The Pennsylvania State University

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FACTORS THAT INFLUENCE BAROTOLERANCE OF *LISTERIA MONOCYTOGENES* AND THE MECHANISM OF INACTIVATION BY HIGH PRESSURE PROCESSING

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by

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ABSTRACT

Listeria monocytogenes is a Gram-positive bacterium that causes the foodborne disease listeriosis. Although listeriosis is rare it is of concern to the food industry due to the severity of the disease and the high fatality rate. Listeriosis is commonly associated with ready-to-eat (RTE) foods, for example soft cheeses. As a result, there is a zero-tolerance policy for the presence of *L. monocytogenes* in RTE foods and many costly food recalls are due to the presence of this pathogen. High pressure processing (HPP) is a non-thermal technology that can be used to pasteurize foods while maintaining their fresh-like qualities. Hydrostatic pressures of up to 700 MPa are applied to inactivate microorganisms in foods, thereby extending shelf life and improving food safety. The mechanism(s) of microbial inactivation by HPP are not understood, but are thought to involve the cell wall, cell membrane, DNA and/or proteins. The aim of this research was to elucidate the mechanism(s) of inactivation of *L. monocytogenes* by HPP in milk. Factors that influence barotolerance of *L. monocytogenes* were also investigated as a way of identifying potential mechanism(s) of inactivation. Initial experiments investigating various cell targets were unsuccessful at elucidating a mechanism of inactivation by HPP. Transmission electron microscopy and other experiments showed that the cell wall and cell membrane of *L. monocytogenes* were not affected by HPP. The effects of growth phase (mid-exponential, late-exponential or mid-stationary) and growth temperature (4, 15, 25, 35 and 43°C) on the inactivation of *L. monocytogenes* by HPP at 400 MPa were investigated. Stationary-phase cells were significantly more barotolerant than mid-exponential-phase cells. Growth temperature also had a significant effect on barotolerance, which generally increased with increasing growth temperature. Tailing

inactivation kinetics were observed in stationary phase cells grown at 35 or 43°C, but not in stationary phase cells grown at 4, 15 or 25°C or exponential-phase cells grown at 4, 15, 25, 35 or 43°C. The effect of water activity on the barotolerance of *L. monocytogenes* was also investigated. Lyophilized cells (starting concentration 7.5 x 10^7 CFU/g) were suspended in water/glycerol solutions or left dry and HP-processed at 600 MPa for 5 min. Dry cells or cells suspended in 100% glycerol showed no inactivation; however cells suspended in 100% water were completely inactivated. Glycerol concentrations greater than 40% ($a_w = 0.86$) significantly increased barotolerance and there was a log-linear relationship between glycerol concentration and $\log_{10}$ CFU/ml. The effect of heat shock on barotolerance was also investigated. *L. monocytogenes* was grown to stationary-phase at 15°C and heat shocked at 48°C. Heat shock significantly enhanced the barotolerance of *L. monocytogenes* at 400 MPa, with 5 min of heat shock conferring maximal barotolerance. Addition of chloramphenicol (a protein synthesis inhibitor) prior to heat shock resulted in barotolerance similar to that of non-heat-shocked cells, indicating that synthesis of heat shock proteins (which are involved in stabilization and/or renaturation of proteins) was responsible for increased barotolerance. The above results indicated that proteins may play an important role in barotolerance. Therefore, differential scanning calorimetry (DSC) was employed to investigate the effect of HPP on proteins in whole cells. Thermograms of non-pressure-treated cells of *L. monocytogenes* showed that the largest peak, which is associated with cellular proteins, occurred at approximately 70°C and was irreversible. Lethal high pressure treatments significantly reduced this peak, indicating that HPP caused protein denaturation in whole cells. While inactivation of *L. monocytogenes* by HPP may be multi-factorial, results indicated that protein denaturation

plays an important or even dominant role in inactivation of *L. monocytogenes*. Understanding the mechanism of inactivation by HPP and the factors that influence barotolerance will aid in the development of process guidelines for the manufacture of safe HP-processed foods.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
a_w	water activity
C	Heat Capacity
ca.	approximately
CAMP	Christie, Atkins, Munch-Petersen
CSP(s)	Cold Shock Protein(s)
CFSAN	Center for Food Safety and Applied Nutrition
CFU	Colony Forming Units
D	Decimal Reduction Time
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
FDA	Food and Drug Administration
g	gram
h	hour
HHP	High Hydrostatic Pressure
HP	High Pressure
HPP	High Pressure Processing
HSP(s)	Heat Shock Protein(s)
HTST	High temperature short time
kDa	Kilodalton
LDH	Lactate Dehydrogenase
log	Logarithimic
mDSC	micro Differential Scanning Calorimetry
min	minute(s)
ml	milliliter
MPa	Mega Pascal
MOX	Modified Oxford Agar
NaCl	Sodium Chloride (salt)
NADH	Nicotinamide-Adenine Dinucleotide (reduced form)
ND	Not Determined or Not Detected
nm	nanometer
NR	Not Reported
oPSU broth	optimized Penn State University broth
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PIP(s)	Pressure Induced Protein(s)
PSB	Phosphate Buffer
PSI	Pounds per Square Inch
r^2	Correlation coefficient

RAE	Ribosome Associated Enthalpy
rRNA	Ribosomal Ribonucleic Acid
RTE	Ready-to-Eat
s	second
S	Svedberg Units
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SFA	Saturated Fatty Acid
TEM	Transmission Electron Microscopy
TSYEA	Tryptic Soya Yeast Extract Agar
TSYEB	Tryptic Soya Yeast Extract Broth
UFA	Unsaturated Fatty Acid
UHT	Ultra Heat Treated
U.K.	United Kingdom
µm	micrometer
U.S.	United States
UV	Ultra Violet
v	volume
w	weight

PREFACE

Chapter 4 "Effect of water activity on inactivation of *Listeria monocytogenes* and lactate dehydrogenase during high pressure processing" was initiated by Melinda Hayman and conducted in collaboration with Dr. Gilles Kouassi. Dr. Kouassi conducted experiments with lactate dehydrogenase and Melinda Hayman conducted experiments with *L. monocytogenes*. The paper was written jointly by Melinda Hayman and Dr. Gilles Kouassi, with Melinda Hayman writing the first draft and completing revisions, and Dr. Gilles Kouassi adding information on lactate dehydrogenase and proteins in all sections of the paper. Figures showing results from *L. monocytogenes* experiments were created by Melinda Hayman, lactate dehydrogenase figures were generated by Dr. Gilles Kouassi.

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CHAPTER ONE

STATEMENT OF THE PROBLEM

Listeria monocytogenes is the etiological agent of listeriosis, a foodborne disease commonly associated with ready-to-eat (RTE) foods, such as soft milk cheeses. *L. monocytogenes* is ubiquitous and resistant to a diverse range of environmental conditions, therefore it is extremely hard to eliminate from food processing environments and RTE foods. High pressure processing (HPP) is a novel method of food processing that can be used to pasteurize pre-packaged food products, and hence eliminate *L. monocytogenes* from RTE foods. However, the mechanism(s) of microbial inactivation by HPP is/are not known. This information might help food scientists to optimize HPP and aid in the development of commercial technologies to inactivate microorganisms by HPP. Current literature on the mechanism(s) of bacterial inactivation by HPP focuses on Gram-negative organisms, such as *Escherichia coli*. The current consensus in the field is that the cell membrane may be the main target of bacterial inactivation by HPP. Therefore, the main objective of this thesis was to investigate the mechanism(s) of inactivation of *L. monocytogenes* by HPP.

Initial experiments directly investigating the effects of pressure on the cell wall, cell membrane and DNA did not indicate that any of these was the primary target of inactivation. Therefore, factors that influence barotolerance of *L. monocytogenes* were investigated as a way to identify potential mechanisms. *L. monocytogenes* was selected as the study organism because of its importance in RTE foods, and because it is one of the most pressure-resistant vegetative foodborne pathogens.

The specific objectives of this project were as follows;

1. To investigate the effects of growth phase and growth temperature on the barotolerance and HP-induced injury of *L. monocytogenes*.

2. To investigate the effects of reduced water activity and lyophilization of the barotolerance of *L. monocytogenes.*

3. To investigate the effect of heat shock on the barotolerance of *L. monocytogenes*.

4. To investigate the role of protein denaturation in the inactivation of *L. monocytogenes* by HPP using differential scanning calorimetry.

As an editorial note, the research chapters in this thesis are written as papers for publication in the style of 'The International Journal of Food Microbiology'. Therefore, there is some repetition in the Introductions and Materials and Methods of the chapters.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Listeria monocytogenes*

2.1.1 Taxonomy & Bacteriology

Bergey's Manual of Systematic Bacteriology classifies the genus *Listeria* as "Regular, nonsporing Gram-positive rods" (Kandler and Weiss, 1986). The genus *Listeria* is comprised of six species, *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri* and *L. grayi*. *L. monocytogenes* was first described in 1926, after causing infection in laboratory animals in Cambridge (Sutherland et al., 2003). *Listeria* are most closely related to the genus *Brochrothrix*, and more distantly related to *Lactobacillus* and *Bacillus*.

L. monocytogenes are regular, short, Gram-positive rods, 0.4-0.5 um in diameter and 0.5-2.0 um in length (Seelinger and Jones, 1986). They are non-sporing, non-acid-fast and non-capsulated. They are aerobic and facultatively anaerobic and have an optimum growth temperature of 30-37°C. *Listeria* cells are catalase positive and oxidase negative. The cells occur singly, in parallel or short chains and may appear coccoid, particularly in clinical cultures. *L. monocytogenes* cells are motile when cultured at 20-25°C and they have a characteristic tumbling motility. On solid media they produce colonies that appear blue-green when observed by obliquely transmitted light (Seelinger and Jones, 1986). *L. monocytogenes* produces β-hemolysin which causes zones of clearance on blood agar and is used to differentiate the species from other *Listeria* in the CAMP test (Seelinger and Jones, 1986). *L. monocytogenes* is the predominant *Listeria*

species associated with human disease; however *L. ivanovii* and *L. seeligeri* may occasionally cause disease (Rocourt et al., 1987; Cummins et al., 1994).

2.1.2 Growth and survival

L. monocytogenes is relatively hardy for a foodborne pathogen; it can grow and survive for long periods of time in many different environments. The organism is psychrotrophic, however the temperature limits for growth are from -0.4 to 50°C and freezing at –18°C injures rather than inactivates the cells (Farber and Peterkin, 1991). *L. monocytogenes* can grow between pH 4.3-9.6, with all strains growing best at neutral to slightly alkaline pH (Seelinger and Jones, 1986). The organism is very resistant to high salt concentrations and can grow in nutrient broth with 10% NaCl, while some strains can tolerate, but not grow in, up to 20% or 30% salt (Seelinger and Jones, 1986). *L. monocytogenes* grows best at a a_w of 0.97, but can grow at a a_w as low as 0.90 (Seelinger and Jones, 1986). *L. monocytogenes* can grow in foods with modified atmospheres or that are vacuum packaged (Sutherland et al., 2003). *L. monocytogenes* does not survive heating at 60°C for 30 minutes, or at 72°C for 15 s, and therefore is eliminated during correct pasteurization processes (MacDonald and Sutherland, 1993).

2.1.3 Ecology

L. monocytogenes is widely distributed in nature, and has been found in water, silage, sewage, mud, vegetation, and in the feces of man and animals (Sutherland et al., 2003). *L. monocytogenes* has also been isolated from animal feed, slaughter house waste and healthy human and animal carriers. Plants grown on soil treated with sewage can be

contaminated with *L. monocytogenes* (Sutherland et al., 2003). As *L. monocytogenes* is a frequent contaminant of raw materials it can be constantly reintroduced into manufacturing environments (Gilbert, 1996). Moist refrigerated conditions, often found in processing environments, allow for its survival and growth. Hygiene within processing plants is important in limiting the contamination of processing equipment (Pritchard et al., 1995).

As a result of the ubiquitous and resilient character of *L. monocytogenes*, the organism frequently enters the human food supply and has been detected in a wide variety of food products, including vegetables, coleslaw, seafood, meat, eggs, poultry, milk, cheese and ice cream (Gilbert, 1996; Notermans et al., 1998).

2.1.4 *L. monocytogenes* and food

L. monocytogenes emerged as a major foodborne pathogen in the 1980s (Sutherland et al., 2003). The first demonstrated outbreak of foodborne listeriosis was due to the consumption of coleslaw in Canada in 1981; however there were several suspected outbreaks of listeriosis prior to 1981 (Farber and Peterkin, 1991). The consumption of contaminated foods is the principal route of transmission of listeriosis, and a diverse range of food types have been shown to be associated with outbreaks and sporadic cases (Gilbert, 1996). High-risk foods include RTE products, such as deli meats, meat paste, chicken, ham, pate, shellfish and soft and surface ripened cheeses, stored at refrigeration temperatures for long periods of time (Sutherland et al., 2003).

2.1.5 Listeriosis

Listeriosis is mainly reported in Western countries, the occurrence is typically sporadic although many outbreaks have been reported (Farber and Peterkin, 1991). Reasons for the emergence of listeriosis in the 1980s include demographic changes, such as the increasing proportion of the immunocompromised and elderly in society, and changes in food production and consumption. Despite the frequency of exposure to *L. monocytogenes*, epidemiological research in several countries (such as the U.S. and U.K.) shows that listeriosis is a relatively rare disease (Notermans et al., 1998)

There are 13 serovars of *L. monocytogenes* that cause human listeriosis, however 95% of human isolates are 1/2a, 1/2b, or 4b, with 4b responsible for 50% of sporadic cases and most major outbreaks (Farber and Peterkin, 1991). Approximately 2500 cases are reported annually in the United States (FDA/CFSAN, 2001). Listeriosis primarily occurs in individuals with underlying illness which leads to suppression of their T-cell-mediated immunity (Farber and Peterkin, 1991). High-risk populations include neonates (and pregnant women), alcoholics, the elderly and those compromised by an underlying illness such as cancer or some condition which results in immunosuppression. Listeriosis is a major public health concern because of the severity and the systemic nature of the disease, the high fatality rate (20-40% of cases), and the long incubation time (up to 90 days, with a mean of 3 to 4 weeks) (Farber and Peterkin, 1991). The highest incidence of listeriosis is in neonates (Farber and Peterkin, 1991). The minimum infectious dose for healthy and at risk populations has not been determined. Healthy people can tolerate a dose of 1,000 to 10,000 cells without becoming infected. From a food safety perspective,

up to 100 CFU per ml or g of food is considered to be 'safe' for consumers (ANZFA, 2001).

The clinical syndromes associated with adult listeriosis include central nervous infections (e.g. meningitis), septicemia, abscesses and primary bacteremia, but can also include endocarditis. Listeriosis can also manifest as focal infections, for example in prosthetics and the cornea (Farber and Peterkin, 1991). Pregnant women are most commonly affected in the third trimester. The infection of the mother may be asymptomatic or characterized by flu-like symptoms. Intrauterine infection of the fetus can also result in abortion, stillbirth or the birth of a septic infant. However, healthy infants can be born from infected or carrier mothers (Farber and Peterkin, 1991). Infected bovines excrete the organism into blood, milk and feces, and the cow usually aborts her calf. *L. monocytogenes* is susceptible to a wide range of antibiotics and penicillin and ampicillin are commonly used to treat listeriosis.

2.1.6 *L. monocytogenes* and dairy products

The ability of *L. monocytogenes* to multiply in milk and survive during the manufacture of some cheeses, including cottage cheese, cheddar and Camembert is of great concern to the dairy industry (Pitt et al., 2000). *L. monocytogenes* has also been shown to survive in other dairy products, including butter, milk and ice cream (Farber and Peterkin, 1991). *L. monocytogenes* has the ability to survive the manufacturing and ripening of many different cheeses, and it is usually concentrated in the curd. *L. monocytogenes* does not grow during the ripening of hard or semi-hard cheeses, but can grow on the surface of soft cheeses, such as Camembert (Farber and Peterkin, 1991). As

L. monocytogenes can survive in soft cheeses during and after manufacture, many countries, including the U.S.A., regulate that only pasteurised milk can be used for the manufacture of soft cheeses. There have been several outbreaks of listeriosis in the U.S. and Europe associated with dairy products (Table 1.1). The outbreaks are due to the use of raw milk in the manufacture of cheese or post-processing contamination, as correct pasteurization of milk inactivates *L. monocytogenes*.

Table 2.1 Dairy related incidences and outbreaks of *Listeria monocytogenes (*adapted from Sutherland et al., 2003, with additional information from Promedmail.org).

Location	Year	Number of cases (deaths and miscarrages)	Food Implicated
East Cambria, England	1981	11	Cream
Massachusetts, USA	1983	49 (14)	Pasteurized milk
Canton de Vaud, Switzerland	1983-87	122 (33)	Mont d'Or cheese, raw milk
California, USA	1985	142 (48)	Jalisco cheese
Philadelphia, USA	1986-87	36 (16)	Ice cream
Los Angeles, USA	1987	11	Butter
Luxemburg	1989	2	Camembert cheese
Denmark	1989-1990	26 (6)	Blue-mold cheese
USA	1994	56 (0)	Pasteurized chocolate milk
France	1995	33	Brie de Meaux cheese
France	1997	14	Pont l'eveque cheese
Finland	1998-1999	25 (6)	Butter
USA	2000-2001	12 (5)	Soft cheese
Canada	2002	19	Soft cheese
Canada	2002	68	Soft cheese
USA (CA)	2002	2	Soft cheese?
Switzerland	2005	10 (5)	Soft tomme cheese
USA	2005	6	Mexican cheese
Czech Republic	2006-07	90 (14)	Soft cheese

2.2 Dairy Products

2.2.1 Introduction

Dairy products are an important part of the diet of many consumers in many countries, and are an excellent source of energy, protein, vitamins and minerals. They include a wide range of products such as milk, cheese, yogurt and ice cream. As milk is highly nutritious, has a high moisture content and a slightly acidic to neutral pH (ca. 6.6), it allows the growth of a wide range of microorganisms. Raw milk can be contaminated with many pathogens and spoilage organisms; therefore it must have a preservation treatment to delay spoilage and prevent disease. Historically milkborne diseases have included anthrax, typhoid fever, scarlet fever, tuberculosis, diphtheria, and foot and mouth disease (Holsinger et al., 1999). As late as 1938, milk borne diseases were still responsible for about 25% of illness associated with food and water in the U.S. (Holsinger et al., 1999). This percentage has been greatly reduced with the introduction of thermal pasteurization of dairy products. However, there have been over 30 cheese-related foodborne illness outbreaks in the USA since 1970, and the organisms implicated are primarily *Salmonella, L. monocytogenes, Escherichia coli, Streptococcus* and *Brucella melitensis* (Altekruse et al., 1998).

2.2.2 Thermal pasteurization of dairy products

Pasteur's discoveries in the 1860-1870's demonstrated that heating liquids improved the keeping quality during storage due to microbial inactivation (Holsinger et al., 1999). For dairy products, especially fluid milk, pasteurization has been the method for ensuring safety and increasing shelf life since early last century. Pasteurization may not destroy all pathogenic microorganisms which may be present in the food product, but it reduces the number to a level at which they do not constitute a significant health hazard. Pasteurized, when used to describe dairy products, means that every particle of the product has been heated in properly operated equipment for a specified temperature and time.

Pasteurization offers many benefits as a preservation technique. There is a good safety record associated with pasteurized milk, at present milk accounts for 1% of the reported disease outbreaks associated with food and water in the U.S., typically due to post processing contamination (Holsinger et al., 1999). Pasteurization also considerably extends shelf life and reliable information is available for predicting the processing parameters (Grant et al., 2000). There is a simple test to determine if the milk has been processed adequately, the absence of alkaline phosphatase activity (Holsinger et al., 1999).

