting fluids on bacterial inactivation during high pressure processing. Food Research Intl. **36:** 661-668.
8. **Bartlett, D. H.** 2002. Pressure effects on in vivo microbial processes. Biochim. Biophys. Acta - Protein Structure and Molecular Enzymology. **1595:** 367-381.

9. **Bertsch, L.L., Bonsen, P.P., and Kornberg, A.** 1969. Biochemical studies of bacterial sporulation and germination. XIV. Phospholipids in *Bacillus megaterium*. J. Bacteriol. **98:** 75-81.

10. **Black, E.P., Setlow, P., Hocking, A.D., Stewart, C.M., Kelly, A.L., and Hoover, D.G.** 2007a. Response of spores to high-pressure processing. Comp. Rev. Food Sci. Food Safety. **6:** 103-119.

11. **Black, E.P., Wei, J., Atluri, S., Cortezzo, D.E., Koziol-Dube, K., Hoover, D.G., and Setlow, P.** 2007b. Analysis of factors influencing the rate of germination of spores of *Bacillus subtilis* by very high pressure. J. Appl. Microbiol. **101:** 65-76.

12. **Black, E.P., Kelly, A.L., and Fitzgerald, G.F.** 2005. The combined effect of high pressure and nisin on microorganisms in milk. Innov. Food Sci. Emerging Tech. **6:** 286-292.

13. **Black, E.P., Koziol-Dube, K., Guan, D., Wei, J., Setlow, B., Cortezzo, D.E., Hoover, D.G., and Setlow, P.** 2005. Factors influencing the germination of *Bacillus subtilis* spores via the activation of nutrient receptors by high pressure. Appl. Environ. Microbiol. **71:** 5879-5887.

14. **Borge, G.I.A., Skeie, M., Sorhaug, T., Langsrud, T., and Granum, E.** 2001. Growth and toxin profiles of *Bacillus cereus* isolated from different food sources. Intl. J. Food Microbiol. **6:** 237-246.

15. **Bridgman, P. W.** 1912. Water under pressure. Proc. American Acad. Arts Sci. **48:** 450-558.

16. **Broda, D.M., DeLacy, K.M., Bell, R.G., Braggins, T.J., and Cook, R.L.** 1996. Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Intl. J. Food Microbiol. **29**:335-352.

17. **Brown, K.** 2000. Control of bacterial spores. Brit. Med. Bulletin. **56:** 58-71.

18. **Bull, M.K., Hayman, M.M., Stewart, C.M., Szabo, E.A., and Knabel, S.J.** 2005. Effect of prior growth temperature, type of enrichment medium, and temperature and time of storage on recovery of *Listeria monocytogenes* following high pressure processing in milk. Intl. J. Food Microbiol. **101:** 53-61.

19. **Buzrul, S., Alpas, H., Largeteau, A., and Demazeau, G.** 2008. Inactivation of *Escherichia coli* and *Listeria innocua* in kiwifruit and pineapple juices by high hydrostatic pressure. Intl. J. Food Microbiol. **124:** 275-278.

20. **Cano, R.J., and Borucki, M.** 1995. Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. Science **268:** 1060-1064.

21. **Carlin, F.** 2011. Origin of bacterial spores contaminating foods. Food Microbiol. **28:** 177-182.

22. **Carrera, M., Zandomeni, R.O., Fitzgibbon, J., and Sagripanti, J.-L.** 2007. Difference between the spore sizes of *Bacillus anthracis* and other *Bacillus* species. J. Appl. Microbiol. **102:** 303-312.

23. **Chatterjee, C., Paul, M., Xie, L., and van der Donk, W.A.** 2005. Biosynthesis and mode of action of lantibiotics. Chem. Rev. **105:** 633-683.

24. **Cheftel, J. C.** 1995. Review: high-pressure, microbial inactivation and food preservation. Food Sci. Tech. Intl. **1:** 75-90.

25. **Chen, S.Q., Tang, Q.Y., Zhang, X.D., Zhao, G.H., Hu, X.S., Liao, X.J., Chen, F., Wu, J.H., and Xiang, H.** 2006. Isolation and characterization of thermo-acidophilic endospore-forming bacteria from the concentrated apple juice-processing environment. Food. Microbiol. **23**: 439-445.

26. **Chirakkal, H., O'Rourke, M., Atrih, A., Foster, S.J., and Moir, A.** 2002. Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination. Microbiol. **148:** 2383-2392.

27. **Chong, P.L.-G., Fortes, P.A.G., and Jameson, D.M.** 1985. Mechanisms of inhibition of (Na, K)-ATPase by hydrostatic pressure studied with fluorescent probes. J. Biol. Chem. **260:** 14484-14490.

28. **Cleveland, J., Montville, T.J., Nes, I.F., and Chikindas, M.L.** 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Intl. J. Food Microbiol. **71:** 1-20.

29. **Coleman, W.H., Chen, D., Li, Y.Q., Cowan, A.E., and Setlow, P.** 2007. How moist heat kills spores of *Bacillus subtilis*. J. Bacteriol. **189:** 8458-8466.

30. **Cortezzo, D.E., and Setlow, P.** 2005. Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. J. Appl. Microbiol. **98:** 606-617.

31. **Cortezzo, D.E., Koziol-Dube, K., Setlow, B., and 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:** 838-852.

32. **Cortezzo, D.E., Setlow, B., and Setlow, P.** 2004. Analysis of the action of compounds that inhibit the germination of spores of *Bacillus* species. J. Appl. Food Microbiol. **96:** 725-741.

33. **Cotter, P.D., Hill, C., and Ross, R.P.** 2005. Bacteriocins: Developing innate immunity for food. Nat. Rev. Microbiol. **3:** 777-788.

34. **Cowan, A.E., Olivastro, E.M., Koppel, D.E., Loshon, C.A., Setlow, B., and Setlow, P.** 2004. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. Proc. Natl. Acad. Sci. USA. **101:** 7733-7738.

35. **Cowan, A.E., Koppel, D.E., Setlow, B., and 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:** 4209-4214.

36. **Crawford, Y.J., Murano, E.A., Olson, D.G., and Shenoy, K.** 1996. Use of high hydrostatic pressue and irradiation to eliminate *Clostridium sporogenes* spores in chicken breast. J. Food Prot. **59:** 711-715.

37. **Czeslik C., Reis O., Winter R., and Rapp G.** 1998. Effect of high pressure on the structure of dipalmitoylphosphatidylcholine bilayer membranes: a synchrotron-X-ray diffraction and FT-IR spectroscopy study using the diamond anvil technique. Chem. Phys. Lipids. **91:** 135-144.

38. **Daubenspeck, J.M., Zeng, H., Chen, P., Dong, S., Steichen, C.T., Krishna, N.R., Pritchard, D.G., and Turnbough, C.L.** 2004. Novel oligosaccharide chains of the collagen-like region of BclA, the major glycoprotein of the exosporium. J. Biol. Chem. **279:** 30945-30953.

39. **Delves-Broughton, J., Blackburn, P., Evans, R.J., and Hugenholtz, J.** 1996. Applications of the bacteriocin, nisin. Antonie van Leeuwenhoek. **69:** 193-202.

40. **Dreyfus, G., Guimaraes-Motta, H., and Silva, J.L.** 1988. Effect of hydrostatic pressure on the mitochondrial ATP synthase. Biochem. **27:** 6704-6710.

41. **Driessen, A.J.M., van den Hooven, H.W., Kuiper, W., van der Kamp, M., Sahl, H.G., Konnigs, R.N.H., and Konnigs, W.N.** 1995. Mechanistic studies of lantibiotic-induced permeabilization of phospholipids vesicles. Biochem. **34:** 1606-1614.

42. **Driks, A.** 2002. *Bacillus subtilis* and its Closest Relatives: from Genes to Cells: Proteins of the spore core and coat. (A. H. Sonenshein, Ed.) Washington: American Society for Microbiology.

43. **Driks, A.** 2002. Overview: development in bacteria: spore formation in *Bacillus subtilis*. Cell. Molec. Life Sci. **59:** 389-391.

44. **Driks, A.** 1999. *Bacillus subtilis* spore coat. Microbiol. Molec. Biol. Rev. **63:** 1-20.

45. **Earnshaw, R.G., Appleyard, J., and Hurst, R.M.** 1995. Understanding physical inactivation processes: Combined preservation opportunities using heat, ultrasound and pressure. Intl. J. Food Microbiol. **28:** 197-219.

46. **Erkmen, O., and Dogan, C.** 2004. Effects of ultra hydrostative pressure on *Listeria monocytogenes* and natural flora in broth, milk, and fruit juices. Intl. J. Food Sci. Tech. **39:** 91-97.

47. **Esty, J.R., and Meyer, K.F.** 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes. J. Infec. Diseases. **31:** 650-664.

48. **Farkas, D.F., and Hoover, D.G.** 2000. High pressure processing. J. Food Sci. **65:** 47-64.

49. **Federal Register, U.S.A.** 1988. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. **53:** 11247-11251.

50. **Gänzle, M. G.** 2009. From gene to function: Metabolic traits of starter cultures for improved quality of cereal foods. Intl. J. Food Microbiol. **134:** 29-36.

51. **Gänzle, M. G.** 2004. Reutericyclin: biological activity, mode of action, and potential applications. Appl. Microbiol. Biotech. **64:** 326-332.

52. **Gänzle, M.G., and Vogel, R.F.** 2003. Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuteri* in an industrial sourdough fermentation. Intl. J. Food Microbiol. **80:** 31-45.

53. **Gänzle, M.G., and Vogel, R.F.** 2003. Studies on the mode of action of reutericyclin. Appl. Environ. Microbiol. **69:** 1305-1307.

54. **Gänzle, M. G., Holtzel, A., Walter, J., Jung, G., and Hammes, W.P.** 2000. Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Appl. Environ. Microbiol. **66:** 4325-4333.

55. **Gänzle, M.G., Hertel, C., and Hammes, W.P.** 1995. Microbial physiology and gene regulation: Emerging principles and applications: Antimicrobial activity in lactobacilli from sourdough. W. Scheffers. Ed. Delft: Delft University Press.

56. **Gao, Y.-L., and Ju, X.-R.** 2008. Exploiting the combined effects of high pressure and moderate heat with nisin on inactivation of *Clostridium botulinum* spores. J. Microbiol. Meth. **72:** 20-28.

57. **Gao, Y., Ju, X.R., and Jiang, H.H.** 2006a. Studies on inactivation of *Bacillus subtilis* spores by hydrostatic pressure and heat using desing of experiments. J. Food Eng. **77:** 672-679.

58. **Genest, P.C., Setlow, B., Melly, E., and Setlow, P.** 2002. Killing of spores of *Bacillus subtilis* by peroxynitrite appears to be caused by membrane damage. Microbiol. **148:** 307-314.

59. **Gerhardt, P., and Marquis, R.E.** 1989. Regulation of prokaryotic development: Spore thermoresistance mechanisms. (I. S. Smith, Ed.) Washington, USA: American Society for Microbiology.

60. **Ghosh, S., Setlow, B., Wahome, P.G., Cowan, A.E., Plomp, M., Malkin, A.J., and Setlow, P.** 2008. Characterization of spores of *Bacillus subtilis* that lack most coat layers. J. Bacteriol. **190:** 6741-6748.

61. **Gould, G.W., and Jones, M.V.** 1989. Mechanisms of action of food preservation procedures: Combination and synergistic effects. (G. Gould, Ed.) London: Elsevier Applied Science.

62. **Gratia, A.** 1925. Sur un remarquable exemple d'antagonisme entre deux souches de *Colibacille*. C. R. Soc. Biol. **93:** 1040-1041.

63. **Griffiths, K.K., and Setlow, P.** 2009. Effects of modification of membrane lipid composition of *Bacillus subtilis* sporulation and spore properties. J. Appl. Microbiol. **106:** 2064-2078.

64. **Hasper, H.E., Kramer, N.E., Smith, J.L., Hillman, J.D., Zachariah, C., Kuipers, O.P., de Krujiff, B., and Breukink, E.** 2006. Mechanism of action for lantibiotic peptides that target lipid II. Science **313:** 1636-1637.

65. **Hauben, K.J.A., Bernaerts, K., and Michiels, C.W.** 1998. Protective effect of calcium on inactivation of *Escherichia coli* by high hydrostatic pressure. J. Appl. Microbiol. **85:** 678-684.

66. **Hauben, K., Wuytack, E., Soontjens, C., Michiels, C.** 1996. High pressure transient sensitization of Escherichia coli to lysozyme and nisin by disruption of outer-membrane permeability. J. Food Protec. **59:** 350-355.

67. **Hendrickx, M., and Knorr, D.** 2001. Ultra-high pressure treatments of foods. New York, USA: Plenum Publishers.

68. **Henriques C.P, and Moran A.O.** 2007. Structure, assembly, and function of the spore surface layers. Ann. Rev. Microbiol. **61:** 555-588.

69. **Heremans, K., and Smeller, L.** 1998. Protein structure and dynamics at high pressure. Biochim. Biophys. Acta. **1386:** 353-370.

70. **Hirsch, A., Grinsted, E., Chapman, H.R., and Mattick, A.T.R.** 1951. A note on the inhibition of an anaerobic sporeformer in Swiss-type cheese by a nisin-producing *Streptococcus*. J. Dairy Sci. **18:** 205-206.

71. **Hite, B. H.** 1914. The effects of pressure on certain microorganisms encountered in the preservation of fruits and vegetables. Vol. 146. Morgantown, West Virginia, USA: University Agriculture Experiment Station.

72. **Hite, B.H., Giddings, N.J., and Weakly, C.E.** 1899. The effects of pressure in the preservation of milk: a preliminary report. Vol. 58. Morgantown, West Virginia, USA: West Virginia Agricultural Experiment Station.

73. **Höltzel, A., Gänzle, M.G., Nicholson, G.J., Hammes, W.P., and Jung, G.** 2000. The first low molecular weight antibiotic from lactic acid bacteria: reutericylin, a new tetramic acid. Angewandte Chemie-Intl. Ed. **39:** 2766-2768.

74. **Hoover, D.G., Metrick, C., Pepinean, A.M., Forkas, D.F., and Knorr, D.** 1989. Biological effects of high hydrostatic pressure on food microorganisms. Food Tech. **3:** 99-107.

75. **Hudson, K.D., Corfe, B.M., Kemp, E.H., Feavers, I.M., Coote, P.J., and Moir, A.** 2001. Localization of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. J. Bacteriol. **183:** 4317-4322.

76. **Hummer, G., Garde, S., Garcia, A.E., Paulaitis, M.E., and Pratt, L.R.** 1998. The pressure dependence of hydrophobic interactions is consistent with the observed pressure denaturation of proteins. Proc. Natl. Acad. Sci. **95:** 1552-1555.

77. **Igarashi, T., and Setlow, P.** 2005. Interaction between individual protein components of the GerA and GerB nutrient receptors that trigger germination of *Bacillus subtilis* spores. J. Bacteriol. **187:** 2513-2518.

78. **Janssen, F.W., Lund, A.J., and Anderson, L.E.** 1958. Calorimetric assay for dipicolinic acid in bacterial spores. Science **127:** 26-27.

79. **Jones, C.A., Padula, N.L., and Setlow, P.** 2005. Effect of mechanical abrasion on the viability, disruption and germination of spores of *Bacillus subtilis*. J. Appl. Microbiol. **99:** 1484-1494.

80. **Kalchayanand, N., Dunne, C.P., Sikes, A., and Ray, B.** 2003. Inactivation of bacterial spores by combined action of hydrostatic pressure and bacteriocins in roast beef. J. Food Safety. **23:** 219-233.

81. **Kalchayanand, N., Sikes, A., Dunne, C.P., and Ray, B.** 1998. Interaction of hydrostatic pressure, time and temperature of pressurization and pediocin AcH on inactivation of foodborne bacteria. J. Food Protec. **61:** 425-431.

82. **Kennedy, M.J., Reader, S.L., and Swierczynski, L.M.** 1994. Preservation records of microorganisms: evidence of the tenacity of life. Microbiology **140:** 2513-2529.

83. **Kim, J., and Foegeding, P.M.** 1993. Ecology and controls in foods. A. Hauschild, Ed. New York, USA: Marcel Dekker, Inc.

84. **Klobutcher, L.A., Ragkousi, K., and Setlow, P.** 2006. The *Bacillus subtilis* spore coat provides "eat resistance" during phagosomal predation by the protozoan *Tetrahymena thermophila*. Proc. Natl. Acad Sci. USA. **103:** 165-170.

85. **Knasi, G., and Hillier, J.** 1949. Preliminary observations on the germination of the endospore in *Bacillus megatherium* and the structure of the spore coat. J. Bacteriol. **57:** 23-29.

86. **Knorr D., Heinz V., and Buckow R.** 2006. High pressure application for food biopolymers. Biochim. Biophys. Acta. **1764:** 619-631.

87. **Kort, R., O'Brien, A.C., van Stokkum, I.H.M., Oomes, S.J.C.M., Crielaard, W., Hellingwerf, K.J., and Brul, S.** 2005. Assessment of heat resistance of bacterial spores from food product isolates by fluorescence monitoring of dipicolinic acid release. Appl. Environ. Microbiol. **71:** 3556-3564.

88. **Lai, E.M., Phadke, N.D., Kachman, M.T., Giorno, R., Vazquez, S., Vazquez, J.A., Maddock, J.R., and Driks, A.** 2003. Proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*. J. Bacteriol. **185:** 1443-1454.

89. **Lee, M.J., Dong-Ho, B., Dong-Ha, L., Ki-Hyo, J., Deog-Hwan, O., and Sang-Do, H.** 2006. Reduction of *Bacillus cereus* in cooked rice treated with sanitizers and disinfectants. J. Microbiol. Biotech. **16:** 639-642.

90. **Lee, S.W., and Sim, S.J.** 2006. Increased heat resistance of *Geobacillus stearothermophilus* spores heat-shocked during sporulation. J. Microbiol. Biotech. **16:** 633-636.

91. **Leistner, L., and Gorris, L.G.M.** 1995. Food preservation by hurdle technology. Trends Food Sci. Tech. **6:** 41-46.

92. **Leuschner, R.G.K., Weaver, A.C., and Lillford, P.J.** 1999. Rapid particle size distribution analysis of *Bacillus* spore suspensions. Colloids Surfaces. **13:** 47-57.

93. **Li, B., Yu, J.P.J., Brunzelle, J.S., Moll, G.N., van der Donk, W.A., and Nair, S.K.** 2006. Structure and mechanism of the lantibiotic cyclase involved in nisin biosynthesis. Science **311:** 1464-1467.

94. **Lopez-Pedemonte, T.J., Roig-Sagues, A.X., Trujillo, A.J., Capellas, M., and Guamis, B.** 2003. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. J. Dairy Sci. **86:** 3075-3081.

95. **Loshon, C.A., Melly, E., Setlow, B., and Setlow, P.** 2001. Analysis of the killing of spores of *Bacillus subtilis* by a new disinfectant, Sterilox(R). J. Appl. Microbiol. **91:** 1051-1058.

96. **Lubelski J., Rink R., Khusainov R., Moll G.N., and Kuipers O.P.** 2008. Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. Cell. Molec. Life Sci. **65:** 455-476.

97. **Lund, B. M.** 1990. Foodborne disease due to *Bacillus* and *Clostridium* species. Lancet. **336:** 982-986.

98. **Magge, A., Granger, A.C., Wahome, P.G., Setlow, B., Vepachedu, V.R., Loshon, C.A., Peng, L., Chen, D., Li, Y., and Setlow, P.** 2008. Role of dipicolinic acid in the germination, stability, and viability of spores of *Bacillus subtilis*. J. Bacteriol. **190:** 4798-4807.

99. **Magoon, C. A.** 1926. Studies Upon Bacterial Spores. J. Infec. Dis. **38:** 429-439.

100. **Maisnier-Patin, S., and Richard, J.** 1996. Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentrations. FEMS Microbiology Letters. **140:** 29-35.

101. **Mantovani, H.C., and Russel, J.B.** 2001. Nisin resistance of *Streptococcus bovis*. Appl. Environ. Microbiol. **67:** 808-813.

102. **Margosch D., Ehrmann M.A., Buckow R., Heinz V., Vogel R.F., and Gänzle M.** 2006. High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. Appl. Environ. Microbiol. **72:** 3476-3481.

103. **Margosch, D., Ehrmann, M.A., Gänzle, M.G., and Vogel, R.F.** 2004a. Comparison of pressure and heat resistance of *Clostridium botulinum* and other endospores in mashed carrots. J. Food Protec. **67:** 2530-2537.

104. **Margosch, D., Gänzle, M.G., Ehrmann, M.A., and Vogel, R.F.** 2004b. Pressure inactivation of *Bacillus* endospores. Appl. Environ. Microbiol. **70:** 7321-7328.

105. **Masschalck, B., Garcia-Graells, C., Van Haver, E., and Michiels, C.W.** 2000. Inactivation of high pressure resistant *Escherichia coli* by lysozyme and nisin under high pressure. Innov. Food Sci. Emerging Tech. **1:** 39-48.

106. **Matches, J.R., Walker, H.W., and Ayres, J.C.** 1964. Phospholipids in vegetative cells and spores of *Bacillus polymyxa*. J. Bacteriol. **87:** 16-23.

107. **Matser, A.M., Krebbers, B., van den Berg, R.W., and Bartels, P.V.** 2004. Advantages of high pressure sterilization on quality of food products. Trends Food Sci. Tech. **15:** 79-85.

108. **Mattick, A.T.R., and Hirsch, A.** 1947. Further observations on an inhibitory substance (nisin) from lactic streptococci. Lancet. **2:** 5-7.

109. **Melly, E., Cowan, A.E., and Setlow, P.** 2002. Studies on the mechanism of killing of *Bacillus subtilis* spores by hydrogen peroxide. J. Appl. Microbiol. **93:** 316-325.

110. **Meyer, R.S., Cooper, K.L., Knorr, D., and Lelieveld, H.L.M.** 2000. High-pressure sterilization of foods. Food Tech. **54:** 67-72.

111. **Mills, G., Earnshaw, R., and Patterson, M.F.** 1998. Effects of high hydrostatic pressure on *Clostridium sporogenes* spores. Letters Appl. Microbiol. **26:** 227-230.

112. **Moerman, F., Mertens, B., Demey, L., and Huyghebaert, A.** 2001. Reduction of *Bacillus subtilis*, *Bacillus stearothermophilus* and *Streptococcus faecalis* in meat batters by temperature-high pressure pasteurization. Meat Sci. **59:** 115-125.

113. **Morgan, S.M., Ross, R.P., Beresford, T., and Hill, C.** 2000. Combination of hydrostatic pressure and lacticin 3147 causes increased killing of *Staphylococcus* and *Listeria*. J. Appl. Microbiol. **88:** 414-420.

114. **Naim, F., Zareifard, M.R., Zhu, S., Huizing, R.H., Grabowski, S., and Marcotte, M.** 2008. Combined effects of heat, nisin and acidification on the inactivation of *Clostridium sporogenes* spores in carrot-alginate particles: From kinetics to process validation. Food Microbiol. **25:** 936-941.

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

116. **Nicholson, W.L.** 2002. Roles of *Bacillus* endospores in the environment. Cell. Molec. Life Sciences. **59:** 410-416.

117. **Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P.** 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Molec. Biol. Rev. **64:** 548.

118. **Nikolopoulou, M., and Vary, J.C.** 1987. Lipid composition of *Bacillus megaterium* spores and spore membranes. Lipids. **22:** 698-703.

119. **Okazaki, T., Kakugawa, K., Yoneda, T., Suzuki, K.** 2000. Inactivation behaviour of heat-resistant bacterial spores by thermal treatments combined with high-hydrostatic pressure. Food Sci. Tech. Research. **6:** 204-207.

120. **Paidhungat, M., and Setlow, P.** 2002. Spore germination and outgrowth. In *Bacillus subtilis* and its closest relatives: from genes to cells. J. L. Hoch, Ed. Washington, DC, USA: American Society for Microbiology.

121. **Paidhungat, M., Setlow, B., Daniels, W.B., Hoover, D., Papafragkou, E., and Setlow, P.** 2002. Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. Appl. Environ. Microbiol. **68:** 3172-3175.

122. **Paidhungat, M., and Setlow, P.** 2001. Localization of a germinant receptor protein (GerBA) to the inner membrane of *Bacillus subtilis* spores. J. Bacteriol. **183:** 3982-3990.

123. **Paidhungat, M., Setlow, B., Driks, A., and Setlow, P.** 2000. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. J. Bacteriol. **182:** 5505-5512.

124. **Paredes-Sabja, D. and Sarker, M.R.** 2011. Host serum factor triggers germination of *Clostridium perfringens* spores lacking the cortex hydrolysis machinery. J. Med. Microbiol. **60:** 1734-1741.

125. **Patazca, E., Koutchma, T., and Ramaswamy, H.S.** 2006. Inactivation kinetics of *Geobacillus stearothermophilus* spores in water using high-pressure processing at elevated temperatures. J.Food Sci. **71:** 110-116.

126. **Patterson, M. F.** 2005. Microbiology of pressure-treated foods. J. Appl. Microbiol. **98:** 1400-1409.

127. **Patterson, M.F., and Kilpatrick, D.J.** 1998. The combined effect of high hydrostatic pressure and mild heat on inactivation of pathogens in milk and poultry. J. Food Protec. **61:** 432-436.

128. **Patterson, M.F., Quinn, M., Simpson, R., and Gilmour, A.** 1995. Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. J. Food Protec. **58:** 524-529.

129. **Pellegrino, P.M., Fell, N.F. Jr., and Gillespie, J.B.** 2002. Enhanced spore detection using dipicolinate extraction techniques. Anal. Chim. **455:** 167-177.

130. **Piggot, P.J., and Hilbert, D.W.** 2004. Sporulation of *Bacillus subtilis*. Curr. Op. Microbiol. **7:** 579-586.

131. **Popham, D. L.** 2002. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. Cell. Molec. Life Sci. **59:** 426-433.

132. **Racine, F.M. and Vary, J.C.** 1980. Isolation and properties of membranes from *Bacillus megaterium* spores. J. Bacteriol. **143:** 1208-1214.

133. **Rajan, S., Ahn, J., Balasubramaniam, V.M., and Yousef, A.E.** 2006. Combined pressure-thermal inactivation kinetics of *Bacillus amyloliquefaciens* spores in egg patty mince. J. Food Protec. **69:** 853-860.

134. **Rasanayagam, V., Balasubramaniam, V.M., Ting, E., Sizer, C.E., Bush, C., and Anderson, C.** 2003. Compression heating of selected fatty food materials during high pressure processing. J. Food Sci. **68**:254-259.