However, there are also several disadvantages associated with pasteurization of milk products. There have been several large outbreaks of foodborne disease associated with post-processing contamination. Pasteurization does not inactivate all microorganisms in milk, for example bacterial spores survive high temperature short time pasteurization (HTST, 72°C for 15 s), and therefore milk requires refrigeration. *Bacillus*

cereus spores survive HTST pasteurization, and can grow at temperatures as low as 7°C, causing quality defects in milk and reducing the shelf life. Additionally, thermal processes can affect the flavor, texture and color of food and destroy vitamins in the food (Grant et al., 2000). American consumers typically dislike ultra-heat-treated (UHT) milk due to its severely altered taste. Heat affects milk enzymes and proteins, such as rennet and whey, and sugars. This can lead to compromised taste, texture and quality in products such as cheese (Kelly, 2000).

2.2.3 Inactivation of microorganisms by heat

The precise mechanism by which microorganisms are inactivated by heat has yet to be elucidated. However, there are several studies demonstrating that multiple targets in the cell are damaged by heat and that inactivation by heat may be multifactorial. Membrane damage of heated cells has been demonstrated indirectly through the leaking of UV-absorbing materials. Iandolo and Ordal (1966) demonstrated loss of nucleic acids, amino acids and proteins from *Staphylococcus aureus* due to thermal injury. DNA damage may also contribute to microbial inactivation by heat. Single-strand and double-strand breaks in DNA in heated cells has been reported (Bridges et al., 1969; Uchida et al., 1977). Heat inhibits protein synthesis, perhaps due to ribosome denaturation (Iandolo and Ordal, 1966; Anderson et al., 1991). Loss of magnesium due to heat is thought to contribute to ribosome denaturation (Hurst and Hughes, 1978). Many cellular proteins are inactivated by heat, which may lead to bacterial inactivation (Rosenberg et al., 1971). However, there is no direct relationship between any one specific protein and inactivation.

2.3 High pressure processing

2.3.1 Introduction

High pressure processing (HPP), also referred to as high hydrostatic pressure or ultra high pressure, has been known to be a potential food preservation technique for over one hundred years. However, the technology to process food in this way on a commercial scale did not exist until recently. High pressure (HP) has been applied during the twentieth century for the production of ceramics, carbon graphite, plastics, steels, and superalloys. New synthetic materials, including synthetic diamonds and polycrystalline cubic boron hydride, may also be manufactured at high pressure conditions (Hoover et al., 1989). In the late 1980's the potential of HPP as a food preservation technique was re-investigated, starting in Japan. Dr. Dallas Hoover at the University of Delaware was among the first to relaunch the investigation of application of HP to foods in the U.S. The first HP-treated food products were launched in Japan in 1991. A range of pressure-treated foods are currently available in Japan, including fruit juice preparations, jams, salad dressings, fruit juices, rice cakes and raw squid. There are several HP-treated food products available in the U.S., including guacamole, salsa, oysters and deli meats. HP-treated foods are also available in Europe.

Pressures in the range of 300-700 MPa are used to pressure treat foods (Stewart and Cole, 2001). Typically a batch system is used, which consists of a pressure vessel with a 35-300 liter capacity and a pressure generating device. Food is pre-packaged in flexible packaging and loaded into the vessel, which is firmly closed. A pressure medium, usually water, is pumped into the vessel until the desired pressure is reached. Then pumping is stopped, the valves are closed and the pressure is maintained without

further energy input (Farr, 1990; Grant et al., 2000). The cycling time is relatively short, generally no more than 15 minutes, depending on the desired application. The time and pressure magnitude of the HP-treatment is dependent on the target microbes and the type of food. As the pressure is hydrostatic, and hence equal from all sides, the product shape is not distorted. Liquid and pumpable foods are processed using a semi-continuous system, where the liquid itself is the pressure-transmitting fluid.

2.3.2 Principles and effects on chemical reactions

The effects of HP are explained by two principles. One is that of Le Chatelier, according to which any phenomenon accompanied by a decrease in volume will be enhanced by pressure (Gross and Jaenicke, 1994). The other is the Isostatic Principle, which states that pressure is transmitted instantly and uniformly independent of the size and geometry of the food (Smelt, 1998). Key effects of HP include the inactivation of microorganisms, protein denaturation, enzyme activation or inactivation, and retention of quality and freshness retention (Knorr, 1993). HP does not affect covalent bonds, but does affect other types of chemical bonds, including hydrogen, hydrophobic and ionic bonds (Tewari et al., 1999). Ion pairs in aqueous solution are strongly destabilized by HP. A decrease in volume favors the dissociation of ionic interactions as each ion arranges water molecules in its vicinity more densely than bulk water (Gross and Jaenicke, 1994). Similarly, the exposure of hydrophobic groups to water disturbs the loosely packed structure of pure water and leads to a hydrophobic solvation layer which is assumed to be more densely packed (Gross and Jaenicke, 1994).

2.3.3 Benefits and limitations of high pressure processing in foods

HPP exhibits several benefits over thermal processing of foods. The main benefits of HPP are the extension of shelf life while maintaining the fresh qualities of the food product. Unlike thermal processing, pressure treatment is not time or mass dependant, therefore processing time is reduced (Zimmerman and Bergman, 1993). As pressure is transmitted instantly and uniformly the food is preserved evenly, without any particle escaping preservation (Kelly, 2000). Post-processing contamination can be eliminated by pre-packaging food products prior to processing, therefore increasing safety and shelf life. As HPP does not affect covalent bonds (in contrast to heat), many small molecules in foods, such as many flavor compounds and vitamins, are left intact (Farr, 1990; Tewari et al., 1999). Hence, the taste and quality of many HP-treated foods are superior to those that are thermally processed. Notable examples are fruit products, such as juices and jams. Often these products have significant vitamin loss, flavor loss, and texture and color changes during thermal processing, but may be HP-treated with minimal changes to these attributes. The products have increased consumer appeal and they may be labelled as 'natural' and preservative free, although the FDA is still deliberating as to whether 'fresh' can be used to market HP-processed foods (IFT HPP workshop, Philadelphia 2005). HPP can be used to extend the shelf life of 'raw' products, such as oysters, fish and fruit without resulting in a cooked appearance or cooked flavor (Ananth et al., 1998; Amanatidou et al., 2000). At pressures lower than those required to inactivate microorganisms, enzymes can be activated to improve food characteristics, for example HPP can increase tenderness in meats (Ananth et al., 1998).

However, there are several limitations in the high pressure processing of foods. HPP cannot be applied to all food products for several reasons. The texture and color of some foods are adversely affected by HPP (Cheftel, 1995). In particular, foods with high interior air volume, such as whole fruits and vegetables, may exhibit texture changes and shape distortion (Lucore et al., 2000). High protein foods, for example meat and fish, can take on an irreversible cooked appearance during HPP. Bacterial spores are not inactivated by HPP alone (at ambient temperatures) at 300 to 700 MPa, therefore sterile and shelf-stable foods are difficult to produce (Smelt, 1998; Anonymous, 2005). Consequently a combination of hurdles (i.e. different treatments) is often required to prevent microbial growth in HP-treated foods, for example acidification, heating during HPP, or refrigeration following HPP (Hoover et al., 1989; Gould, 1996; Garcia-Graells et al., 2000; Anonymous, 2005). Presently the high cost of HPP equipment prohibits many food companies from purchasing/leasing the equipment. Generally manufacturers desire short processing times to ensure maximum throughput of the product, therefore very high pressures (e.g. 600 MPa) are desired. However, pressure of this magnitude may not be economical due to the high cost of the equipment and increased metal fatigue (Kalchayanand et al., 1998). Currently HP-treated foods are more expensive than their thermally processed counterparts; therefore they need to show considerable benefits in taste and quality to entice the consumer to pay more.

2.3.4 High pressure and microorganisms

Many factors influence the sensitivity of microorganisms to pressure, including the magnitude of pressure, pressurization time, food temperature, microbial cell type, cell growth phase, cell growth temperature, suspending menstruum during and following HPP, pH, and the presence of antimicrobial compounds in the suspending media. The effects of pressure on microorganisms in food are also influenced by food constituents, such as fat and various sugars.

Typically microbial inactivation increases as magnitude of pressure and/or as exposure time is increased (Gervilla et al., 1997; Simpson and Gilmour, 1997c; Ritz et al., 2000b; Hayman, 2001; Chen and Hoover, 2003; Guan et al., 2005). Additionally, the temperature at which HP-treatment occurs affects the extent of inactivation (Ritz et al., 2000b). Cells become increasingly sensitive to HPP as the processing temperature is raised above 35°C (Gervilla et al., 1997; Simpson and Gilmour, 1997c; Alpas et al., 1999; Alpas and Bozoglu, 2000). HPP at temperatures near 0°C can also increase the rate of microbial inactivation (Ritz et al., 2000b).

Cell species and type also affect HP-resistance. Typically it is thought that Gram-positive bacteria are more barotolerant than Gram-negative bacteria, which in turn are more resistant than eukaryotic cells such as yeasts and molds (Casedei, 2000). Recently Chen et al. (2006) conducted a study on the pressure resistance of eight common pathogenic foodborne bacteria, including *L. monocytogenes*, *Staphylococcus aureus*, *Salmonella* and *E. coli* O157:H7. They found no clear trend between Gram-stain type and HP-resistance, reporting that *Shigella flexneri* (a Gram-negative rod) was the most HP-resistant bacterium of those surveyed (Chen et al., 2006). Bacterial spores are

extremely HP-resistant, and are not inactivated by pressures under 1000 MPa at ambient temperatures, however they can be inactivated by combining high temperature and high pressure (Cheftel, 1995; Smelt, 1998). Investigation of viral inactivation by pressure is a relatively unexplored field, however it is known that their pressure resistance varies considerably (Hoover et al., 1989).

Stationary phase bacteria and yeast are more resistant to pressure treatment than exponentially growing cells (Isaacs et al., 1995; Brul et al., 2000; Pagan and Mackay, 2000; McClements et al., 2001; Hayman et al., 2007a), and this will be discussed in more detail in Section 2.3.8. Considerable pressure resistance between bacterial strains of the same species has been observed, but is unexplained (Patterson et al., 1995; Benito et al., 1999; Murano et al., 1999; Alpas and Bozoglu, 2000; Hayman, 2001; McClements et al., 2001; Tay et al., 2003). There may be a correlation between pressure resistance and resistance to other stresses. It has been reported that heat resistant microorganisms are also more resistant to pressure, but there are many exceptions (Benito et al., 1999).

The menstruum in which microorganisms are suspended can affect inactivation and survival during and after HP-treatment (Cheftel, 1995). Some food products have baroprotective effects in comparison to HP-treatment in peptone diluent or laboratory media. Milk has a baroprotective effect in comparison to phosphate buffer (Patterson et al., 1995; Simpson and Gilmour, 1997b; Garcia-Graells et al., 2000). Milk fat in particular has been associated with lowering the lethality of HP-treatment (Garcia-Graells et al., 1999). Recently Black et al. (2007) reported that minerals in milk, specifically calcium, magnesium, phosphate and citrate, contribute significantly to the baroprotective effect of milk, possibly by acting as buffers and preventing pH decrease during HPP.

2.3.5 Mechanisms of inactivation of microorganisms by HPP

The mechanism(s) responsible for microbial inactivation by HPP are still not completely understood (Hauben et al., 1997). Many cellular targets have been reported to be involved, including the cell wall (Brul et al., 2000), cytoplasmic membrane (Pagan and Mackay, 2000; Ritz et al., 2002), nucleic acids (Mackey et al., 1994), ribosomes (Niven et al., 1999) and various proteins and enzymes (Smelt et al., 1994; Simpson and Gilmour, 1997a; Wouters et al., 1998; Ritz et al., 2000a). Pressure affects cellular morphology, for example intracellular gas vacuoles can collapse and some organisms form long filaments (Welch et al., 1993; Aertsen et al., 2004a). Motile organisms may lose motility due to loss of flagella (Casedei, 2000).

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have been used by several research groups to investigate the effect of HPP on microbial morphology. Mackey et al. (1994) utilized TEM to investigate the effects of pressure on *Salmonella enterica* serovar Thompson (Gram-negative) and *L. monocytogenes*. They observed marked differences in the appearance of both cell types following HPP. In *L. monocytogenes* they observed circular areas in the cytoplasm that were lighter in contrast (i.e. less electron dense) than the rest of the cytoplasm, usually adjacent to the cytoplasmic membrane. They speculated that the lighter areas were devoid of ribosomes, but this may not be the case (their pictures were overexposed and hence show too much contrast, and they did not use high magnifications to closely examine the lighter areas). *S. enterica* serovar Thompson appeared to lose cytoplasmic material due to HPP, indicating that cell lysis had occurred. In both organisms the DNA was condensed in the center of the cell. Extreme condensation of DNA in HP-treated

microbial cells has also been reported by Hayman (2001) and Park et al. (2001). TEM micrographs of *Lactobacillus* following HPP at 600 MPa show a gap between the cell wall and the cytoplasmic membrane (Park et al., 2001), while this was not seen in *L. monocytogenes* (Mackey et al., 1994; Hayman, 2001). SEM of *L. monocytogenes* following HPP revealed that there was some scarring on the surface of the cells, but little change in overall morphology (Ritz et al., 2002).

i. Outer membrane, cytoplasmic membrane and cell wall

One of the limitations with some of the research investigating the effect of HPP on bacterial membranes is that the authors do not differentiate between the outer membrane and the cytoplasmic membrane when investigating Gram-negative organisms. The composition and functions of these membranes vary, and therefore it is very possible that they may behave differently under pressure. It has been suggested that membrane fluidity is decreased and membrane thickness is increased in organisms surviving HPP due to tighter packing (Fernandes et al., 2004), although this is contrary to reports that intracellular volume increases (Tholozan et al., 2000).

Pagan and Mackey (2000) examined HP-induced membrane damage in three strains of *Escherichia coli* after HPP at 100 to 600 MPa for 8 min. They measured membrane damage by the ability to plasmolyse in the presence of 0.75 M NaCl and staining with propidium iodide (PI). Exponential-phase cells were completely inactivated at pressures of 200 MPa and above (a 6-$\log_{10}$ reduction). Cells lost the ability to plasmolyse when HP-treated at 200 MPa and higher, which was not surprising as the cells were dead. PI uptake in exponential-phase cells was apparent after treatment at 100

(one strain) or 200 MPa (two strains), and uptake increased as magnitude of pressure increased, plateauing between 300 and 400 MPa. It is interesting to note that PI uptake increased in cells HP-treated at 400 MPa in comparison to cells treated at 200 MPa, but decrease in viability was the same for cells HP-processed at 200-600 MPa. Stationary-phase *E. coli* were more barotolerant than exponential-phase cells, requiring 500-600 MPa to achieve a 6-$\log_{10}$ reduction in two of the three strains, with one strain displaying a 2-$\log_{10}$ reduction after 8 min at 600 MPa. The osmotic response in stationary-phase cells did not correlate with inactivation, for example one of the strains lost the ability to plasmolyse at 100 MPa even though no inactivation was evident. Stationary-phase cells did not stain with PI when the dye was added after HPP. However, when PI was added prior to pressurisation cells took up the dye. PI uptake in stationary-phase cells plateaued at 400 MPa, even though inactivation increased when the pressure was increased to 500 or 600 MPa. Pagan and Mackey (2000) concluded that membrane damage in exponential-phase cells cannot be resealed, and therefore there is a permanent loss of membrane integrity which leads to cell death. Stationary-phase cells were permeable during pressure treatment, but were able to reseal the membranes post-HP-treatment, even when the cells were dead. A transient increase in membrane permeability in stationary-phase cells did not necessarily lead to cell death.

Ritz et al. (2000a) used SDS-PAGE to investigate the effect of HPP on the membrane proteins of *Salmonella enterica* serovar Typhimurium. They reported that there were no proteins associated with the outer membrane following HPP at 600 MPa. After HPP at 400 MPa, the protein content of the outer membrane was less than that of the control, but there were still many proteins associated with the membrane. The loss of

outer membrane proteins was enhanced by lowering the pH from 7.0 to 5.6 during HPP. There also appeared to be a loss of protein content from the inner membrane, which was more pronounced at 600 MPa than 350 MPa; however the effect was not as dramatic as that seen in the outer membrane.

Brul et al. (2000) investigated the effect of HPP (15 min at 100-300 MPa) on the cell wall and membrane of *Saccharomyces cerevisiae*. They hypothesised that the cell wall may protect the underlying membrane from compression; however mutants with various mutations in cell wall-related genes were no more HP-sensitive than the wild-type isolates. They did not find a quantitative correlation between PI uptake and inactivation. For example, following HPP at 100 MPa most of the cells stained with PI even though they were still viable, and following HPP at 300 MPa cells were not viable but did not stain with PI. However, they still concluded that the cell membrane was an important target in HPP inactivation of yeasts. They also speculated that membrane damage was reversible, but found no difference in staining when they applied PI before or after staining. They also investigated whether repair of the membrane required protein synthesis, but reported that de novo protein synthesis was not involved in membrane repair.

ii. **Enzymes and Proteins**

Several investigators have suggested that HPP inactivates cells by interfering with protein conformation. For example, Timson and Short (1965) suggested that HPP destroys biological systems because of protein precipitation. Suzuki and Taniguchi

(1972) suggested that HPP damages biological systems because HPP enhances protein-protein hydrophobic interactions.

Pressure induced protein denaturation was first reported by Bridgeman in 1914 (Gross and Jaenicke, 1994). HPP affects the tertiary and quaternary structure of proteins, causing dissociation, and promotes unfolding and misassembly (Gross and Jaenicke, 1994). Oligomeric proteins are dissociated at pressures lower than those required to unfold the individual subunits of complex (Prehoda et al., 1998). In general, single-chain proteins do not undergo denaturation at pressures below 400 MPa (when HP-processed at ambient temperature and neutral pH). Dissociation of multimeric proteins occurs at pressures lower than 400 MPa (Gross and Jaenicke, 1994). Denaturation of proteins by pressure results in volume changes, although the difference in partial molar mass of the folded and unfolded states may be small (Prehoda et al., 1998). The volume occupied by the unfolded state is smaller.

Compact proteins with low flexibility show low compressibility and higher stability to HP and vice versa (Gross and Jaenicke, 1994). The β-domains of proteins are more stable against pressure-induced unfolding than the α-domains (Gross and Jaenicke, 1994). The binding of a ligand to the protein can drastically increase its pressure resistance, as ligand binding may be accompanied by a significant increase in packing density of the protein (Gross and Jaenicke, 1994). Therefore, studies investigating the effect of HPP on purified enzymes may not represent what occurs in whole cells. Conversely, oxidation of proteins increases their compressibility (Kaminsky and Richards, 1992). Interestingly, loss of activity in HP-treated enzymes may occur at lower pressures than those needed to cause dissociation of multimeric proteins. For example,

Masson and Balny (1990) demonstrated loss of activity of butyrylcholinesterase at 20-100 MPa; however, dissociation did not occur until 320 MPa.

Enzymes vary widely in their ability to withstand exposure to high pressure. Not only do enzymes that vary in catalytic functions vary in their ability to withstand high pressure, but it has also been reported that similar enzymes produced by different organisms may differ with respect to barotolerance (Zobell and Kim, 1972). Simpson and Gilmour (1997a) investigated barotolerance of 13 metabolic enzymes in three strains of *L. monocytogenes* with differing barotolerance. They reported a wide difference in barotolerance between enzymes. However, there was no difference in enzyme barotolerance associated with the different strains. They noted that the most pressure-sensitive enzymes were multimeric. They concluded that the thirteen selected enzymes did not contribute to the variation of pressure resistance between the three strains studied.

iii. Homeostasis

Several reports have demonstrated that HPP affects various aspects of cellular physiology, including intracellular pH and cell volume (Wouters et al., 1998; Tholozan et al., 2000). Wouters et al. (1998) investigated the effect of HPP (250 MPa) on the morphology, ΔpH (pH_{in}-pH_{out}) and F_0F_1ATPase activity of *Lactobacillus plantarum*. They reported that cells grown at pH 5.0 were more barotolerant than cells grown at pH 7.0. They isolated inside-out membrane vesicles and measured F_0F_1ATPase following HPP. Membrane activity, determined by rate of ATP hydrolysis, increased following HPP at 10, 80 or 120 min at 250 MPa. HPP for 240 min at 250 MPa lead to a slight decrease in F_0F_1ATPase activity. Pressure treatment resulted in a decrease in ΔpH in

whole cells, indicating that the regulation of internal pH, controlled by F_0F_1ATPase, may be impaired. TEM did not reveal any changes in membrane morphology. They concluded that disruption of F_0F_1ATPase activity is involved in HPP inactivation of *L. plantarum*.

Tholozan et al. (2000) examined the effect of HPP (150-600 MPa for 10 min) on various physiological features of *L. monocytogenes* and *S. enterica* serovar Typhimurium. *S. enterica* serovar Typhimurium was more barosensitive than *L. monocytogenes.* They reported that HPP at 325 MPa in citrate buffer (pH 5.6) or 425 MPa in phosphate buffer (pH 7.0) resulted in an increase in the cellular volume of *L. monocytogenes*. There was no change in the intracellular volume of *S. enterica* serovar Typhimurium following HPP up to 600 MPa. There was a small decrease in δpH when cells of either species were HP-processed in phosphate buffer at 400-600 MPa. HPP in citrate buffer (pH 5.6) at 275 MPa and above resulted in a decrease of ΔpH in *S . enterica* serovar Typhimurium, with no ΔpH (i.e. $pH_{in} = pH_{out}$) after processing at 350 MPa. HPP at 400 MPa led to a complete efflux of intracellular potassium in both *L. monocytogenes* and *S . enterica* serovar Typhimurium in both buffers. HPP at 400 to 600 MPa also led to a loss of membrane potential, with loss occurring at lower pressures in the citrate buffer. HPP at 300 to 400 MPa resulted in a decrease of intracellular ATP concentration in both organisms, with a ~90% decrease at 400 MPa. Scanning electron microscopy showed that *Salmonella* cells were crumpled and dimpled following HPP at 350 MPa. *L. monocytogenes* cells were the same shape and size following HPP at 400 MPa, but had scars on the cell surface. Tholozan et al. (2000) hypothesised that cell death by HPP is due to extensive membrane permeabilization. Furthermore, the major cellular ATP-

driven potassium uptake system is damaged, which leads to a low concentration of intracellular ATP.

iv. Ribosomes

Pressures greater than 68 MPa inhibit protein synthesis *in vitro*, but the inhibition is reversible after pressure release (Schwartz and Landau, 1972; Smith et al., 1975). Niven et al. (1999) used differential scanning calorimetry to examine the effect of HPP (50-250 MPa) on ribosome conformation in *E. coli*. Niven et al. (1999) aimed to establish a link between ribosome-associated enthalpy (RAE) and cell viability. A decrease in RAE indicated that the ribosomes may have become inactive, dissociated, or changed conformation. RAE decreased following HPP at 200 or 250 MPa, but not after HPP at 50-150 MPa. Viability was slightly reduced in cells HPP at 50-150 MPa, but RAE increased.

2.3.6 Effects of HPP on gene expression and protein synthesis

Welsh et al. (1993) grew *E. coli* at 55 MPa and reported that 55 proteins were induced. These were termed pressure-induced-proteins (PIPs). Of these 11 were heat shock proteins (HSPs; ClpB, ClpP, Lon, RpoH, DnaK, GroEL, GroES, Grp, E, G21.0 and F10.1) and 4 were cold shock proteins (CSPs; G41.2, RecA, HNS and F10.6). The overall rate of protein synthesis in HP-treated cells was 16% of that of the control, indicating that the synthesis of many proteins was inhibited. The largest increase in PIP induction was seen 60-90 min following pressure. There was no protein synthesis when

pressure was increased to 111 MPa. They also reported that growth of *E. coli* at 55 MPa increased cell size and caused filamentation.

Robey et al. (2001) investigated the role of RpoS activity in the variation of barotolerance in *E. coli*. They postulated that increased barotolerance of stationary-phase cells, in comparison to exponential-phase-cells, was due to expression of *rpoS,* a sigma factor that activates more than 30 genes, some of which are involved in stationary-phase stress survival. Using two strains they compared the pressure resistance of the wild type and a *rpoS* null mutant. The mutants were more sensitive to HPP. When they restored *rpoS* activity through complementation via a plasmid, the barotolerance of the mutant returned to a level similar that that of the wildtype. They also aimed to correlate RpoS activity, measured indirectly as *lux* expression, with barotolerance in six wild type strains. Lux expression did not increase in all isolates as cells entered stationary phase. Although they reported that the two highest lux expressors were also the most barotoleant, there was no information on cell numbers. They concluded that *rpoS* plays a role in pressure resistance, but that microbial inactivation by pressure is multifactorial.

Fernandes at al. (2004) used whole genome microarray hybridization to investigate the effect of HPP at 50-200 MPa for 30 min on gene expression in *S. cerevisiae*. They did not correlate the microarray results to viability. They reported that out of 6200 known genes in *S. cerevisiae*, 5% were affected by pressure treatment. A total of 131 genes were >2-fold induced while 143 suffered a < 2-fold downregulation. Most of the pressure up-regulated mRNAs (~45%) corresponded to uncharacterized open reading frames (ORF), and even among the characterized genes, almost half had unknown function. Many of the unknown up-regulated proteins had transmembrane

domains. The three most up-regulated genes were an uncharacterized open reading frame YER067W followed by two genes that code for small HSPs, HSP30 and HSP12. HSP 12 is activated by heat shock and a wide range of environmental stresses, while HSP30 is more specifically regulated by organic acid stress, high ethanol concentration and entry into stationary-phase. For genes with known functions they reported that most of the up-regulated genes were involved in metabolism (particularly carbon metabolism), while many of the down-regulated genes were involved in protein synthesis.

Wemekamp-Kamphiuis et al. (2002) applied a low level HP-treatment (200 MPa for 10 min) to exponentially growing *L. monocytogenes* and measured levels of CSPs. They reported that the levels of CSP1 and CSP2 were 3.5 and 2 fold higher in HP-treated cells than in the controls. They also reported that cold shocked cells (10°C for 4 hours) were more pressure resistant (at 200-350 MPa for 10 min) than cells grown at 37°C (both were in the exponential-phase of growth), however there was no statistical comparison.

Aerstsen et al (2004b) reported that the SOS response was induced in *E. coli* following HPP at 100 MPa. The SOS response is typically induced upon DNA damage, for example after UV irradiation. The SOS response leads to the termination of DNA replication, and involves transcription of over 40 genes that are involved in stabilization of single-stranded DNA, base or nucleotide excision repair, recombinational repair, and control of cell division (Aertsen et al., 2004b). Aerstsen et al (2004b) demonstrated that HPP induced the RecA and LexA-dependent expression of *urvA, recA*, and *sulA*. There were differences between pressure-induced and the classical SOS response; the pressure-induced response was not triggered in response to DNA damage (Aertsen et al., 2004b). The authors suggested that HPP causes disassembly of the DNA replication fork, leading

to exposed ssDNA, which in turn activates RecA and hence the SOS response. They also suggested that the SOS response may explain the filamentation seen in piezosensitive bacteria grown under elevated pressure.

2.3.7 Injury and recovery of microorganisms following HPP

The phenomenon of HP-induced injury in microorganisms and subsequent recovery has been reported by several research groups (Hayman, 2001; Bozoglu et al., 2004; Bull et al., 2005). Injury can be defined as the difference in colony forming units (CFUs) on non-selective and selective agar, or solid media with and without salt (Jordan et al., 2001). In the laboratory *L. monocytogenes* is usually grown on Tryptic Soya Agar (TSB) or TSA with Yeast Extract (TSYEA). These media are non-selective and support the growth of a wide variety of microorganisms. When background flora are anticipated Modified Oxford agar (MOX) can be used to select for *L. monocytogenes*. Selective ingredients in MOX prevent the growth of injured *L. monocytogenes* cells. Healthy cells of *L. monocytogenes* are not inhibited by MOX and CFU per ml or g will be equal on MOX and TSYEA.

Injury caused by HPP in acid foods such as fruit juices may result in further reduction of microbial numbers following HPP (Jordan et al., 2001). However, cells in low acid foods, such as milk, may recover from HP-induced injury (Hayman, 2001; Bull et al., 2005). This has important food safety implications, as injured cells may not be detected immediately following HPP, but may subsequently recover and cause foodborne infection. There have been no mechanistic studies investigating the HP-induced injury of microorganisms published to date. It appears that injury is largely dependant on the

magnitude of pressure; when *L. monocytogenes* is HP-processed in milk injured cells are generated at 300-400 MPa, but not when pressure is increased to 600 MPa (Hayman, 2001; Bozoglu et al., 2004; Bull et al., 2005).

Bozoglu et al. (2004) investigated HPP-induced injury in *L. monocytogenes*, *E. coli, Salmonella enterica* serovar Enteritidis and *S. aureus* in 1% UHT milk. They investigated the effects of magnitude of pressure (350, 450 or 550 MPa), milk storage temperature following HPP (4, 22 and 30°C) and time of storage on the recovery of HP-injured cells (up to four weeks). When milk was sampled immediately after HPP at 350 MPa *L. monocytogenes* grew on both MOX and TYSEA. When milk was sampled immediately after HPP at 450 MPa, *L. monocytogenes* grew on TSYEA, but not on MOX. Bozoglu et al. (2004) designated this condition as injury one (I1), and saw repair (i.e. growth on MOX) within one day at all storage temperatures. Following processing at 550 MPa they did not recover colonies on either agar immediately following processing, they designated these cells as having type two injury (I2). Cells were able to grow on MOX and TSYEA within 1 day when stored at either 22 or 30°C, or within 6 days when stored at 4°C. Bozoglu et al. (2004) postulated that I1 might be structural damage to the cell membrane or wall, and that I2 might be an unspecified metabolic injury. They also postulated that the target of injury may differ with magnitude of pressure, with ribosomes the primary target below 400 MPa, and cell membranes the primary target above 400 MPa.

Bull at al. (2005) investigated the effect of storage temperature (4, 15 or 30°C) on the recovery of injured *L. monocytogenes* following HPP at 450 MPa in skim milk. They

reported recovery typically within 24-72 h of processing; storage at 15°C gave maximal recovery of injured cells.

De Lamo-Castellvi et al. (2005) investigated the recovery of four strains of *Yersinia enterocoliticia* following HPP (300, 400 and 500 MPa) in skim milk. Samples were stored at 8°C for up to 15 days. After processing at 500 MPa, cells could not be detected in the milk until four days after processing, but by day 15 there was greater than 5-$\log_{10}$ CFU/ml in all samples.

2.3.8 Effects of growth phase and growth temperature on the barotolerance of microorganisms

Stationary-phase bacterial and yeast cells are more barotolerant than exponential-phase cells (Isaacs et al., 1995; Casadei et al., 2002; Hayman et al., 2007a). Isaacs et al. (1995) postulated that increased barotolerance in stationary-phase cells may be due to their smaller size and more spherical shape, or due to the accumulation of cell components such as proteins or carbohydrates. Proteins synthesized as part of the stationary-phase response may also contribute to barotolerance (Robey et al., 2001). Prior growth temperature also affects barotolerance of microorganisms, including *L. monocytogenes, E. coli, Bacillus cereus* and *Pseudomonas fluorescens* (McClements et al., 2001; Casadei et al., 2002; Bull et al., 2005; Hayman et al., 2007a).

Bull et al. (2005) reported that stationary-phase *L. monocytogenes* grown at 43°C were more barotolerant (to 400 MPa) than cells grown at 15°C. McClements et al. (2001) reported that exponential- and stationary-phase *L. monocytogenes* grown at 30°C

were more barotolerant (to 400 MPa) then cells grown at 8°C. The effect of growth temperature on barotolerance on *L. monocytogenes* is strain dependent, as some strains show very little difference in barotolerance when grown at different temperatures (McClements et al., 2001).

Membrane composition changes with growth temperature to maintain a liquid-crystalline state (Annous et al., 1997). This phenomenon may play a role in growth temperature-dependant barotolerance of *L. monocytogenes*. There are several changes that bacteria can make to membrane fatty acids to change membrane fluidity. These are: the type of branching at methyl end i.e. anteiso (a; lower melting point) or iso (i; higher melting point), chain length and degree of unsaturation. There are species-specific differences in the mode of adaptation to growth at low temperatures. Annous et al. (1997) reported that the main fatty acids in mid-exponential-phase *L. monocytogenes* grown at 37°C were a-$C_{15:0}$, a-$C_{17:0}$ and i-$C_{15:0}$. At 5°C the major changes were a large decrease in a-$C_{17:0}$ content, an increase in a-$C_{15:0}$, and a general shortening of fatty acids in the profile. They noted that when reducing growth temperature from 45 to 20°C generally there was a shortening of fatty acids in the membrane of *L. monocytogenes*, and when decreasing growth temperature from 20 to 5°C, there was continued shortening as branching switched from iso to anteiso.

Casadei et al. (2002) investigated the relationship between growth temperature, membrane fluidity and barotolerance in *E. coli*. They hypothesized that increased membrane fluidity would confer pressure resistance. Cells were grown at 10, 20, 30, 37 and 43°C and HP- processed for 5 min at pressures ranging from 100 MPa to 600 MPa. They reported that pressure resistance in exponential-phase cells was maximal in cells

grown at 10°C, and then decreased with increasing growth temperature (i.e. cells grown at 45°C were the most HP-sensitive). However, no statistical comparisons were made between treatments. Stationary-phase cells were more barotolerant than exponential-phase cells, and pressure variation between cells grown at different temperatures was more apparent. Pressure resistance was maximal in cells grown at 30 and 37°C, before decreasing (in order) in cells grown at 45, 20 and then 10°C. As the growth temperature was increased from 10 to 45°C the proportion of saturated fatty acids (SFA) in the membrane increased, while the proportion of unsaturated fatty acids (UFA) decreased. Hence, the fluidity index (the ratio of UFA to SFA) decreased as the growth temperature was increased from 10 to 45°C; this occurred in both exponential- and stationary-phase cells. No correlation was observed between membrane fluidity and pressure resistance in stationary-phase cells; however there may have been evidence for a relationship between the two factors in exponential-phase cells. Casadei et al. (2002) speculated that the cell membrane plays a role in the inactivation of exponential-phase *E. coli*. However physiological changes that occur when the cells enter stationary-phase may override the effect of membrane fluidity on pressure.

2.3.9 Effect of water activity on microbial inactivation by HPP

While reduced water activity can inhibit the growth of microorganisms, it can also protect them from other environmental stresses, such as heat (Gould, 1996). Decreasing a_w has also been found to increase the resistance of microorganisms to high pressures (Oxen and Knorr, 1993), however this effect depends on the solute used to depress water

activity (Patterson, 2005). The mechanism for the protective effect of reduced a_w on microbial inactivation by HPP is not known, but may be due to protein stabilization (Moussa et al., 2006).

The protective effect of reduced a_w on HP-inactivation of microbial cells has been reported by several authors. For example, Simpson and Gilmour (1997b) showed that inactivation of *Listeria monocytogenes* was significantly reduced when cells were suspended in an olive oil mixture (30% v/v oil). However, this effect was only significant with one strain, and not with two others (Simpson and Gilmour, 1997b). Hayman et al. (2004) reported that the pressure resistance of *L. monocytogenes* was increased as the salt concentration of the medium was increased, but a_w was not measured. Morales at al. (2006) showed that the a_w of cheese significantly affected the pressure resistance of *L. monocytogenes*. Moussa et al. (2006) investigated the effects of low temperature and water activity on the inactivation of *E. coli* by HPP. They used solutions with a_w s of 0.850, 0.992, and ca. 1.000 (the solutions were, in order; water/glycerol, Luria-Bertani broth without any adjustment to a_w and distilled water). Lowered a_w reduced the inactivation of *E. coli*, and was particularly protective when cells were HP-processed at -20°C. They showed that the compressibility of water and water-glycerol solutions (up to 40% glycerol w/w) was different over the range 100-400 MPa, but did not measure compressibility at 600 MPa. They demonstrated that the difference in compressibility becomes smaller as the pressure increases.

2.3.10 Heat shock, cold shock and HPP

Increased barotolerance resulting from sublethal heat shock has been reported in the yeast *Saccharomyces cerevisiae* (Iwahashi et al., 1991; Iwahashi et al., 1997) and the Gram-negative bacterium *E. coli* (Aertsen et al., 2004b). Moreover, stress proteins are synthesized in response to sublethal pressure shock (Welch et al., 1993; Wemekamp-Kamphuis et al., 2002; Fernandes et al., 2004). *E. coli* expresses about 55 proteins upon exposure to sublethal pressure (55 MPa); 11 of these were HSPs (Welch et al., 1993). *S. cerevisiae* has also been reported to express HSPs in response to mild pressure treatment (Fernandes et al., 2004). CSPs are also induced by pressure (Wemekamp-Kamphuis et al., 2002).

Iwahashi et al. (1991) reported that the addition of cycloheximide, an inhibitor of protein synthesis in yeast, prior to heat shock attenuated the induction of barotolerance and thermotolerance in *S. cerevisiae*. These findings indicate that de novo synthesis of HSPs during heat shock contributes to increased barotolerance in *S. cerevisiae.*

Aertson et al. (2004b) reported that three heat shock genes, *lon, dnaK*, *clpPX* (encoding the Lon protease, the DnaK chaperone, and the ClpPX protease) and the sigma factor *rpoH* were induced in *E. coli* after exposure to pressure (150 MPa). The genes are regulated by σ^{32}. They did not investigate the induction of these genes beyond pressures of 150 MPa as most of the cells were inactivated. They showed that heat shock at 50°C for 15 min increased pressure resistance at 250 MPa. *dnaK* transcription paralleled the development of increased pressure resistance. They reported that pressure shock did not increase heat or pressure resistance. They found that the heat shock genes were expressed in basal levels in pressure-resistant mutants (except *rpoH*). They also found

that some heat shock proteins were more strongly expressed in pressure-resistant mutants during growth at 37°C (no exposure to pressure). These proteins were DnaK, GroEl, GroES, GrpE, ClpB and HtpG.

2.3.11 Tailing

Microorganisms often show "tailing" inactivation kinetics when subjected to high pressure. This phenomenon has been reported by many researchers and is unlikely to be an artefact of poor experimental method. Tailing has been described as rapid inactivation at the early stage of treatment followed by a reduced inactivation rate as time is increased (Tay et al., 2003). Tailing inactivation kinetics during exposure to HPP has been reported with Gram-positive and Gram-negative bacteria, as well as with viruses and eukaryotic cells (Avsaroglu et al., 2006).

Metrick et al. (1989) was one of the first groups to publish data on tailing inactivation kinetics due to high pressure. Inactivation curves of *Salmonella* in phosphate buffer at 275 and 350 MPa displayed tailing. They conclude that a small portion of the population was more resistant to pressure, but did not speculate why.