135. **Raso, J., and Barbosa-Canovas, G.V.** 2003. Nonthermal preservation of foods using combined processing techniques. Crit. Rev. Food Sci. Nutr. **43:** 265-285.

136. **Ray, B.** 2002. The encyclopedia of environmental microbiology. G. Britton, Ed. New York, USA: John Wiley & Sons Inc.

137. **Ray, B., Kalchayanand, N., Dunne, P., and Sikes, A.** 2001. Novel processing control technologies in the food industry. F. D. Deak, Ed. Amsterdam, Netherlands: IOS Press.

138. **Reddy, N.R., Tetzloff, R.C., and Skinner, G.E.** 2010. Effect of media, additives, and incubation conditions on the recovery of high pressure and heat-injured *Clostridium botulinum* spores. Food Microbiol. **27:** 613-617.

139. **Reddy, N.R., Tetzloff, R.C., Solomon, H.M., and Larkin, J.W.** 2006. Inactivation of *Clostridium botulinum* nonproteolytic type B spores by high pressure processing at moderate to elevated high temperatures. Innov. Food Sci. Emerging Tech. **7:** 169-175.

140. **Reddy, N.R., Solomon, H.M., Tetzloff, R.C., and Rhodehamel, E.J.** 2003. Inactivation of *Clostridium botulinum* Type A spores by high-pressure processing at elevated temperature. J. Food Protec. **66:** 1402-1407.

141. **Reddy, N.R., Solomon, H.M., Fingerhut, G.A., Balasubramaniam, V.M., and Rhodehamel, E.J.** 2000. Inactivation of *Clostridium botulinum* type E spores in a model buffer or food system by high pressure processing. Presented at the Annual Meeting of the Institute of Food Technologists. Dallas, TX.

142. **Redmond, C., Baillie, L.W., Hibbs, S., Moir, A.J., and Moir, A.** 2004. Identification of proteins in the exosporium of *Bacillus anthracis*. Microbiol. **150:** 355-363.

143. **Ritz, M., Tholozan, J.L., Federighi, M., and Pilet, M.F.** 2002. Physiological damages of *Listeria monocytogenes* treated by high hydrostatic pressure. Intl. J. Food Microbiol. **79:** 47-53.

144. **Ritz, M., Jugiau, F., Rama, F., Courcoux, P., Semenou, M., and Federighi, M.** 2000. Inactivation of *Listeria monocytogenes* by high hydrostatic pressure: effects

and interactions of treatment variables studies by analysis of variance. Food Microbiol. **17:** 375-382.

145. **Roberts, C.M., and Hoover, D.G.** 1996. Sensitivity of *Bacillus coagulans* spores to combinations of high pressure, heat, acidity and nisin. J. Appl. Bacteriol. **81:** 363-368.

146. **Rocken, W., and Spicher, G.** 1993. Fadenziehende Bakterien - Vorkommen, Bedeutung Gegenmassnahmen. Getreide, Mehl und Brot. **47:** 30-35.

147. **Rodriguez, A.C., Larkin, J.W., Dunn, J., Patazca, E., Reddy, N.R., Alvarez-Medina, M., Tetzloff, R., and Fleischman, G.J.** 2004. Model of the inactivation of bacterial spores by moist heat and high pressure. J. Food Sci. **69:** 367-374.

148. **Ross A.I.V., Griffiths M.W., Mittal G.S., and Deeth H.C.** 2003. Combining nonthermal technologies to control foodborne microorganisms. Intl. J. Food Microbiol. **89:** 125-138.

149. **Rovere, P., Miglioli, L., Lonneborg, N.G., Scaramuzza, N., and Gola, S.** 1998. Modeling and calculation of the sterilizing effect in high pressure heat-treatments. Industria Conserve. **73:** 303-315.

150. **Salkinoja-Salonen, M.S., Vuorio, R., Andersson, M.A., Kampfer, P., Andersson, M.C., Honkanen-Buzalski, T., and Scoging A.C.** 1999. Toxigenic strains of *Bacillus licheniformis* related to food poisoning. Appl. Environ. Microbiol. **65:** 4637-4645.

151. **San Martin, M.F., Barbosa-Canovas, G.V., Swanson, B.G.** 2002. Food processing by high hydrostatic pressure. Crit. Rev. Food Sci. Nutr. **46:** 627-645.

152. **Setlow, B., Atluri, S., Kitchel, R., Koziol-Dube, K., and Setlow, P.** 2006. Role of dipicolinic acid in the resistance and stability of spores of *Bacillus subtilis*. J. Bacteriol. **188:** 3740-3747.

153. **Setlow, B., McGinnis, K.A., Ragkousi, K., and Setlow, P.** 2000. Effects of major spore-specific DNA binding proteins on *Bacillus subtilis* sporulation and spore properties. J. Bacteriol. **182:** 6906-6912.

154. **Setlow, B., and Setlow, P.** 1980. Measurements of the pH within dormant and germinated bacterial spores. Proc. Natl. Acad. Sci. USA. **77:** 2474-2476.

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

156. **Setlow, P.** 2003. Spore germination. Curr. Op. Microbiol. **6:** 550-556.

157. **Setlow, P.** 2000. Bacterial stress responses: resistance of bacterial spores. (G. a.-A. Storz, Ed.) Washington: American Society for Microbiology.

158. **Setlow, P.** 1994. Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. J. Appl. Bacteriol. **76:** 49S-60S.

159. **Setlow, P**. 1983. The Bacterial spore: Germination and outgrowth. (Vol. 2). London: Academic Press.

160. **Shah, I.M., Laaberki, M.H., Popham, D.L., and Dworkin, J.** 2008. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell **135:** 486-496.

161. **Shapiro, R.L., Hatheway, C., and Swerdlow, D.L.** 1998. Botulism in the United States: a clinical and epidemiologic review. Ann. Intern. Med. **129:** 221-228.

162. **Shigeshia, T., Ohmori, T., Seito, A., Taji, S., and Hayashi, R.** 1991. Effects of high hydrostatic pressure on characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. Intl. J. Food Microbiol. **12:** 107-216.

163. **Sila, D.N., Smout, C., Elliot, F., Van Loey, A., and Hendrickx, M.** 2006. Non-enzymatic depolymerization of carrot pectin: Toward a better understanding of carrot texture during thermal processing. J. Food Sci. **71:** E1-E9.

164. **Smelt, J.P.P.M., Hellemons, J.C., Wouters, P.C., and van Gerwen, S.J.C.** 2002. Physiological and mathematical aspects in setting criteria for decontamination of foods by physical means. Intl. J. Food Microbiol. **78:** 57-77.

165. **Smith, L.D.S., and Williams, B.L.** 1984. *Clostridium perfringens*: The pathogenic anaerobic bacteria. Springfield, Illinois, USA: Charles C. Thomas Publishers.

166. **Splittstoesser, D.F., Churey, J.J., and Lee, C.Y.** 1994. Growth characteristics of aciduric sporeforming bacilli isolated from fruit juices. J. Food Protec. **57:** 1080-1083.

167. **Starin, W. A.** 1926. Viability and toxicogenic power of small numbers of spores of *Cl. Botulinum* subjected to different temperatures. J. Infec. Diseases. **38:** 101-105.

168. **Stewart, C.M., Dunne, C.P., Sikes, A., and Hoover, D.G.** 2000. Sensitivity of spores of *Bacillus subtilis* and *Clostridium sporogenes* PA3679 to combinations of high hydrostatic pressure and other processing parameters. Innov. Food Sci. Emerging Tech. **1:** 49-56.

169. **Steyn, C.E., Cameron, M., and Witthuhn, R.C.** 2011. Occurrence of *Alicyclobacillus* in the fruit processing environment- A review. Intl. J. Food Microbiol. **147:** 1-11.

170. **Swerdlow, B.M., Setlow, B., and Setlow, P.** 1981. Levels of H+ and other monovalent cations in dormant and germinating spores of *Bacillus megaterium*. J. Bacteriol. **148:** 20-29.

171. **Tagg, J.R., Dajani, A.S., and Wannamaker, L.W.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40:** 722-756.

172. **Taylor, L.Y., Cann, D.D., and Welch, B.J.** 1990. Antibotulinal properties of nisin in fresh fish packaged in an atmosphere of carbon dioxide. J. Food Protec. **53:** 953-957.

173. **Timson, W.J., and Short, A.J.** 1965. Resistance of microorganisms to hydrostatic pressure. Biotech. Bioeng. **7:** 139-159.

174. **Ting, E., Balasubramaniam, V.M., and Raghubeer, E.** 2002. Determining thermal effects in high pressure processing. Food Tech. **56:** 31-35.

175. **Todd, S.J., Moir, A.J., Johnson, M.J., and Moir, A.** 2003. Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. J. Bacteriol. **185:** 3373-3378.

176. **Ulmer H.M., Herberhold H., Fahsel S., Ganzle M.G., Winter R., and Vogel R.F.** 2002. Effects of pressure-induced membrane phase transitions on inactivation of HorA, on ATP-dependant multidrug resistance transporter, in *Lactobacillus plantarum*. Appl. Environ. Microbiol. **68:** 1088-1095.

177. **Vepachedu, V.R., and Setlow, P.** 2007. Role of SpoVA proteins in release of dipicolinic acid during germination of *Bacillus subtilis* spores triggered by dodecylamine or lysozyme. J. Bacteriol. **189:** 1565-1572.

178. **Vepachedu, V.R., and Setlow, P.** 2005. Localization of SpoVAD to the inner membrane of spores of *Bacillus subtilis*. J. Bacteriol. **187:** 5677-5682.

179. **Vreeland, R.H., Rosenzweig, W.D., and Powers, D.W.** 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature **407:** 897-900.

180. **Waller, L.N., Fox, N., Fox, K.F., Fox, A., and Price, R.L.** 2004. Ruthenium red staining for ultrastructural visualization of a glycoprotein layer surrounding the spore of *Bacillus anthracis* and *Bacillus subtilis*. J. Microbiol. Meth. **58:** 23-30.

181. **Whitehead, H.R.** 1933. A substance inhibiting bacterial growth, produced by certain strains of lactic streptococci. Biochem. J. **27:** 1793-1800.

182. **Wiencek, K.M., Klapes, N.A., and Forgrding, P.M.** 1990. Hydrophobicity of *Bacillus* and *Clostridium* spores. Appl. Environ. Microbiol. **56:** 2600-2605.

183. **Wilson, M.J., and Baker, R.** 1997. High temperature/ultra-high pressure sterilization of low acid foods. PCT IB9601500.

184. **Winter, R. and Jeworrek, C.** 2009. Effect of pressure on membranes. Soft Matter. **5:** 3157-3173.

185. **Wuytack, E.Y., and Michiels, C.W.** 2001. A study on the effects of high pressure and heat on *Bacillus subtilis* at low pH. Intl. J. Food Microbiol. **64:** 333-341.

186. **Wuytack, E.Y., Soons, J., Poschet, F., and Michiels, C.W.** 2000. Comparative study of pressure- and nutrient-induced germination of *Bacillus subtilis* spores. Appl. Environ. Microbiol. **66:** 257-261.

187. **Wuytack, E.Y., Boven, S., and Michiels, C.W.** 1998. Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressure. Appl. Environ. Microbiol. **64:** 3220-3224.

188. **Xiao, Y., Francke, C., Abee, T., Wells-Bennik, M.H.J.** 2011. Clostridial spore germination versus bacilli: Genome mining and current insights. Food Microbiol. **28:** 266-274.

189. **Xie, L., Miller, L.M., Chatterjee, C., Averin, O., Kelleher, N.L., and van der Donk, W.A.** 2004. Lacticin 481: In vitro reconstitution of lantibiotic synthetase activity. Science **303:** 679-681.

190. **Yuste, J., Mor-Mur, M., Capellas, M., and Pla, R.** 1999. Pressure- vs. heat-induced bacterial stress in cooked poultry sausages: A preliminary study. Letters Appl. Microbiol. **29:** 233-237.

191. **Zein, M., and Winter, R.** 2000. Effect of temperature, pressure, and lipid acyl chain length on the structure and phase behaviour of phospholipid-gramicidin bilayers. Phys. Chem. Chem. Phys. **2:** 4545-4551.

## 2. Effects of Nisin and Reutericyclin on Resistance of Endospores of *Clostridium* spp. to Heat and High Pressure[1]

### 2.1 Introduction

Bacterial endospores are a dormant form of bacteria ubiquitous in the environment. Endospores are composed of multiple layers that confer resistance to adverse conditions, including UV radiation, heat, and chemicals (3, 12, 18, 24, 39). Endospore dormancy can last millions of years (7). Germination and outgrowth of endospores can be induced by exposure to nutrients, muropeptides, chemicals, high temperature, and pressure (28, 38, 40). Within the core of an endospore, dipicolinic acid (DPA) forms a complex with divalent cations that excludes water, which in turn contributes to thermal resistance (16, 39). As an endospore germinates, DPA is released from its core (38). DPA is also released when an endospore's structural integrity is compromised by chemicals, heat, or high pressure (38). Measurement of DPA has been used to examine the inactivation kinetics of *Bacillus* spp. endospores following thermal and pressure treatments (19, 22). Loss of DPA contributes to the loss of endospore thermal resistance (19, 22).

The ubiquity and inherent resistance of endospores makes them a safety concern for food processors. *Clostridium botulinum* is the most significant threat (9, 12, 18, 24). The use of thermal processing to eliminate endospores in foods compromises organoleptic properties. High-hydrostatic pressure (HHP) has shown promise as an alternative for inactivating endospores. Pressure-assisted thermal processing (PATP) makes use of pressure, from 200 to 800 MPa, in combination with heat to enhance endospore inactivation (1, 15, 34). However, a 5-log reduction of resistant clostridial

[1] David Gebhardt and Linda Ho are acknowledged for their contribution to the inactivation profiles of endospores presented within this chapter.

endospores is achieved only by processes that operate at high pressure in combination with 120°C (4, 24, 44).

Application of antimicrobial compounds, such as nisin and the tetramic acid reutericyclin (13, 21), in combination with moderate heat and pressure, may be suitable to achieve minimal processing of foods and control of endospore outgrowth and viability. Nisin, a pore-forming lantibiotic produced by *Lactococcus lactis*, enhanced the inhibitory effects of heat and pressure on *Bacillus* endospores (20, 36). Reutericyclin is a tetramic acid that acts as a proton-ionophore with potent bactericidal activity against Gram-positive organisms, including endospores of *Bacillus* spp. (14). However, only limited data are available on the inactivation of clostridial endospores by combined application of nisin, pressure, and temperature (15). Effects of reutericyclin have not been evaluated.

The aim of this study was to examine the effects of high-pressure, temperature, and nisin or reutericyclin on survival of endospores of selected *Clostridium* spp. *C. sporogenes* ATCC 7955 was chosen for its heat-resistant endospores and genetic similarity to *C. botulinum* Group I (9); *C. beijerinckii* ATCC 8260 was chosen due to implications of involvement in blown-pack meat spoilage and growth at refrigeration temperature (4 °C) (6); *C. difficile* 3195 is a gastrointestinal human pathogen. *B. amyloliquefaciens* FAD 11/2 was used as a pressure-resistant reference strain (24). Nisin and reutericyclin were selected because both antimicrobials target cell membranes but have different modes of activity. Loss of DPA from endospore populations was quantified by fluorescence spectroscopy in the presence of terbium (Tb) ions, which form stable fluorescent complexes (19, 37). 4'6-Diamidino-2-phenylindole (DAPI) was used to determine the accessibility of endospore DNA to chemical compounds (11).

## 2.2 Materials and Methods

### 2.2.1 Strains, Growth and Sporulation Conditions.

*Clostridium beijerinckii* ATCC 8260, *Clostridium difficile* 3195 (CanBiocin Inc., Edmonton, AB), *Clostridium sporogenes* ATCC 7955, and *Bacillus amyloliquefaciens* FAD 11/2 were inoculated from endospore stocks, maintained at -80°C in 60% glycerol, into Reinforced Clostridial Media (RCM; Difco, Becton, Dickinson and Company, Sparks, USA) broth and incubated anaerobically (*Clostridium* spp.) or aerobically (*B. amyloliquefaciens* FAD 11/2) at 37°C. Weihenstephan Südhang (WSH) media was prepared as described previously (22). Cultures were sporulated by plating onto the surface of WSH agar that was incubated anaerobically or aerobically at 37°C. Following 14 d of incubation, sporulation was confirmed by phase-contrast microscopy in which ~95% endospore purity was observed. Endospores were harvested from the surface of WSH agar plates by washing the surface of the plates with sterile 0.9% saline. Harvested endospores were washed five times by centrifugation at 2,700 x *g* and re-suspended in sterile 0.9% saline. Endospore suspensions were standardized to $OD_{600}$ values of 0.5, 1.5, and 2.0 in sterile 0.9% saline. Endospore suspensions were plated onto RCM agar and incubated anaerobically or aerobically at 37°C for 6 d to verify that increasing $OD_{600}$ values corresponded to increasing endospore population densities. An $OD_{600}$ of 2.0 equated to approximately $10^8$ endospores $mL^{-1}$ for each *Clostridium* sp. and *B. amyloliquefaciens* FAD 11/2. Endospore stocks were stored at -20°C. Sterile 0.9% saline was used as the endospore suspension medium throughout subsequent experiments.

### 2.2.2 Minimum Inhibitory Concentration (MIC).

Nisin (Chrisaplin, Chr. Hansen HS, Hørsholm, Denmark) was diluted in aqueous 0.05% acetic acid solution and stored at -20°C. Reutericyclin was purified by solvent extraction from the culture supernatant of *Lactobacillus reuteri* LTH2584 as described

(13), and stored in 80:20 isopropanol:water solution at -20°C. The concentration of reutericyclin was determined by a critical dilution assay using *Lactobacillus sanfranciscensis* as an indicator strain (13). Critical dilution assays (13) were used to determine the MIC of nisin and reutericyclin against endospores and vegetative cells of *Clostridium* spp. MIC procedures were carried out in an anaerobic hood. In brief, 100 µL of RCM broth was added to each well of a microtitre plate. Nisin or reutericyclin stock solutions (100 µL) were added to separate wells and serially diluted. Stationary phase cultures of *Clostridium* spp. or endospore suspensions with an $OD_{600}$ 2.0 were diluted to one-tenth of the initial concentration in RCM broth, and microtitre plates were inoculated with 50 µL of the diluted culture. Plates were incubated anaerobically at 37°C for 24 h (vegetative cells) or 6 d (endospores).

**2.2.3 Thermal Treatment of Endospores.**

Nisin and reutericyclin were mixed with endospore suspensions ($OD_{600}$ 2.0) at 16x the endospore MIC values (16 mg $liter^{-1}$ and 6.4 mg $liter^{-1}$, respectively). Endospore suspensions with antimicrobials were heat-sealed in borosilicate glass capillary tubes (Disposable Micropipets, Fisher Scientific, Toronto, Canada) and kept on ice overnight until treatment. Thermal treatments were performed in an oil bath. Samples were placed into pre-heated canola oil at 90˚C, 100˚C, and 120˚C for up to 60 min. Samples were stored on ice for <60 min following treatment until extracted from glass capillaries. Samples were plated onto RCM agar, and incubated anaerobically or aerobically at 37°C for 6 d.

**2.2.4 High Hydrostatic Pressure (HHP) Treatments of Endospores.**

Nisin and reutericyclin were mixed with endospore suspensions ($OD_{600}$ 2.0) at 16X the endospore MIC values (16 mg $liter^{-1}$ and 6.4 mg $liter^{-1}$, respectively). Mixed solutions were heat-sealed in Tygon tubing (Application Specific Tubing, Saint-Gobain

Performance Plastics, Pittsburg, USA) using a hair-straightener (TONI&GUY®, Model TGST2976F, London, UK). Samples were kept on ice overnight until treatment. To achieve isothermal conditions during pressure holding times, adiabatic heating during compression was compensated for in accordance with heating curves of pressurized water. Sealed samples were preheated in a water bath to a temperature corresponding to (target temperature – adiabatic heating). For treatments at 90°C, samples were conditioned at 74°C for 5 min prior to pressurization. Samples were placed in vessels of a U111 Unipress (Warsaw, Poland) conditioned at 90°C, compressed to 600 MPa in 1 min (compression rate unavailable), and held at this pressure and temperature for up to 60 min. Pressure vessels had dimensions of 12 x 58 mm. Temperature control of the pressure vessels was achieved using an external circulating propylene glycol system (LAUDA Proline, Delran, USA). Pressure was released in 30 sec following treatment (depressurization rate unavailable). Samples were stored on ice for <60 min following treatment, plated onto RCM agar, and incubated anaerobically or aerobically at 37°C for 6 d.

**2.2.5 Terbium-DPA Fluorescence Assay.**

The protocol described by Kort et al. (19) was adapted to a 96-well microtitre plate. Control samples to determine maximum DPA release were obtained for each endospore crop using samples heated at 120°C for 60 min. Standardized endospore suspensions were subjected to HHP or thermal treatments as described above. Samples were centrifuged (10,600 x *g* for 4 min.) to remove insoluble material. Following centrifugation, supernatants were transferred into a 96-well microtitre plate and mixed with an equal volume of 20 mM terbium chloride in Tris buffer (pH 7.5). Fluorescence measurements were done in a spectrofluorometer (Varioskan Flash, Thermo Electron Corporation, Nepean, Canada) with excitation and emission wavelengths of 270 and 545

nm, respectively. Results were calculated as % DPA release relative to control samples prepared from the same endospore suspension. Fluorescence was recorded as relative fluorescence units (RFU).

### 2.2.6 4', 6-Diamidino-2-phenylindole (DAPI) Fluorescence Assay.

Endospores were dyed with DAPI as outlined by Den Blaauwen et al (11). Samples were immobilized on glass slides using 2% agarose (Sigma-Aldrich, St Louis, MO). Visualization was done using a fluorescence microscope (Zeiss AXIOVision Imager, Toronto, Canada) with a Hoechst filter set (Ex. 365 nm; Em. 445 ± 50 nm). The fluorescence intensity of five individual endospores was measured and integrated per treatment (data not shown). All sample measurements were calibrated against background intensities. Vegetative cells dosed with DAPI were used as maximal fluorescence intensity controls.

## 2.3 Results

### 2.3.1 MICs of Nisin and Reutericyclin for Vegetative Cells and Endospores of *Clostridium* spp.

The MIC of nisin and reutericyclin against *Clostridium* spp. vegetative cells and endospores was determined (Table 2-1). The concentration of nisin required for inhibition

**Table 2-1:** Minimum inhibitory concentration of nisin and reutericyclin against vegetative cells and endospores of *Clostridium* spp.[a]

| | Nisin (mg liter$^{-1}$) | | Reutericyclin (mg liter$^{-1}$) | |
|---|---|---|---|---|
| | Vegetative Cells | Endospores | Vegetative Cells | Endospores |
| *C. sporogenes* | 0.23 ± 0.084 | 1.11 ± 0.48 | 0.33 ± 0.12 | 0.40 ± 0.00 |
| *C. beijerinckii* | 1.30 ± 0.00 | 1.09 ± 0.38 | 0.33 ± 0.12 | 0.27 ± 0.12 |
| *C. difficile* | 5.20 ± 0.00 | 3.47 ± 1.50 | 0.67 ± 0.23 | 0.53 ± 0.23 |

[a] Values represent mean ± standard deviation of three replicates

of vegetative cells was similar to that required for inhibition of endospores of all *Clostridium* spp., except *C. sporogenes* (Table 2-1). There was no significant difference in the MIC of reutericyclin between vegetative cells and endospores of *Clostridium* spp. The MIC of reutericyclin against *Clostridium* spp. endospores and vegetative cells were lower than those of nisin, except for *C. sporogenes* vegetative cells (Table 2-1).

### 2.3.2 Correlation of DPA-Release to Endospore Inactivation.

Terbium-DPA fluorescence assays were done to establish a relationship between endospore density and DPA levels following lethal thermal treatments of endospores. Suspensions of *C. beijerinckii* endospores, standardized to $OD_{600}$ values of 0.25, 0.5, 1.0, and 2.0, were heated at 120°C for 60 min. As the $OD_{600}$ values increased, the amount of DPA released from the endospores increased (data not shown). Treatment of endospores at 120ºC caused a near complete DPA release in under a minute for all three *Clostridium* spp. (Figure 2-1).

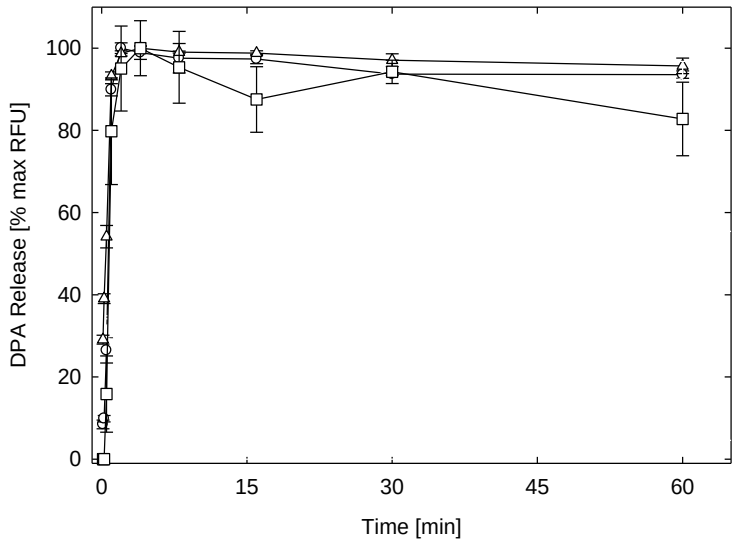


**Figure 2-1:** Release of DPA from endospores of *C. difficile* (○), *C. sporogenes* (Δ), or *C. beijerinckii* (□) standardized to $OD_{600}$ 2.0 and heated at 120ºC. Symbols represent means ± standard deviations of three independent replicates.

### 2.3.3 Heat Resistance of Clostridial Endospores.

At 100ºC, *C. beijerinckii* and *C. difficile* endospores were inactivated within 25 min of treatment (Figure 2-2). Near-maximal DPA release was achieved for *C. difficile* and *C. beijerinckii* after 60 min. The heat inactivation profile for *C. sporogenes* exhibited a tailing effect. In comparison to the other strains, *C. sporogenes* exhibited a higher resistance and a higher level of DPA release at 100ºC with a near-maximal DPA release occurring at 30 minutes. *C. sporogenes* and *C. beijerinckii* were selected for subsequent experiments to determine the combined sporicidal effects of high temperature, high pressure, and antimicrobial compounds. *C. difficile* was omitted from further investigation as its response to heat was similar to other *Clostridium* spp.