Patterson et al. (1995) demonstrated tailing inactivation kinetics when *E. coli, S. aureus* and *L. monocytogenes* were HP processed in buffer, UHT milk and chicken at 600 MPa. Simpson and Gilmour (Simpson and Gilmour, 1997b) showed that tailing occurred in some strains of *L. monocytogenes*, but not others. Tailing occurred when cells were treated in phosphate buffered saline (PBS) and was especially prominent when cells were HPP at 400 and 450 MPa. Kalachayanand et al. (1998) also reported tailing in various

pathogenic and spoilage organisms in peptone. Benito et al. (1999) demonstrated tailing of *E. coli* HPP at 500 MPa in PBS.

Tay et al. (2003) reported tailing inactivation kinetics with pressure-sensitive and pressure-resistant strains of *L. monocytogenes* grown at 37°C. When cells were processed at 350 MPa tailing occurred after 10 min of processing. They postulated that non log-linear inactivation kinetics was consistent with pressure affecting multiple targets within the cell, rather than lethality being due to a single target. They were not able to recover barotolerant cells by regrowth of those cells in the tail despite repeated attempts.

Vurma et al. (2006) reported tailing of *L. monocytogenes* HP-processed in buffer at various pressures (up to 700 MPa). They demonstrated that the addition of phenolic compounds, particularly *tert*-butylhydroquinone, reduced the tailing effect.

Chen (Chen, 2007) conducted a survey of six foodborne pathogens and demonstrated tailing inactivation kinetics in all when HP-processed in UHT milk. Tailing generally occurred after 5- to 6-$\log_{10}$ reductions and was particularly pronounced in the inactivation curves of *L. monocytogenes*.

Tailing may be influenced by the menstruum in which the microorganisms are suspended. Chen (2007) showed that tailing occurred when six foodborne pathogens were HP-processed in milk, but that tailing did not occur in peptone water. However, other groups have shown tailing in peptone and buffers, as described previously in this section. Growth temperature and growth phase also affect tailing. Tailing occurred at 400 MPa when *L. monocytogenes* was grown at 35 and 43°C, but not when cells were grown at 4, 15 or 25°C (Hayman et al., 2007a).

The mechanisms that lead to tailing are unknown; however there are several possible explanations. There may be a small group of genetically distinct mutants within the population that are more barotolerant than the majority of the population (Karatzas et al., 2002; Hauben et al., 1997). Another hypothesis is that HP itself causes tailing by inducing the synthesis of PIPs, which subsequently protect the cell against HP inactivation. Another explanation could be the presence of stress proteins, such as HSPs, in high concentrations in a small percentage of the population before HPP.

If there is a pressure-resistant sub-population, regrowth of tailing cells should yield a population with higher pressure resistance than the parent population. Several groups have investigated this possibility. Metrick et al. (1989), Patterson et al. (1995), Ulmer el al. (2000) and Tay et al. (2003) reported that regrown "tail" bacteria did not show increased pressure resistance.

Pressure-resistant mutants of *L. monocytogenes* and *E. coli* have been isolated by Hauben et al. (1997). *E. coli* was HP-treated at 280 MPa for 15 min, and the survivors were grown at 30, 37 or 42°C. They subjected the cells to 18 rounds of increasing higher pressure, up to 450 MPa. When the resulting cultures were exposed to 800 MPa for 15 min there was a $< 3\text{-log}_{10}$ reduction, while there was a $> 9\text{-log}_{10}$ reduction of the control cells. The pressure-resistant strains displayed the same re-tailing behaviour after two subsequent sub-culturings. When they only did one round of pressure treatment and re-culturing (only two colonies) they did not find a significant difference in the pressure resistance of the regrown culture. Hence, they concluded that the pressure-resistant isolates were mutants, with multiple mutations, however they did not identify these mutations. They also showed increased heat resistance in two of the three isolates, but

only at 58 and 60°C, but not at 62°C. Karatzas and Bennik (2002) were also able to obtain HP-resistant *L. monocytogenes* mutants. In both these cases there was not a pressure-resistant sub-population, but rather there was one pressure-resistant mutant in the original population.

Noma et al. (2006) investigated the mechanism of tailing in HP-processed *E. coli* 0157:H7 at 300 MPa. They reported that a majority of cells were inactivated in the first 30 min, after which there existed a small subpopulation (ca. 10 cells) that persisted even after an hour of pressurization. Addition of chloramphenicol to the cells during pressure treatment did not reduce tailing. When they regrew the cells from the tail and re-pressurized they showed that the 'tail-culture' cells were more pressure resistant than the original culture (a ~2-$\log_{10}$ vs. 6-$\log_{10}$ CFU/g reduction). It is unclear whether all of the tailing cells were more pressure resistant, or if the enhanced pressure resistance was due to the presence of one mutant.

Biphasic inactivation kinetics during heat inactivation has been reported by Humpheson et al. (1998). They reported that tailing only occurred in high-density (> 1 x 10^7 CFU/ml) stationary phase cells. If the initial inoculum was reduced below 1 x 10^7 CFU/ml log-linear inactivation kinetics occurred. They calculated that the tailing cells represented 1 in 10^4 or 10^5 cells in the total initial population. After one round of re-growth or tailing cells following heating there was no enhanced heat resistance. They reported that addition of chloramphenicol (100 μg/ml) prior to heating reduced tailing. This indicates that tailing may have been due to synthesis of heat shock proteins during heating.

Biphasic inactivation kinetics during heating may display a shoulder followed by exponential killing (Obuchi et al., 1998), which as also been reported in high pressure inactivation kinetics. These two rates of inactivation, slow and then fast, have been attributed to two critical, targets involved in inactivation (Obuchi et al., 1998; 2000). Alternatively, Miles (2006) suggested that biphasic inactivation kinetics may be due to the number of copies of the critical component per cell. For example, ribosomes are present at ca. 10,000 per cell. The cell would still survive if most of the ribosomes were still functioning, resulting in a shoulder and die when the number of ribosomes fell below a critical level. These theories may also explain the presence of tailing in high pressure inactivation curves.

2.3.12 Modeling inactivation of microorganisms by HPP

There are many challenges to modeling the inactivation of microorganisms by pressure. For a start, there are wide differences in pressure resistance between different strains of organisms. This can make it difficult to predict the correct processing parameters (Patterson et al., 1995; Alpas et al., 1999; Tay et al., 2003). Inactivation also depends on the food in which the organism is treated. Some food matrices such as milk provide protection against high pressure, while acidic foods such as orange juice enhance inactivation (Alpas and Bozoglu, 2000; McClements et al., 2001). Furthermore, the mechanism underlying inactivation of microorganisms is unknown. Finally, tailing inactivation kinetics means that a D value cannot be generated using a traditional log-linear equation. There has been much work in the last ten years in trying to find suitable models to describe non-linear microbial inactivation.

Lee at al. (2001) used a biphasic model to describe the tailing inactivation kinetics of *E. coli* in whole egg subjected to HPP. There was tailing inactivation in the range 250-400 MPa in cells HP-processed at 5°C. To model the data they simply fit two log-linear lines to the curve. They had postulated that changes to the egg during processing may lead to tailing, as they found that egg protein coagulation usually corresponded to the beginning of tailing in cells processed at 5°C, but not 25°C.

Tailing inactivation kinetics has also been reported for inactivation of lactococcal bacteriophage by HPP (Avsaroglu et al., 2006). Of the models tested, Weibull model resulted in the best fit of the data. De Lamo-Castellví et al. (2005) studied inactivation of *Yersinia enterocolitica* in UHT skim milk and demonstrated tailing at 500 MPa. They used a quadratic model to fit the data. Panagou et al. (2007) studied the inactivation of *Pediococcus damnosus* by HPP. Pronounced tailing was observed at 600 and 650 MPa. The Weibull and biphasic models produced better fits than a linear model.

Chen (2007) conducted a survey of six foodborne pathogens and demonstrated tailing inactivation kinetics in all of them. Tailing generally occurred after 5- to 6-$\log_{10}$ reductions and was particularly pronounced in *L. monocytogenes*. The log-logistic model was the best model in all cases. Guan et al. (2004) reported that the log-logistic model provided the best fit to describe the tailing inactivation of *Salmonella* in milk.

Klotz et al. (2007) reported that plotting log survivors against the square root of time provided a good fit for many (ca. 70%) HPP inactivation curves. However, this model could not accommodate tailing, and tailing curves were fit by simply excluding the data points from the tail from the model. Based on their model Klotz et al. (2007) suggest that HPP inactivation of microorganisms may be dependent on a diffusion

process. They also suggest that tailing inactivation kinetics may be due to water loss for the cell during HPP or due to a slow change in the cell membrane, which leads to enhanced pressure resistance. They point out that there is a wide diversity in HP microbial inactivation curves and it is unlikely that a single model will account for all circumstances.

2.3.13 Combination treatments in HPP

Combination treatments, such as the application of heat or bacteriocins in conjunction with pressure, may be able to enhance inactivation of some microorganisms, and therefore may enhance safety and shelf life. The lactoperoxidase system in conjunction with HP (400 to 600 MPa) enhanced inactivation of *L. innocua, Enterococcus faecalis* and *L. plantarum*, but did not enhance inactivation of *E. coli, Salmonella* and *Pseudomonas fluorescens* (Garcia-Graells et al., 2003). Vurma et al. (2006) demonstrated that addition of the phenolic compound *tert*-butylhydroquinone prior to HPP increased the lethality of HP against *L. monocytogenes*.

2.3.14 Applications of HPP in the manufacture of dairy products

Initial studies show that HPP holds promise as a method of preserving milk. There are few modifications on renneting properties, native milk enzymes, and small molecules, such as flavors and nutrients (Kelly, 2000). One of the applications of HP-treated milk is for the production of premium soft cheeses, which are preferentially made from raw milk. High-pressure treatment may improve the properties of other dairy products such as yoghurt.

Pasteurization attenuates or activates the activity of milk enzymes such as the plasmin/plasminogin complex, lipases or alkaline phosphatase, and also produces slight denaturation of serum proteins and slight modifications in milk rennetability. Texture is an extremely important attribute in the identity of cheese. Pasteurization leads to cheese with a higher moisture content, which in turn is linked to lower scores for firmness (as compared to raw milk cheeses). There are rheological difference between raw and pasteurized milk cheese, which is attributed to interactions between whey protein and casein, to differences in the percentage of α_s-casein degradation, and to differences in dry matter content (Buffa et al., 2001b).

Rennet coagulation properties of milk can be enhanced by pressure. Cheeses made from HP-treated milk were reported to have higher moisture levels, and higher NaCl and lactose levels by Trujillo et al. (2000). HPP can help in reducing the great variability in moisture within a block of cheese or between different blocks of cheese, and generate new and desirable textures. HPP can accelerate the brining or ripening in certain cheeses and increase the shelf life of cheese, particularly when used in combination with preservatives such as nisin (Trujillo et al., 2000).

Buffa et al (2001) observed some chemical differences between raw milk, pasteurized milk, and HPP milk cheeses. On day one the HPP milk cheese had a higher moisture content and higher total free amino acid content than the other two types. Additionally on day 45 of sampling, the pH of the raw milk cheese was lower than that of the pasteurized or HPP milk cheeses. These findings agree with those of Trujillo et al. (1999). Buffa et al (2001) reported that there were no differences in salt or fat content and total nitrogen between HPP milk cheese and raw milk cheese. At the end of ripening

(45 days), the microbial counts obtained from pasteurized and HPP milk cheese were very similar.

Buffa et al. (2001b) reported that there were textural differences between raw, pasteurized and HPP milk cheeses. The differences were more pronounced at the beginning of ripening. The raw milk and HPP cheeses were firmer and less fracturable than pasteurized milk cheeses, while pasteurized and HPP milk cheeses were less cohesive. HPP milk cheeses had the most regular and close protein matrix, which closely resembled the microstructure of the raw milk cheeses. The microstructure of the pasteurized milk cheeses had an open structure with irregular cavities.

Buffa et al., (2001c) assessed the effect of milk treatment on lipolysis in goat milk cheeses. Lipolysis is extensive in hard cheeses and is essential for correct flavor development. The main free fatty acids (FFAs) present in milk were palmitic, myristic, capric and stearic acids. Raw and HPP (500 MPa for 20 min) milk cheeses had similar levels of lipolysis over 60 days, whereas pasteurized milk cheeses had no significant overall increase in FFAs. There were no differences in sensory scores for aroma, taste, bitterness, saltiness and spicy flavors between HPP and raw milk goat cheeses.

Capellas et at., (2001) also examined the effect of HPP on the physico-chemical characteristics (composition, whey loss, texture, color and microstructure) of goat milk cheese. HPP at 500 MPa did not significantly affect the cheese composition in comparison to raw milk cheese. However, there were some minor differences in the water retention capacity, texture and color, microstructure, non-protein nitrogen and the whey loss.

2.4. Differential Scanning Calorimetry

2.4.1 Introduction

Differential Scanning Calorimetry (DSC) measures the production (exothermic) or absorption (endothermic) of heat that occurs in a sample during a fixed rate of temperature increase or decrease. When the sample undergoes a physical transformation such a phase transition, more (or less) heat will need to flow to the sample in comparison to a reference in order to maintain both at the same temperature. Whether more or less heat flows to the sample depends on whether the process is exothermic or endothermic. For example, as a solid sample melts to a liquid more heat will need to flow to the sample to increase its temperature at the same rate as the reference. This is due to the absorption of heat by the sample as it undergoes the endothermic phase transition from solid to liquid. Likewise, as the sample undergoes exothermic processes (such as crystallization) less heat is required to raise the sample temperature. By observing the difference in heat flow between the sample and reference, differential scanning calorimeters are able to measure the amount of heat absorbed or released during such transitions.

DSC is a very sensitive method that can measure very small changes in specific heat (Cp). The results are plotted as a thermograph, with Cp on the y-axis and temperature on the abscissa. Positive deflections of Cp represent heat absorbed due to endothermic reactions; negative deflections are heat released due to exothermic reactions. The sample is scanned to determine at which temperatures transitions occur, and the sample can be rescanned to see whether the transitions are reversible or irreversible. A reference, typically buffer, is used to measure the relative Cp. The midpoints of the peaks are referred to as T_m. DSC can be used to investigate the folding and stability of

biomolecules, including proteins, nucleic acids and lipids. Typically proteins give broad transitions and lipids give sharp transitions. DSC is usually used to characterize isolated biopolymers, but has been successfully applied to live cells. Unlike many techniques in microbiology, DSC gives instantaneous information about the physiology of bacterial cells.

Gómez et al (1995) modeled the heat capacity of proteins in order to investigate the role of protein structure in heat capacity. Their results indicated that most of the heat capacity associated with a protein comes from its primary covalent structure, and contributions from vibrational frequencies arising from the stretching and bending modes of each covalent bond and internal rotations. At 25°C close to 85% of the heat capacity of a protein can be attributed to its primary structure. Hydration of the protein can account for 25-40% of the total heat capacity. Finally, noncovalent interactions arising from tertiary structure and side-groups accounts for ~3% of total heat capacity at 25°C. Unfolded proteins have a greater heat capacity than native proteins due to the exposure to the solvent of apolar groups that are buried in the native state. The heat capacity of proteins is also proportional to their molecular weight.

2.4.2 Using DSC to study microorganisms

Lepock at al (1990) used DSC to investigate the relationship between maximum growth temperature and thermal transitions in *Bacillus* spp. They used thermophilic, mesophilic and psychrotrophic strains and aimed to identify the critical targets for heat-induced inhibition of growth. They reported that they were able to resolve transitions for lipid melting, protein denaturation and DNA denaturation. Lipid transitions varied

according to growth temperature, and occurred at 22-33°C below the T_m. The transition temperature for DNA melting was 88-92°C, which was 22-32°C above T_m. The transition for DNA was partially reversible. The main part of the profile was associated with three irreversible peaks, which the authors attributed to protein denaturation. The onset temperature of these peaks was dependent on growth temperature. Lepock et al. (1990) pointed out that the peaks may not represent a distinct transition in a single component of the cell, but rather a sum of many components. They concluded that there was a correlation between the onset of protein denaturation and the maximum growth temperature for *Bacillus* spp. They also showed that growth temperature affects the temperature in which transitions in lipids and DNA occur.

Mackey et al (1991) used DSC to investigate whole cells and cell components of *E. coli*. They were able to elucidate ten peaks in whole cells. The T_ms in the thermograms of whole cells and thermograms of the isolated cellular constituents did not occur at the same temperatures; they reasoned that cell components may behave differently in buffer and in the cytoplasm. Although they tried to isolate "pure" cell components and run them individually to determine their T_m, running the isolated cell components did not always result in a single peak. Furthermore, some of the isolated cell components were contaminated with other cell components. For example the ribosome fraction had considerable protein contamination. They attributed melting of membrane lipids with a broad, partly reversible, peak that occurred at 20-40°C. DNA denaturation occurred at 95°C and was also partly reversible. The largest peak on the thermograph had a T_m of 71°C and they suggested that ribosomes were associated with this peak. However, this peak was also present in the ribosome-free extract, indicating that it is

probably protein. As proteins constitute ~55% of the cell mass it makes sense that the largest peak in the thermograph is protein. Other peaks reported by Mackey et al. (1991) are described in Table 2.

Anderson et al (1991) employed DCS to investigate the effect of salt on the thermal inactivation of *L. monocytogenes*. There was an excellent correlation between loss of viability and the first peak on the thermograph, T_m=68.6°C. Addition of salt to the buffer during DSC shifted the T_m of the first peak to a higher temperature. They showed that following heating of the cells for 2 or 5 min at 60°C, the first peak was not present. They point out that as the first peak was the largest peak, it is probably due to proteins as they are present in high concentrations in the cell. Thermographs revealed five peaks, details are in Table 2. Anderson et al. (1991) concluded that internal solute concentration, and/or water content are major factors in thermal tolerance of *Listeria* and other mesophilic bacteria.

Belliveau et al. (Belliveau et al., 1992) investigated thermal inactivation of *Bacillus* spores using DSC. Thermograms of whole spores showed three major endothermic peaks and one exothermic peak. The second endothermic peak, at 100°C, was associated with loss of cell viability. They calculated that the total enthalpy of the dormant spore was 25.9 J. Thermograms of activated spores and germinated cells were very different to those of dormant spores. They concluded that a crucial protein is the primary target in the heat killing of dormant spores, and its denaturation is represented by the second peak in the thermogram.

Mackey et al. (1993) used DSC to investigate thermal inactivation of *E. coli* and *Bacillius stearothermophilus*. They aimed to determine the temperature at which thermal

inactivation started, and link this to specific targets in the cell. They reported that 99% of *E. coli* were inactivated at 63°C, which corresponded to the first peak in the thermogram. For *Bacillius stearothermophilus,* 99% of the cells were inactivated at 76°C which corresponded to the first and second peaks of the thermogram. They concluded that denaturation of the 30S ribosomal subunit may play an important role in thermal inactivation, using the results of Mackey et al. (1991) to identify the peak.

Stephens and Jones (1993) investigated the effect of high salt and heat shock on the thermotolerance of *L. monocytogenes* using DSC. They reported that heat shock increased the stability of the first peak (T_m = 66.5°C) to subsequent heating, as compared to non-heat-shocked cells. Heat shock did not affect any other peaks. Addition of salt prior to heating resulted in the absence of the first peak from the thermogram, instead the second peak (T_m = 72°C) was much larger, indicating that it incorporated both peaks one and two. They concluded that denaturation of the 30S ribosomal subunit may play an important role in thermal inactivation, also using the results of Mackey et al. (1991) to identify the peak.

Teixeira et al (1997) used DSC to investigate thermal injury of *Lactobacillus bulgaricus*. They aimed to correlate loss of viability with specific denaturation events in the cell. They used several methods to investigate heat injury, including sensitivity to various antibiotics. They had seven peaks in the thermographs of whole cells, as outlined in Table 2. They also ran cell sub-fractions of ribosomes, lipids and cell walls. The largest peak occurred at 73°C, and they concluded that it represented ribosomes. They concluded that at temperatures below 65°C the cell membrane is the main target of

thermal inactivation. However, at 65°C and above ribosome and/or protein denaturation and cell wall damage may be the main cause of death.

Niven et al (1999) followed up on the work conducted by Mackey et al. (1991). They used DSC to investigate the effect of HPP on *E. coli*. They claimed to show a link between ribosome-associated enthalpy and cell death. Interesting, they did not detect the same four peaks over 61-81°C as reported by Mackey et al. (1991), but reported only two peaks. They concluded that changes in ribosome conformation due to HPP leads to cell death.

Mohácsi-Farkas et al. (1999) utilized DSC to study the thermal stability of *L. monocytogenes, E. coli and L. plantarum*. They also investigated the effects of pH and NaCl concentration on heat resistance of these bacteria. In contrast to Anderson et al. (1991), they reported that increasing the salt concentration to ~10% shifted the T_m of the peaks in the thermogram to lower temperatures, indicating increased thermal sensitivity. They showed an excellent correlation between the third peak in the thermogram, T_m= 66°C for *L. monocytogenes*, 63°C for *E. coli* or 56°C for *L. plantarum*, and loss of viability. However, the second and fourth peak also showed extremely high correlations to loss of cell viability. Mohácsi-Farkas et al. (1999) speculated that the third peak may correspond to protein denaturation during ribosome melting. They also demonstrated that the buffer employed and the scanning rate can affect the thermograph.

Obuchi et al. (2000) tried to identify critical targets in the thermal inactivation of *S. cerevisiae* using DSC. They hypothesized that biphasic inactivation kinetics in *S. cerevisiae* (due to shoulders) was due to the inactivation of two separate critical targets in the cell during heating. They were unable to determine the components responsible for

biphasic inactivation kinetics, but they did find that inactivation of the two components occurred just before and after the second peak in the thermogram. They concluded that the microsomal fraction of yeast cells may contain the critical targets for thermal inactivation.

Bayles et al. (2000) utilized DCS to investigate cold shocked *L. monocytogenes*. They demonstrated that cells grown at 37°C and cold shocked at 0°C for 3 h were more heat sensitive to 60°C than non-cold shocked cells. DSC thermograms showed two main peaks with T_ms at 67.5 and 73.5°C. T_ms in cold shocked cells shifted down to 66.8 and 72.1°C, the later being significantly different from the control. Antibiotic treatment, with kanamycin or tetracycline, also shifted the T_ms. They conclude that the ribosomes are involved in thermal resistance, and that ribosomes are altered by cold shock.

Lee and Kaletunç (2002b) used DSC to investigate thermal inactivation of *E. coli* and *L. plantarum*. They used plate count data and colorimetric data to investigate the heat resistance of ribosomes in the two organisms. They observed six major peaks in the thermogram, the main peak had a T_m of 70°C in *E. coli* or 63°C in *L. plantarum*. Isolated ribosomes resulted in a thermogram with one peak, T_m= 74°C in *E. coli* or 71°C in *L. plantarum*. Pre-treatment of the cells at high temperatures in the DSC changed the appearance of the subsequent thermograph; as the pre-treatment temperature was increased the peaks became smaller. Pre-treatment at 85°C and above practically eliminated all of the peaks. There was a correlation between loss of viability and the second peak on the thermogram, they attributed the peak to ribosomes. Furthermore, there was a relationship between heat resistance and the shape of the thermographs.

Peaks for *L. plantarum*, which had lower heat resistance, had lower T_ms than those of *E. coli*.

Lee and Kaletunç (2002a) used DSC to determine D (decimal reduction time; the time needed to reduce the population by one log) and z (temperature change required for a 1-log reduction in D) values for the thermal inactivation of *E. coli*. The D values calculated using both the apparently enthalpy and viability data for cells heat treated in the DSC were similar to D values obtained from isothermal treatment. They also showed that pre-heating the cells significantly altered the shape of the thermograms.

Table 2.2 Summary of DCS results from microbial studies

Study	Organism	Treatment	Tm of endothermic peaks (°C)	Reversible?	Cell constituent (Speculated)
Lepock et al (1990)	*Bacillus* spp (6 strains)	Varying growth temperatures	25-30	N	Lipid
			50-65	N	Metabolism
			34-73	N	Protein
			54-81	N	Protein
			71-87	N	Protein
			88-92	Y	DNA
Mackey et al (1991)	*E. coli*	Whole cells and cell components	20-40	NR*	Lipid
			47	NR	Ribosome 30S subunit, proteins
			61	NR	NR
			71	NR	NR
			75	NR	NR
			81	NR	NR
			95	NR	DNA
			95	NR	Cell wall
			118	NR	Cell envelope
			125	NR	DNA?
Anderson et al (1991)	*L. monocytogenes*	Thermal inactivation Salt concentration	68.6	N	NR
			75.3	N	NR
			81.5	N	NR
			92-93	Y	DNA
Belliveau et al (1992)	*Bacillus* dormant spores	None	56	N	Heat of germination
			100	N	Protein
			114	N	Spore coat
			119 (exo)	N	NR

Study	Organism	Treatment	Tm of endothermic peaks (°C)	Reversible?	Cell constituent (Speculated)
Belliveau et al (1992)	Bacillus vegetative cells	None	72.3	N	NR
			82.5	N	NR
			91	Y	DNA
			107	N	NR
			105.3	N	NR
Teixeira et al (1997)	*Lactobacillus bulgaricus*	Thermal inactivation	35	N	Cell wall
			51	Y	Lipids
			61	NR	NR
			73	Y	Ribosomes
			80	NR	Ribosomes
			89	Y	NR
			100	N	DNA
			112	N	NR
Niven et al (1999)	*E. coli*	None	69	NR	Ribosomes
			81	NR	Ribosomes
Mohácsi-Farkas et al (1999)	*L. monocytogenes*	None	~25	N	NR
			~65	N	NR
			~73	N	Protein denaturation (in ribosomes)
			~80	N	NR
			~88	Y	DNA

Study	Organism	Treatment	Tm of endothermic peaks (°C)	Reversible?	Cell constituent (Speculated)
Bayles et al (2000)	*L. monocytogenes*	Cold shock and thermal inactivation	67.5	NR	30S subunit
			73.4	NR	50S subunit and 70S particle
Lee and Kaletunç (2002)	*E. coli/ L. plantarum*	None	56/-	NR	Ribosome 30S subunit
			70/63	NR	NR
			77/75	NR	NR
			94/93	NR	NR
			105/100	NR	Cell wall
			120/-	NR	NR

* NR = Not Reported

2.5 Stress Responses in Bacterial Cells

2.5.1 The general stress response

Stresses encountered by microorganisms vary in magnitude and outcome. Stress may be mild, not resulting in reduced cell numbers but arresting the growth rate. Moderate stress arrests microbial growth rate and may also result in reduced cell viability. Damage to cellular components by stresses may also sensitize the cells to mildly deleterious factors, a phenomenon commonly referred to as injury. Injury is usually detected when stress-exposed cells become sensitive to selective agents that healthy cells readily survive (Yousef and Courtney, 2003).

Activation of the general stress response usually results in reduced growth rate or entry into stationary phase (Hengge-Aronis, 1999). The best characterized general stress response systems are controlled by the alternative sigma factors, σ^s and σ^B. *E. coli*, which uses σ^s, is the model for Gram-negative bacteria, and *B. subtilis*, which uses σ^B, is the model for Gram-positive bacteria (Yousef and Courtney, 2003). The general stress response induces multiple physiological changes in the cell including resistance to multiple stresses, the accumulation of storage compounds, changes in the cell envelope composition and altered overall morphology (Hengge-Aronis, 1999).

General stress response in Gram-positive bacteria is controlled by σ^B, a transcription factor which is activated by a diverse range of stresses to control the synthesis of more than 100 stress proteins (Price, 2000). Genes controlled by σ^B are involved in protecting cellular DNA, protein and lipid against the deleterious effects of various stresses (Price, 2000). The expression of genes under σ^B control can account for 25-35% of new protein synthesis under stress conditions. Many genes are under the dual

control of σ^B and another σ factor (Price, 2000). σ^B transcription is activated in response to two broad classes of stresses: (i) energy stress, and (ii) environmental stress (Price, 2000). It is currently thought that these two classes of stress are conveyed to σ^B by separate signal transduction pathways (Price, 2000). Many general stress proteins require σ^B for full expression and loss of σ^B leads to increased sensitivity to multiple stresses (Price, 2000).

σ^B activity is negatively controlled by the association of a specific anti-σ factor, RsbW (regulator of sigma-B). Upon release from RsbW, σ^B associates with RNA polymerase core enzyme to direct the transcription of the general stress response. RsbW is in turn regulated by the anti-anti-σ factor RsbV (Price, 2000). This network functions by a "partner switching" mechanism, in which the key protein-protein interactions are controlled by serine phosphorylation and dephosphorylation (Price, 2000). Only one other σ factor *in B. subtilis* is known to be regulated by the partner switching mechanism, the σ^F factor which is involved in sporulation (Price, 2000).

The *sigB* operon contains eight genes related to σ^B function, and the order is identical in *Bacillus subtilis* and *L. monocytogenes* (Price, 2000). A σ^A-like promoter controls the *sigB* operon while an internal σ^B dependent promoter is responsible for the induction of four downstream genes. Three of these genes encode RsbV, RsbW and σ^B. The fourth product is RsbX, which appears to function in a negative feedback loop that dampens continued environmental signalling (Price, 2000).

Some of the genes under σ^B control are themselves known to code for regulatory proteins (Price, 2000). These include ClpP and ClpC, which play essential roles in adaptive responses such as motility, competence development, and sporulation (Price,

2000). ClpC belongs to the HSP 104 family of heat shock proteins, members of which have a dual function: they manifest chaperone activity of their own and are also involved in targeting dispensable or damaged proteins for degradation by a protease moiety such as ClpP. ClpC could therefore serve to sort damaged proteins, restoring the less impaired and presenting the terminally damaged for destruction by the ClpP protease. Consistent with this role, growth of *clpP* and *clpC* null mutants is impaired at elevated temperatures.

Protection of cellular protein, DNA, and lipid against reactive oxygen species following growth limiting energy or environmental stress are thought to be a major role of the σ^B regulon (Price, 2000). In addition to ClpC and ClpP, the known or suspected functions of a number of σ^B-dependent genes are consistent with this role (Price, 2000). KatB and KatX catalases are specifically expressed under growth-limiting conditions (Price, 2000).

In general, bacterial sensing of environmental changes in not well understood. Some stresses may affect folding of mRNA or proteins, or change a protein's half-life, resulting in changes in gene expression (Yousef and Courtney, 2003). For example, the CspA mRNA involved in cold tolerance is extremely unstable at 37°C, but dramatically stabilized at lower temperatures (Yousef and Courtney, 2003). Levels of certain metabolites, such as guanosine phosphates, may also trigger the synthesis of stress-related proteins (Yousef and Courtney, 2003). Two-component signal transduction systems, consisting of membrane-associated sensor kinase and an intracellular response regulator, have been implicated in the sensing of and response to some stresses. In *B. subtilis* a two-component system is involved in the expression of cold-inducible genes (Aguilar et al., 1998).

2.5.2 Cold Shock

Exposure to cold temperatures induces a number of physiological changes in microorganisms. These include changes in the membrane fatty acid composition to promote optimum membrane fluidity (Nichols et al., 2002), synthesis of DNA- and RNA-binding proteins that counteract the stabilizing effect of cold temperatures on nucleic acid secondary structures (Yousef and Courtney, 2003), and importation of compatible solutes (Wemekamp-Kamphuis et al., 2002). *L. monocytogenes* transports the compatible solutes betaine and carnitine in response to low temperatures (Wemekamp-Kamphuis et al., 2002).

There are two classes of proteins involved in the response to cold, cold shock proteins (Csps) and cold-shock acclimation proteins (Caps). Csps are rapidly and transiently over-expressed in response to cold. Caps are synthesized during continuous growth at cold temperatures; they are rapidly induced, but remain over-expressed several hours after temperature downshift. A slow temperature downshift results in the synthesis of both (Yousef and Courtney, 2003).

Cold shock also causes stabilization of the hydrogen bonds in nucleic acid secondary structures resulting in reduced efficiency of translation, transcription and DNA synthesis. CspA, the major cold shock protein in *E. coli*, is proposed to regulate gene expression by functioning as an RNA chaperone at low temperatures (Yousef and Courtney, 2003).

2.5.3 Heat shock

All organisms contain HSPs and this family of proteins is highly conserved amongst prokaryotic and eukaryotic organisms. HSPs probably do not contribute to thermotolerance during heating, but they are required for recovery from heat stress. Heat shock proteins can be synthesized within 1 min of exposure to sublethal heat. Heat causes damage to macromolecular cell components, thus the main function of heat-induced stress proteins is to repair or destroy these damaged components so they do not interrupt cellular metabolism. Many heat-induced stress proteins are protein chaperones that assist in folding and assembly of heat-damaged proteins (e.g. GroEL and DnaK) or are ATP-dependent proteases that degrade damaged proteins (e.g. Lon and ClpAP).

In *E. coli*, induction of HSPs occurs primarily due to a transient increase in σ^{32} (encoded by *rpoH*). The increase in σ^{32} results primarily from two distinct events: (i) the translational induction due to the temperature melting of the *rpoH* mRNA secondary structure, and (ii) transient stabilization of σ^{32}, which is normally unstable (Yura et al., 2000). There is a second regulon involved in the heat shock response, which is controlled by σ^{E}.

Gram-positive bacteria differ markedly from Gram-negative bacteria in their regulation of heat shock response (Yousef and Courtney, 2003). Four classes of heat shock genes have been identified in *B. subtilis,* which is representative for other low G + C Gram-positive bacteria. Class I consists of the *dnaK* and *groE* operons, which encode the major chaperones DnaK, DnaJ, GrpE, GroEL and GroES (Yura et al., 2000). These genes are specifically induced by heat shock. Expression of the genes is controlled by σ^{A}-dependent promoters which are under the negative regulation of HrcA. The HrcA

repressor exerts its activity through binding to the operator, a conserved 9-bp inverted repeat with a 9-bp spacer, called CIRCE (controlling IR of chaperone expression). This regulatory system is widespread within the bacterial kingdom and has been described in more than 40 different species. Both the DnaK-DnaJ and GroEL-GroES chaperones are likely to play major roles in assisting protein folding in all bacteria (Yura et al., 2000).

Class II genes encode the general stress proteins whose synthesis is induced by either environmental or metabolic stresses. These are the largest group of heat-induced genes in *B. subtilis* with more than 50 members (Yura et al., 2000). The heat shock induction of Class II genes are wholly under σ^B control (Price, 2000). These genes are frequently under the control of another σ factor. It is common for the second promoter to have σ^A-like recognition sequences, but dependence of the second promoter on σ^F, σ^H, and σ^X has also been reported (Price, 2000).

Class III genes include the CtsR regulon which encodes the *clpP* and *clpC* genes. This operon is under dual control of an upstream σ^B-dependent promoter and a downstream σ^A–like promoter. The highly conserved Clp proteins are negatively regulated by the CstR repressor (Price, 2000). CstR binds to a specific sequence in the promoter region upstream of *clp* genes, *clpP, clpE* and *clpC*. These three genes are components of the Clp protease system which degrades damaged proteins. Class III genes are important in the survival and virulence of pathogens (Yura et al., 2000).

Finally, Class IV includes the rest of the heat-inducible genes such as *htpG*, *ftsH*, and *lon*, controlled by as yet undefined mechanisms.

The increased synthesis of HSPs usually occurs 5 to 60 min after heat shock and declines with the onset of normal protein synthesis 60 to 90 min after return to normal

temperatures (Juneja and Novak, 2003). For mesophilic bacteria, temperatures between 45 to 50°C are optimum for the development of the heat shock response. Molecular chaperones constitute 15 to 20% of the total cellular protein in response to elevated temperatures. The primary function of the classic chaperones, *E. coli* DnaK, DnaJ, GrpE, GroEL (Hsp60) and GroES is to bind to and stabilize polypeptides already present in cells, modulate protein folding pathways to prevent misfolding or aggregation of proteins, and promote refolding and proper assembly.

2.5.4 Stress response and heat shock in *L. monocytogenes*

The available evidence suggests that the σ^B regulon is not directly involved in the interaction of *L. monocytogenes* with its host but rather σ^B's function is important for environmental persistence (Price, 2000). The *sigB* operon has an identical gene order to *B. subtilis*. Loss of σ^B function has no obvious effect on the spread of *L. monocytogenes* to mouse liver or spleen following intragastric or intraperitoneal infection. Cold stress induces σ^B activity. σ^B also contributes to enhanced osmotolerance, and acid resistance.

Sublethal heat shock of *L. monocytogenes* increases thermotolerance to subsequent lethal heat treatments. The exact mechanism of this action is not understood. *L. monocytogenes* has been shown to produce 12 to 14 types of HSPs.

Pagan et al (1997) reported that heat shock at 40, 43 or 46°C all increased resistance of *L. monocytogenes* to subsequent heating at 65°C. Thermotolerance increased with the duration of heat shock, up to 120 min. In cells grown at 37°C thermotolerance increased fourfold; in cells grown at 4°C it increased 7 fold. They also

showed that addition of salt to the plating medium inhibited the growth of injured cells. Although heat shock increased the D value, there was no effect on the z value.

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CHAPTER THREE

THE EFFECTS OF GROWTH TEMPERATURE AND GROWTH PHASE ON THE INACTIVATION OF *LISTERIA MONOCYTOGENES* IN WHOLE MILK SUBJECT TO HIGH PRESSURE PROCESSING*

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3.1 Abstract

The aim of this study was to explore the effect of a wide range of growth temperatures, growth phases and plating media on the inactivation of *Listeria monocytogenes* by high pressure processing (HPP). In part one, *L. monocytogenes* was grown to mid-stationary phase at 4, 15, 25, 35 or 43°C, inoculated into whole UHT milk at ~10^7 CFU/ml and high pressure processed at 400 MPa at room temperature (20-25°C). Afterward, the HPP milk was plated on Tryptic Soy Yeast Extract Agar (TSYEA) and Modified Oxford Agar (MOX) to determine the degree of injury. For part two, cells were grown to mid-exponential, late-exponential or mid-stationary-phase at 15 or 43°C and processed in the same way. Time to reach a 5-$\log_{10}$ reduction was determined and data were analyzed by ANOVA. The results from part one showed that both growth temperature and plating medium had a significant effect ($P < 0.001$) on the inactivation of stationary-phase *L. monocytogenes* by HPP. Tukey's pairwise comparisons revealed that the effects of all temperatures, except 35 and 43°C, were significantly different ($P < 0.05$). Cells grown at 15°C were most sensitive to HPP, followed by cells grown at 4, 25 or 35°C, with cells grown at 43°C appearing to be most resistant. Inactivation of cells grown at 4, 15 or 25°C followed first order kinetics, whereas cells grown at 35 or 43°C displayed nonlinear inactivation kinetics due to tailing. In part two, both growth phase and plating medium had significant effects on the inactivation ($P \leq 0.001$) of *L. monocytogenes* by HPP. Cells grown at 15°C to mid-stationary-phase were significantly more resistant than cells grown at the same temperature to mid- or late-exponential-phase ($P < 0.05$). There was no significant difference between mid- and late-exponential-phase cells grown at 15°C. When cells were grown at 43°C, mid-exponential-phase cells were

significantly more sensitive ($P < 0.05$) than either late-exponential or mid-stationary-phase cells, with no difference between late-exponential or mid-stationary-phase cells. It was postulated that membrane composition, stationary-phase proteins and/or stress proteins may affect pressure resistance.