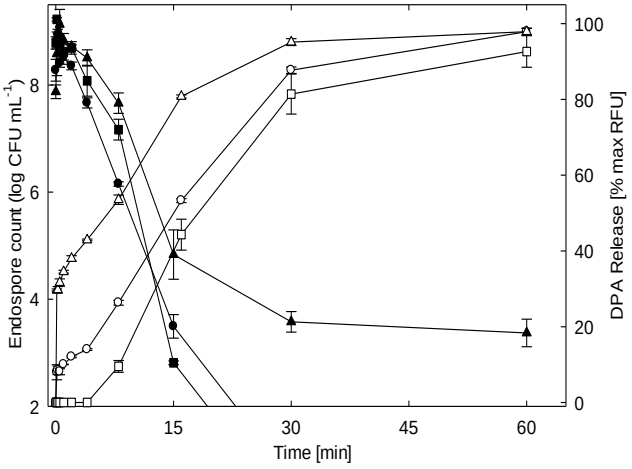


**Figure 2-2:** Survival and release of DPA from endospores of *C. difficile* (●, ○), *C. sporogenes* (▲, Δ), and *C. beijerinckii* (■, □). Endospore suspensions were standardized to $OD_{600}$ 2.0 and subjected to treatment at 100ºC. Solid symbols: log CFU $mL^{-1}$; Open symbols: % max RFU. Lines dropping below the x-axis indicate values below the detection limit of 2 log CFU $mL^{-1}$. Symbols represent means ± standard deviations of three independent replicates.

### 2.3.4 Endospore Inactivation by High Temperature and Antimicrobials.

Combined thermal and antimicrobial treatments were done to establish kinetics of inactivation and DPA release for comparison to thermal treatments. No increased

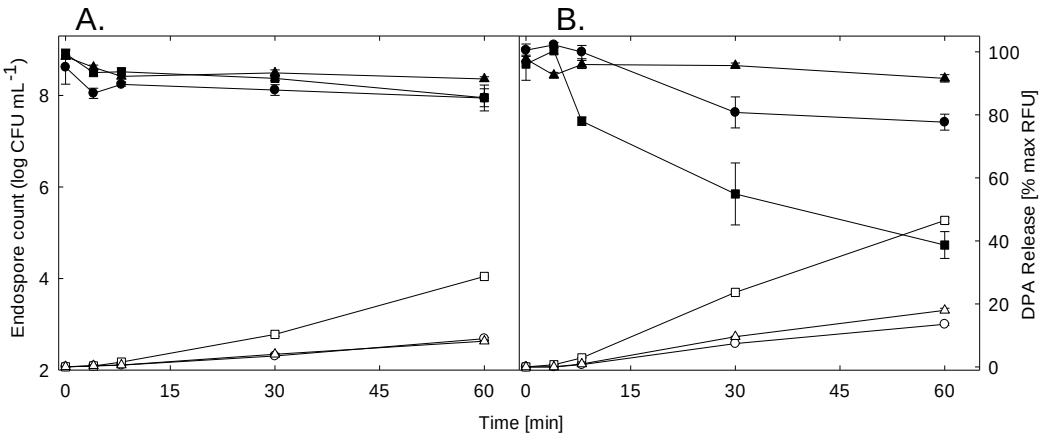


**Figure 2-3:** Survival and release of DPA from endospores of *C. beijerinckii* (A) and *C. sporogenes* (B) standardized to $OD_{600}$ 2.0 and heated to 90ºC in the presence of 6.4 mg liter$^{-1}$ reutericyclin (▲, Δ), 16 mg liter$^{-1}$ nisin (■, □), or in the absence of antimicrobials (●, ○). Solid symbols: log CFU/mL; open symbols: % max RFU. Symbols represent means ± standard

inactivation of *C. beijerinckii* endospores was observed when heated in combination with nisin and 90ºC relative to 90ºC alone, but an increase in DPA levels was observed (Figure 2-3A). The application of nisin and heat at 90ºC to endospores of *C. sporogenes* resulted in a more rapid inactivation in comparison to that observed for endospores of *C. sporogenes* at 90ºC without nisin, and in comparison to *C. beijerinckii* (Figure 2-3). The amount of DPA released was higher when endospores were treated with a combination of nisin and 90ºC relative to 90ºC alone. It is important to note that the DPA profiles are represented on a linear scale, whereas the inactivation of endospores is represented on a logarithmic scale. Addition of reutericyclin to endospores of *C. sporogenes* and *C. beijerinckii* had no effect on DPA release or endospore inactivation (Figure 2-3).

### 2.3.5 Endospore Inactivation by High-Pressure, High Temperature, and Antimicrobials.

Combined treatments of high-pressure, thermal, and antimicrobials were done to establish kinetics of inactivation and DPA release for comparison to thermal treatments. In the absence of antimicrobials, both *C. sporogenes* and *C. beijerinckii* endospores treated at 600 MPa and 90ºC displayed more rapid inactivation compared to endospores heated at 100ºC (Figure 2-2) or 90ºC (Figure 2-3). Both *Clostridium* spp. exhibited a complete release of DPA in the time it took the high-pressure equipment to ramp up to 600 MPa (1 minute) (Figure 2-4).

Nisin and reutericyclin initially accelerated inactivation of *C. beijerinckii* endospores at 600 MPa and 90ºC, but both compounds induced a tailing effect (Figure 2-4A). Addition of nisin resulted in a more rapid inactivation of *C. sporogenes* at 600 MPa and 90°C relative to 90ºC alone (Figure 2-4B). The presence of reutericyclin enhanced the survival of endospores of *C. sporogenes* endospores (Figure 2-4B).

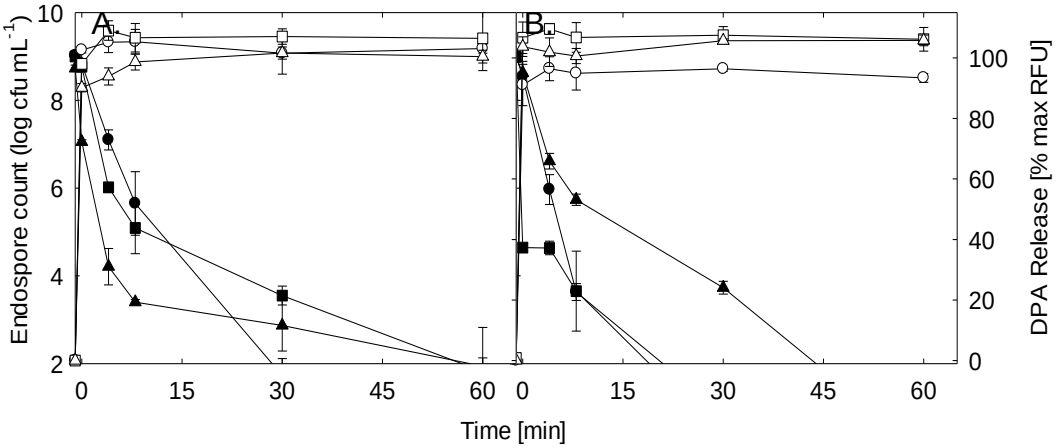


**Figure 2-4:** Survival and release of DPA from endospores of *C. beijerinckii* (A) and *C. sporogenes* (B) standardized to $OD_{600}$ 2.0 and treated at 600MPa and 90ºC in the presence of 6.4 mg liter$^{-1}$ reutericyclin (▲, Δ), 16 mg liter$^{-1}$ nisin (■, □), or in the absence of antimicrobials (●, ○). Solid symbols: log CFU/mL; open symbols: % max RFU. Lines dropping below the x-axis indicate values below the detection limit of 2 log CFU mL$^{-1}$. Symbols represent means ± standard deviations of three independent replicates.

Because the effects of nisin and reutericyclin on endospore survival appeared to be species- or strain-specific, the effect of these antimicrobials on *B. amyloliquefaciens* FAD 11/2 was assessed (Figure 2-5). *B. amyloliquefaciens* was more resistant to heat or pressure than any of the *Clostridium* spp. investigated in this study. Reutericyclin had no effect on endospore survival at 90°C, or at 600 MPa and 90°C. Nisin accelerated endospore inactivation at 90°C, or at 600 MPa and 90°C when compared to treatments with reutericyclin or without antimicrobials. In comparison to treatments at 90°C, pressure accelerated endospore inactivation in the absence of antimicrobials or in the presence of reutericyclin. However, relative to 90°C, pressure application was capable of slowing down endospore inactivation when nisin was present.

### 2.3.6 DAPI Fluorescence in Treated Endospores.

Endospore permeability was assayed by determination of the accessibility of DAPI to endospore DNA after treatment. Untreated endospores of *C. sporogenes* and *C.*

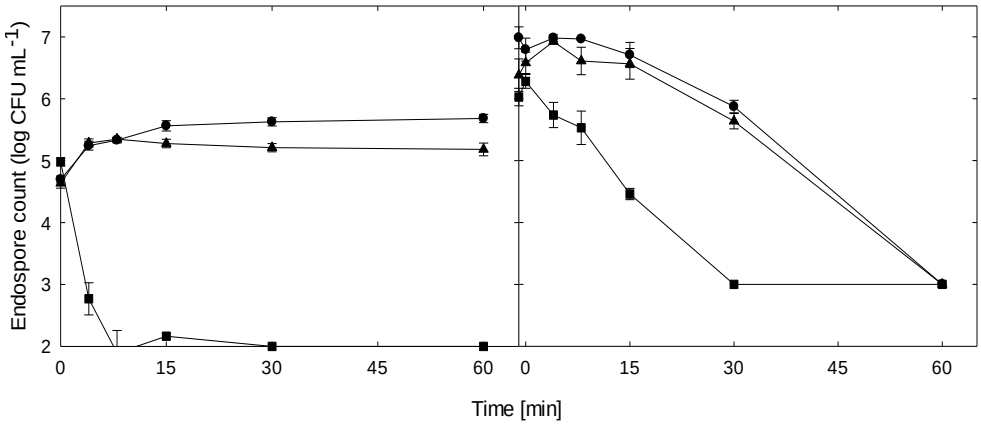


**Figure 2-5:** Survival of endospores of *B. amyloliquefaciens* FAD 11/2 standardized to $OD_{600}$ 2.0 and treated at 90ºC (A) or 90ºC and 600 MPa (B). Endospores were treated in presence of 6.4 mg liter$^{-1}$ reutericyclin (▲), 16 mg liter$^{-1}$ nisin (■), or in the absence of antimicrobials (●). Symbols represent means ± standard deviations of three independent replicates.

*beijerinckii* did not stain with DAPI, indicating that the endospore was impermeable to DAPI (Figure 2-6 and data not shown). *C. sporogenes* and *C. beijerinckii* endospores fluoresced after all treatments at 90ºC and 600 MPa (Figure 2-6A). An average value of 76 ± 19% and 70 ± 23% fluorescence intensity was recorded for endospores relative to vegetative cells of *C. sporogenes* and *C. beijerinckii*, respectively. One hundred percent fluorescence intensity as observed in vegetative cells was not expected, as endospores are markedly smaller than vegetative cells. Following treatment at 90ºC for 0 to 60 min, endospores of both *C. sporogenes* and *C. beijerinckii* did not exhibit increased fluorescence after being exposed to DAPI (Figure 2-6B and data not shown). No fluorescence was observed among samples heated at 90ºC in the presence of reutericyclin, or in the control samples. However, slight fluorescence was observed in endospores treated at 90ºC in the presence of nisin (Figure 2-6B), indicating that nisin increased the permeability of the endospore to DAPI.

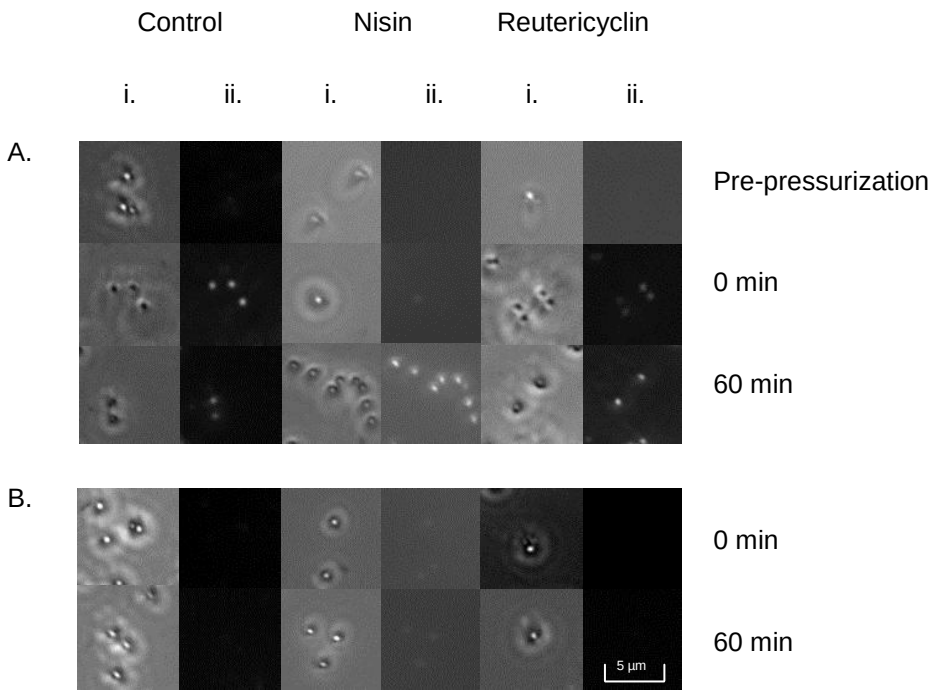


**Figure 2-6:** Photos of endospores of *C. sporogenes* dyed with DAPI following 0 and 60 minutes of thermal and high-pressure treatment. (A.) = 90°C and 600 MPa; (B.) = 90°C. (i), phase contrast microscopy; (ii) fluorescence microscopy to detect DAPI fluorescence. Several photographs were taken for each treatment and five endospores were selected from each treatment photo. The fluorescence intensities of the five endospores were integrated and standardized against background interference and vegetative cell controls for each photo. Comparable results were obtained with endospores of *C. beijerinckii* (data not shown).

## 2.4 Discussion

This study suggests that the membrane-active antimicrobial agents nisin and reutericyclin have divergent effects on the heat or heat and pressure mediated inactivation of clostridial endospores. Release of DPA from clostridial endospores, as well as their permeability to DAPI, suggests that this divergent activity of nisin and reutericyclin was related to their mode of action.

Sensitivity of clostridial cells and endospores to nisin falls within the previously observed MIC range for *Clostridium* spp. endospores, ranging from approximately 0.17 mg/L to 59 mg/L (2, 25, 26, 32). Differences in sensitivity to nisin between cells and

endospores of the same species are common (26, 25). The MICs of reutericyclin towards Clostridia are not previously reported, but our data are comparable to MICs of *Bacillus* spp. and other Gram-positive bacteria (14). In keeping with previous data, reutericyclin sensitivity of endospores was comparable to vegetative cells (13). Remarkably, the MIC of reutericyclin towards *C. difficile*, 1 µM or less, was substantially lower when compared to the MIC of synthetic tetramic acids, 30 µM, probably reflecting the higher hydrophobicity of reutericyclin (13, 42). Reutericyclin and nisin were used in high temperature / high pressure experiments at a concentration exceeding their MIC 16-fold, corresponding to about 5 µM nisin and 18 µM reutericyclin, respectively.

Endospores of *Bacillus* spp. release DPA during thermal treatments or high temperature / high pressure treatments at 98°C to 140°C, and measurement of DPA release was proposed as a rapid screening tool for the heat resistance of *Bacillus* endospores (19, 22). However, pressure-induced release of DPA from endospores of *C. botulinum* was observed only after inactivation of more than 90% of the endospore population (23). Release of DPA from *C. beijerinckii* and *C. sporogenes* endospores at 90°C in this study was quite comparable (30 and 50% loss of DPA, respectively, after 60 min treatments). However, sporicidal effects of the same treatments differed by 4-log. The current results do not support the use of DPA release profiles as a measure of endospore inactivation for comparison between strains. However, they do allow for intra-strain comparison of the effects of high temperature or high temperature / high pressure on endospore inactivation. *C. sporogenes* and *C. beijerinckii* endospores released DPA after treatment at 90°C and 600 MPa with or without antimicrobials; these treatments also permeabilised endospore walls to DAPI. Thermal treatments without nisin did not allow access of DAPI to the endospore cytoplasm, and did not result in an appreciable DPA release. Release of DPA thus corresponded to endospore permeability to DAPI.

Addition of nisin enhanced inactivation of clostridial endospores in both thermal, and high temperature / and high-pressure treatments, in keeping with literature data (1, 15, 20, 36), and the expectation that a combination of several antagonistic principles (nisin, heat, pressure) leads to additive or synergistic effects. Nisin activity against endospores at high temperature conditions was reflected by accelerated release of DPA, and an increased DAPI uptake (Figures 2-3 and 2-6). These results are consistent with the hypothesis that nisin, a pore forming lantibiotic, forms pores in membranes of resting endospores under high temperature conditions. However, the magnitude of the synergistic effect of nisin was strongly dependent on the species and decreased in the order *B. amyloliquefaciens* > *C. sporogenes* > *C. beijerinckii*. For endospores of *C. beijerinckii*, the initial synergistic inactivation effect of nisin with high temperature / high pressure was followed by tailing during later stages of the pressure-death time curve.

In contrast to nisin, and in contrast to the expectation that the combination of several antagonistic principles enhance sporicidal or bactericidal effects, reutericyclin did not enhance endospore inactivation, DPA release, or permeability to DAPI by high temperature or high temperature / high pressure treatments (Figures 2-3, 2-4, and 2-6). Reutericyclin protected endospores of *C. sporogenes* during high temperature or high temperature / high pressure treatments, confirming interaction of the antimicrobial compound with the endospore envelope. Nisin and reutericyclin target cell membranes, but exert antimicrobial activity by different mechanisms. Nisin forms pores of a size expected to accommodate entry of water or DAPI, and the release of DPA (21), whereas reutericyclin acts as a proton ionophore (14). Ionophore activity does not directly facilitate DPA release and DAPI uptake by endospores. Moreover, the assay systems used here and elsewhere (13) to determine reutericyclin activity against germinating endospores does not determine whether reutericyclin is active against resting endospores.

The mode of action of ionophores necessitates translocation of ions from the outer leaflet of the cytoplasmic membrane to the inner leaflet. Translocation may be inhibited by biophysical properties of the endospores' inner membrane. Lipids of endospore inner membranes are known to be immobile, with evidence suggesting that a compressed state is responsible (10).

In conclusion, endospore inactivation by high temperature or high temperature / high pressure processes may be accelerated by membrane-active antimicrobials. However, the effect of antimicrobial compounds present during high temperature / high-pressure treatments on endospore inactivation appears to depend on their mode of action. Nisin, an antimicrobial that facilitates the release of DPA via pores, accelerated endospore inactivation but reutericyclin, a proton ionophore, appears to have antagonistic effects. Increasing demand for minimally-processed, safe food products merits consideration of mechanisms responsible for endospore resistance. With a more definitive understanding of endospore physiology, particularly the properties of endospore membranes and responses to processing, highly specific inactivation strategies can be designed.

## 2.5 References


1. **Ahn J., Balasubramaniam V.M. and Yousef A.E.** 2007. Inactivation kinetics of selected aerobic and anaerobic bacterial spores by pressure-assisted thermal processing. Int. J. Food Microbiol. **113**:321-329.

2. **Bartoloni, A., Mantella, A., Goldstein, B.P., Dei, R., Benedetti, M., Sbaragli, S., and Paradisi, F.** 2004. In-vitro activity of nisin against clinical isolates of *Clostridium difficile*. J. Chemo. **16**:119-121.

3. **Beaman TC, Gerhardt P** (1986) Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaption. Appl. Environ. Microbiol. **52**:1242–1246.

4. **Black, E.P., P. Setlow, A.D. Hocking, C.M. Stewart, A.L. Kelly, and D.G. Hoover.** 2007. Response of spores to high-pressure processing. Comp. Rev. Food Sci. Food Safety. **6:**103-119.

5. **Black, E.P., Linton, M., McCall, R.D., Curran, W., Fitzgerald, G.F., Kelly, A.L., and Patterson, M.F.** 2008. The combined effects of high pressure and nisin on germination and inactivation of *Bacillus* spores in milk. J. Appl. Microbiol. **105**:78-87.

6. **Broda D.M., DeLacy K.M., Bell R.G., Braggins T.J., and Cook R.L.** 1996. Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Int. J. Food Microbiol. **29**:335-352.

7. **Cano R.J. and Borucki M.K**. 1995. Revival and identification of bacterial spores in 25-million year-old to 40-million year-old Dominican amber. Science. **268**:1060-1064.

8. **Carrera M., Zandomeni R.O., Fitzgibbon J., and Sagripanti J.L.** 2006. Difference between the spore sizes of *Bacillus anthracis* and other *Bacillus* species. J. Appl. Microbiol. **102**:303-312.

9. **Collins, M.D., and East, A.K.** 1998. Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins. J. Appl. Microbiol. **84**□□□□□□

10. **Cowan, A.E., Olivastro, E.M., Koppel, D.E., Loshon, C.A., Setlow, B., and Setlow, P.** 2004. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. Proc. Natl. Aca. Sci. USA **101**:7733-7738.

11. **Den Blaauwen T., Buddlemeijer N., Aarsman M.E.G., Hameete C.M., and Nanninga N.** 1999. Timing of FtsZ assembly in *Escherichia coli*. J. Bacteriol. **181**:5167-5175.

12. **Esty J.R. and Meyer K.F.** 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes. J. Infect. Dis. **31**:650-664.

13. **Gänzle M.G., Höltzel A., Walter J., Jung G., Hammes W.P.** 2000. Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH 2584. Appl. Environ. Microbiol. **66**:4325-4333.

14. **Gänzle M.G.** 2004. Reutericyclin: biological activity, mode of action, and potential applications. Appl. Microbiol. Biotechnol. **64**:326-332.

15. **Gao Y and Ju X.** 2008. Exploiting the combined effects of high pressure and moderate heat with nisin on inactivation of *Clostridium botulinum* spores. Microbiol. Meth. **72**:20-28.

16. **Gerhardt, P., and R. E. Marquis.** 1989. Spore thermoresistance mechanisms, p. 43–63. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of Prokaryotic Development. American Society for Microbiology, Washington, DC.

17. **Henriques A.O and Moran C.P.** 2007. Structure, assembly, and function of the spore surface layers. Annu. Rev. Microbiol. **61**:555-588.

18. **Johnson E.A. and Bradshaw M.** 2001. *Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective. Toxico. **39**:1703-1722.

19. **Kort R., O'Brien A.C., van Stokkum I.H.M., Oomes S.J.C.M., Crielaard W., Hellingwerf K.J., and Brul S.** 2005. Assessment of heat resistance of bacterial spores from food product isolates by fluorescence monitoring of dipicolinic acid release. Appl. Environ. Microbiol. **71**:3556-3564.

20. **Lopez-Pedemonte T.J., Roig-Sagues A.X., Trujillo A.J., Capellas M, and Guamis B.** 2003. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. J. Dairy Sci. **86**:3075-3081.

21. **Lubelski J., Rink R., Khusainov R., Moll G.N., and Kuipers O.P.** 2008. Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. Cell. Mol. Life Sci. **65**:455-476.

22. **Margosch D., Gänzle M.G., Ehrmann M.A., and Vogel R.F.** 2004a. Pressure inactivation of *Bacillus* endospores. Appl. Environ. Microbiol. **70**:7321-7328.

23. **Margosch D., M. A. Ehrmann, M. G. Gänzle, and R. F. Vogel.** 2004b. Comparison of pressure and heat resistance of *Clostridium botulinum* and other endospores in mashed carrots. J. Food Prot. **67**:2530–2537.

24. **Margosch D., Ehrmann M.A., Buckow R., Heinz V., Vogel R.F., and Gänzle M.G.** 2006. High-pressure mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. Appl. Environ. Microbiol. **72**:3476-3481.

25. **Mazzotta, A.S., Crandall, A.D., and Montville, T.J.** 1997. Nisin resistance in *Clostridium botulinum* spores and vegetative cells. Appl. Environ. Microbiol. **63**:2654-2659.

26. **Meghrous, J., Lacroix, C., and Simard, R.E.** 1999. The effects on vegetative cells and spores of three bacteriocins from lactic acid bacteria. Food Microbiol. **16**:105-114.

27. **Moeller R, Setlow P, Reitz G, Nicholson W.L.** 2009. Roles of small, acid-soluble spore proteins and core water content in survival of *Bacillus subtilis* spores exposed to environmental solar UV radiation. Appl. Environ. Microbiol. **75**: 5202-5208.

28. **Moir A.** 2006. How do spores germinate? J. Appl. Microbiol. **101**:526-530.

29. **Montville T.J., De Siano T., Nock A., Padhi S., and Wade D.** 2006. Inhibition of *Bacillus anthracis* and potential surrogate Bacilli growth from spore inocula by nisin and other antimicrobial peptides. J. Food Prot. **10**:2529-2533.

30. **Naim, F., Zareifard, M.R. Zhu, S., Huizing, R.H., Grabowski, S., and Marcotte, M.** 2008. Combined effects of heat, nisin and acidification on the inactivation of *Clostridium sporogenes* spores in carrot-alginate particles: From kinetics to process validation. Food Microbiol. **25**:936-941.

31. **Pellegrino P.M., Fell N.F., and Gillespie J.B.** 2002. Enhanced spore detection using dipicolinate extraction techniques. Analytica Chimica Acta. **455**:167-177.

32. **Rayman, K., Aris, B., and Hurst, A.** 1981. Nisin: a possible alternative to adjunct of nitrite in the preservation of meats. Appl. and Environ. Microbiol. **41**:375-380.

33. **Reddy, N. R., H. M. Solomon, G. A. Fingerhut, E. J. Rhodehamel, V. M. Balasubramaniam, and S. Palaniappan.** 1999. Inactivation of *Clostridium botulinum* type E spores by high pressure processing. J. Food Safety. **19:**277-288.

34. **Reddy NR, Solomon HM, Tezloff RC, Rhodehamel EJ**. 2003. Inactivation of *Clostridium botulinum* type A spores by high pressure processing at elevated temperatures. J. Food Prot. **66**:1402–1407.

35. **Reddy N.R., Tetzloff R.C., Solomon H.M., and Larkin J.W.** 2006. Inactivation of *Clostridium botulinum* nonproteolytic type B spores by high pressure processing at moderate to elevated high temperatures. Innov. Food Sci. and Emerg. Tech. **7**:169-175.

36. **Roberts C.M. and Hoover D.G.** 1996. Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity and nisin. J. Appl. Bact. **81**:363-368.

37. **Rosen D.L.** 1997. Bacterial spore detection and determination by use of terbium dipicolinate photoluminescence. Anal. Chem. **69**:1082-1085.

38. **Setlow P.** 2003. Spore germination. Current Opinion in Microbiol. **6**:550-556.

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

40. **Shah I.M., Laaberki M.H., Popham D.L., and Dworkin J.** 2008. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell **135**:486-496.

41. **Taylor, S.L., and Somers, E.B.** 1985. Evaluation of the antibotulinal effectiveness of nisin-nitrite combinations in culture medium and chicken frankfurter emulsions. J. Food Prot. **48**:949-952.

42. **Ueda, C., K. Tateda, M. Horikawa, S. Kimura, Y. Ishii, K. Nomura, K. Yamada, T. Suematsu, Y. Inoue, M. Ishiguro, S. Miyairi, and K. Yamaguchi.** 2010. Anti-*Clostridium difficile* potential of tetramic acid derivatives from *Pseudomonas aeruginosa* quorum-sensing autoinducers. Antimicrob. Agents Chemother. **54:**683-688.

43. **Walter, J., C. Schwab, D.M. Loach, M.G. Gänzle and G.W. Tannock.** 2008. Glucosyltransferase A (GtfA) and inulosucrase (Inu) of *Lactobacillus reuteri* TMW1.106 contribute to cell aggregation, in vitro biofilm formation, and colonization of the mouse gastrointestinal tract. Microbiology. **154**:72-80.

44. **Zhu SM, Naim F, Marcotte M, Ramaswamy H., and Shao Y.** 2008. High-pressure destruction kinetics of *Clostridium sporogenes* spores in ground beef at elevated temperatures. Int. J. Food Microbiol. **126**:86-92

# 3. Labeling Endospores of *Clostridium* spp. with the Fluorescent Probe LAURDAN[1]

## 3.1 Introduction

Bacterial endospores are widely distributed within the environment (13, 54), and are a significant problem for the food industry. Endospores of *Clostridium* spp. are known to cause food spoilage and foodborne illness (9, 11, 18, 72). Endospores resist thermal processing and 121°C is required for extending the shelf life of foods (40, 65). Pressure-assisted thermal sterilization (PATS) has been examined as an alternative to thermal processing for endospore control (51, 84). Unfortunately, variability in endospore species' resistance to PATS means high temperatures are still required for food processing (47, 48, 64, 65).

Resistance of endospores to heat and pressure is attributed primarily to low water activity within endospore cores (7, 46, 47, 48). A calcium-dipicolinic acid (Ca-DPA) matrix maintains a low water activity within the core (75). Integrity of the Ca-DPA matrix is maintained by the inner membrane's activity as a barrier to the entry of water from the external environment (56, 75). The release of DPA coincides with rehydration of the core, and facilitates endospore inactivation by heat and pressure (7, 46, 47, 48). A better understanding of endospore inner membranes during and after physical and chemical treatments will yield information useful in designing specific inactivation strategies for deployment in the food industry.

Researchers have attempted to develop rapid endospore detection techniques using fluorescent probes (80). Malachite green can be used to detect endospores in solution, but requires that endospores be permeabilised and thus compromises any

[1] Christian Denter is acknowledged for his contribution to the confocal laser scanning fluorescence microscopy work presented within this chapter.

information about the inner membrane state (27). Other fluorescence techniques used in endospore research have focused primarily on germination or sporulation.

Fluorescent dyes have been used extensively to examine germination. Fluorescent dyes used in this manner depend on the extent to which an endospore membrane is permeable, and give no other information about the membrane itself. The terbium ion is often used to complex with DPA in inactivation studies as a measure of thermal resistance and germination (37, 69). Fluorescent dyes such as SYTO 16, an indicator of germination, and propidium iodide, an indicator of membrane damage in vegetative cells (12, 25, 81, 82), are only useful for labeling the interior of endospores deficient of Ca-DPA (4). The same is true of SYTO 9, Hoechst 33342, and carboxyfluorescein diacetate (CFDA) (20).

Endospore sporulation studies typically employ green fluorescent protein (GFP), or an analogue. Protein fluorophores exhibit great utility in studying protein localization and gene expression during sporulation, specifically in *Bacillus* spp. (2, 18, 30, 31, 34, 35, 48, 51, 63, 70, 84). GFP has been used to assess sporulation heterogeneity of *Bacillus subtilis* cell populations (33), endospore coat protein assembly (86), coat protein localization (71), as well as lipolytic enzyme localization (46). Yellow fluorescent protein (YFP) has been used to assess localization of a peptidoglycan synthesis protein to membranes during sporulation (65). In one study, GFP was used to assess endospore core pH and degree of dehydration during high pressure, thermal processing that induced germination (89). GFP and its analogues require oxygen for maturation of the fluorophore and resulting fluorescence (68). Thus, GFP can only be expressed in aerobic endospore-forming bacteria such as *Bacillus* spp. and not *Clostridium* spp. The oxygen requirement of this probe precludes its use to examine membrane properties of endospores of *Clostridium* spp.

The membrane dye N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl-hexatrienyl) pyridinium dibromide has been successfully used to dye *Bacillus subtilis* cells during sporulation (61). This dye preferentially labels membranes, and was able to reveal septal biogenesis and engulfment mechanisms of the sporulation process (61). Following this, endospores of *Bacillus subtilis* and *Bacillus megaterium* containing membrane-specific fluorescent dyes were successfully generated without altering the sporulation process (19). The fluorescent probes dibromide (FM-4-64) and di-4-ANEPPS insert into endospore inner membranes and were used to examine inner membrane fluidity during germination (19). Fluorescence recovery after photobleaching (FRAP) of di-4-ANEPPS, in addition to volumetric measurements using FM-4-64, determined that inner membranes of endospores are immobile, with fluidity returning upon germination (19).

FM-4-64 is useful for volumetric assessment of individual endospores, but its spectra does not directly give any information about the inner membrane state. FRAP analysis of di-4-ANEPPS, in contrast, is exquisitely powerful in that it measures membrane fluidity of individual endospores (19, 37, 38). However, FRAP analysis cannot rapidly assess inner membrane fluidity of endospore populations. A fluorescence protocol for assessing inner membrane properties of populations during physical and chemical trials has not been established.

The goal of this study was to develop a method to measure inner membrane fluidity of bacterial endospore populations of *Clostridium* spp. Endospore membranes were integrated with 6-dodecanoyl-2-dimethylaminonaphthalene (LAURDAN). LAURDAN has been used in assessing membrane fluidity of vegetative cells, but not endospores (70, 82). Shifts in the fluorescence spectrum of LAURDAN accurately indicate differences in membrane fluidity (55), and its use in this respect has been well established (20, 30, 31, 58, 59, 60, 62, 79). *Clostridium sporogenes* ATCC 7955 was

chosen for its heat-resistant endospores and genetic similarity to *Clostridium botulinum* Group I (15). *Clostridium beijerinckii* ATCC 8260 was chosen due to potential involvement in blown-pack meat spoilage and growth at refrigeration temperature (4 °C) (10). Endospores containing LAURDAN will hereafter be denoted as WSH-L endospores within this manuscript.

## 3.2 Materials and Methods

### 3.2.1 Bacterial Strains and Growth Conditions.

*Clostridium beijerinckii* ATCC 8260, and *Clostridium sporogenes* ATCC 7955 cells were inoculated from frozen stocks, held at -80°C in 60% glycerol, into Reinforced Clostridial Media (RCM; Difco, Sparks, USA) broth and incubated at 37°C, anaerobically, overnight.

### 3.2.2 Sporulation and Harvesting Endospores.

Weihenstephan Sudhang (WSH) media was prepared as described by Margosch et al (46). Endospores containing LAURDAN (Invitrogen, Cat. No. D-250) (WSH-L endospores) were generated by plating 400 μL of LAURDAN-saturated ethanol onto individual WSH agar plates, allowing ethanol to evaporate in the absence of light, and then plating 100 μL of fully grown RCM broth. Plates were incubated anaerobically at 37°C, in absence of light, for 14 days. Sporulation was confirmed using phase-contrast microscopy in which ~95% endospore purity was observed. Endospores were harvested from the surface of WSH agar plates by washing with sterile 0.9% saline solution. Harvested endospores were centrifuged at 2,700 xg for 5 min, and suspended in sterile 0.9% saline. Care was taken to discard the top layer of each cell pellet. Endospore stock solutions were standardized to $OD_{600}$ values of 0.5 using sterile 0.9% saline. Control endospore stock solutions were generated as above, omitting addition of LAURDAN-

saturated ethanol to WSH agar plates. Standardized endospore stocks were stored at -20°C. Sterile 0.9% saline was used as the endospore suspension medium in subsequent manipulations.

### 3.2.3 Removal of Endospore Coat and Outer Membrane.

Aliquots (1 mL) of WSH-L endospore stock solutions were centrifuged at 2,700 xg for 10 min and supernatant removed. Pellets were suspended in a solution of 0.5% SDS, 0.1 M dithiothreitol, 0.1 M NaCl (pH 10, adjusted with 1 M NaOH) for 3 hours at 37°C as described by Fitz-James (24). Following incubation, endospores were centrifuged at 2,700 x*g* for 10 min and pellets suspended in sterile 0.9% saline to an $OD_{600}$ of 0.5. Samples were kept on ice until needed.

### 3.2.4 Lipid Extraction of *Clostridium* Cells and Endospores.

Lipid extraction was done according to a modified protocol using comparable solvents (7). Pellets (0.25 g) of cells and decoated endospores of *C. sporogenes* ATCC 7955 and *C. beijerinckii* ATCC 8260 were mixed with 3.75 mL of chloroform, 7.5 mL of methanol, and 3 mL of 50 mM $K_2HPO_4$ buffer solution (pH 7.4) in a screw cap vial. Samples were homogenized on a tissue-tearor for 3 min until pellets were dispersed in the solvent. Samples were subjected to brief sonication in a water bath before homogenization to prevent formation of aggregates. Samples were agitated on a shaker for 1 h at 200 rpm. Equal volumes of chloroform and 50 mM $K_2HPO_4$ buffer solution (pH 7.4) were added to each sample to generate a biphasic system. Sample containers were placed on a shaker for 30 min at 200 rpm, and centrifuged at 4000 x*g* for 20 min. In each sample, the upper aqueous, non-lipid contaminants were carefully aspirated and the lower organic lipid phase transferred to a separate Teflon-lined, screw cap vial. These samples were evaporated to dryness under nitrogen gas, and the lipid film suspended in a small volume of 2:1 chloroform: methanol (v/v). Samples where stored at -20°C until needed.

### 3.2.5 Preparation of Fatty Acid Methyl Esters (FAMEs).

Preparation of FAMEs was done according to Christie (14). A known amount of total lipids or triacylglycerol in 2:1 chloroform: methanol (v/v) was taken together with heptadecanoic acid (17:0, internal standard). The mixture was evaporated under nitrogen gas, and 1 ml of 2% sulphuric acid in methanol (v/v, methylating agent) was added. The mixture was incubated at 80 °C for 1 h, cooled on ice for 10 min, and neutralized by 0.5 mL 0.5% sodium chloride solution. Fatty acid methyl esters were extracted by addition of 2 x 2 mL aliquots of hexane and vortexing. The two layers were allowed to separate. The upper hexane layer was recovered. Samples were sent for gas chromatographic analysis, for quantification of fatty acids, by the Wishart Lab Group (University of Alberta, Department of Biological Sciences, Edmonton, Alberta, Canada).

### 3.2.6 Transmission Electron Microscopy (TEM) of Endospores.

Control and decoated WSH-L endospore stock solutions were centrifuged at 2,700 x*g* for 5 minutes. Pellets were prefixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) at room temperature for 1 hr. Prefixed samples were centrifuged at 2,700 x*g* for 5 min and washed in cacodylate buffer (pH 7.2) for 15 min three times. Samples were then fixed in 1-2 % osmium tetroxide ($OsO_4$) in cacodylate buffer (pH 7.2) at room temperature for 1-3 hs, and washed in distilled water. Washed samples were dehydrated in series using 50%, 70%, 80%, 90%, and absolute ethanol for 15 min each. Two additional dehydration steps using absolute ethanol were done for 10 min each. Absolute ethanol was replaced with propylene oxide, incubated at room temperature for 10 min, followed by two more propylene oxide washes of 10 min each. Samples were then embedded in Araldite CY212 / propylene oxide mixture for 1 h, followed by a pure Araldite CY212 mixture overnight in a vacuum desiccator. Polymerization was done at 60˚C for 2 days. Samples were

observed using a Philips – FEI transmission electron microscope (Morgagni 268) operating at 80 kV.

### 3.2.7 Two-Photon Confocal Laser Scanning Microscopy (CLSM) of WSH-L Endospores.

Control WSH-L and decoated WSH-L endospores (10 µL) were allowed to dry onto the surface of quartz glass slides. Two-photon excitation microscopy experiments were recorded by a confocal laser-scanning microscope (Biorad MRC 1024, extended for multi-photon excitation) coupled via a side-port to an inverted microscope (Nikon, Eclipse TE-300 DV, infinity corrected optics) enabling fluorescence excitation in the focal plane of an objective lens (Nikon Plan Apochromat 60x, water immersion, NA = 1.2, collar rim corr.). By focusing a high peak power laser light source on a diffraction-limited spot, through a high numerical aperture objective, the high photon densities required for two-photon absorption were achieved. Only fluorophores in areas with high photon flux were excited. This offered the advantage that two-photon absorption did not occur in areas above and below the focal plane because of insufficient photon flux densities, and allowed a sectioning effect without the use of emission pinholes as in confocal microscopy. Fluorescence in the PMT-channels (emission bandpass filter at 405 nm/(FWHM 35 nm) and at 460 nm, respectively) was acquired simultaneously using a Ti-Sap-Laser (Coherent, Mira 900-F, 76 MHz repetition rate, ca. 250 fs pulse width, pumped by a 5 W Verdi) tuned to 800 nm for two-photon excitation of LAURDAN. Image acquisition was controlled by LaserSharp2000 software (formerly Biorad, now Zeiss). Images were monitored with a frame rate of ca. 1 Hz, at a resolution of 512 x 512 pixels, and at lowest average laser power of about 10 mW.

### 3.2.8 Fluorescence Measurement of Endospores and Cells.

Vegetative cells and control endospores were labeled with LAURDAN as described previously (50). Overnight cell cultures grown in RCM broth were centrifuged, at 2,700 x*g* for 5 min, and suspended in sterile 0.9% saline twice. Ethanol containing LAURDAN was added to a suspension of cells, and unlabeled endospores, to a final concentration of 40 μM. Staining with LAURDAN was done in the dark at 30°C for 30 min. Suspensions of vegetative cells, labeled endospores, and WSH-L endospores were washed twice by centrifuging at 2,700 x*g* for 5 min and suspending in sterile 0.9% saline. Suspensions (100 μL) were aliquotted into 96-well microtitre plates in triplicate. Generalized polarization (GP) values of solutions were calculated using emission values at 440 nm and 490 nm following excitation at 360 nm using a spectrofluorometer (Varioskan Flash, Thermo Electron Corporation, Nepean, Canada).

$$GP = (I_{440}-I_{490})/(I_{440}+I_{490}) \quad (3)$$

Fluorescence emission spectra of labeled, unlabeled, and WSH-L endospores were measured following excitation at 360 nm using a specrtrofluorometer (SynergyMx, BioTek, Winooski, USA). Fluorescence emission spectra of labeled and unlabeled cells and WSH-L endospores were measured following excitation at 360 nm using a different spectrofluorometer (Varioskan Flash, Thermo Electron Corporation, Nepean, Canada). The fluorescence emission spectra of 10 mM LAURDAN suspended in 95% ethanol was measured as above. Little to no fluorescence was observed in unlabeled controls.

## 3.3 Results

### 3.3.1 Endospore Viability Following Sporulation in LAURDAN.

Viability of endospores labeled with LAURDAN was assessed to determine whether LAURDAN labeling adversely affected physiology. Control and LAURDAN-labeled endospore stock solutions were plated on RCM agar and incubated at 37°C

anaerobically for 6 days. No difference was observed between colony counts of control and LAURDAN-labeled endospores (data not shown).

### 3.3.2 Removal of Outer Membranes of WSH-L Endospores.

Endospores contain two membranes, both of which can bind to fluorescent dyes, and removal of the outer membrane was done to examine differences in endospores containing both membranes, and those with only the inner membrane (61). Transmission electron microscopy was used to examine the extent to which removal of the outer membrane affected morphology of WSH-L endospores. Endospores of *Clostridium* spp. that contained LAURDAN were subjected to a decoating protocol and compared to untreated WSH-L endospores using TEM. The decoating protocol successfully removed the outer membrane and protein coat of endospores of both *C. beijerinckii* and *C. sporogenes* (24) (Figure 3-1).

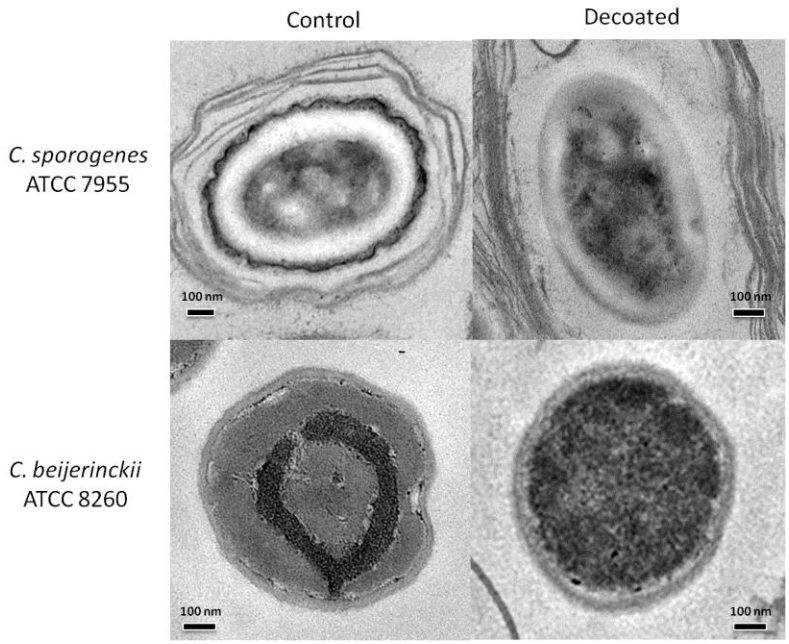


**Figure 3-1.** Transmission electron microscopy (TEM) photos of endospores of *C. sporogenes* and *C. beijerinckii* with outer membrane and protein coat removed.

The exosporium of endospores of *C. sporogenes* was severely disrupted, but not entirely removed (Figure 3-1). Little to no exosporium was observed surrounding endospores of *C. beijerinckii* before or after decoating (Figure 3-1). Decoating endospores did not appear to alter their size relative to untreated endospores (Figure 3-1).

### 3.3.3 Lipid Composition of *Clostridium* spp. Cells and Endospores.

Lipid composition of cells and endospores was assessed to compare levels of unsaturated fatty acids to LAURDAN GP measurements. The cells of *C. beijerincki* ATCC 8260 and *C. sporogenes* ATCC 7955 contained significantly greater quantities of short chain fatty acids (SCFAs) than respective control and decoated endospores (Table 3-1). Endospores of *C.* sporogenes ATCC 7955 contained significantly more SCFAs and saturated fatty acids (SFAs) than those of *C. beijerinckii* ATCC 8260 (Table 3-1).

**Table 3-1:** Total fatty acid content of cells and endospores of *Clostridium sporogenes* ATCC 7955 and *Clostridium beijerinckii* ATCC 8260. Short chain (SCFA) and long chain fatty acids (LCFA), as well as saturated (SFA) and unsaturated fatty acids (USFA), were compared between cells and endospores.

| Fatty Acid | *Clostridium sporogenes* ATCC 7955 | | | *Clostridium beijerinckii* ATCC 8260 | | |
|---|---|---|---|---|---|---|
| | Cells | Control Endospores | Decoated Endospores | Cells | Control Endospores | Decoated endospores |
| | Mol% FA /mg lipid ± Std. Dev. | Mol% FA /mg lipid ± Std. Dev. | Mol% FA /mg lipid ± Std. Dev. | Mol% FA /mg lipid ± Std. Dev. | Mol% FA /mg lipid ± Std. Dev. | Mol% FA /mg lipid ± Std. Dev. |
| 12:0 | 0.208 ± 0.046 | 0.0633 ±0.017 | 0.0765 ± 0.017 | 0.287 ± 0.038 | 0.164 ± 0.038 | 0.243 ± 0.12 |
| 13:0 | 0.110 ± 2.3x10^-3 | 0.0662 ± 4.7x10^-3 | 0.0684 ± 7.2x10^-3 | 0.0869 ± 0.012 | 0.0789 ± 4.7x10^-3 | 0.0977 ± 0.019 |
| 14:0 | 28.4 ± 1.3 | 27.2 ± 0.17 | 29.8 ± 2.4 | 28.6 ± 5.3 | 23.7 ± 1.5 | 22.1 ± 0.83 |
| i15:0 | 0.124 ± 0.024 | 0.182 ± 2.9x10^-3 | 0.152 ± 2.0x10^-3 | 0.0977 ± 0.018 | 0.162 ± 0.014 | 0.171 ± 0.012 |
| a15:0 | 2.85 ± 0.26 | 0.371 ± 0.087 | 0.268 ± 0.016 | 1.73 ± 0.22 | 0.541 ± 0.18 | 0.926 ± 0.30 |
| 15:0 | 2.01 ± 0.098 | 2.02 ± 0.050 | 1.85 ± 0.093 | 1.64 ± 0.24 | 1.40 ± 0.089 | 1.42 ± 0.082 |
| i16:0 | 5.22 ± 0.34 | 4.89 ± 0.24 | 3.57 ± 0.33 | 6.67 ± 1.0 | 3.85 ± 0.35 | 3.30 ± 0.10 |
| 16:0 | 44.6 ± 1.6 | 47.2 ± 0.66 | 45.5 ± 1.3 | 44.2 ± 1.1 | 48.3 ± 2.8 | 47.1 ± 0.95 |
| 16:1 | 4.95 ± 0.90 | 4.83 ± 0.37 | 4.96 ± 0.74 | 4.72 ± 0.67 | 3.83 ± 0.39 | 4.97 ± 0.75 |
| i17:0 | 0.097 ± 0.033 | 0.123 ± 0.025 | 0.155 ± 3.0x10^-3 | 0.430 ± 0.089 | 0.132 ± 0.015 | 0.143 ± 0.053 |
| 17:0cy | 1.34 ± 0.23 | 1.61 ± 0.24 | 1.98 ± 0.54 | 2.14 ± 0.63 | 1.62 ± 0.56 | 2.42 ± 0.52 |
| 18:0 | 3.88 ± 0.53 | 4.76 ± 0.23 | 4.59 ± 0.74 | 3.45 ± 0.34 | 6.22 ± 0.41 | 6.22 ± 1.4 |
| 18:1 | 1.36 ± 0.39 | 1.49 ± 0.16 | 1.68 ± 0.15 | 2.63 ± 0.044 | 1.70 ± 0.13 | 2.18 ± 0.31 |
| 18:1(9E) | 2.80 ± 0.25 | 3.59 ± 0.35 | 2.79 ± 0.31 | 2.93 ± 0.32 | 6.56 ± 0.61 | 6.03 ± 0.33 |
| 18:2 | 0.250 ± 0.090 | 0.264 ± 0.079 | 0.313 ± 0.067 | 0.0836 ± 9.4x10^-3 | 0.347 ± 0.097 | 0.530 ± 0.090 |
| 19:0cy | 0.463 ± 0.083 | 0.613 ± 0.084 | 0.723 ± 0.11 | 0.209 ± 0.046 | 0.431 ± 0.10 | 0.947 ± 0.21 |
| 19:0 | 0.858 ± 0.25 | 0.352 ± 0.038 | 0.995 ± 0.20 | 0.226 ± 0.016 | 0.380 ± 0.072 | 0.722 ± 0.25 |
| 20:0 | 0.139 ± 0.099 | 0.134 ± 0.011 | 0.170 ± 0.046 | 0.0535 ± 0.027 | 0.162 ± 0.040 | 0.162 ± 0.078 |
| 22:0 | 0.0550 ± 0.020 | 0.0617 ± 0.010 | 0.0766 ± 0.034 | 0.0208 ± 5.3x10^-3 | 0.0706 ± 6.0x10^-3 | 0.0760 ± 0.040 |
| 22:1(13) | 0.300 ± 0.085 | 0.179 ± 0.096 | 0.241 ± 0.021 | 0.0693 ± 0.021 | 0.328 ± 0.048 | 0.330 ± 0.21 |

P-Values of t-Tests Comparing Fatty Acid Ratios Within Species

| Ratios Compared | *Clostridium sporogenes* ATCC 7955 | | *Clostridium beijerinckii* ATCC 8260 | |
|---|---|---|---|---|
| | SCFA / LCFA | SFA / USFA | SCFA / LCFA | SFA / USFA |
| Cells:Control Endospores | 0.053 | 0.293 | <0.001* | 0.037**** |
| Cells:Decoated Endospores | 0.063 | 0.595 | <0.001**** | 0.009**** |
| Control:Decoated Endospores | 0.795 | 0.543 | 0.323 | 0.168 |

P-Values of t-Tests Comparing Fatty Acid Ratios Between *Clostridium* spp.

| | SCFA / LCFA | SFA / USFA |
|---|---|---|
| Control : Control Endospores | <0.001**** | 0.014**** |
| Decoated : Decoated Endospores | 0.002**** | 0.005**** |

***** Statistically significant ($P < 0.05$).

### 3.3.4 Two-Photon CLSM of WSH-L Endospores.

Two-photon CLSM images of control and decoated WSH-L endospores of *Clostridium* spp. were used to examine the fluorescence quality of individual endospores. Two-photon CLSM provided a highly sensitive means of visualizing individual WSH-L endospores (Figure 3-2).