Key Words: High pressure processing (HPP); *Listeria monocytogenes*; Whole milk; Growth temperature; Growth phase; Injury, Inactivation kinetics

3.2 Introduction

High pressure processing (HPP) is a non-thermal food preservation method that utilizes hydrostatic pressures in the range of 300-700 MPa (Stewart and Cole, 2001). Effects of HPP on foods include the inactivation of microorganisms, protein denaturation, enzyme activation or inactivation, and retention of quality and freshness (Knorr, 1993). HPP causes disruption of hydrophobic and ionic bonds but not covalent bonds, so many small molecules in foods, including flavor compounds and vitamins, are left intact (Farr, 1990). Foods processed by high pressure may then be of superior quality as compared with their thermally processed counterparts. HPP can therefore be used to meet current consumer demands for 'fresh', preservative free and minimally processed foods. HPP-treated salsas, guacamole, oysters and deli meats are available in the U.S. Despite numerous outbreaks of foodborne illness associated with the consumption of raw milk and raw milk cheeses (Headrick et al., 1998; Mazurek et al. 2003), many consumers believe that raw milk has superior taste and is healthier than pasteurized milk. In recent years consumer consumption of raw milk and raw milk products has increased (Gish,

2004). Therefore, a potential application of HPP could be for the elimination of pathogens from raw milk and allow for the production of safe non-thermally pasteurized soft cheeses.

Listeria monocytogenes is a ubiquitous foodborne pathogen which causes the rare, but potentially fatal, disease listeriosis. The elderly, pregnant women and immunocompromised individuals are at a much higher risk of contracting listeriosis than the general community (Farber and Peterkin, 1991). Listeriosis is most commonly associated with consumption of raw milk, soft cheeses, hot dogs and deli meats (Sutherland and Porritt, 1997). *L. monocytogenes* is Gram positive, rod shaped, facultatively anaerobic, psychrotrophic and relatively resistant to salt, drying and low pH (Seelinger and Jones, 1986). Due to its ability to grow at refrigeration temperatures in many ready-to-eat (RTE) foods, it is extremely important that *L. monocytogenes* be eliminated from these foods.

A variety of factors can affect the sensitivity of microorganisms to HPP, including the species and strain of the microorganism (Alpas et al., 1999; Benito et al., 1999; Robey et al., 2001), the growth temperature and growth phase prior to HPP (McClements et al., 2001; Casadei et al., 2002; Bull et al., 2005), the composition of the suspending medium (Patterson et al., 1995; Simpson and Gilmour 1997), the presence of antimicrobial compounds in the suspending medium (Patterson and Kilpatrick, 1998; Alpas et al., 2000; Chen and Hoover, 2003), and the magnitude and duration of the applied pressure (Simpson and Gilmour, 1997; Alpas et al., 1998; Lucore et al., 2000; Ritz et al., 2000; Jordan et al., 2001). The effects of pressure on microorganisms in foods are also influenced by food components such as fat, sugars and minerals (Garcia-Graells

et al., 1999, Black et al., 2007). Very little has been reported on the effect of prior growth conditions on the pressure resistance of microorganisms. It has been shown that growth temperature affects the pressure sensitivity of *L. monocytogenes, Bacillus cereus* and *Pseudomonas fluorescens* in milk (McClements et al., 2001) and *Escherichia coli* in broth (Casadei et al., 2002). McClements et al. (2001) reported that exponential- and stationary-phase cells of *L. monocytogenes* grown at 30°C were more resistant to HPP at 400 MPa than cells grown at 8°C. Bull et al. (2005) recently reported that stationary phase cells of *L. monocytogenes* grown at 43°C were more resistant to HPP (400 MPa) than cells grown at 15°C.

L. monocytogenes can be injured during high pressure processing, which is usually detected by growth of cells on non-selective, but not on selective media (McClements et al., 2001). It is important to detect HPP-injured cells, as they can recover rapidly in milk during storage (Hayman, 2001; Bozoglu et al., 2004; Bull et al., 2005). McClements et al. (2001) reported that counts of *L. monocytogenes* on a non-selective agar were higher than on a selective agar following HPP treatment of UHT milk.

The objective of this study was to explore the effect of a wider range of growth temperatures and growth phases on inactivation of *L. monocytogenes* subject to HPP as determined on both a selective and a non-selective medium. In addition, enumeration rather than enrichment was used to determine the inactivation kinetics of *L. monocytogenes* in UHT whole milk by high pressure processing.

3.3 Materials and Methods

A strain of *L. monocytogenes* serotype 4b (ATCC 19115) was maintained in glycerol stock at -80ºC. All media were obtained from Difco (Becton, Dickinson and Company, Sparks, MD, USA) unless otherwise stated. A loopful of glycerol stock was streaked onto Tryptic Soy Agar supplemented with 0.6% Yeast Extract (TSYEA). The plate was incubated at 35°C overnight, and then one colony was inoculated into 10 ml of Tryptic Soy Broth supplemented with 0.6% Yeast Extract (TSYEB) which was then incubated at 35°C for 18-24 hours. The resulting culture was diluted to ~10^7 CFU/ml in 0.1% peptone water, and 1 ml of diluted culture was added to 100 ml of TSYEB.
For part one broths were incubated at 4, 15, 25, 35 or 43°C, for 2 weeks, 72 h, 24-26 h, 14-16 h or 14 h respectively, to obtain mid-stationary-phase cells (growth curves at each growth temperature were determined in advance by both plating and absorbance, data not shown). Growth phase was subsequently confirmed by absorbance at 625 nm in all experiments. One ml of stationary-phase culture was added to 100 ml Paramlat UHT whole milk at 4°C to obtain an initial inoculum of ~10^7 CFU/ml.

Tips were aseptically cut off 3.1 ml plastic transfer pipettes (Sigma-Aldrich, Milwaukee, WI, USA) and 5 ml of inoculated milk was aseptically added to each plastic pipette bulb using a sterile syringe. Pipette bulbs were heat sealed by melting in a Bunsen flame and pressing with a heat sealer (MP-12, Impulse; Midwest Pacific, St. Louis, MO, USA). Sealed pipette bulbs were placed in 4”x 6” stomacher bags (VWR, West Chester, PA, USA) containing 1% chlorine and then heat sealed.

Samples were processed in a 2-L HPP unit (Avure Technologies, Kent, WA, USA) at 400 MPa for various times at room temperature (20-25°C), with water as the

pressure-transmitting fluid. It took ~100 s to reach 400 MPa and decompression occurred in < 10 s. There were 4-6 replicates for each time/growth temperature combination. Samples were kept on ice before and after processing and were plated within two hours of processing. Samples were spread on plates of TSYEA and Modified Oxford Agar (MOX), which where incubated at 35°C for 48 h. Samples were diluted with 0.1% peptone water before plating.

For part 2 cells were grown to mid-exponential, late-exponential or mid-stationary-phases at 15°C for 30, 50 or 72 h respectively; or at 43°C for 5, 10 or 14 h respectively. When incubation was complete, 30 ml of each culture was centrifuged at 4°C for 15 min at 13,000 x *g*, the pellet suspended in 0.1% peptone water, and then added to UHT whole milk to achieve an initial inoculum of ~ 10^7 CFU/ml. Samples were processed, and *L. monocytogenes* were enumerated, as described above.

In part one the mean number of cells in inoculated milk samples incubated at 4, 15, 25 or 35°C was ~10^7 CFU/ml and the initial concentration of cells grown at 43°C was ~10^8 CFU/ml. In part two the mean number of cells was ~10^7 CFU/ml in all samples.

For part one, D values were calculated where lines were log-linear by taking the inverse of the slopes of trend lines generated in Microsoft Excel. These rates were compared by one way ANOVA and Tukey's pairwise comparisons using Minitab software (State College, PA, USA). For all data in parts one and two, times to obtain a 5-$\log_{10}$ reduction (X) was calculated using the formula $\frac{Y_2 - Y}{X_2 - X} = \frac{Y - Y_1}{X - X_1}$, where Y is log CFU/ml after a 5-$\log_{10}$ reduction, X_1 is the time that resulted in less than a 5-$\log_{10}$ reduction, X_2 is the time that resulted in greater than a 5-$\log_{10}$ reduction, Y_1 is CFU/ml at

X_1 and Y_2 is CFU/ml at X_2). Data were analyzed using the ANOVA general linear model. The model using data from part one was Time to achieve 5-$\log_{10}$ reduction = Overall mean + Growth Temperature + Media +Media*Temperature + Error. The model using data from part two was Time to achieve 5-$\log_{10}$ reduction = Overall mean + Growth Phase + Media +Media*Growth Phase + Error. Tukey's pairwise comparisons were also generated using Minitab. The critical value was set at $\alpha = 0.05$.

The number of injured cells was defined as the difference between CFU/ml determined on TSYEA and CFU/ml determined on MOX. Percent injury was defined as (injured cells ÷ total cells) x 100.

3.4 Results

Low levels of inactivation of *L. monocytogenes* occurred during the come-up time, with less than 1-$\log_{10}$ inactivation observed in all samples. Some of the curves exhibited log-linear and others non-log-linear inactivation kinetics (Figures 3.1, 3.2 & 3.3), therefore regression was not used to compare the curves. Instead, a time-to-inactivation approach was used; with times for a 5-$\log_{10}$ reduction in numbers of *L. monocytogenes* being used to compare the results statistically. Curves were assumed to be log-linear over the time to achieve a 5-$\log_{10}$ reduction.

ANOVA of the data from part one showed that both growth temperature and plating medium significantly affected the time to obtain a 5-$\log_{10}$ reduction ($P < 0.001$), but the interaction between growth temperature and medium was not significant. Cells grown at 15°C were the most sensitive to HPP at 400 MPa (Fig. 3.1). Pairwise comparisons showed that times to achieve a 5-$\log_{10}$ reduction were different ($P < 0.05$)

for all growth-temperature pairs except 35°C and 43°C. Inactivation of *L. monocytogenes* grown at 4, 15 and 25°C and processed at 400 MPa followed first order kinetics (Fig. 3.1). However, inactivation of cells grown at 35 and 43°C did not follow first order kinetics, due to tailing. Inactivation rates were calculated where log-linear inactivation was observed. For numbers recorded on TSYEA the mean D values were 27.0 s at 4°C, 16.4 s at 15°C and 42.0 s at 25°C. ANOVA confirmed that inactivation rates were different ($P < 0.001$), and the Tukey test confirmed that all pairs of rates were different ($P < 0.05$). On MOX the average D values were 24.2, 13.3 and 34.0 s, at 4, 15 and 25°C respectively, and growth temperature significantly affected inactivation rate ($P < 0.001$). The Tukey test showed incubation at 15°C to be different from incubation at 4 or 25 °C, but incubation at 4 or 25°C were not different from each other.

Results from part two showed that both growth phase and plating medium significantly affected time to achieve a 5-$\log_{10}$ reduction ($P \leq 0.001$). The interaction between growth phase and plating medium was not significant at 15 or 43°C. Cells grown at 15°C to mid-stationary-phase were the most pressure resistant on both types of media, and were significantly more resistant ($P < 0.05$) than cells grown to the mid- or late-exponential-phase (Fig. 3.2). There was no significant difference in pressure resistance between mid-exponential-phase and late-exponential-phase cells. The results differed slightly when cells were grown at 43°C. Mid-exponential-phase cells were significantly ($P < 0.05$) more pressure sensitive than late-exponential-phase and mid-stationary-phase cells, which were not significantly different from each other with respect to pressure sensitivity. Inactivation of mid-exponential-phase cells grown at 43°C was

log-linear ($R^2 > 0.75$); whereas tailing ($R^2 < 0.75$) was generally observed in late-exponential and mid-stationary-phase cells (Fig. 3.3).

Parts one and two both investigated the effect of a selective vs. a non-selective medium on the time determined for a 5-$\log_{10}$ reduction of *L. monocytogenes* by HPP. Time to reach a 5-$\log_{10}$ reduction was always less with numbers determined on MOX, indicating that injury occurred regardless of growth temperature or growth phase. Counts were the same on TSYEA and MOX initially and at 1 second. In general, injury increased as processing time increased (Tables 3.1 and 3.2). Injury was also influenced by growth phase (Table 3.2).

Figure 3.1 Effects of growth temperature and plating medium on the numbers of *Listeria monocytogenes* recovered after high pressure processing (HPP) at 400 MPa. *L. monocytogenes* was grown to mid-stationary phase in Tryptic Soy Yeast Extract Broth at 4°C (●), 15°C (■), 25°C (▲), 35°C (♦) or 43°C (▼). Whole UHT milk was inoculated with *L. monocytogenes* to ~10^7 CFU/ml and HPP at 400 MPa was applied at ambient temperature (20-25°C) for various times. After HPP milk samples were spread on plates of Tryptic Soy Yeast Extract Agar (A) or Modified Oxford Agar (B) which were incubated at 35°C for 48 h.

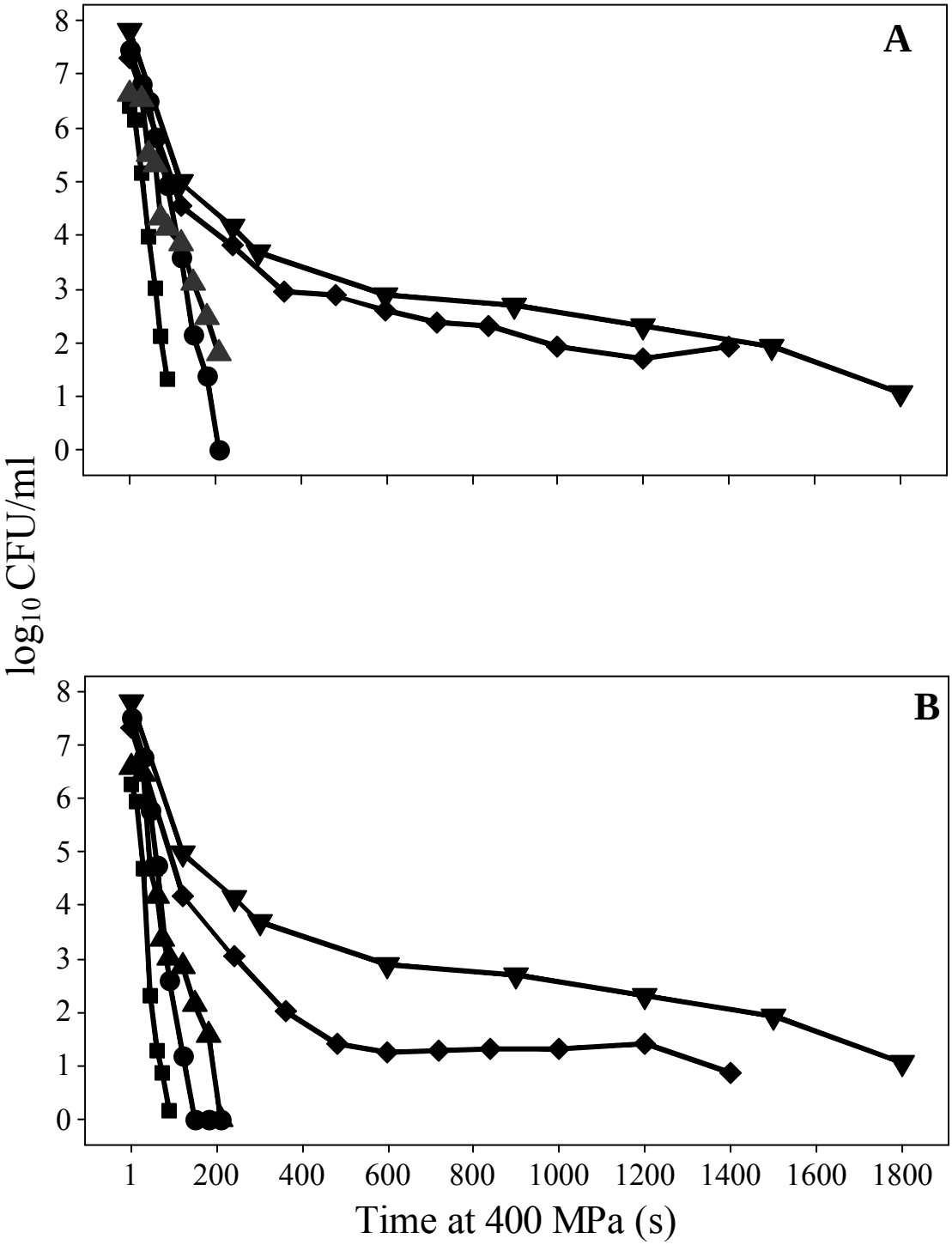
A
B
log10 CFU/ml
Time at 400 MPa (s)
0
1
2
3
4
5
6
7
8
1
200
400
600
800
1000
1200
1400
1600
1800

Figure 3.2 Effects of growth phase and plating medium on the numbers of *Listeria monocytogenes* recovered from UHT whole milk after high pressure processing (HPP) at 400 MPa. *L. monocytogenes* was grown in Tryptic Soy Yeast Extract Broth to mid-exponential (●), late-exponential (▲) or mid-stationary-phase (■) at 15°C. Whole UHT milk was inoculated with *L. monocytogenes* to ~10^7 CFU/ml and HPP at 400 MPa was applied for up to 180 s at ambient temperature (20-25°C). After HPP milk samples were plated onto Tryptic Soy Yeast Extract Agar (A) or Modified Oxford Agar (B) and incubated at 35°C for 48 h.