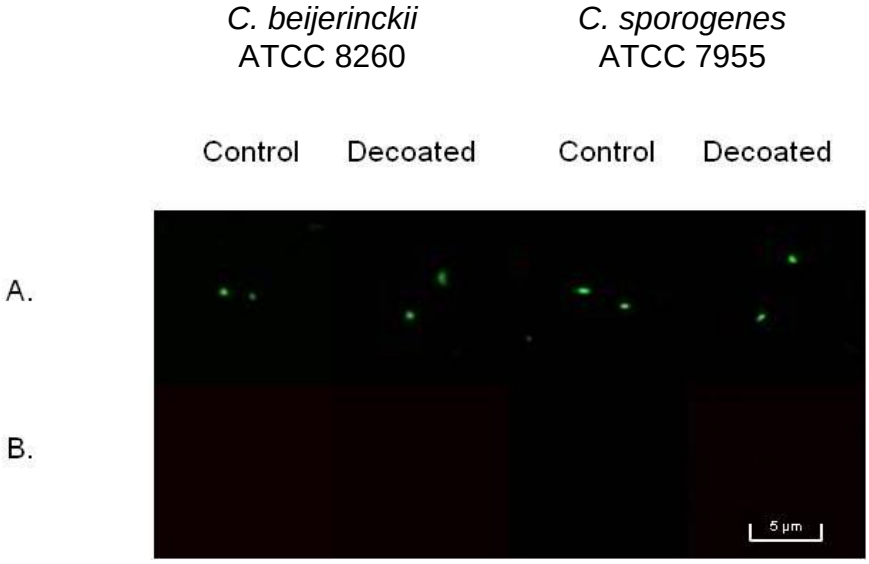


**Figure 3-2.** Two-photon confocal laser scanning microscopy photos of WSH-L endospores of *Clostridium* spp. before and after decoating. Samples were visualized after at (A) 405 nm and (B) 460 nm, using bandpass filters, following two-photon excitation.

Fluorescence was observed for both control and decoated WSH-L endospores of both *Clostridium* spp. when a 405 nm bandpass filter was used (Figure 3-2). The 405 nm bandpass filter allowed high (440 nm) and low (490 nm) energy emissions to be detected. Fluorescence observed using this filter confirms WSH-L endospores fluoresce individually. No fluorescence was observed when a 460 nm bandpass filter was applied (Figure 3-2). The 460 nm bandpass filter excludes the low energy emission (490 nm) from being detected. Decoating did not adversely affect fluorescence of WSH-L endospores.

### 3.3.5 Fluorescence Emission Spectra.

The fluorescence emission spectra of WSH-L endospores (formed during sporulation) and endospores labeled with LAURDAN after sporulation were recorded, before and after washing with 0.9% saline, for comparison of dye retention. Unlabeled endospores of both *Clostridium* spp. exhibited slight background fluorescence, which was subtracted from subsequent fluorescence readings. Endospores of both *Clostridium* spp. exhibited a higher overall fluorescence intensity when labeled after sporulation in comparison to WSH-L endospores (Figures 3-3 and 3-4), which take up LAURDAN during sporulation. However, endospores labeled with LAURDAN after sporulation also exhibited variable retention of fluorescence intensity after being washed in 0.9% saline. Endospores of *C. sporogenes* exhibited a decrease in fluorescence intensity (Figure 3-4C) after washing, whereas endospores of *C. beijerinckii* did not (Figure 3-3C). The WSH-L endospores for both *Clostridium* spp. retained their original fluorescence intensity after being washed with 0.9% saline (Figures 3-3E and 3-4E). The WSH-L endospores were selected for use in subsequent experiments due to this favorably consistent retention of fluorescence after washing.

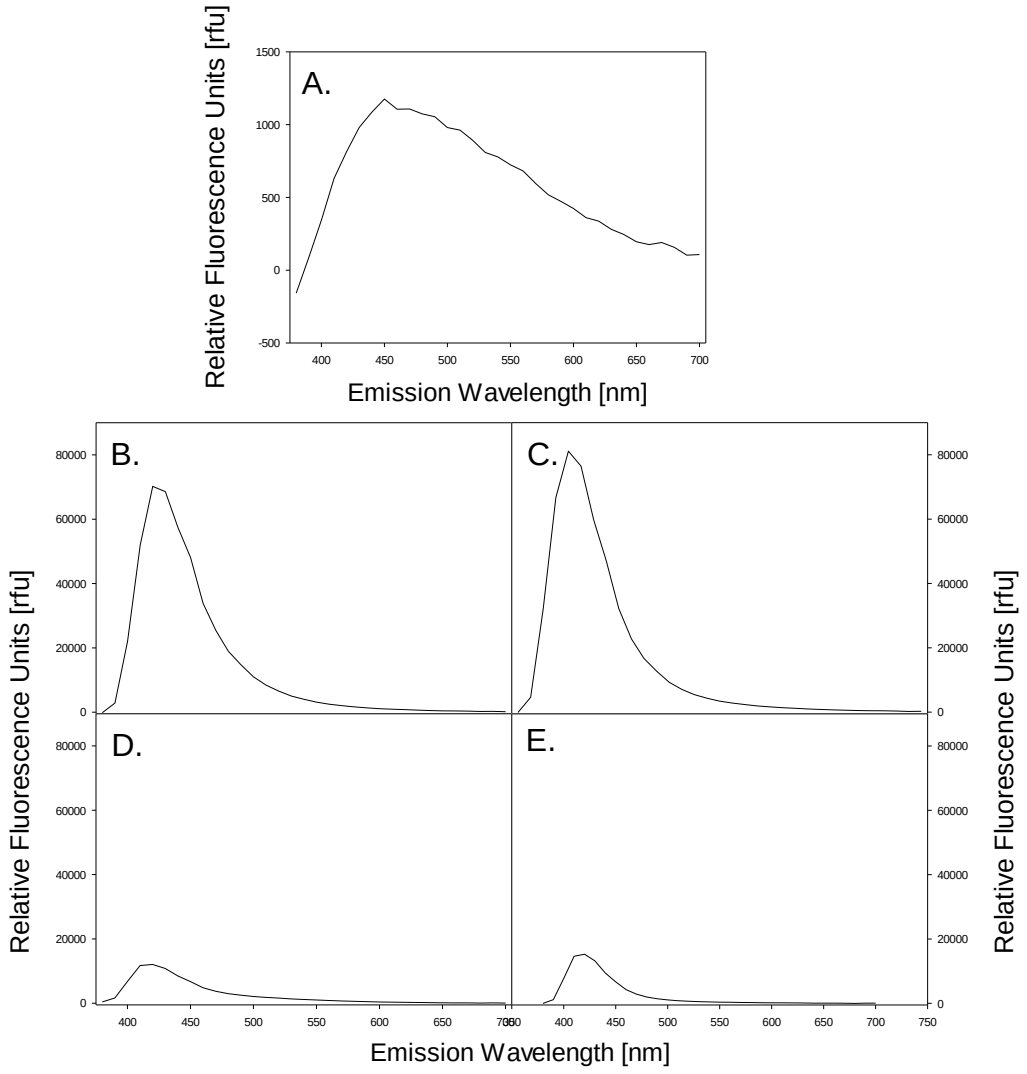


**Figure 3-3.** Fluorescence emission spectra of endospores of *C. beijerinckii* labeled after sporulation or during sporulation (WSH-L endospores) with LAURDAN. Samples were visualized following excitation at 360 nm using a spectrofluorometer. (A) Unlabeled endospores; (B) endospores labeled after sporulation; (C) endospores labeled after sporulation and washed with 0.9% saline; (D) WSH-L endospores; (E) WSH-L endospores washed with 0.9% saline.

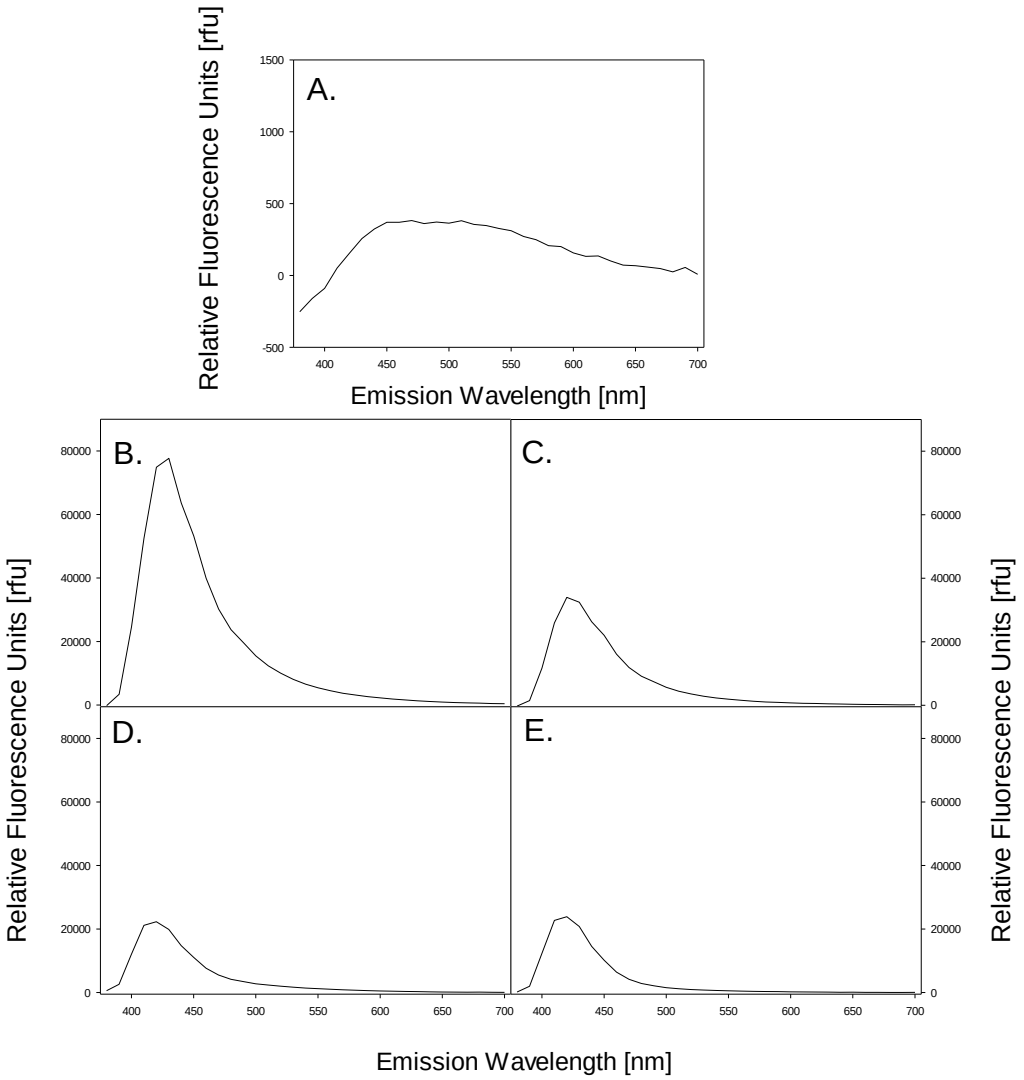


**Figure 3-4.** Fluorescence emission spectra of endospores of *C. sporogenes* labeled after sporulation or during sporulation (WSH-L endospores) with LAURDAN. Samples were visualized following excitation at 360 nm using a spectrofluorometer. (A) Unlabeled endospores; (B) endospores labeled after sporulation; (C) endospores labeled after sporulation and washed with 0.9% saline; (D) WSH-L endospores; (E) WSH-L endospores washed with 0.9% saline.

Fluorescence emission spectra, of cells and WSH-L endospores of *Clostridium* spp., were recorded for comparison to a control LAURDAN fluorescence emission spectrum. Suspension of LAURDAN in 95% ethanol exhibited a characteristic emission peak between 450 and 500 nm (data not shown). Unlabeled cells of *Clostridium* spp. did not exhibit detectable fluorescence emission spectra when excited at 360 nm (data not shown). Labeled cells and WSH-L endospores of *Clostridium* spp. exhibited detectable fluorescence emission spectra characteristic of LAURDAN labeling (Figure 3-5).

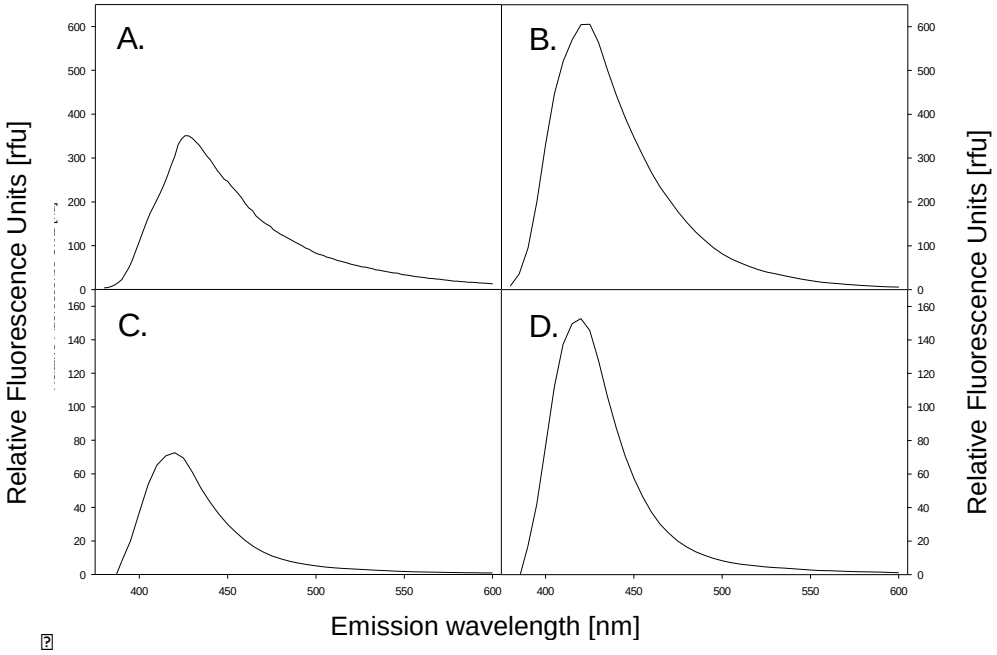


**Figure 3-5.** Fluorescence emission spectra of cells and endospores of *Clostridium* spp. labeled with LAURDAN. Samples were visualized following excitation at 360 nm using a spectrofluorometer. (A) Cells of *C. beijerinckii* ATCC 8260; (B) cells of *C. sporogenes* ATCC 7955; (C) endospores of *C. beijerinckii* ATCC 8260; (D) endospores of *C. sporogenes* ATCC 7955.

### 3.3.6 Generalized Polarization Values of WSH-L Endospores and LAURDAN-Labeled Cells.

Generalized polarization values were calculated to quantitatively compare fluorescence of LAURDAN-labeled cells to WSH-L endospores, and to determine whether decoating quantitatively affected fluorescence of endospores. Generalized

polarization values of control WSH-L endospores were not significantly different from GP values of decoated WSH-L endospores for both strains of *Clostridium* spp. (Table 3-2). The GP values of both untreated and decoated WSH-L endospores were substantially higher than those of labeled cells (Table 3-2).

**Table 3-2.** Generalized polarization values of cells and endospores of *Clostridium* spp. labeled with LAURDAN and suspended in 0.9% saline.[a]

| | | Control | Decoated |
|---|---|---|---|
| | Cells | Endospores | Endospores |
| *C. beijerinckii* | 0.35 ± 0.00 | 0.73 ± 0.00 | 0.74 ± 0.027 |
| *C. sporogenes* | 0.31 ± 0.012 | 0.77 ± 0.010 | 0.77 ± 0.031 |

[a] Values represent mean ± standard deviation of three replicates.

## 3.4 Discussion

This study established a protocol for labeling and measuring inner membrane fluidity of endospores of *Clostridium* spp. The effect of removing the outer membrane of endospores on LAURDAN fluorescence was measured using two-photon CLSM and GP values. Measuring inner membrane fluidity has potential utility for determining effects of membrane-specific antimicrobials, high pressure, and heat on endospores during and after exposure.

Transmission electron microscopy confirms that decoating of WSH-L endospores of *Clostridium* spp. effectively removes the outer membrane, and induces visible changes consistent with decoating of other species of endospores (5, 24). The core, inner membrane, germ cell wall, cortex, outer membrane and protein coat are visible in both *C. sporogenes* ATCC 7955 and *C. beijerinckii* ATCC 8260, and are comparable to those observed in endospores of *Bacillus cereus* and *Bacillus megaterium* (5, 24). Endospores of *C. sporogenes* ATCC 7955 possess an exosporium, whereas those of *C. beijerinckii* ATCC 8260 do not. The presence of an exosporium is species-dependent, and not an

essential component of endospore resistance (40). Labeling endospores of *Clostridium* spp. with LAURDAN does not appear to cause any overt physical alterations. LAURDAN is a fluorophore that preferentially accumulates within hydrophobic regions, and it is possible that the outer membrane of endospores sequesters enough of the fluorophore to influence measurements (60, 70, 82). Decoating of endospores, therefore, allows the examination of the influence of the outer membrane on LAURDAN fluorescence.

Two-photon CLSM reveals WSH-L endospores of *Clostridium* spp. successfully retain LAURDAN following sporulation. Fluorescence visualization using two-photon confocal excitation is preferable to single photon excitation because it does not severely photobleach samples (3, 55). Decoated and control WSH-L endospores of *C. sporogenes* ATCC 7955 and *C. beijerinckii* ATCC 8260 fluoresce upon two-photon excitation. Decoating does not appear to adversely affect WSH-L endospore fluorescence. The absence of WSH-L endospore fluorescence when using a 460 nm bandpass filter, and the fluorescence observed when using a 405 nm bandpass filter suggest LAURDAN is in a highly ordered environment. Fluorescence differences observed between bandpass filters during two-photon CLSM are likely due to the dehydrated state of WSH-L endospores during readings. Having been dried onto slides, there is a minimum of water present to facilitate energetic loss of LAURDAN during probe excitation and reorientation within membranes (60, 70, 82).

The LAURDAN emission spectra measured in cells and endospores of labeled clostridia are consistent with the spectra of pure LAURDAN. Membranes of cells and endospores of *Clostridium* spp. can successfully be labeled with LAURDAN. Endospores that are labeled during sporulation (WSH-L endospores) appear to retain LAURDAN more consistently after washing than those labeled after sporulation. Thus, WSH-L endospores are preferable for experimental use. Fluorescence spectroscopy using

LAURDAN has been used to differentiate between gel and liquid crystalline phases of living cells (82). In doing so, values are calculated from LAURDAN emission readings taken from cell populations in liquid suspension. Theoretically, GP values range from -1.0 to +1.0 (70). GP values for LAURDAN in membranes of liquid phases typically range from -0.3 to 0.3, and those in the gel phase from 0.5 to 0.6 (70). Experimentally, however, values typically range from -0.3 to 0.6 (60). The highest GP values are usually observed in pure lipid suspensions during gel state measurements (60). Generalized polarization values of decoated WSH-L endospores of *Clostridium* spp. do not differ substantially from those containing the outer membrane. This suggests the outer membrane of endospores does not sequester LAURDAN to an extent capable of substantially affecting GP calculations for endospore populations.

The GP values we report for clostridial endospores are high, and indicate a high level of order consistent with a gel phase membrane. These high GP values agree with lipid immobility observed in inner membranes of endospores of *Bacillus* spp. reported previously (19). Inner membranes exist in a compressed state, with surface areas expanding 1.5-fold upon germination in the absence of lipid synthesis (19). Membrane lipids of endospores of *Bacillus* spp. are similar in both quality and quantity to those of vegetative cells (7, 55, 64). Endospores of *Clostridium* spp. in this study differ significantly from vegetative cells in content of SCFA sans SFAs; however, the extent to which this difference of content may influence GP measurements is unclear.

In conclusion, endospores containing LAURDAN can successfully be generated on WSH agar, harvested, and GP values measured. Two-photon CLSM and 96-well microtitre plate-scale spectrofluorometry are capable of assessing WSH-L endospore fluorescence of individual endospores and populations, respectively. Outer membranes of WSH-L endospores can be removed without adversely affecting fluorescence, and do not appear to sequester LAURDAN in amounts that significantly interfere with readings.

WSH-L endospores exhibit high GP values consistent with a high degree of inner membrane lipid packing and immobility. WSH-L endospores may provide a means of assessing endospore membrane fluidity changes during and after a variety of food-processing parameters, including exposure to high temperature, pressure, and antimicrobials.

## 3.5 References


1. **Ahn, J., Balasubramaniam, V.M., and Yousef, A.E.** 2007. Inactivation kinetics of selected aerobic and anaerobic bacterial spores by pressure-assisted thermal processing. Int. J. Food Microbiol. **113**:321-329.
2. **Asai, K., Takamatsu, H., Iwano, M., Kodama, T., Watabe, K., and Ogasawara, N.** 2001. The *Bacillus subtilis* yabQ gene is essential for formation of the spore cortex. Microbiology UK. **147**:919-927.
3. **Bagatolli, L.A., Sanchez, Hazlett, T., and Gratton, E.** 2003. Giant vesicles, laurdan, and two-photon fluorescence microscopy: Evidence of lipid lateral separation in bilayers. Meth. Enzym. **360**:481-500.
4. **Baier, D., Reineke, K., Doehner, I., Mathys, A., and Knorr, D.** 2011. Fluorescence-based methods for the detection of pressure-induced spore germination and inactivation. High Pressure Research. **31**:110-115.
5. **Beaman, T.C., and Gerhardt, P.** 1986. Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation. Appl. Environ. Microbiol. **52**:1242-1246.
6. **Belliveau, B.H., Beaman, T.C., Pankratz, H.S., and Gerhardt, P.** 1992. Heat killing of bacterial spores analyzed by differential scanning calorimetry. J. Bacteriol. **174**:4463-4474.
7. **Bertsch, L.L., Bonsen, P.P., and Kornberg, A.** 1969. Biochemical studies of bacterial sporulation and germination. XIV. Phospholipids in *Bacillus megaterium*. J. Bacteriol. **98**:75-81.
8. **Black, E.P., Setlow, P., Hocking, A.D., Stewart, C.M., Kelly, A.L., and Hoover, D.G.** 2007. Response of spores to high-pressure processing. Com. Rev. Food Sci. Food Safety. **6**:103-119.

9. **Borge, G.I.A., Skeie, M., Sorhaug, T., Langsrud, T., and Granum, E.** 2001. Growth and toxin profiles of *Bacillus cereus* isolated from different food sources. Int. J. Food Microbiol. **6**:237-246.

10. **Broda, D.M., DeLacy, K.M., Bell, R.G., Braggins, T.J., and Cook, R.L.** 1996. Psychrotrophic *Clostridium* spp. Associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Intl. J. Food Microbiol. **29**:335-352.

11. **Brown, K.** 2000. Control of bacterial spores. Brit. Med. Bulletin. **56**:58-71.

12. **Bunthof, C.J., van den Braak, S., Breeuwer, P., Rombouts, F.M., and Abee, T.** 1999. Rapid fluorescence assessment of the viability of stressed *Lactococcus lactis*. Appl. Environ. Microbiol. **65**:3681-3689.

13. **Cano, R.J., and Borucki, M.** 1995. Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. Science. **268**:1060-1064.

14. **Carlin, F.** 2011. Origin of bacterial spores contaminating foods. Food Microbiol. **28**:177-182.

15. **Christie, W.** 2003. *Lipid Analysis – 3rd Edition*. Bridgewater: The Oily Press.

16. **Collins, M.D., and East, A.K.** 1998. Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins. J. Appl. Microbiol. **84**:5-17.

17. **Cortezzo, D.E., Setlow, B., and Setlow, P.** 2004. Analysis of the action of compounds that inhibit the germination of spores of *Bacillus* species. J. Appl. Food Microbiol. **96**:725-741.

18. **Costa, T., Steil, L., Martins, L.O., Volker, U., and Henriques, A.O.** 2004. Assembly of an oxalate decarboxylase produced under sigma(K) control into the *Bacillus subtilis* spore coat. J. Bacteriol. **186**:1462-1474.

19. **Cowan, A.E, Olivastro, E.M., Koppel, D.E., Loshon, C.A., Setlow, B., and Setlow, P.** 2004. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. Proc. Natl. Academy Sci. USA. **101**:7733-7738.

20. **Cronin, U.P. and Wilkinson, M.G.** 2008. Monitoring changes in germination and permeability of *Bacillus cereus* endospores following chemical, heat and enzymatic treatments using flow cytometry. J. Rapid Methods Automation Microbiol. **16**:164-184.

21. **Cuevas, F.J., Jameson, D.M., and Sotomayor, C.P.** 2006. Modulation of reconstituted pig kidney $Na^+/K^+$-ATPase activity by cholesterol in endogenous lipid vesicles: Role of lipid domains. Biochemistry. **45**:13855-13868.

22. **Driks, A.** 2002. Overview: development in bacteria: spore formation in *Bacillus subtilis*. Cell. Mol. Life Sci. **59**:389-391.

23. **Esty, J.R., and Meyer, K.F.** 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes. J. Infec. Diseases. **31**:650-664.

24. **Fitz-James, P.C.** 1971. Formation of protoplasts from resting spores. J. Bacteriol. **105**:1119-1136.

25. **Ganzle, M.G. and Vogel, R.F.** 2001. On-line fluorescence determination of pressure-mediated outer membrane damage in *Escherichia coli*. System. Appl. Microbiol. **24**:477-485.

26. **Gao, Y.-L., and Ju, X.-R.** 2008. Exploiting the combined effects of high pressure and moderate heat with nisin on inactivation of *Clostridium botulinum* spores. J. Microbiol. Meth. **72**:20-28.

27. **Guilford, J. and Pavel, L.** 2007. Poly(methacrylic acid) enhances emission of dye stain extracted from bacterial spores: Fluorimetric quantitation in aqueous samples. J. Photochem. Photobiol. A-Chemistry. **189**:147-152.

28. **Harris, F.M., Best, K.B., and Bell, J.D.** 2002. Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order. Biochim. Biophys. Acta. **1565**:123-128.

29. **Harris, F.M., Smith, S.K., and Bell, J.D.** 2001. Physical properties of erythrocyte ghosts that determine susceptibility to secretory phospholipase A2. J. Biol. Chem. **276**:22722-22731.

30. **Hilbert, D.W., Chary, V.K., and Piggot, P.J.** 2004. Contrasting effects of sigma(E) on compartmentalization of sigma(F) activity during sporulation of *Bacillus subtilis*. J. Bacteriol. **186**:1983-1990.

31. **Hinc, K., Nagorska, K., Iwanicki, A., Wegrzyn, G., Seror, S.J., and Obuchowski, M.** 2006. Expression of genes coding for GerA and GerK spore germination receptors is dependent on the protein phosphatase PrpE. J. Bacteriol. **188**:4373-4383.

32. **Johnson, E.A., and Bradshaw, M.** 2001. *Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective. Toxico. **39**:1703-1722.

33. **de Jong, I. G., Veening, J.W., and Kuipers, O.P.** 2010. Heterochronic phosphorelay gene expression as a source of heterogeneity in *Bacillus subtilis* spore formation. J. Bacteriol. **192**:2053-2067.