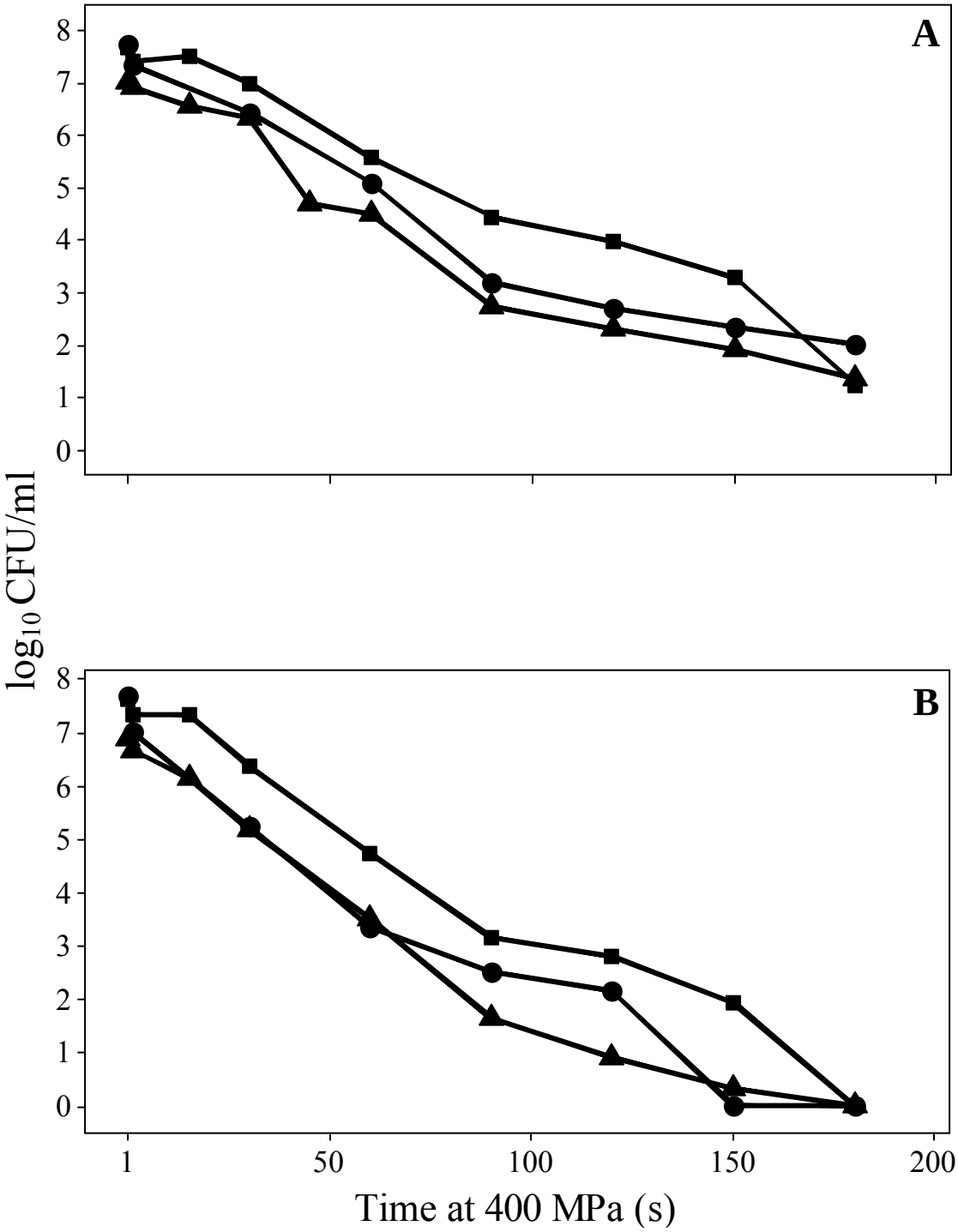
A
B
log10CFU/ml
0
1
2
3
4
5
6
7
8
1
50
100
150
200
Time at 400 MPa (s)

Figure 3.3 Effects of growth phase and plating medium on numbers of *Listeria monocytogenes* recovered from UHT whole milk after high pressure processing (HPP) at 400 MPa. *L. monocytogenes* was grown in Tryptic Soy Yeast Extract Broth to mid-exponential (●), late-exponential (▲) or mid-stationary-phase (■) at 43°C. Whole UHT milk was inoculated with *L. monocytogenes* to ~10^7 CFU/ml and HPP at 400 MPa was applied for up to 1500 s at ambient temperature (20-25°C). After HPP milk samples were spread on plates of Tryptic Soy Yeast Extract Agar (A) or Modified Oxford Agar (B) and incubated at 35°C for 48 h.

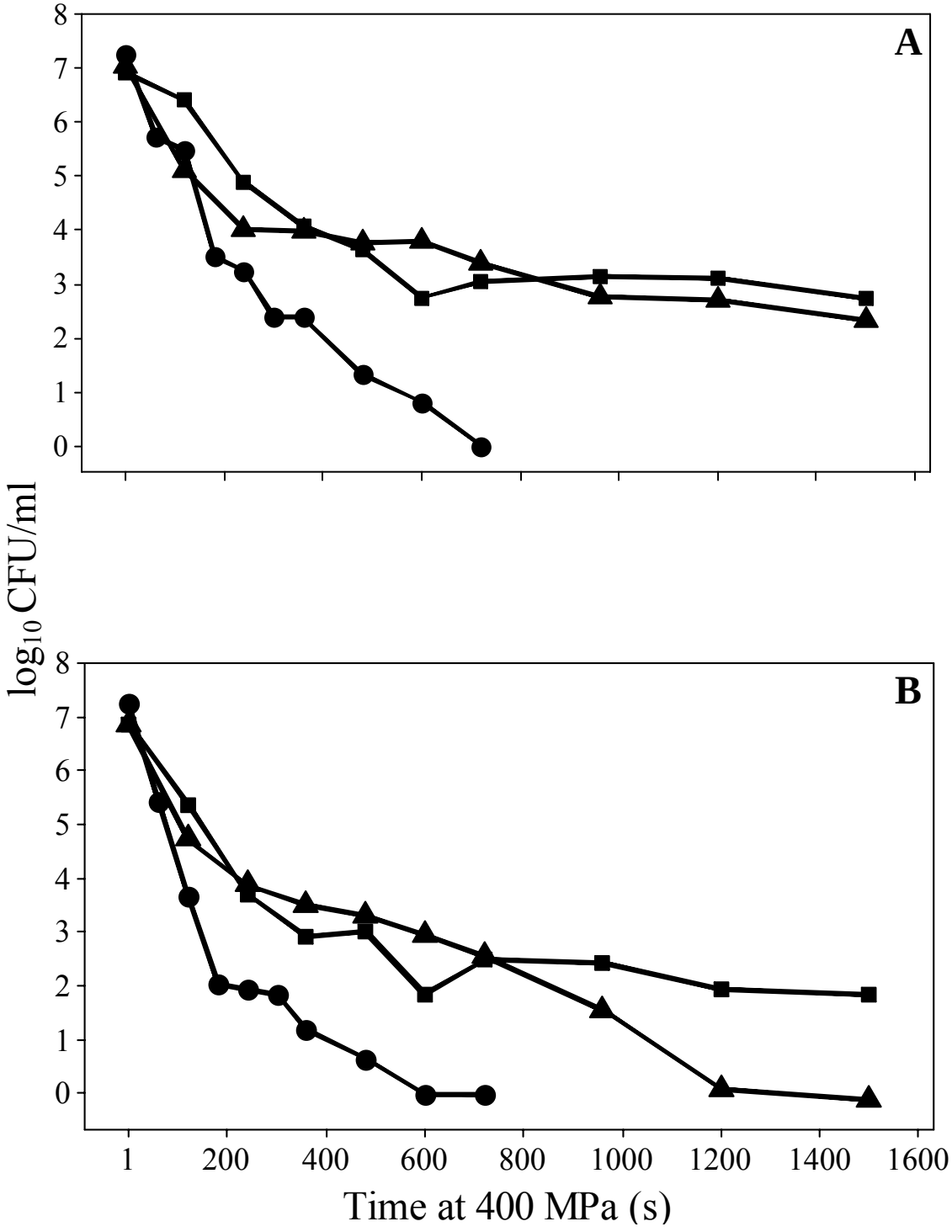
A
B
log10 CFU/ml
Time at 400 MPa (s)
0
1
2
3
4
5
6
7
8
1
200
400
600
800
1000
1200
1400
1600

Table 3.1 Effects of growth temperature on percent injury of stationary-phase *Listeria monocytogenes* recovered after high pressure processing (HPP) of UHT milk. Whole UHT milk was inoculated with stationary phase *L. monocytogenes* to ~10^7 CFU/ml and HPP was applied for various times at 400 MPa at ambient temperature (20-25 °C). After HPP, milk samples were spread on plates of Tryptic Soy Yeast Extract Agar (TSYEA) or Modified Oxford Agar (MOX) which were incubated at 35°C for 48 h. The number of injured cells was defined as the difference between numbers recovered on TSYEA and MOX. Percent injury was defined as (injured cells ÷ total cells) x 100

Time at 400	Growth temperature (°C)				
MPa (s)	4	15	25	35	43
1	-8.3	29.9	50.1	0.4	14.6
15	ND	37.4	ND	ND	ND
30	7.6	64.8	23.2	ND	ND
45	79.8	97.8	84.3	ND	ND
60	91.5	98.1	92.5	ND	ND
75	ND	94.0	87.9	ND	ND
90	99.5	92.8	91.5	ND	ND
120	99.6	ND	90.2	54.7	ND
150	99.3	ND	88.3	ND	ND
180	95.7	ND	ND	ND	ND
210	0.0	ND	ND	ND	ND
240	ND	ND	ND	82.3	ND
300	ND	ND	ND	ND	73.2
360	ND	ND	ND	87.7	ND
480	ND	ND	ND	96.5	ND
600	ND	ND	ND	95.1	78.4
720	ND	ND	ND	92.1	ND
840	ND	ND	ND	89.9	ND
900	ND	ND	ND	ND	96.9
1000	ND	ND	ND	75.0	ND
1200	ND	ND	ND	48.5	99.3
1400	ND	ND	ND	90.8	ND
1500	ND	ND	ND	ND	99.1
1800	ND	ND	ND	ND	91.1

[a]ND= Not determined

Table 3.2. Effects of growth temperature and growth phase on percent injury of *Listeria monocytogenes* recovered after high pressure processing (HPP) of UHT milk. Whole UHT milk was inoculated with mid-exponential, late-exponential or mid-stationary-phase *L. monocytogenes* (grown at 15 or 43°C) to ~10^7 CFU/ml and HPP was applied for various times at 400 MPa at ambient temperature (20-25°C). After HPP, milk samples were spread on plates of Tryptic Soy Yeast Extract Agar (TSYEA) or Modified Oxford Agar (MOX) which were incubated at 35°C for 48 h. The number of injured cells was defined as the difference between the numbers recovered on TSYEA and MOX. Percent injury was defined as (injured cells ÷ total cells) x 100

Time (s) at 400 MPa	Growth temperature 15°C			Growth temperature 43°C		
	Mid-Exponential	Late-Exponential	Mid-Stationary	Mid-Exponential	Late-Exponential	Mid-Stationary
1	52.6	43.2	-1.8	4.8	34.0	6.9
15	ND[a]	61.4	29.0	ND	ND	ND
30	93.4	92.6	74.4	ND	ND	ND
45	ND	ND	ND	ND	ND	ND
60	98.2	89.3	85.3	49.5	ND	ND
90	78.8	91.7	94.7	ND	ND	ND
120	72.1	96.0	93.2	98.5	57.5	90.5
150	99.5	97.4	95.6	ND	ND	ND
180	99.1	95.5	94.1	96.5	ND	ND
240	ND	ND	ND	95.2	24.8	93.1
300	ND	ND	ND	70.9	ND	ND
360	ND	ND	ND	93.5	68.5	92.8
480	ND	ND	ND	79.1	65.5	75.8
600	ND	ND	ND	84.6	85.5	87.4
720	ND	ND	ND	0.0	85.1	72.2
960	ND	ND	ND	ND	93.6	80.0
1200	ND	ND	ND	ND	99.8	93.4
1500	ND	ND	ND	ND	99.7	87.0

[a]ND= Not determined

3.5 Discussion

The results demonstrate the important effects of prior growth temperature on the kinetics of inactivation of *L. monocytogenes* during HPP. Pressure resistance was greatest in stationary-phase cells grown at 35°C, which is near the optimal growth temperature (Nichols et al., 2002) or 43°C, which is close to the maximum growth temperature (Seelinger and Jones, 1986). Casadei et al. (2002) examined the effect of growth temperature on pressure inactivation of stationary-phase *E. coli* and they reported that pressure-resistance varied with growth temperature in the order: 30 or 37 > 20 > 45 > 10°C. The results obtained with *L. monocytogenes* and *E. coli* show that prior growth temperature should be an important part of experimental designs for investigations of the inactivation of microorganisms by HPP. These findings have practical implications also. It is possible that bacteria from animal sources may be more pressure resistant than cells from environmental sources, due to the different temperatures at which cells grow in these environments. If HPP was to be used for cheese-making, it may be prudent to keep the processing plant chilled and refrigerate milk for a day or more before HP processing to sensitize the microorganisms in the milk to HPP, but this would need to be verified with further research.

It is not known why pressure resistance of *L. monocytogenes* increases with increasing growth temperature, but differences in cell membrane composition could play a role. It has been demonstrated that the bacterial cell membrane is affected by HPP (Pagan and Mackey, 2000; Ritz et al., 2001). As growth temperature decreases, cell membrane fatty acids of *L. monocytogenes* become shorter and branching changes from iso to anteiso to maintain a liquid-crystalline state (Annous et al., 1997). This may

increase sensitivity to HPP by reducing membrane flexibility. Stationary-phase cells grown at 15°C were more pressure-sensitive than cells grown at 4°C. *L. monocytogenes* adapts to growth at low temperatures by increasing the intracellular concentration of solutes such as glycine betaine, carnitine and proline (Becker et al., 2000); glycine and L-carnitine have been reported to increase pressure resistance of *L. innocua* (Smiddy et al., 2004).

Our finding that growth phase affects pressure resistance is also in agreement with previous research. McClements et al. (2001) reported that when *L. monocytogenes* was grown at 30°C stationary-phase cells were more pressure resistant than exponential-phase cells. When cells were grown at 8°C it appeared that stationary-phase cells also were more pressure resistant (McClements et al., 2001). Similar to our findings, Casadei et al. (2002) also found that stationary-phase cells of *E. coli* were more pressure resistant than exponential-phase cells. However, in contrast to our results, they reported that pressure resistance of exponential-phase *E. coli* was optimal when cells were grown at 10°C, whereas pressure resistance in stationary phase cells was optimal in cells grown at 30 or 37°C. Isaacs et al. (1995) demonstrated that mid-exponential-phase *E. coli* were much more pressure sensitive than stationary-phase cells. They postulated that increased pressure resistance in stationary-phase cells may be due to their smaller size and more spherical shape, or the accumulation of cell components such as proteins or carbohydrates. Different growth temperature/growth phase effects were observed in the present study. When cells were grown at 15°C mid-stationary cells were significantly more pressure resistant than either mid- or late exponential-phase cells, In contrast, when cells were grown at 43°C the pressure resistance of late-exponential and mid-stationary

cells was similar. Stationary-phase proteins confer resistance to many stresses and may contribute to increased pressure resistance in *L. monocytogenes,* but why this differs with growth temperature is uncertain. Growth of cells at 43°C may result in greater concentrations of stationary-phase proteins in late-exponential-phase cells than in cells grown to the same phase at 15°C.

Our finding of injury of *L. monocytogenes* by HPP is in agreement with previous reports (Patterson et al., 1995; Hayman, 2001; Jordan et al., 2001; Bozoglu et al., 2004; Bull et al., 2005). We confirmed that HPP-induced injury occurred in cells grown at all of the temperatures and at all of the growth phases that were tested, and that a majority of viable cells are injured following processing at 400 MPa. As injured cells can recover in milk (Hayman, 2001; Bozoglu et al; 2004; Bull et al, 2005), detection of injured cells following processing is very important. Optimized Penn State University broth (oPSU) has been demonstrated to enhance detection of pressure-injured cells (Bull et al., 2005). A list of ingredients in oPSU broth can be found in Knabel (2002).

Tailing of survival curves following thermal and nonthermal processes has been demonstrated by many researchers (Metrick et al., 1989; Patterson et al., 1995). However the causes of tailing and the factors that affect it are not well understood. Tailing did not occur in stationary-phase cells grown at 4, 15 or 25°C, but did occur in cells grown at 35 or 43°C, demonstrating the influence of growth temperature on tailing. We also showed that tailing did not occur with exponential-phase cells grown at 43°C. McClements et al. (2001) reported tailing in exponential-phase cells grown at 30°C. It is not known whether tailing is caused by a pressure-resistant subpopulation or is an artifact of HPP

treatment. Tailing may be due to inactivation of multiple targets in cells grown at 35 or 43°C.

Further research is needed to investigate why growth temperature affects pressure resistance and why growth at elevated temperatures causes tailing.

3.6 Acknowledgements

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CHAPTER FOUR

EFFECT OF WATER ACTIVITY ON INACTIVATION OF *LISTERIA MONOCYTOGENES* AND LACTATE DEHYDROGENASE DURING HIGH PRESSURE PROCESSING*

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4.1 Abstract

The aim of this study was to investigate the effect of water activity (a_w) on the inactivation of *Listeria monocytogenes* and lactate dehydrogenase (LDH) during high pressure processing (HPP). For microbial inactivation lyophilized cells of *L. monocytogenes* 19115 were left dry or were suspended in 10 ml of 0.1% peptone water, 10 ml of glycerol, or mixtures of glycerol and peptone water. All samples of various a_ws were high pressure (HP) processed at ambient temperature at 600 MPa for 300 s. Following HPP, samples were serially diluted in 0.1% peptone and spread-plated on Tryptic Soy agar supplemented with Yeast Extract. For enzyme inactivation, 4.2 mg of lyophilized LDH was suspended in 2 ml of 100 mM phosphate buffer (pH 7.4), 2 ml of peptone water or glycerol, or in 2 ml mixtures of glycerol and peptone water. A lyophilized sample with no added liquid was also included. All enzyme samples were subjected to HPP as described above. After HPP, LDH was diluted to 0.28 µg/ml in 100 mM phosphate buffer (pH 7.4). LDH activity was assessed by measuring the change in concentration of β-NADH as a function of time. Dynamic light scattering analysis (DLS) was performed to examine the size distribution, polydispersity, and hydrodynamic radius of LDH before and after HPP. No significant difference in CFU/g was observed between lyophilized cells not subjected to HPP and lyophilized cells subjected to 600 MPa for 300 s ($P < 0.05$). However, lyophilized cells that were suspended in 100% to 60% peptone water showed a ~7.5-$\log_{10}$ reduction when subjected to HPP. Survival of *L. monocytogenes* following HPP significantly increased ($P < 0.05$) when the peptone water concentration was decreased below 60% ($a_w \sim 0.8$). DLS results revealed that LDH suspended in buffer underwent aggregation following HPP (600 MPa, 5 min).

Inactivation rate constants obtained using a first–order kinetic model indicated that untreated and high pressure processed lyophilized LDH had similar activities. When LDH was subject to HPP in solutions containing glycerol, enzyme activity decreased as the water content increased ($r^2 = 0.95$). Lyophilization completely protected *L. monocytogenes* and LDH from inactivation by high pressure. Furthermore, enzyme activity and cell survival increased as water activity was decreased. We postulate low a_w results in protein stabilization, which prevents protein denaturation and cell death during HPP.

Keywords: High pressure processing (HPP); *Listeria monocytogenes*; Lactate dehydrogenase; Glycerol; Lyophilization; Water Activity

4.2 Introduction

High pressure processing (HPP) is a non-thermal food preservation method that has garnered considerable interest in the last two decades because of its ability to preserve foods while maintaining their fresh-like qualities. Pressures of 300-700 MPa, which inactivate vegetative cells but not bacterial spores, are typically used for food preservation (Stewart and Cole, 2001). Combination treatments, typically heat and high pressure, can be used to inactivate bacterial spores (Meyer et al., 2000; Anon, 2005; Patterson, 2005). HPP causes disruption of hydrophobic and ionic bonds but not covalent bonds, so many small molecules in foods, including flavor compounds and vitamins, are left intact (Farr, 1990). The mechanism of microbial inactivation by HPP remains uncertain as many targets in the cell have been speculated to be involved,

including the cell wall (Brul et al., 2000) cytoplasmic membrane (Pagan and Mackey, 2000; Ritz et al., 2002), nucleic acids (Mackey et al., 1994), ribosomes (Niven et al., 1999) and various proteins (Simpson and Gilmour, 1997a; Wouters et al., 1998; Ritz et al., 2000).

Listeria monocytogenes is a ubiquitous foodborne pathogen which causes the disease listeriosis. Listeriosis primarily affects the elderly, pregnant women and immunocompromised individuals and has a high case fatality rate (~20-30%) (Farber and Peterkin, 1991). Listeriosis is most commonly associated with consumption of raw milk, soft cheeses, hot dogs and deli meats (Sutherland and Porritt, 1997). *L. monocytogenes* is Gram-positive, rod shaped, facultatively anaerobic, psychrotrophic and relatively resistant to salt, drying and low pH (Seelinger and Jones, 1986). As *L. monocytogenes* is capable of growing at refrigeration temperatures it is of concern in ready-to-eat foods which support its growth, such as soft cheeses and deli meats.