34. **Ju, J.L., Luo, T.Q., and Haidenwang, W.G.** 1997. *Bacillus subtilis* pro-sigma(E) fusion protein localizes to the forespore septum and fails to be processed when synthesized in the forespore. J. Bacteriology. **179**:4888-4893.

35. **Ju, J.L. and Haidenwang, W.G.** 2003. Tethering of the *Bacillus subtilis* sigma(E) proprotein to the cell membrane is necessary for its processing but insufficient for its stabilization. J. Bacteriol. **185**:5897-5900.

36. **Kennedy, M.J., Reader, S.L., and Swierczynski, L.M.** 1994. Preservation records of microorganisms: evidence of the tenacity of life. Microbiology. **140**:2513-2529.

37. **Koppel, D.E.** 1980. Lateral diffusion in biological membranes: A normal-mode analysis of diffusion on a spherical surface. Biophys. J. **30**: 187-192.

38. **Koppel, D.E.** 1985. Normal-mode analysis of lateral diffusion on a bounded membrane surface. Biophys. J. **47**:337-347.

39. **Kort, R., O'Brien, A.C., van Stokkum, I.H.M., Oomes, S.J.C.M., Crielaard, W., Hellingwerf, K.J., and Brul, S.** 2005. Assessment of heat resistance of bacterial spores from food isolates by fluorescence monitoring of dipicolinic acid release. Appl. Environ. Microbiol. **71**:3556-3564.

40. **Koshikawa, T., Beaman, T.C., Pankratz, H.S., Nakashio, S., Corner, T.R., and Gerhardt, P.** 1984. Resistance, germination, and permeability correlates of *Bacillus megaterium* spores successively divested of integument layers. J. Bacteriol. **159**: 624-632.

41. **Lee, M.J., Dong-Ho, B., Dong-Ha, L., Ki-Hyo, J., Deog-Hwan, O., and Sang-Do, H.** 2006. Reduction of *Bacillus cereus* in cooked rice treated with sanitizers and disinfectants. J. Microbiol. Biotech. **16**:639-642.

42. **Lund, B.M.** 1990. Foodborne disease due to *Bacillus* and *Clostridium* species. Lancet. **336**:982-986.

43. **Margosch, D., Ehrmann, M.A., Buckow, R., Heinz, V., Vogel, R.F., and Gänzle, M.** 2006. High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. Appl. Environ. Microbiol. **72**:3476-3481.

44. **Margosch, D.**, **Ehrmann, M.A., Gänzle, M.G., and Vogel, R.F.** 2004a. Comparison of pressure and heat resistance of *Clostridium botulinum* and other endospores in mashed carrots. J. Food Protection. **67**:2530-2537.

45. **Margosch, D., Gänzle, M.G., Ehrmann, M.A., and Vogel, R.F.** 2004b. Pressure inactivation of *Bacillus* endospores. Appl. Environ. Microbiol. **70**:7231-7238.

46. **Masayama, A., Kuwana, R., Takamatsu, H., Hemmi, H., Yoshimura, T., Watabe, K., and Moriyama, R.** 2007. A novel lipolytic enzyme, YcsK (lipC), located in the spore coat of *Bacillus subtilis*, is involved in spore germination. J. Bacteriol. **189**:2369-2375.

47. **Matches, J.W.** 1964. Phospholipids in vegetative cells and spores of *Bacillus polymyxa*. J. Bacteriol. **87:**16-23.

48. **McBride, S.M., Rubio, A., Wang, L., and Haidenwang, W.G.** 2005. Contributions of protein structure and gene position to the compartmentalization of the regulatory proteins sigma(E) and SpoIIE in sporulating *Bacillus subtilis*. Molec. Microbiol. **57**:434-451.

49. **Meyer, R.S., Cooper, K.L., Knorr, D., Lelieveld, H.L.M.** 2000. High-pressure sterilization of foods. Food. Tech. **54**:67-72.

50. **Molina-Höppner, A., Doster, W., Vogel, R.F., and Gänzle, M.G.** 2004. Protective effect of sucrose and sodium chloride for *Lactococcus lactis* during sublethal and lethal high-pressure treatments. Appl. Env. Microbiol. **70**:2013-2020.

51. **Murakami, T., Haga, K., Takeuchi, M., and Sato, T.** 2002. Analysis of the *Bacillus subtilis* spoIIIJ gene and its paralogue gene, yqjG. J. Bacteriol. **184**:1998-2004.

52. **Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P.** 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. **64**:548-572.

53. **Nicholson, W.L.** 2002. Roles of *Bacillus* endospores in the environment. Cell. Mol. Life Sci. **59**:410-416.

54. **Nicolini, C., Celli, A., Gratton, E., and Winter, R.** 2006. Pressure tuning of the morphology of heterogeneous lipid vesicles: a two-photon-excitation fluorescence microscopy study. Biophys. J. **91**:2936-2942.

55. **Nikolopoulou, M.** 1987. Lipid composition of *Bacillus megaterium* spores and spore membranes. Lipids. **22:**698-703.

56. **Paidhungat, M.S.** 2000. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. J. Bacteriol. **182**: 5505-5512.

57. **Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R.M., and Gratton, E.** 1991. Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of laurdan fluorescence. Biophys. J. **60**:179-189.

58. **Parasassi, T., Giusti, A.M., Raimondi, M., and Gratton, E.** 1995. Abrupt modifications of phospholipid bilayer properties at critical cholesterol concentrations. Biophys. J. **68**:1895-1902.

59. **Parasassi, T., Krasnowska, E.K., Bagatolli, L., and Gratton, E.** 1998. Laurdan and prodan as polarity-sensitive fluorescent membrane probes. J. Fluor. **8**:365-373.

60. **Piggot, P.J., and Hilbert, D.W.** 2004. Sporulation of *Bacillus subtilis*. Curr. Op. Microbiol. **7**:579-586.

61. **Pogliano, J., Osborne, N., Sharp, M.D., Abanes-De Mello, A., Perez, A., Sun, Y.L., and Pogliano, K.** 1999. A vital stain for studying membrane dynamics in bacteria: a novel mechanism controlling septation during *Bacillus subtilis* sporulation. Mol. Microbiol. **31**:1149-1159.

62. **Powalska, E., Janosch, S., Kinne-Saffran, E., Kinne, R.K.H., Fontes, C.F.L., Mignaco, J.A., and Winter, R.** 2007. Fluorescence Spectroscopic Studies of Pressure Effects on $Na^+$, $K^+$ ATPase Reconstituted into Phospholipid Bilayers and Model Raft Mixtures. Biochemistry. **46**:1672-1683.

63. **Price, K.D. and Losick, R.** 1999. A four-dimensional view of assembly of a morphogenetic protein during sporulation in *Bacillus subtilis*. J. Bacteriology. **181**:781-790.

64. **Racine, F.** 1980. Isolation and properties of membranes from *Bacillus megaterium* spores. J. Bacteriol. **143**:1208-1214.

65. **Real, G., Fay, A., Eldar, A., Pinto, S.M., Henriques, A.O., and Dworkin, J.** 2008. Determinants for the subcellular localization and function of a nonessential SEDS protein. J. Bacteriol. **190**:363-376.

66. **Reddy, N.R., Solomon, H.M., Fingerhut, G.A., Balasubramaniam, V.M., and Rhodehamel, E.J.** 1999. Inactivation of *Clostridium botulinum* Type E spores in a model buffer or food system by high pressure processing. J. Food Saf. **19**:277-288.

67. **Reddy, N.R., Solomon, H.M., Tetzloff, R.C., and Rhodehamel, E.J.** 2003. Inactivation of *Clostridium botulinum* Type A spores by high-pressure processing at elevated temperature. J. Food Prot. **66**:1402-1407.

68. **Reid, B.G. and Flynn, G.C.** 1997. Chromophore formation in green fluorescent protein. Biochemistry. **36**:6786-6791.

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

70. **Resnekov, O., Alper, S., and Losick, R.** 1996. Subcellular localization of proteins governing the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. Genes to Cells. **1**:529-542.

71. **Ritsuko, K., Hiromu, T., and Kasuhito, W.** 2007. Expression, localization and modification of YxeE spore coat protein in *Bacillus subtilis*. J. Biochem. **142**:681-689.

72. **Salkinoja-Salonen, M.S., Vuorio, R., Andersson, M.A., Kampfer, P., Andersson, M.C., Honkanen-Buzalski, T., and Scoging, A.C.** 1999. Toxigenic strains of *Bacillus licheniformis* related to food poisoning. Appl. Environ. Microbiol. **65**:4637-4645.

73. **Sanchez, S.A., Tricerri, M.A., Gunther, G., and Gratton, E.** 2007. Laurdan generalized polarization: from cuvette to microscope (Ed: Mendez-Vilas, A., and Diaz, J.). Modern Research and Educational Topics in Microscopy. Pg. 1007-1014.

74. **Schantz, E.J., and Johnson, E.A.** 1992. Properties and use of botulinum toxin and other microbial neurotoxins in medicine. Microbiol. Rev. **56**:80-99.

75. **Scott, A.B., and Suzuki, D.** 1988. Systemic toxicity of botulinum toxin by intramuscular injection in the monkey. Movement Disor. **3**:333-335.

76. **Setlow, P.** 2003. Spore germination. Curr. Op. Microbiol. **6**:550-556.

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

78. **Shapiro, R.L., Hatheway, C., and Swerdlow, D.L.** 1998. Botulism in the United States: a clinical and epidimiologic review. Ann. Intern. Med. **129**:221-228.

79. **Sotomayor, C.P., Aguilar, L.F., Cuevas, F.J., Helms, M.K., and Jameson, D.M.** 2000. Modulation of pig kidney $Na^+/K^+$-ATPase activity by cholesterol: Role of hydration. Biochemistry **39**:10928-10935.

80. **Taylor, L.C., Tabacco, M.B., and Gillespie, J.B.** 2001. Sensors for detection of calcium associated with bacterial endospore suspensions. Analytica Chimica Acta. **435**:239-246.

81. **Ulmer, H.M., Gänzle, M.G., and Vogel, R.F.** 2000. Effects of high pressure on survival and metabolic activity of *Lactobacillus plantarum* TMW1.460. Appl. Environ. Microbiol. **66**:3966-3973.

82. **Ulmer, H.M., Heinz, V., Gänzle, M.G., Knorr, D., and Vogel, R.F.** 2002. Effects of pulsed electric fields on inactivation and metabolic activity of *Lactobacillus plantarum* in model beer. J. Appl. Microbiol. **93:**326-335.

83. **Ulmer, H.M., Herberhold, H., Fahsel, S., Gänzle, M.G., Winter, R., and Vogel, R.F.** 2002. Effects of pressure-induced membrane phase transitions on inactivation

of HorA, on ATP-dependent multidrug resistance transporter, in *Lactobacillus plantarum*. Appl. Environ. Microbiol. **68**:1088-1095.

84. **Van Ooiij, C., Eichenberger, P., and Losick, R.** 2004. Dynamic patterns of subcellular protein localization during spore coat morphogenesis in *Bacillus subtilis*. J. Bacteriology. **186**:4441-4448.

85. **Vreeland, R.H., Rosenzweig, W.D., and Powers, D.W.** 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature. **407**:897-900.

86. **Wang, K.H., Isidro, A.L, Domingues, L., Eskandarian, H.A., McKenney, P.T., Drew, K., Grabowski, P., Chua, M.H., Barry, S.N., Guan, M., Bonneau, R., Henriques, A.O., and Eichenberger, P.** 2009. The coat morphogenetic protein SpoVID is necessary for spore encasement in *Bacillus subtilis*. Molec. Microbiol. **74**:634-649.

87. **Wilson, M.J., and Baker, R.** 1997. High temperature/ulta-high pressure sterilization of low acid foods. PCT IB9601500.

88. **Winter, R., and Jeworrek, C.** 2009. Effect of pressure on membranes. Soft Matter. **5**:3157-3173.

89. **Wuytack, E.Y. and Michiels, C.W.** 2001. A study on the effects of high pressure and heat on *Bacillus subtilis* spores at low pH. Int. J. Food Microbiol. **64**:333-341.

# 4. Membrane Fluidity of Endospores of *Clostridium* spp. During Pressure-Assisted Thermal Processing in Combination with Antimicrobials[1]

## 4.1 Introduction

Endospores resist inactivation by thermal and pressure-assisted thermal processing used in extending storage life, and ensuring safety of foods (23, 40, 46, 47, 48, 64, 65). Multiple, distinct layers compose an endospore, and contribute to resistance and metabolic dormancy (75). The core of an endospore contains a matrix of calcium and dipicolinic acid (Ca-DPA) that keeps water activity low (74). This low water activity is thought to be the main contributor to endospore thermal resistance (75). Compromising membrane integrity of endospores rehydrates the core, and facilitates subsequent inactivation of endospores by heat, pressure, and antimicrobials (29, 42, 50, 74). Pressure-assisted thermal sterilization (PATS) has been examined as an alternative to thermal processing for inactivation of endospores (84, 51). PATS utilizes pressure (200 to 800 MPa) and temperature (90 – 120°C) to achieve inactivation of endospores (1, 28, 65). Pressure-assisted thermal sterilization forces the release of DPA, rehydrates cores of endospores, and allows for thermal inactivation (7, 48, 47, 46). Variability in resistance to PATS exists among endospores of *Clostridium botulinum*, and temperatures required for inactivation range from 50 – 120°C (47, 48, 64, 65). Furthermore, high pressures in combination with heat have also been shown to stabilize endospores (46).

Use of antimicrobials in concert with PATS in food processing may enhance inactivation of endospores at lower temperatures, thus maintaining product safety and quality. Some bacteriocins and the tetramic acid reutericyclin exhibit suitable antimicrobial activity (26, 43). Of the bacteriocins, nisin is a pore-forming lantibiotic

[1] Professor Roland Winter is acknowledged for his contribution to the fourier-transform infrared spectroscopy work presented within this chapter.

produced by *Lactococcus lactis* subspecies *lactis*, and enhances inactivation of endospores by heat and pressure (28, 41, 68, 81). Reutericyclin, a proton-ionophore produced by *Lactobacillus reuteri*, exhibits bactericidal activity against Gram-positive organisms, including endospores (27). Both nisin and reutericyclin target cell membranes, though they exert antimicrobial activity by very different mechanisms (27, 43). Inactivation of endospores in the presence of nisin and reutericyclin may be due to the release of Ca-DPA caused by PATS or heat, which leads to regained sensitivity to the antimicrobial compound. Alternatively, antimicrobials may increase loss of Ca-DPA and subsequently facilitate denaturation of protein by heat and pressure. Endospores possess an outer and inner membrane (6, 49, 56, 63). The inner membrane exists in a compressed state, with the surface area expanding 1.5-fold upon germination in the absence of lipid synthesis (19). Lipids within the inner membrane of endospores are immobile, regaining mobility only upon germination (19).

An improved understanding of the effect of pressure, nisin, and reutericyclin on membranes of endospores will facilitate the development of a strategy to enhance control of endospores using PATS. Effects of nisin and reutericyclin on vegetative cells are well documented (26, 35). Although both are effective against endospores, little is known about their physiological effects on inner membranes of endospores, or about these membranes in general.

Measurement of membrane fluidity using LAURDAN with nisin and reutericyclin has been done using cells of *Lactobacillus reuteri* (72). Shifts in the fluorescence spectrum of LAURDAN accurately indicate differences in membrane fluidity (55), and its use in this respect has been well established (58, 60, 59, 79, 20, 30, 62, 31). Previous work has shown that endospores can successfully be labeled with LAURDAN, and that the outer membrane of endospores does not appreciably sequester the fluorescent dye (Chapter 3, this thesis). The goal of this study was to examine effects

of high pressure, temperature, nisin, and reutericyclin on membrane fluidity of endospores of food-relevant *Clostridium* spp. Nisin and reutericyclin were selected due to their food-related, lactic acid bacteria (LAB) origins, efficacy against endospores, membrane-specific activity, and different modes of action (21, 27, 28, 34, 41, 68, 81). *Clostridium sporogenes* ATCC 7955 was chosen for its heat-resistant endospores and genetic similarity to *Clostridium botulinum* (15). *Clostridium beijerinckii* ATCC 8260 was chosen due to potential involvement in blown-pack meat spoilage and growth at 4°C (10). Endospores containing LAURDAN are hereafter denoted as WSH-L endospores.

## 4.2 Materials and Methods

### 4.2.1 Bacterial Strains and Growth Conditions.

Cells of *Clostridium beijerinckii* ATCC 8260, and *Clostridium sporogenes* ATCC 7955 were grown by inoculating Reinforced Clostridial Media (RCM; Difco, Sparks, USA) broth with endospores maintained at -80°C in 60% glycerol. Inoculated broth was incubated at 37°C, overnight, in anaerobic conditions to produce a cell culture.

### 4.2.2 Sporulation and Harvesting Endospores.

Weihenstephan Sudhang (WSH) media was prepared as described by Margosch et al (46). Endospores containing 6-dodecanoyl-2-dimethylaminonaphthalene- (LAURDAN) (Invitrogen) (WSH-L endospores) were generated as described previously (Chapter 3, this thesis). LAURDAN-saturated ethanol (400 μL) was plated onto individual WSH agar plates, the ethanol allowed to evaporate in the absence of light, and 100 μL of fully-grown cultures were plated onto the surface in an anaerobic hood. Plates were incubated at 37°C, in absence of light in an anaerobic hood for 14 days. Control endospore stocks were generated as above, omitting addition of LAURDAN-saturated ethanol. Following incubation, sporulation was confirmed using phase-contrast

microscopy in which ~95% endospore purity was observed. Endospores were harvested from the surface of WSH agar plates by washing with sterile 0.9% saline. Harvested endospores were centrifuged at 2,700 x*g*, and suspended in sterile 0.9% saline. Care was taken to discard the top layer of slurry of each cell pellet, as this contained the majority of cell debris. Endospore stock solutions were standardized to $OD_{600}$ values of 0.5 for WSH-L endospores, and 2.0 for control endospores, with sterile 0.9% saline. The lower $OD_{600}$ value was used for WSH-L endospores to provide accurate LAURDAN fluorescence readings. Stock endospore solutions were plated on RCM agar and incubated at 37°C anaerobically for 6 days to verify endospore viability. Standardized endospore stocks were stored at -20°C until needed. Sterile 0.9% saline was used as the endospore suspension medium throughout subsequent experiments.

### 4.2.3 Preparation and Storage of Antimicrobial Compounds.

Nisin (Chrisaplin, Chr. Hansen HS, Hørsholm, Denmark) was diluted in 0.05% acetic acid and stored at -20°C. Reutericyclin was purified from the culture supernatant of *Lactobacillus reuteri* LTH2584 and antimicrobial activity quantified as described previously (26). Reutericyclin was stored in a 80:20 ispropanol:water solution at -20°C.

### 4.2.4 Preparation of Dipalmitoylphosphatidylcholine (DPPC) Vesicles Containing LAURDAN.

DPPC was weighed into Eppendorf tubes (1.5 mL) to 0.5 mg, and suspended in 100 µL chloroform. LAURDAN in chloroform (1.36 µL of 1 mM) was added. The chloroform was evaporated using nitrogen gas. Samples were freeze-dried overnight in absence of light. Freeze-dried samples were suspended in 1 mL of distilled water and warmed to 65°C for 15 min in a sonicating waterbath. Samples were frozen in liquid nitrogen for 5 min, followed by warming to 65°C in a waterbath; this step was repeated four times. Aliquots (293 µL) were mixed with 707 µL distilled water and kept at -20°C

until scanned (ISS K2 Multifrequency Fluorometer, spindle press and pressure gauge). Nisin and reutericyclin were mixed with DPPC solutions at 16 mg/L and 6.4 mg/L, respectively, prior to fluorescence measurements. GP values were calculated using emission values at 440 nm and 490 nm following excitation at 360 nm.

$$GP = (I_{440}-I_{490})/(I_{440}+I_{490}) \quad (3)$$

Theoretically, GP values range from -1.0 to +1.0 (70). Experimentally, the values typically range from -0.3 to 0.6 (60). High values are attributed to pure lipid suspensions, such as DPPC during gel state measurements (60). GP in liquid phases typically range from -0.3 to 0.3, and gel phase from 0.5 to 0.6 (70).

**4.2.5 Fourier-Transform Infrared (FT-IR) Spectroscopy.**

Samples were prepared as described previously (25). Endospore stock solutions were washed five times with distilled water and centrifuged at 2,700 x*g* between washes. A pipette tip was used to transfer pelleted endospores onto calcium-fluoride windows ($CaF_2$, 21 mm diameter, 3 mm thickness). Distilled water (10 µL) water was added to the pellet, and a pipette tip was used to smear the pellet into a thin film covering the FT-IR cell window. The film was allowed to dry for 8 hs at 50°C. The dried sample film was sealed between another $CaF_2$ window and absorbance spectra measured using a Nicolet 5700 FT-IR spectrometer (Thermo Electron Corporation). Settings were as follows: 256 scans/sample, 2 nm resolution, 0.964 $cm^{-1}$ data spacing, KBr beamsplitter, range of 4000-1111 nm, velocity of 1.8988, aperture: 9, apodization: Happ-Genzel, phase correction: Mertz. Temperature control for 90°C at ambient pressure was achieved using a Julabo F-32 external circulating water bath. Temperatures were increased at a rate of 5°C/10 min, allowing 5 min for equilibration and FTIR scans. Samples were cooled to 4°C before measurements were initiated, followed by heating to and holding at 90°C for 60 min. The

Julabo F-32's external temperature probe was used to confirm sample temperature equilibration.

### 4.2.6 *In situ* GP Measurements.

Nisin and reutericyclin were mixed with WSH-L endospore stock solutions ($OD_{600}$ 0.5) at 16X MIC values reported previously for endospores of the same *Clostridium* spp. (Chapter 2, this thesis) (16 mg/L and 6.4 mg/L respectively). Aliquots of 1 mL were sealed in quartz vials using Dura Seal (Diversified Biotech, Lab Sealing Film, Sigma Aldrich, Cat. No. DS5-150) and a rubber o-ring. Quartz vials were placed in a custom high-pressure vessel (approximately 10.5 x 12 mm) and pressurized to 200 MPa, followed by heating to 90˚C (ISS K2 Multifrequency Fluorometer, spindle press and pressure gauge). Ethanol (95%) was used as the pressurizing medium. Samples were held at 90˚C for 60 min, after which the temperature was decreased to 4˚C. Temperatures were achieved using an external circulating water system (Julabo F-25). GP values were measured 10 times every 12 seconds. Samples were frozen at -20 °C overnight before rescanning at 21°C for 5 min.

### 4.2.7 *Ex situ* GP Measurements.

Nisin and reutericyclin were mixed with WSH-L endospore stock solutions ($OD_{600}$ 0.5) at 16X MIC values reported previously for endospores of the same *Clostridium* spp. (Chapter 2, this thesis) (16 mg/L and 6.4 mg/L respectively). Aliquots (120 µL) were sealed in Tygon tubing (Application Specific Tubing, Saint-Gobain Performance Plastics, Pittsburg, USA) using a hair-straightener (TONI&GUY®, Model TGST2976F, London, UK). Samples were kept on ice until needed. Compensation for adiabatic heating was done, in accordance with heating curves of pressurized water, by preheating sealed samples in a water bath at 74°C for 5 minutes prior to pressurization. Samples were placed in vessels of a U111 Unipress (Warsaw, Poland) and pressurized to

600 MPa in 1 min. at 90 ˚C, and held at this pressure and temperature for no longer than 60 min. Temperatures were achieved using an external circulating propylene glycol system (LAUDA Proline, Delran, USA). Samples were stored on ice overnight following treatment until extracted from sample vessels for GP measurements (Varioskan Flash, Thermo Electron Corporation, Nepean, Canada).

## 4.3 Results

### 4.3.1 Generalized Polarization Measurements of DPPC Vesicles.

Generalized polarization measurements of DPPC vesicles were done to establish effects of heat, pressure, and antimicrobials, on membrane fluidity of an established vesicle system. Generalized polarization values of LAURDAN in model DPPC membrane preparations were recorded during thermal and high pressure thermal treatments to establish effects of nisin and reutericyclin on membrane phase transitions. Heating to 90°C resulted in a rapid decrease in GP values below detectable limits for all treatments (Figure 4-1A).

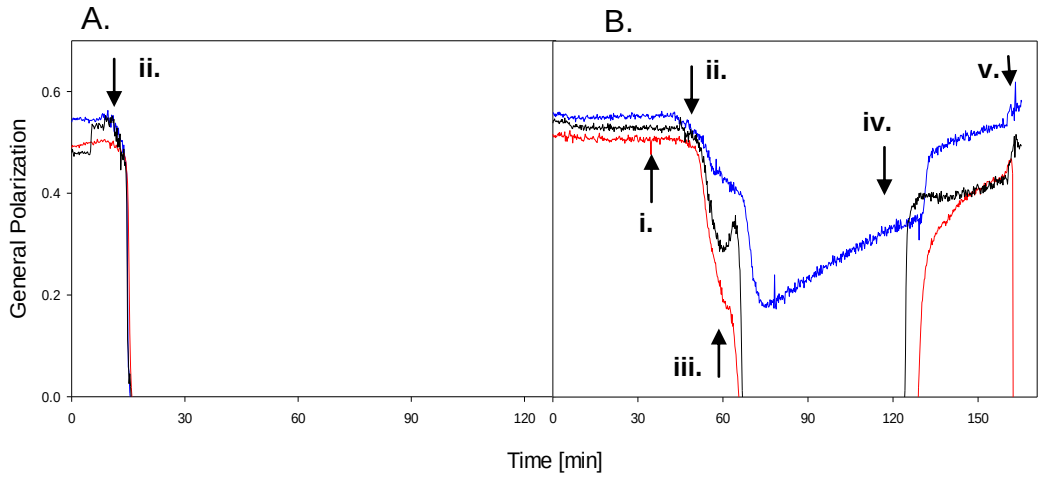


**Figure 4-1.** Generalized polarization of dipalmitoylphosphatidylcholine-LAURDAN vesicles treated at 90˚C (A) or 90˚C and 200 MPa (B). Samples were treated in the presence of 6.4 mg liter$^{-1}$ reutericyclin (red), 16 mg liter$^{-1}$ nisin (blue), or in the absence of antimicrobials (black). Measurements were taken every 12 sec. Displayed values are the average of 10 measurements. (i) Initiation of heating after 200 MPa achieved; (ii) drop in GP values corresponding to 40 - 45˚C; (iii) 90˚C achieved; (iv) cooling to 4˚C initiated; (v) depressurization.

Generalized polarization values began decreasing between 40 and 45°C for all thermal treatments, and were not recovered upon cooling to 4°C. Heating to 90°C at 200 MPa caused a rapid decrease in GP values of control samples and samples containing reutericyclin; the presence of nisin mitigated the decrease in GP values **(**Figure 4-1B). Generalized polarization values began decreasing between 40 and 45°C for all high pressure thermal treatments. When samples were depressurized, all GP values with the exception of those of the reutericyclin treatment returned to initial levels (Figure 4-1).

### 4.3.2 Fourier-Transform Infrared Spectroscopy Scans of Endospores.

Fourier-transform infrared spectroscopy of endospores of *Clostridium* spp. during thermal treatments was used as an alternative method to confirm membrane transitions observed at 90°C using LAURDAN. Both *Clostridium* spp. exhibited a shift at wavenumber 2849 /cm to 2853 - 2854 /cm when heated with or without nisin or reutericyclin (Figure 4-2 and 4-3; A, C, and E). Processing of spectra to the inverted second derivative confirmed an upward wavenumber shift for both *Clostridium* spp. for all treatments (Figure 4-2 and 4-3; B, D, and F).

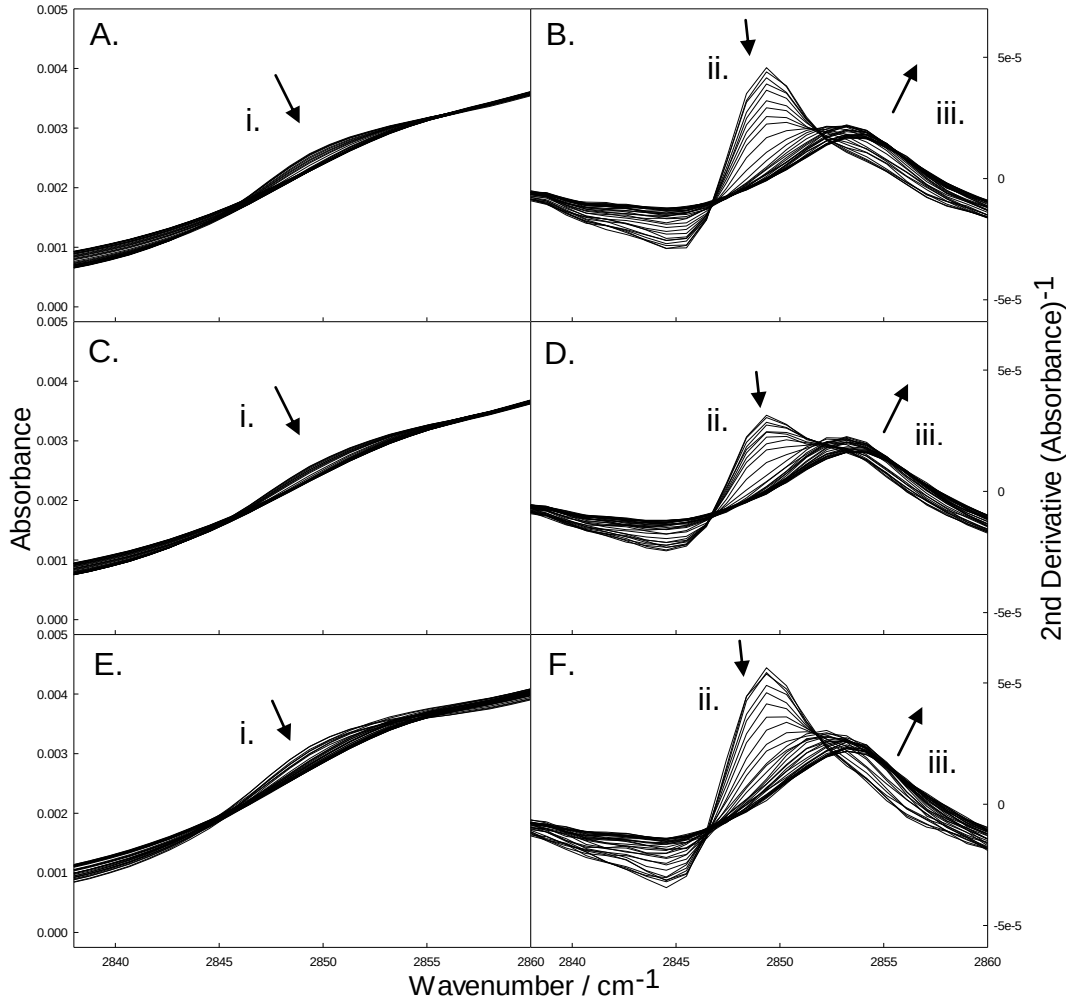


**Figure 4-2.** Fourier-transform infrared spectroscopy (A, C, E) and second derivative spectra (B, D, F) of WSH-L endospores of *C. beijerinckii* heated to 90˚C. Samples were heated in the absence of antimicrobials (A, B), the presence of 16 mg liter$^{-1}$ nisin (C, D), or the presence of 6.4 mg liter$^{-1}$ reutericyclin (E, F). Measurements were taken every 10 min as samples were heated at a rate of 5˚C /10min. (i) $CH_2$ asymmetrical stretching absorbance; (ii) gel state membrane; (iii) fluid state membrane.

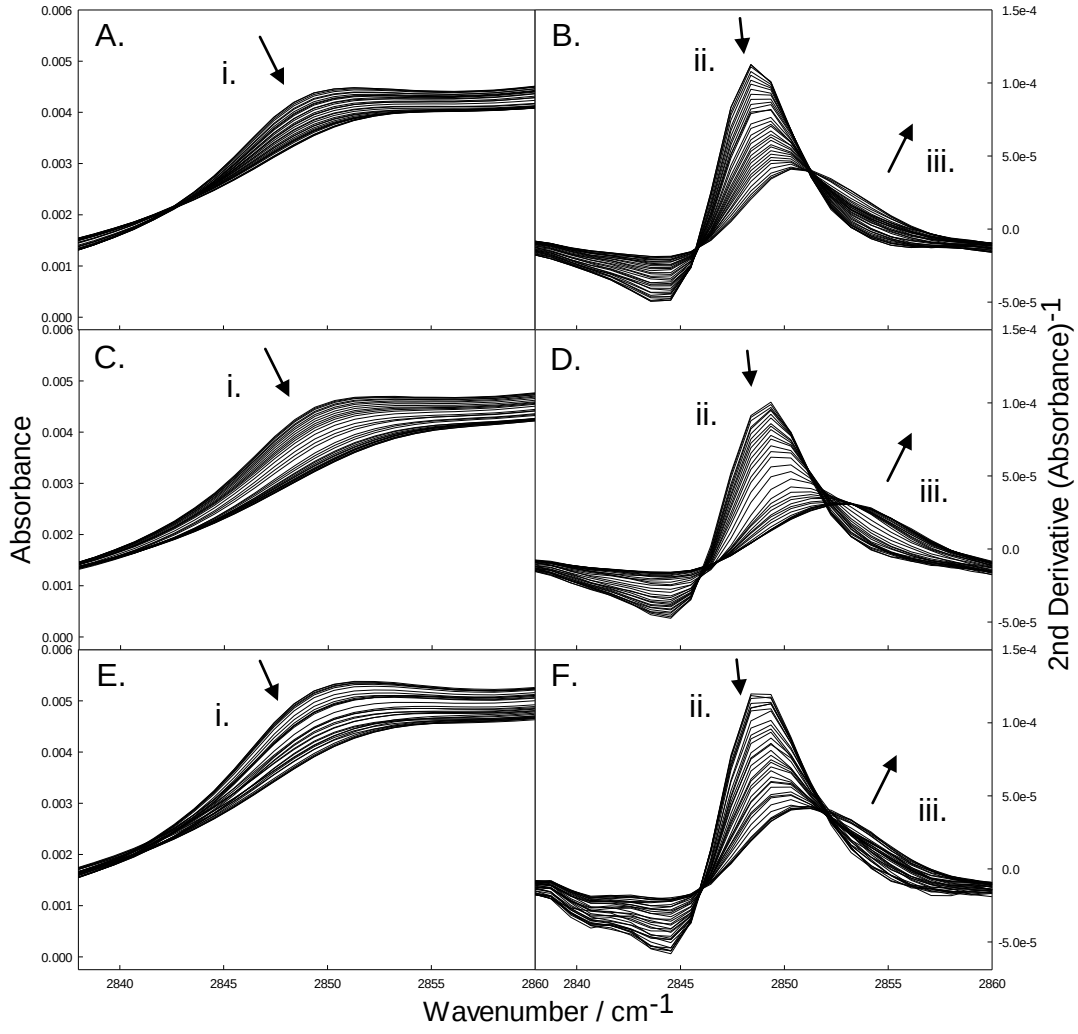


**Figure 4-3.** Fourier-transform infrared spectroscopy (A, C, E) and second derivative spectra (B, D, F) of WSH-L endospores of *C. sporogenes* heated to 90˚C. Samples were heated in the absence of antimicrobials (A, B), the presence of 16 mg liter$^{-1}$ nisin (C, D), or the presence of 6.4 mg liter$^{-1}$ reutericyclin (E, F). Measurements were taken every 10 min as samples were heated at a rate of 5˚C /10min. (i) $CH_2$ asymmetrical stretching absorbance; (ii) gel state membrane; (iii) fluid state membrane.

### 4.3.3 *In situ* GP Measurements of WSH-L Endospores.

Generalized polarization values of WSH-L endospores, exposed to nisin or reutericyclin during 90°C or 90°C/200 MPa, were measured to assess membrane fluidity during treatments. Heating to 90°C at 200 MPa temporarily stabilized GP values for both

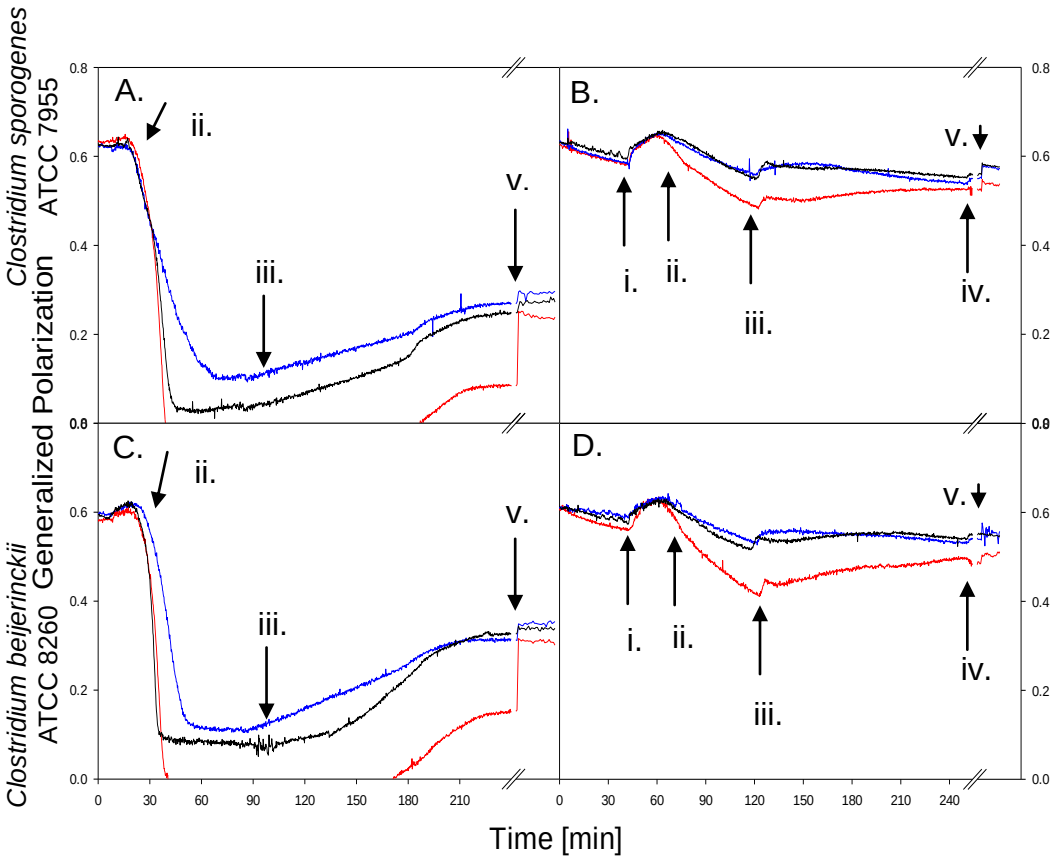


**Figure 4-4.** Generalized polarization of WSH-L endospores of *C. sporogenes* and *C. beijerinckii* standardized to $OD_{600}$ 0.5 and treated at 90 ˚C (A and C) or 90 ˚C and 200 MPa (B and D). Samples were treated in the presence of 6.4 mg liter$^{-1}$ reutericyclin (red), 16 mg liter$^{-1}$ nisin (blue), or in the absence of antimicrobials (black). Measurements were taken every 12 sec during treatments. Displayed values are the average of 10 measurements. (i) Initiation of heating after 200 MPa achieved; (ii) drop in GP values corresponding to 70 - 75˚C; (iii) cooling to 4˚C initiated; (iv) depressurization; (v) initiation of scans following overnight freezing at -20˚C.

*Clostridium* spp. (Figure 4-4 B and D). A sharp decline and subsequent plateau in GP values was observed in WSH-L endospores of both *Clostridium* spp. (Figure 4-4). Application of 90°C alone produced a more pronounced decrease in GP values than 90°C/200 MPa for both strains (Figure 4-4). The GP values of the 90°C treatments did

not return to initial levels upon cooling to 4°C (Figure 4-4, A and C). During the 90°C hold the presence of nisin kept GP values higher than those of the control and reutericyclin treatments in both *Clostridium* spp. (Figure 4-4, A and C). Depressurization resulted in a temporary increase in GP values **(**Figure 4-4).

### 4.3.4 *Ex situ* GP Measurements of WSH-L Endospores.

Generalized polarization values of WSH-L endospores, exposed to nisin or reutericyclin during 90°C or 90°C/600 MPa, were calculated to assess membrane fluidity after treatment. Generalized polarization values during treatments at 90°C/600 MPa were not available; therefore, GP values were measured after treatment. Treatments of 90°C resulted in lowered GP values, following cooling, in both control and reutericyclin

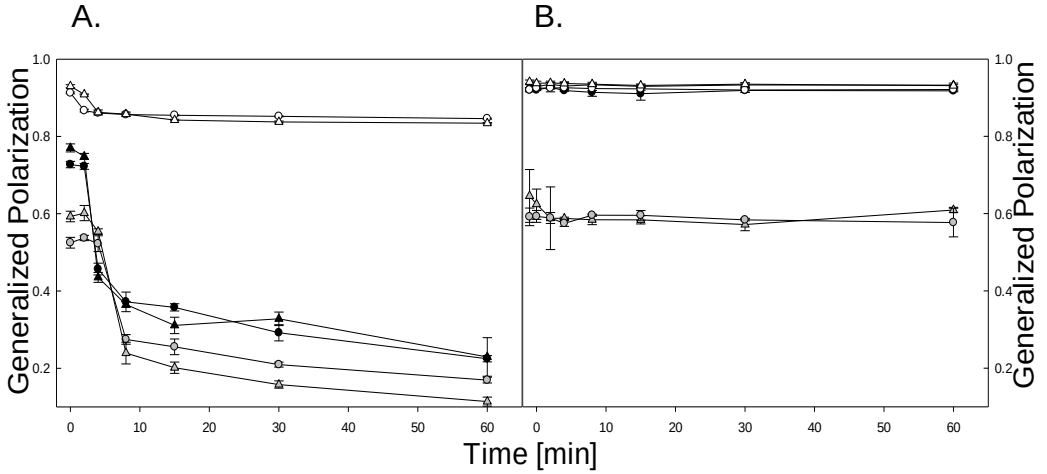


**Figure 4-5.** Generalized polarization of WSH-L endospores of *C. sporogenes* (▲) and *C. beijerinckii* (●) standardized to $OD_{600}$ 0.5 and treated at 90 ˚C (A) or 90 ˚C and 600 MPa (B). Samples were treated in the presence of 6.4 mg liter$^{-1}$ reutericyclin (grey), 16 mg liter$^{-1}$ nisin (white), or in the absence of antimicrobials (black). Measurements were taken after holding samples overnight at 4˚C. Displayed values are the average ± standard deviations of 3 measurements.

treatments of both strains (Figure 4-5). The presence of reutericyclin resulted in lower GP values than the control treatments at 90°C and at 90°C/600 MPa (Figure 4-5). The presence of nisin mitigated a decline in GP values in samples heated at 90°C for both *Clostridium* spp. (Figure 4-5A). Treatment of endospores of *Clostridium* spp. at 90°C/600

MPa had no effect on GP values for all treatments of both *Clostridium* spp., relative to initial GP values (Figure 4-5B**)**.

## 4.4 Discussion

Heat and pressure have opposing effects on clostridial endospore membranes, and these effects can be enhanced by the membrane-active antimicrobials nisin and reutericyclin. Generalized polarization values of clostridial endospores, as well as melting curves of a model DPPC membrane system, suggest a combination of nisin and pressure mitigate the fluidizing effects of heat. Endospores can maintain calcium-dipicolinic acid (Ca-DPA) in their cores despite a disordered membrane, and Ca-DPA can be released, and inactivation can occur, without fluidizing the inner membrane.

Spectral shifts in LAURDAN during temperature changes can be monitored to confirm phase transitions in living cells (70, 82). Measurement of biological membrane fluidity using LAURDAN with nisin and reutericyclin has been done using cells of *Lactobacillus reuteri* (72). However, assessing membrane fluidity in endospores using LAURDAN is a recent methodology (Chapter 3, this thesis). In this study, melting of the DPPC model membrane system occurred between 40 and 45°C. This agrees with the established melting temperature for this system (30). The presence of nisin or reutericyclin did not appear to affect onset of DPPC vesicle melting temperature when heated to 90°C at 200 MPa or ambient pressure. Nisin appeared to facilitate the stabilization of the DPPC system. Readings of LAURDAN during membrane phase transitions can be corroborated using FT-IR (62).

Fourier-transform infrared spectroscopy scans of cells can yield information on membrane phase transitions, conformational states of lipids, protein denaturation, and hydration effects (33, 38, 82). Infrared and LAURDAN fluorescence spectroscopy have been used to assess the thermodynamic phase state of cytoplasmic membranes during

pressure treatments (82). With this and the DPPC model membrane system in mind, lipid phase transitions of clostridia endospores were assessed while heating to 90°C in the presence of nisin or reutericyclin. Infrared and LAURDAN readings confirm a gel state of inner membranes and a phase shift caused by temperature. The wavenumber 2850 /cm is indicative of $CH_2$ asymmetrical bond stretching, and a shift to a higher wavenumber is consistent with a membrane phase transition- in this case, melting (38, 67, 78). Endospores of both *C. beijerinckii* ATCC 8260 and *C. sporogenes* ATCC 7955 exhibited this characteristic wavenumber shift. Fluorescence readings of LAURDAN in the DPPC model system, combined with the FT-IR wavenumber shifts in clostridial endospores, were taken to substantiate the use of LAURDAN GP values to assess membrane fluidity of endospores in the presence of nisin and reutericyclin.

In situ GP measurements of clostridial endospores heated to 90°C are congruent with those of the model DPPC vesicle system. Reutericyclin causes an increase in inner membrane fluidity within clostridial endospores, as indicated by a lower GP value relative to control and nisin treatments. Reutericyclin preferentially interacts with the hydrophobic interior of lipid bilayers, and has been shown to increase membrane fluidity in cells of *Lactobacillus reuteri* (72). A return to initial GP values was not achieved upon cooling, or overnight freezing and rescanning at room temperature. Unlike the DPPC membrane system in which vesicle disintegration was not reversed upon cooling, GP values of clostridial endospores are able to return to levels indicative of a fluid membrane state. This suggests that 90°C is capable of permanently altering the highly ordered state of the inner membrane of endospores. Application of pressure greatly diminishes this disordering effect.

In situ GP measurements of clostridial endospores heated to 90°C at 200 MPa highlight the antagonistic effect of pressure on fluidization of membranes. A high degree of order, consistent with gel-state membranes, was maintained for all treatments of both

*Clostridium* spp. Although reutericyclin counteracts this ordering effect to a degree, it was not to an extent that caused a deviation from the gel-state membrane. In comparison, the DPPC system exhibited melting curves similar to 90°C treatments, once pressure was achieved, except in the presence of nisin. Presence of nisin facilitated resistance to fluidization within the DPPC system during the 90°C/200 MPa treatment.

Ex situ GP measurements of clostridial endospores at 90°C/600 MPa further illustrated the protective effects of pressure on the order of membranes of endospores during heating, and allow for comparison to endospore inactivation. Endospores of both *Clostridium* spp. maintained GP values consistent with that of a gel state membrane after exposure to 90°C/600 MPa. Ex situ GP measurements at 90°C are consistent with in situ measurements, with the exception of samples treated in the presence of nisin. Endospores of both *Clostridium* spp. exhibited a return to high GP values ex situ, and thus a high degree of membrane order. It is possible that the difference in in situ and ex situ GP measurements of nisin treatments at 90°C can be attributed to sample handling. Ex situ treatments were held on ice overnight until extraction could take place, whereas in situ measurements were measured during treatment or after overnight freezing at -20°C. Membranes in stored ex situ samples may be capable of slow molecular rearrangements and a return to an ordered membrane state, whereas freezing of the in situ samples overnight would have prevented this. It is likely that nisin facilitates a return to an ordered state by virtue of its mode of action. Nisin forms tetrameric pores, contingent on suitable aggregation of lipids in proximity, thereby possibly introducing order to a membrane (43).

Opposing effects of heat and pressure on endospore membrane fluidity, in combination with inactivation kinetics of previous work (Chapter 2, this thesis), confirmed that clostridial endospores are capable of maintaining a dehydrated, calcium-dipicolinic acid (Ca-DPA) core despite a fluidized membrane. In previous work, the

inactivation profiles and release of DPA of Clostridial endospores was assessed using identical conditions, methods, and bacterial strains (Chapter 2, this thesis). In the case of thermal treatments, in which all GP values of clostridial endospores dropped, a Ca-DPA core was maintained despite a highly fluidized membrane. A decline in GP values is indicative of membrane disruption and accessibility of LAURDAN to surrounding water molecules (2, 58). In other words, water actively interacts with LAURDAN in the lipid bilayer at 90°C, but the inner membrane itself retains its barrier properties. The present findings suggest that lipid bilayers of endospores may lose all measurable rigidity and yet maintain a Ca-DPA rich core, and thereby retain thermal resistance, although inactivation can occur (Chapter 3, this thesis). Viability of clostridial endospores is largely unaffected during the 90°C treatments, unless nisin is present in the case of *C. sporogenes* ATCC 7955; the presence of nisin during thermal treatment can result in a 90% inactivation of endospores (Chapter 2, this thesis).

Conversely, high pressure thermal processing was capable of forcing the release of the majority of Ca-DPA from clostridial endospores without severely compromising membrane fluidity (Chapter 2, this thesis). The presence of nisin during high pressure thermal processing had no effect on membrane fluidity, but inactivation of endospores and levels of released Ca-DPA were high (Chapter 2, this thesis). This suggests that pressure-mediated release of Ca-DPA occurs predominantly via protein channels localized in endospore inner membranes. The combination of nisin and 90°C facilitated release of DPA from clostridial endospores; reutericyclin and 90°C did not (Chapter 2, this thesis). In situ GP measurements of identical treatments confirmed these treatments exhibit similar membrane disruption, without DPA release, and that nisin's pore-forming activity is the most likely explanation for DPA expulsion.

In conclusion, applying high pressure can counteract the fluidizing effects of heat on clostridial endospore inner membrane fluidity. Furthermore, this study demonstrates

that the antimicrobials nisin and reutericyclin reach the inner membranes of endospores and influence membrane fluidity. When coupled with the inactivation profiles the GP values suggest that nisin is more or less capable of exerting antimicrobial activity, whereas reutericyclin does not. Reutericyclin facilitates increased disorder within membranes during thermal and combined thermal and high pressure treatments. Nisin facilitates a return to a highly ordered membrane state following high pressure, thermal processing. These findings indicate that clostridial endospores can be inactivated by heat and pressure without a necessary fluidization of the inner membrane.

## 4.5 References


1. **Ahn, J., Balasubramaniam, V.M., and Yousef, A.E.** 2007. Inactivation kinetics of selected aerobic and anaerobic bacterial spores by pressure-assisted thermal processing. Int. J. Food Microbiol. **113**:321-329.
2. **Bagatolli, L.A., Parasassi, T., Fidelio, G.D., and Gratton, E.** 1999. A Model for the Interaction of 6-Lauroyl-2-(*N,N*-dimethylamino)naphthalene with Lipid Environments: Implications for Spectral Prperties. Photochem. Photobiol. **70:**557-564.
3. **Bagatolli, L.A., Sanchez, Hazlett, T., and Gratton, E.** 2003. Giant vesicles, laurdan, and two-photon fluorescence microscopy: Evidence of lipid lateral separation in bilayers. Meth. Enzym. **360**:481-500.
4. **Beaman, T.C., and Gerhardt, P.** 1986. Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation. Appl. Environ. Microbiol. **52**:1242-1246.
5. **Belliveau, B.H., Beaman, T.C., Pankratz, H.S., and Gerhardt, P.** 1992. Heat killing of bacterial spores analyzed by differential scanning calorimetry. J. Bacteriol. **174**:4463-4474.
6. **Bertsch, L.L., Bonsen, P.P., and Kornberg, A.** 1969. Biochemical studies of bacterial sporulation and germination. XIV. Phospholipids in *Bacillus megaterium*. J. Bacteriol. **98**:75-81.
7. **Black, E.P., Setlow, P., Hocking, A.D., Stewart, C.M., Kelly, A.L., and Hoover, D.G.** 2007. Response of Spores to High-Pressure Processing. Com. Rev. Food Sci. and Food Safety. **6**:103-119.

8. **Borge, G.I.A., Skeie, M., Sorhaug, T., Langsrud, T., and Granum, E.** 2001. Growth and toxin profiles of *Bacillus cereus* isolated from different food sources. Int. J. Food Microbiol. **6**:237-246.

9. **Bosak, T., Losick, T.M., and Pearson, A.** 2008. A polycyclic terpenoid that alleviates oxidative stress. Proc. Natl. Acad. Sci. USA. **105**:6725-6729.

10. **Broda, D.M., DeLacy, K.M., Bell, R.G., Braggins, T.J., and Cook, R.L.** 1996. Psychrotrophic *Clostridium* spp. Associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Intl. J. Food Microbiol. **29**:335-352.

11. **Brown, K.** 2000. Control of bacterial spores. Brit. Med. Bulletin. **56**:58-71.

12. **Cano, R.J., and Borucki, M.** 1995. Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. Science. **268**:1060-1064.

13. **Carlin, F.** 2011. Origin of bacterial spores contaminating foods. Food Microbiol. **28**:177-182.

14. **Christie, W.** 2003. *Lipid Analysis – 3rd Edition*. Bridgewater: The Oily Press.

15. **Collins, M.D., and East, A.K.** 1998. Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins. J. Appl. Microbiol. **84**:5-17.

16. **Cortezzo, D.E., and Setlow, P.** 2005. Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. J. Appl. Microbiol. **98**:606-617.

17. **Cortezzo, D.E., Koziol-Dube, K., Setlow, B, and 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**:838-852.

18. **Cortezzo, D.E., Setlow, B., and Setlow, P.** 2004. Analysis of the action of compounds that inhibit the germination of spores of *Bacillus* species. J. Appl. Food Microbiol. **96**:725-741.

19. **Cowan, A.E, Olivastro, E.M., Koppel, D.E., Loshon, C.A., Setlow, B., and Setlow, P.** 2004. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. Proc. Natl. Aca. Sci. USA. **101**:7733-7738.

20. **Cuevas, F.J., Jameson, D.M., and Sotomayor, C.P.** 2006. Modulation of reconstituted pig kidney $Na^+/K^+$-ATPase activity by cholesterol in endogenous lipid vesicles: Role of lipid domains. Biochem. **45**:13855-13868.

21. **Driessen, A.J.M., van den Hooven, H.W., Kuiper, W., van der Kamp, M., Sahl, H.G., Konnigs, R.N.H., and Konnigs, W.N.** Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. Biochemistry. **34**:1606-1614.

22. **Driks, A.** 2002. Overview: development in bacteria: spore formation in *Bacillus subtilis*. Cell. Mol. Life Sci. **59**:389-391.

23. **Esty, J.R., and Meyer, K.F.** 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes. J. Infec. Diseases. **31**:650-664.

24. **Fitz-James, P.C.** 1971. Formation of protoplasts from resting spores. J. Bacteriol. **105**:1119-1136.

25. **Foster, N.S., Thompson, S.E., Valentine, N.B., Amonette, J.E., Johnson, T.J.** 2004. Identification of sporulated and vegetative bacteria using statistical analysis of fourier transform mid-infrared transmission data. Appl. Spectro. **58**:203-211.

26. **Gänzle, M.G., Holtzel, A., Walter, J., Jung, G., and Hammes, W.P.** 2000. Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Appl. Environ. Microbiol. **66**:4325-4333.

27. **Gänzle, M.G.** 2004. Reutericyclin: biological activity, mode of action, and potential applications. Appl. Microbiol. Biotechnol. **64**:326-332.

28. **Gao, Y.-L., and Ju, X.-R.** 2008. Exploiting the combined effects of high pressure and moderate heat with nisin on inactivation of *Clostridium botulinum* spores. J. Microbiol. Meth. **72**:20-28.

29. **Genest, P.C., Setlow, B., Melly, E., and Setlow, P.** 2002. Killing of spores of *Bacillus subtilis* by peroxynitrite appears to be caused by membrane damage. Microbiology **148**:307-314.

30. **Harris, F.M., Best, K.B., and Bell, J.D.** 2002. Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order. Biochim. Biophys. Acta. **1565**:123-128.

31. **Harris, F.M., Smith, S.K., and Bell, J.D.** 2001. Physical properties of erythrocyte ghosts that determine susceptibility to secretory phospholipase A2. J. Biol. Chem. **276**:22722-22731.

32. **Henriques, C.P., and Moran, A.O.** 2007. Structure, assembly, and function of the spore surface layers. Ann. Rev. Microbiol. **61**:555-588.

33. **Herberhold, H., Marchal, S., Lange, R., Scheyhing, C.H., Vogel, R.F., and Winter, R.** 2007. Characterization of the pressure-induced intermediate and unfolded state of red-shifted green fluorescent protein – a static and kinetic FTIR, UV/VIS and fluorescence spectroscopy study. J. Mol. Biol. **330**:1154-1164.

34. **Holtzel, A., Gänzle, M.G., Nicholson, G.J., Hammes, W.P., and Jung, G.** 2000. The first low molecular weight antibiotic from lactic acid bacteria: reutericyclin, a new tetramic acid. Angewandte Chemie-International Edition. **39**:2766-2768.

35. **Hurst, A.** 1981. Nisin. Adv. Appl. Microbiol. **27**:85-123.

36. **Johnson, E.A., and Bradshaw, M.** 2001. *Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective. Toxico. **39**:1703-1722.

37. **Kennedy, M.J., Reader, S.L., and Swierczynski, L.M.** 1994. Preservation records of microorganisms: evidence of the tenacity of life. Microbiology. **140**:2513-2529.

38. **Kilimann, K.V., Doster, W., Vogel, R.F., Hartmann, C., and Gänzle, M.G.** 2006. Protection by sucrose against heat-induced lethal and sublethal injury of *Lactococcus lactis*: an FT-IR study. Biochim. Biophys. Acta. **1764**:1188-1197.

39. **Kim, J., and Foegeding, P.M.** 1993. Ecology and controls in foods (Ed: Hauschild, A.H.W. and Dodds, K.L.). Pg. 121-176. New York, USA: Marcel Dekker, Inc.

40. **Lee, M.J., Dong-Ho, B., Dong-Ha, L., Ki-Hyo, J., Deog-Hwan, O., and Sang-Do, H.** 2006. Reduction of *Bacillus cereus* in cooked rice treated with sanitizers and disinfectants. J. Microbiol. Biotech. **16**:639-642.

41. **Lopez-Pedemonte, T.J., Roig-Sagues, A.X., Trujillo, A.J., Capellas, M., and Guamis, B.** 2003. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. J. Dairy. **86**:3075-3081.

42. **Loshon, C.A., Melly, E., Setlow, B., and Setlow, P.** 2001. Analysis of the killing of spores of *Bacillus subtilis* by a new disinfectant, Sterilox®. J. Appl. Microbiol. **91**:1051-1058.

43. **Lubelski, J., Rink, R., Khusainov, R., Moll, G.N., and Kuipers, O.P.** 2008. Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. Cell. Mol. Life Sci. **65**:455-476.

44. **Lund, B.M.** 1990. Foodborne disease due to *Bacillus* and *Clostridium* species. Lancet **336**:982-986.

45. **Magoon, C.A.** 1926. Studies Upon Bacterial Spores. J. Infec. Dis. **38**:429-439.

46. **Margosch, D., Ehrmann, M.A., Buckow, R., Heinz, V., Vogel, R.F., and Gänzle, M.** 2006. High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. Appl. Environ. Microbiol. **72**:3476-3481.

47. **Margosch, D.**, **Ehrmann, M.A., Gänzle, M.G., and Vogel, R.F.** 2004a. Comparison of pressure and heat resistance of *Clostridium botulinum* and other endospores in mashed carrots. J. Food Protection. **67**:2530-2537.

48. **Margosch, D., Gänzle, M.G., Ehrmann, M.A., and Vogel, R.F.** 2004b. Pressure inactivation of *Bacillus* endospores. Appl. Environ. Microbiol. **70**:7231-7238.

49. **Matches, J.W.** 1964. Phospholipids in vegetative cells and spores of *Bacillus polymyxa*. J. Bacteriol. **87:**16-23.

50. **Melly, E., Cowan, A.E., and Setlow, P.** 2002. Studies on the mechanism of killing of *Bacillus subtilis* spores by hydrogen peroxide. J. Appl. Microbiol. **93**:316-325.

51. **Meyer, R.S., Cooper, K.L., Knorr, D., Lelieveld, H.L.M.** 2000. High-pressure sterilization of foods. Food. Tech. **54**:67-72.

52. **Minh, H.N.T., Perrier-Cornet, J.M., Gervais, P., and Champion, D.** 2010. New insight into the thermal properties and the biological behaviour of the bacterial spores. Food Biophys. **5**:238-246.

53. **Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P.** 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. **64**:548-572.

54. **Nicholson, W.L.** 2002. Roles of *Bacillus* endospores in the environment. Cell. Mol. Life Sci. **59**:410-416.

55. **Nicolini, C., Celli, A., Gratton, E., and Winter, R.** 2006. Pressure tuning of the morphology of heterogeneous lipid vesicles: a two-photon-excitation fluorescence microscopy study. Biophys. J. **91**:2936-2942.

56. **Nikolopoulou, M.** 1987. Lipid composition of *Bacillus megaterium* spores and spore membranes. Lipids. **22:**698-703.

57. **Op den Kamp, J.A.F., Redai, I., and van Deenen, L.L.M.** 1969. Phospholipid composition of *Bacillus subtilis*. J. Bacteriol. **99**:298-303.

58. **Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R.M., and Gratton, E.** 1991. Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. Biophys. J. **60**:179-189.

59. **Parasassi, T., Giusti, A.M., Raimondi, M., and Gratton, E.** 1995. Abrupt modifications of phospholipid bilayer properties at critical cholesterol concentrations. Biophys. J. **68**:1895-1902.

60. **Parasassi, T., Krasnowska, E.K., Bagatolli, L., and Gratton, E.** 1998. Laurdan and prodan as polarity-sensitive fluorescent membrane probes. J. Fluor. **8**:365-373.

61. **Piggot, P.J., and Hilbert, D.W.** 2004. Sporulation of *Bacillus subtilis*. Curr. Op. Microbiol. **7**:579-586.

62. **Powalska, E., Janosch, S., Kinne-Saffran, E., Kinne, R.K.H., Fontes, C.F.L., Mignaco, J.A., and Winter, R.** 2007. Fluorescence spectroscopic studies of pressure effects on $Na^+$, $K^+$ ATPase reconstituted into phospholipid bilayers and model raft mixtures. Biochem. **46**:1672-1683.

63. **Racine, F.** 1980. Isolation and properties of membranes from *Bacillus megaterium* spores. J. Bacteriol. **143**:1208-1214.

64. **Reddy, N.R., Solomon, H.M., Fingerhut, G.A., Balasubramaniam, V.M., and Rhodehamel, E.J.** 1999. Inactivation of *Clostridium botulinum* Type E spores in a model buffer or food system by high pressure processing. Presented at the Annual Meeting of the Institute of Food Technologists. J. Food Saf. **19**:277-288.

65. **Reddy, N.R., Solomon, H.M., Tetzloff, R.C., and Rhodehamel, E.J.** 2003. Inactivation of *Clostridium botulinum* Type A spores by high-pressure processing at elevated temperature. J. Food Prot. **66**:1402-1407.

66. **Reddy, N.R., Tetzloff, R.C., Solomon, H.M., and Larkin, J.W.** 2006. Inactivation of *Clostridium botulinum* nonproteolytic type B spores by high pressure processing at moderate to elevated high temperatures. Innov. Food Sci. Emerg. Tech. **7**:169-175.

67. **Reis, O., Winter, R., and Zerda, T.W.** 1996. The effect of high external pressure on DPPC-cholesterol multilamellar vesicles: a pressure-tuning Fourier transform infrared spectroscopy study. Biochim. Biophys. Acta. **1279**:5-16.

68. **Roberts, C.M., and Hoover, D.G.** 1996. Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity and nisin. J. Appl. Bact. **81**:363-368.

69. **Salkinoja-Salonen, M.S., Vuorio, R., Andersson, M.A., Kampfer, P., Andersson, M.C., Honkanen-Buzalski, T., and Scoging, A.C.** 1999. Toxigenic strains of *Bacillus licheniformis* related to food poisoning. Appl. Environ. Microbiol. **65**:4637-4645.

70. **Sanchez, S.A., Tricerri, M.A., Gunther, G., and Gratton, E.** 2007. Laurdan generalized polarization: from cuvette to microscope (Ed: Mendez-Vilas, A., and Diaz, J.). Modern Research and Educational Topics in Microscopy. Pg. 1007-1014.

71. **Schantz, E.J., and Johnson, E.A.** 1992. Properties and use of botulinum toxin and other microbial neurotoxins in medicine. Microbiol. Rev. **56**:80-99.

72. **Schwab, C., and Gänzle, M.G.** 2006. Effect of membrane lateral pressure on the expression of fructosyltransferases in *Lactobacillus reuteri*. Sys. Appl. Microbiol. **29**:89-99.

73. **Scott, A.B., and Suzuki, D.** 1988. Systemic toxicity of botulinum toxin by intramuscular injection in the monkey. Movement Disor. **3**:333-335.

74. **Setlow, P.** 2003. Spore germination. Curr. Op. Microbiol. **6**:550-556.

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

76. **Shapiro, R.L., Hatheway, C., and Swerdlow, D.L.** 1998. Botulism in the United States: a clinical and epidimiologic review. Ann. Intern. Med. **129**:221-228.

77. **Smith, L.D.S., and Williams, B.L.** 1984. *Clostridium perfringens*: The pathogenic anaerobic bacteria. Springfield, Illinois, USA: Charles C. Thomas Publishers.

78. **Snyder, G.R.** 1961. Vibrational spectra of crystalline n-paraffins. J. Mol. Spectrosc. **7**:116-144.

79. **Sotomayor, C.P., Aguilar, L.F., Cuevas, F.J., Helms, M.K., and Jameson, D.M.** 2000. Modulation of pig kidney $Na^+/K^+$-ATPase activity by cholesterol: Role of hydration. Biochem. **39**:10928-10935.

80. **Starin, W.A.** 1926. Viability and toxicogenic power of small numbers of spores of *Cl. Botulinum* subjected to different temperatures. J. Infec. Dis. **38**:101-105.

81. **Stewart, C.M., Dunne, C.P., Sikes, A., and Hoover, D.G.** 2000. Sensitivity of spores of *Bacillus subtilis* and *Clostridium sporogenes* PA3679 to combinations of high hydrostatic pressure and other processing parameters. Inn. Food Sci. Emerg. Tech. **1**:49-56.

82. **Ulmer, H.M., Herberhold, H., Fahsel, S., Gänzle, M.G., Winter, R., and Vogel, R.F.** 2002. Effects of pressure-induced membrane phase transitions on inactivation of HorA, on ATP-dependant multidrug resistance transporter, in *Lactobacillus plantarum*. Appl. Environ. Microbiol. **68**:1088-1095.

83. **Vreeland, R.H., Rosenzweig, W.D., and Powers, D.W.** 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature **407**:897-900.

84. **Wilson, M.J., and Baker, R.** 1997. High temperature/ulta-high pressure sterilization of low acid foods. PCT IB9601500.

85. **Winter, R., and Jeworrek, C.** 2009. Effect of pressure on membranes. Soft Matter. **5**:3157-3173.

## 5. General Discussion and Conclusions

Bacterial endospores, particularly *Clostridium* spp., remain a threat within the food supply to both consumers and producers. Hurdle technology has potential for enhancing the range of products that can be made while maintaining a stringent degree of food safety. However, within the past two decades, two compounding factors are positioning endospores at the forefront of food processing. Novel product categories such as preservative-free, organic, minimally processed, and ready-to-eat meats are being met with increasing demand by consumers. These products can facilitate the germination and outgrowth of endospores- particularly psychrotrophic *Clostridium* spp. in the case of ready-to-eat meats. The second factor relates to the spontaneous development of resistance of bacteria to food-grade antimicrobials such as bacteriocins (33). Resistance to nisin has been reported in *C. botulinum* (11, 12). This is not surprising given nisin's history of use in the food industry, its frequent comparison to antibiotics, and the parallel development of resistant nosocomial pathogens to antibiotics that has occurred in hospital settings. The types of resistance observed in foodborne pathogens correlate with changes in membrane charge, fluidity (7, 16), and cell walls (1, 2, 100, 101). It is thought that the potential for large-scale resistance via use in foods can be reduced through hurdle technology, such as PATS in combination with antimicrobials (33).

As PATS gains acceptance within the industry, it is prudent to examine the true potential for integration into existing hurdle technology. In the current study, a PATS hurdle approach using membrane-specific antimicrobial compounds was shown to effectively enhance inactivation of food-relevant endospores of *Clostridium* spp. Nisin significantly increased inactivation rates of endospores of *Clostridium* spp. treated at 90°C/200 MPa, relative to controls and treatments of 90°C without pressurization. In

contrast, although reutericyclin was able to enhance inactivation kinetics at 90°C/200 MPa, this antimicrobial facilitated tailing effects, and was even prone to enhancing survival during heating at 90°C without pressurization. Differences between the effects of nisin and reutericyclin are most likely a result of their different mode of activity. Although both localize in bacterial membranes, nisin actively forms pores whereas reutericyclin shuttles protons across the membrane through molecular repositioning. The pores formed by nisin enhance the release of DPA from endospores, causing a related loss of thermal resistance as the core of the endospore is rehydrated. Enhanced protein denaturation is facilitated in this manner, and is suspected to be the main reason for enhanced inactivation of endospores. Reutericyclin does not compromise the barrier properties of the membrane of an endospore or lead to a release of DPA. Thus, it does not appreciably enhance inactivation by way of rehydration of the core of an endospore. It is likely that reutericyclin enhances inactivation via hurdle technology; that is to say, reutericyclin exerts antimicrobial activity on heat-injured endospores during germination. Overall, antimicrobial compounds can enhance the efficacy of existing PATS parameters in inactivating endospores in a laboratory setting.

The deployment of antimicrobial compounds with PATS in the food industry comes with two caveats: 1) the combined PATS-antimicrobial approach must be validated in actual food systems and, 2) antimicrobial agents must be carefully selected for this approach in order to eliminate the potential for tailing effects that could lead to endospore survival. The first consideration is not new to the food industry and related research. A given food system can dramatically impact the efficacy of an antimicrobial agent. For example, the use of nisin in a raw meat system intended for PATS processing would be out of the question; nisin is known to complex with glutathione in raw meat, and is subsequently inactivated (14). Testing of proposed antimicrobial agents would be meant to discover such undesirable side effects. The second consideration is unique to

PATS hurdle technology. Based on the findings of the first research objective, it is reasonable to assume that pore-forming compounds are likely better candidates for inactivation of endospores than compounds that require a level of metabolic activity. Selection of an antimicrobial compound for use with heat and pressure necessitates knowledge of the molecular mechanism involved in inactivation of endospores facilitated by said antimicrobial compound.

A more detailed understanding of the physiology of bacterial endospores during PATS would be helpful to accurately begin to assess antimicrobial compounds for possible deployment in the food industry. Current theory of endospores supposes the inner membrane is the primary barrier to rehydration, and thus a barrier to thermodynamic denaturation of vital cytoplasmic proteins leading to inactivation. With this in mind, the second objective of this research was to explain the inactivation mechanism of endospores. Nisin and reutericyclin were deliberately chosen for this project because they both preferentially partition and exert their mode of action within bacterial cell membranes (51, 96). Furthermore, their chemistry is well characterized and may complement gaps in knowledge of endospore physiology (51, 96). At the outset of the second objective, the use of the fluorescent probe LAURDAN to assess membrane fluidity during PATS was proposed, as LAURDAN has been well established in this regard. Endospores of *Clostridium* spp. were sporulated in media infused with LAURDAN and examined for their experimental utility. Qualitative and quantitative analyses of LAURDAN-labeled endospore fluorescence, coupled with a validated decoating protocol, were used to verify the use of these unique endospores for assessment of inner membrane fluidity. High GP values recorded for endospores of *Clostridium* spp. are indicative of a high degree of order, and are corroborated by observations that the lipids of the inner membrane of endospores are in an immobile state (34). Overall, this

method appears to be successful for rapid observation of the inner membranes of entire populations of endospores.

Endospores of *Clostridium* spp. labeled with LAURDAN and subjected to identical trials as in the inactivation kinetics elucidated intriguing information regarding the inner membrane. Treatment at 90°C greatly reduced the order of inner membranes of endospores, and indicates a high level of fluidity. In contrast, 90°C/200 MPa and 90°C/600 MPa appeared to preserve the rigidity of inner membranes, and indicate pressure helps maintain a degree of lipid order. These in situ observations are most notable for the questions they raise about the nature of the inner membrane of endospores when compared with kinetics of inactivation and Ca-DPA release.

During the heating at 90°C a 1-log reduction in endospores occurred. With the current working theory of thermal inactivation of endospores, this means 90% of the population experienced protein denaturation. Or, perhaps 90% of the population of endospores experienced irreversible membrane disruption and died during germination, or a mix of both protein denaturation and irreversible membrane damage occurred. As little to no DPA was lost from the overall population of endospores following treatment, the line of evidence suggests proteins essential to endospore viability, located outside of the core, are denatured. The proteins in question may be membrane-bound germinant receptors and/or CLEs. Endospores with thermally denatured germinant receptors would simply not germinate when exposed to nutrient-rich conditions. It is possible that membrane-bound germinant receptors are not heat-labile, but if CLEs were, the cortex peptidoglycan of endospores would not be degraded to allow rehydration, expansion of the core, and germination.

However, during identical thermal treatments, endospores of *Clostridium* spp. exhibit little to no Ca-DPA release, yet the inner membrane clearly moves from a gel to fluid state. Fluidity of the inner membrane of endospores can be altered independently of

Ca-DPA release during thermal processing. Although it is generally accepted that endospores are inactivated by heat via protein denaturation, there is a growing line of evidence that suggests this issue is far more complex. For example, oxidizing agents can inactivate endospores without causing a subsequent Ca-DPA release (58, 109). Oxidizing agents damage the inner membrane such that endospores lyse during the process of germination (155). The organization of lipids within the inner membranes of endospores may play a critical role in the viability of endospores. Based on GP measurements, endospores of *Clostridium* spp. inactivated by heat may in fact be experiencing irreversible, catastrophic inner membrane damage that leads to subsequent lysing during germination. These endospores would appear as non-viable during plating. The combined high pressure, thermal treatments also indicate that the theory of inactivation of endospores is perhaps more complex.

High pressure combined with heat compromised the thermal resistance of endospores of *Clostridium* spp. without causing membranes to fluidize. This was accompanied by rapid inactivation of endospores along with a complete release of Ca-DPA from cores. This suggests that high pressure, thermal treatments inactivate endospores through a series of events different from that of thermal treatments. The enhanced inactivation of endospores by high pressure, thermal treatments, relative to thermal treatments alone, is likely a result of destruction of components essential to viability within cores of endospores (most likely proteins) in addition to external components. Essential components within the core may have inherent variability in terms of cytoplasmic distribution, based on endospore inactivation curves for both *Bacillus* and *Clostridium* spp. Progressive levels of Ca-DPA release correspond to progressive inactivation of populations of endospores. As well, the release of Ca-DPA most likely occurs by way of endemic membrane protein channels as indicated by high GP values.

How can endospores maintain a Ca-DPA core during thermal treatments despite a highly fluidized membrane? How is Ca-DPA released from endospores during high pressure, thermal treatments despite a highly ordered membrane? To answer these questions, it is the author's opinion that a conceptual parsing of the current working theory of inactivation of endospores must be examined. Specifically, the extent to which a population of endospores is vulnerable to inactivation via membrane damage and denaturation of external proteins, versus denaturation of cytoplasmic proteins upon rehydration.

The objectives of this project have, to an extent, been fulfilled. It is possible to enhance inactivation of food-relevant clostridial endospores using high pressure, thermal treatment in concert with antimicrobial agents. However, those agents must be selected carefully, and have yet to undergo assessment in a food matrix. Inner membranes of endospores show promise as a highly specific target for future control of endospores in food. The second research objective illustrates that inner membrane fluidity of endospore populations show clear disparity between thermal and PATS exposure, and hints at a more complex mechanism behind the destruction of vital components within endospores. The extent to which thermodynamic denaturation of proteins and disruption of inner membranes influence inactivation of endospores remains to be determined.

## 5.1 References


1. **Abachin, E., Poyart, C., Pellegrini, E., Milohanic, E., Fiedler, F., Berche, P., and Trieu-Cuot, P.** 2002. Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listera monocytogenes*. Mol. Microbiol. **43:** 1-14.
2. **Bierbaum, G., and Sahl, H.G.** 1987. Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of N-acetylmuramoyl-L-alanine amidase. J. Bacteriol. **169:** 5452-5458.
3. **Cotter, P.D., Hill, C., and Ross, R.P.** 2005. Bacteriocins: Developing Innate Immunity for Food. Nat. Rev.: Microbiol. **3:** 777-788.
4. **Cowan, A.E., Olivastro, E.M., Koppel, D.E., Loshon, C.A., Setlow, B., and Setlow, P.** 2004. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. Proc. Natl. Acad. Sci. USA. **101:** 7733-7738.
5. **Gänzle, M. G.** 2004. Reutericyclin: biological activity, mode of action, and potential applications. Appl. Microbiol. Biotech. **64:** 326-332.
6. **Genest, P.C., Setlow, B., Melly, E., and Setlow, P.** 2002. Killing of spores of *Bacillus subtilis* by peroxynitrite appears to be caused by membrane damage. Microbiol. **148:** 307-314.
7. **Li, J., Chikindas, M.L., Ludescher, R.D., and Montville, T.J.** 2002. Temperature- and surfactant-induced membrane modifications that alter *Listeria monocytogenes* nisin sensitivity by different mechanisms. Appl. Environ. Microbiol. **68:** 5904-5910.
8. **Lubelski J., Rink R., Khusainov R., Moll G.N., and Kuipers O.P.** 2008. Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. Cell. Molec. Life Sci. **65:** 455-476.

9. **Maisnier-Patin, S., and Richard, J.** 1996. Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentrations. FEMS Microbiology Letters. **140:** 29-35.

10. **Mantovani, H.C., and Russel, J.B.** 2001. Nisin resistance of *Streptococcus bovis*. Appl. Environ. Microbiol. **67:** 808-813.

11. **Mazzotta, A. S., and Montville, T. J.** 1999. Characterization of fatty acid composition, spore germination, and thermal resistance in a nisin-resistance mutant of *Clostridium botulinum* 169B and in the wild-type strain. Appl. Environ. Microbiol. **65:** 659-664.

12. **Mazzotta, A., Crandall, A., and Montville, T.** 1997. Nisin resistance in *Clostridium botulinum* spores and vegetative cells. . Appl. Environ. Microbiol. **63:** 2654-2659.

13. **Melly, E., Cowan, A.E., and Setlow, P.** 2002. Studies on the mechanism of killing of *Bacillus subtilis* spores by hydrogen peroxide. J. Appl. Microbiol. **93:** 316-325.

14. **Rose, N.L., Sporns, P., Stiles, M.E., and McMullen, L.M.** 1999. Inactivation of nisin by glutathione in fresh meat. J. Food Sci. **64:** 759-762.

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

16. **Verheul, A., Russel, N.J., Van, T.H.R., Rombouts, F.M., and Abee, T.** 1997. Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes*. Appl. Environ. Microbiol. **63:** 3451-3457.