L- lactate dehydrogenase (LDH, E.C.1.1.1. 27) is a cytoplasmic enzyme present in essentially all major organ systems in the animal body (Drent et al., 1996), as well as in yeasts (Garvie, 1980) and bacteria (Guiard et al., 1975; Vinals et al., 1995). LDH is a hydrogen transfer enzyme that catalyses the reversible reduction of pyruvate to lactate (Larsen, 2005), with nicotinamide-adenine dinucleotide (NADH) as the hydrogen donor (Drent et al., 1996). The reaction of lactate formation is strongly favored at equilibrium (Drent et al., 1996). Activity of LDH is commonly estimated by measuring the decrease in absorption (due to NADH-disappearance) in monochromatic absorbance photometry (Larsen, 2005).

Enzyme activity relies on the specific three-dimensional conformation of protein molecules and can be affected by changes in their shape, size, association or dissociation of subunits, denaturation or unfolding, and degradation and aggregation (Mozhaev and Martinek, 1982; Yang et al., 1994). Dynamic light scattering (DLS), also known as quasielastic scattering and photocorrelation spectroscopy, has been applied to analyze the size and shape of macromolecules and used to study various inter- and intramolecular interactions, including association, aggregation, gelation, and micellation (Harding et al., 1992). DLS has also been used extensively in studying solutions of many globular proteins, enzymes and hormones, to determine their diffusion coefficients, size and shape characteristics, and aggregation and binding kinetics (Yang et al., 1994).

Cells contain a number of critical proteins including ribosomal proteins, enzymes, and membrane proteins. These proteins assume a myriad of critical functions in the cell. Hence, among many other functions, ribosomal proteins are involved in the synthesis of proteins, membrane proteins regulate the transport of compounds into and out of cells, and enzymes catalyze biological reactions in cells (Campbell, 1991). Thus, life and biological stability in all cells are greatly dependent on the activity of native cellular proteins.

While reduced a_w can inhibit the growth of microorganisms, it can also protect them from other environmental stresses, such as heat (Gould, 1985). Decreasing a_w has also been found to increase the resistance of microorganisms to high pressures (Oxen and Knorr, 1993), however this effect depends on the solute used to depress a_w (Patterson, 2005, Koseki and Yamamoto, 2007). The mechanism for the protective effect of reduced

a_w on microbial inactivation by HPP is not known, but was speculated to be due to protein stabilization (Moussa et al., 2006).

The objective of this study was to investigate the effect of reduced a_w and lyophilization on inactivation of *L. monocytogenes* and lactate dehydrogenase during HPP. In addition, dynamic light scattering was used to determine if HPP caused changes in the structure of LDH.

4.3 Materials and Methods

Lyophilized *L. monocytogenes* serotype 4b (ATCC 19115) was obtained from the American type culture collection. The cotton plugs (containing approximately 4.4 x 10^7 CFU per gram of cotton) were aseptically removed and cut into very small pieces with sterile scissors, mixed together and than weighed into roughly equal portions (~0.2 g). The plugs were used as they were easier to handle and weigh into equal portions than the skim milk pellets. The pieces were placed in 4 x 6" plastic stomacher bags (VWR, West Chester, PA, USA); the weight of cotton in each bag was recorded in order to calculate CFU/g. To obtain cells of various a_ws, the plugs were either left dry or 10 ml of 0.1% peptone diluent [(Difco, Becton, Dickinson and Company, Sparks, MD, USA], or 10 ml of 10, 20, 25, 30, 40, 43, 46, 50, 75%, 100% v/v of glycerol/peptone water was added. Water activity of the peptone water/glycerol solutions, before the addition of cells, was measured using an AquaLab CX-2 water activity meter (Decagon Inc., Pullman, WA, USA). Bags were heat sealed using an Impulse heat sealer (MP-12, Midwest Pacific, St. Louis, MO, USA) and placed into one large plastic bag containing 1% chlorine which was also heat sealed. All *L. monocytogenes* and LDH samples were processed in a 2-L

HPP unit (Avure Technologies, Kent, WA, USA) at 600 MPa for 300 s at room temperature (~22-24°C) with water as the pressure-transmitting fluid. It took ~125 s to reach 600 MPa and decompression occurred in < 10 s. Following processing *L. monocytogenes* samples were diluted with 0.1% peptone diluent and spread on plates of Tryptic Soy Agar supplemented with 0.6% Yeast Extract (TSYEA; Difco). Plates were incubated at 37°C for 48-72 h. The limit of detection was 50 CFU/g. Each treatment was replicated three times.

For enzyme inactivation, a 2.1 mg/ml solution of LDH (Sigma Aldrich, St Louis, MO, USA), was prepared in 100 mM phosphate buffer, pH 7.4 (PSB). Two ml of LDH solution was transferred into a 4 x 6" plastic stomacher bag and sealed. The remaining LDH was freeze-dried for 48 h using a Mobile 12 freeze-dryer (Virtis Company, Cardiner, NY, USA). Various enzyme treatments were prepared by adding 4.2 mg of lyophilized LDH to 2 ml of the following solutions: 10, 20, 30, 40, 50, 75% glycerol containing 0.1% peptone water, 100% glycerol, or PSB alone. A lyophilized 4.2 mg sample without addition of fluid was also included as a treatment. The treatment solutions were transferred into plastic 4 x 6" stomacher bags and heat sealed as described above. The stomacher bags were then placed into one large plastic bag containing water, heat sealed, and subjected to HPP as described above. Following HPP the bags were frozen in liquid nitrogen and then stored at -20°C until enzymatic assays were performed. Each treatment was replicated three times.

DLS was used to examine the size, polydispersity, and hydrodynamic radius measurement of (i) untreated and (ii) HPP LDH (2.1 mg/ml in PSB). Following HPP the solutions were diluted to 2.08 μg/ml (corresponding to 2.1 units/ml) using 100 mM PSB.

One ml of solution placed in 1.5 ml Eppendorf flex-tubes (VWR, Philadelphia, PA, USA) and centrifuged at 15,000 x *g* for 5 min using a table–top Microfuge 18 centrifuge (Beckman Coulter, Fullerton, CA, USA). One hundred µl of supernatant was transferred to a clean tube and then filtered through a Whatman syringe filter, pore size 1 µm (VWR). Fifteen µl of filtrate was loaded into a 1 cm path quartz cuvette and placed into a Viscotek 802 DLS (Houston, TX, USA) for size distribution, polydispersity, and hydrodynamic radius measurement. The DLS was equipped with a class 3B 60 mW laser diode. Ten measurements were made for each sample at a wavelength of 830 nm, and the data were analyzed using Model 802 DLS OmniSize 2.0 software.

Enzyme assays for each LDH treatment were conducted using the LDH enzyme solution (2.08 µg/ml) and a working substrate solution containing 0.11 mM of nicotinamide reduced form (β-NADH), 620 mM of sodium pyruvate, and 10g/l bovine serum albumin (BSA) (all from Sigma Aldrich). The enzyme reaction was started by mixing 0.2 ml of working enzyme solution with 6.8 ml of substrate solution. At 5 min intervals, 50 µl of the reacting solution was taken and absorbance was measured at 340 nm using a model Du 530 spectrophotometer (Beckman Coulter). Three measurements were made for each data point. The change in absorbance was synonymous to the oxidation of β-NADH; hence the LDH activity was measured as the rate of change of the concentration of β-NADH in the reaction medium. A standard curve was constructed by plotting the absorbance of five solutions of β-NADH with known concentrations. LDH activity was calculated using the standard curve.

Data from microbial inactivation were analyzed using Minitab software (Minitab, State College, PA). Data were transformed to $\log_{10}$ CFU/ml and analysis of variance (ANOVA) was performed (Fig. 1). Tukey's test was used to test for differences between treatment pairs. Linear regression was used to analyze for the effect of water/glycerol concentration on the inactivation of *L. monocytogenes* (Fig. 2). The critical limit was set at $\alpha = 0.05$.

The decimal reduction time (D) was used to characterize the rate of LDH inactivation. The relation between the decimal reduction time and the inactivation rate constant is given by the equation $D = 2.303/k$. The inactivation factor was defined as the ratio of the rate constant of untreated LDH versus rate constant of LDH in various treatment media. D was defined as the time in minutes required to reduce the initial enzyme activity by 90% at constant temperature (Fidiloğlu et al, 2005).

A correlation coefficient between the glycerol content of the reaction medium and the decimal reduction time (D) of LDH activity was calculated using Minitab. ANOVA was performed. Tukey's test was used to test for differences between treatment pairs.

4.4 Results

Both lyophilization and reduced water activity significantly reduced the inactivation of *L. monocytogenes* by high pressure processing ($P < 0.001$; Fig. 4.1, Fig. 4.2). When cells were lyophilized (dehydrated by freeze-drying) there was no significant reduction in CFU/g as compared to the control (no HPP) (Fig. 4.1; $p < 0.05$). The approximately 1-$\log_{10}$ reduction observed when cells were subjected to HPP in 100% glycerol was not significantly different from lyophilized/HPP cells ($P < 0.05$), but was

significantly different from control cells (Fig. 4.1; $P > 0.05$). Cells subject to HPP in 100% peptone were not detected after HPP, approximately a 6.5-7.5 $\log_{10}$ reduction in CFU/g (Fig 4.1). This treatment was significantly different than the control, lyophilized/HPP and 100% glycerol/HPP treatment ($P < 0.05$).

To test the effect of various a_ws on inactivation of *L. monocytogenes* by HPP, cells were suspended in various water/glycerol solutions. When cells were subjected to HPP in 0-40% glycerol (i.e. 100-60% peptone water; $a_w = 0.99$-0.86) no survivors were detected following HPP, indicating a 6.5 to 7.5-$\log_{10}$ reduction (Fig. 4.2.A.). However, when the glycerol concentration was increased to 43% (i.e., 57% peptone water, $a_w = 0.83$) there was only a ~2.5-$\log_{10}$ reduction. Therefore, there was a rapid increase in the rate of cell death as a_w was increased from 0.83 to 0.86 (Fig. 4.2.A). When cells were suspended in solutions with a a_w below 0.83 the effectiveness of HPP was significantly reduced ($P < 0.001$)

Figure 4.2.B shows the effect of water activity on LDH activity after HPP at 600 MPa for 5 min. Reaction rate constants (k), decimal reduction time (D), and inactivation factors (i), together with r^2 of pressure inactivation of LDH are presented in Table 4.2. The untreated enzyme had the highest rate constant, 2.81×10^{-2} min^{-1}, while k for the dry enzyme was 2.70×10^{-2} min^{-1}, however these values are not significantly different ($P > 0.05$). The inactivation factor of the dried LDH relative to the untreated was 1.04. When the enzyme was treated in 100% glycerol, $k = 2.01 \times 10^{-2}$ min^{-1}, which was not significantly different than the no-HPP control ($P > 0.05$). Reaction rate constants decreased significantly as the glycerol fraction of the enzyme systems decreased and the water fraction increased ($P < 0.005$). When LDH was treated in 10% glycerol/90%

peptone water, k was 4.0 x 10^{-4} min^{-1}. This value was identical to that of LDH treated in buffer, and the corresponding inactivation factor was 70.3. As the rate constant was reduced due to pressure inactivation, D increased. This increase in D occurred concomitantly with the increase in a_w (reduction in glycerol content) of the medium in which LDH was treated. The correlation coefficient, r^2, between water content and D was 0.96, indicating a strong relationship existed between a_w and inactivation of LDH by HPP.

Figure 4.3 demonstrates the mass distribution diagram of untreated LDH and LDH treated at 600 MPa for 5 min. Untreated LDH exhibited monodispersity confirmed by the presence of a single compound with an hydrodynamic radius (Rh) of 4.6 nm. The molecular weight of this entity was 121 kDa. Monodispersity in untreated LDH is an indication that no aggregation has occurred in the sample. Following HPP at 600 MPa for 5 min, two molecular entities originating from LDH were detected; the first had an Rh of 4.3 nm and the second had an Rh of 67.1 nm. The presence of these entities indicated that the enzyme was aggregated following pressure treatment. The sizes of the entities are presented in Table 4.1. The entity with an Rh of 4.3 nm was attributable to LDH based on its MW (103.7 kDa). The second entity had a MW of 68487.5 kDa and can be assigned to an aggregate formed from native LDH molecules after pressure treatment.

Figure 4.1 Effect of lyophilization and subsequent addition of 100% peptone water or 100% glycerol on the destruction of *Listeria monocytogenes* by high pressure processing. Cotton plugs containing lyophilized *L. monocytogenes* were cut into small pieces and ~0.2 g portions were placed in plastic stomacher bags. The cotton was left dry or 10 ml of liquid was added. Bags were heat sealed and samples were pressurized at 600 MPa for 5 min at ambient temperature. Following processing samples were diluted with 0.1% peptone diluent and spread on plates of Tryptic Soy Agar with Yeast Extract. Plates were incubated at 37°C for 48-72 h. The limit of detection was 50 CFU/g. The experiment was triplicated. ND = not detected.

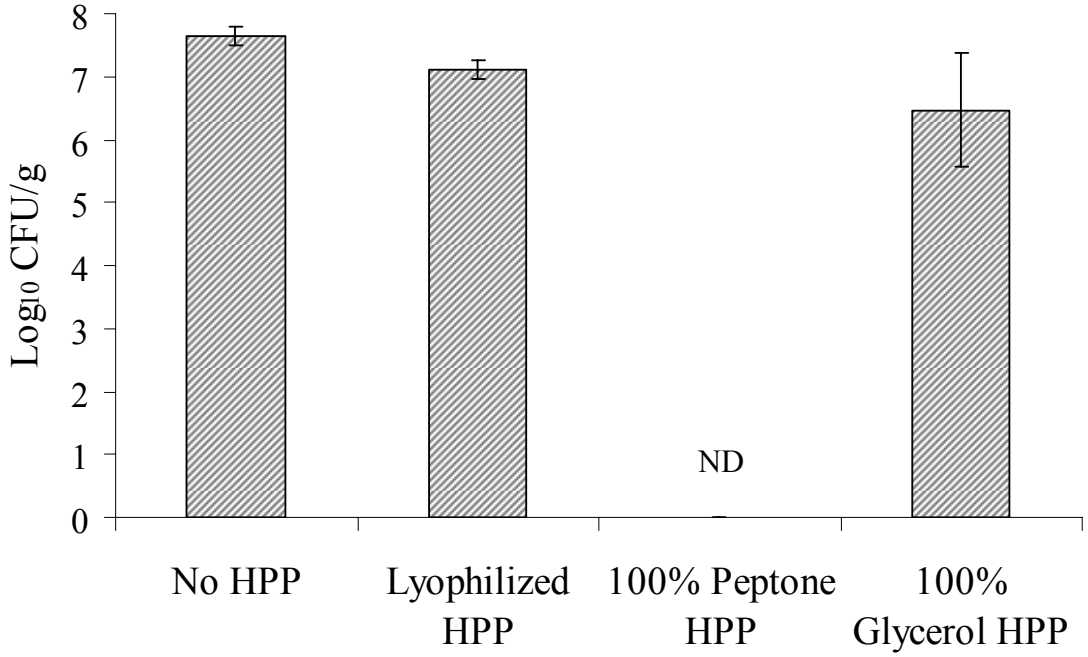
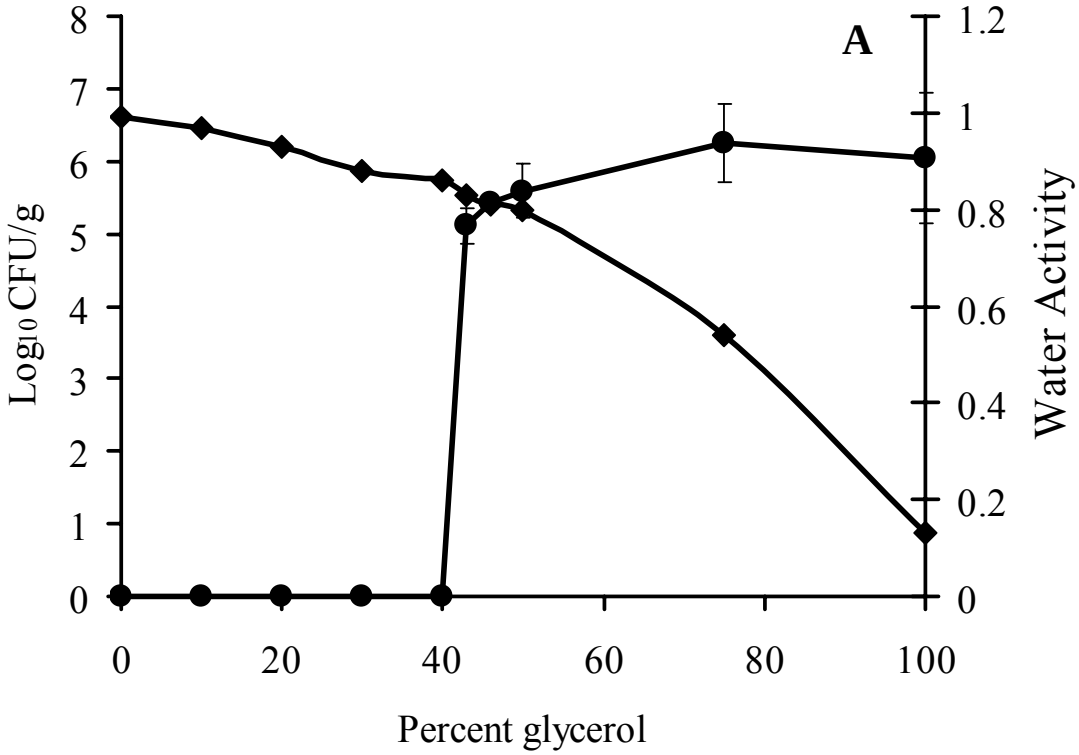

8
7
6
5
4
3
2
1
0
Log10 CFU/g
ND
No HPP
Lyophilized HPP
100% Peptone HPP
100% Glycerol HPP

Figure 4.2.A. Effect of water activity on inactivation of *Listeria monocytogenes* subject to high pressure processing in glycerol solutions. Cotton plugs containing lyophilized *L. monocytogenes* were cut into small pieces and ~0.2 g portions were placed in plastic stomacher bags. Glycerol/peptone water solutions were added to the cotton. Bags were sealed and samples were pressurized at 600 MPa for 5 min at ambient temperature. Following processing samples were diluted with 0.1% peptone diluent and spread on plates of Tryptic Soy Agar with Yeast Extract. Plates were incubated at 37°C for 48-72 h. The limit of detection was 50 CFU/g. The experiment was triplicated. Key: (♦) Water activity; (●) $\log_{10}$ CFU/g.

Figure 4.2.B. Effect of water activity on the rate of inactivation of lactate dehydrogenase (LDH) by high pressure processing (HPP). 4.2 mg of lyophilized LDH was suspended in 2 ml of 100 mM phosphate buffer (pH 7.4), 2 ml of peptone water or glycerol, or in 2 ml mixtures of glycerol and peptone water. All enzyme samples were HP processed at 600 MPa for 5 min. After HPP a 0.208 μg/ml LDH solution in 100 mM phosphate buffer (pH 7.4) was prepared for analysis. LDH activity was assessed by measuring the change in concentration of β-NADH as a function of time. At 5 min intervals, 50 μl of the reacting solution was taken and absorbance was measured at 340 nm using a model Du 530 spectrophotometer.


A
Log10 CFU/g
Water Activity
Percent glycerol
0
1
2
3
4
5
6
7
8
0
0.2
0.4
0.6
0.8
1
1.2
0
20
40
60
80
100

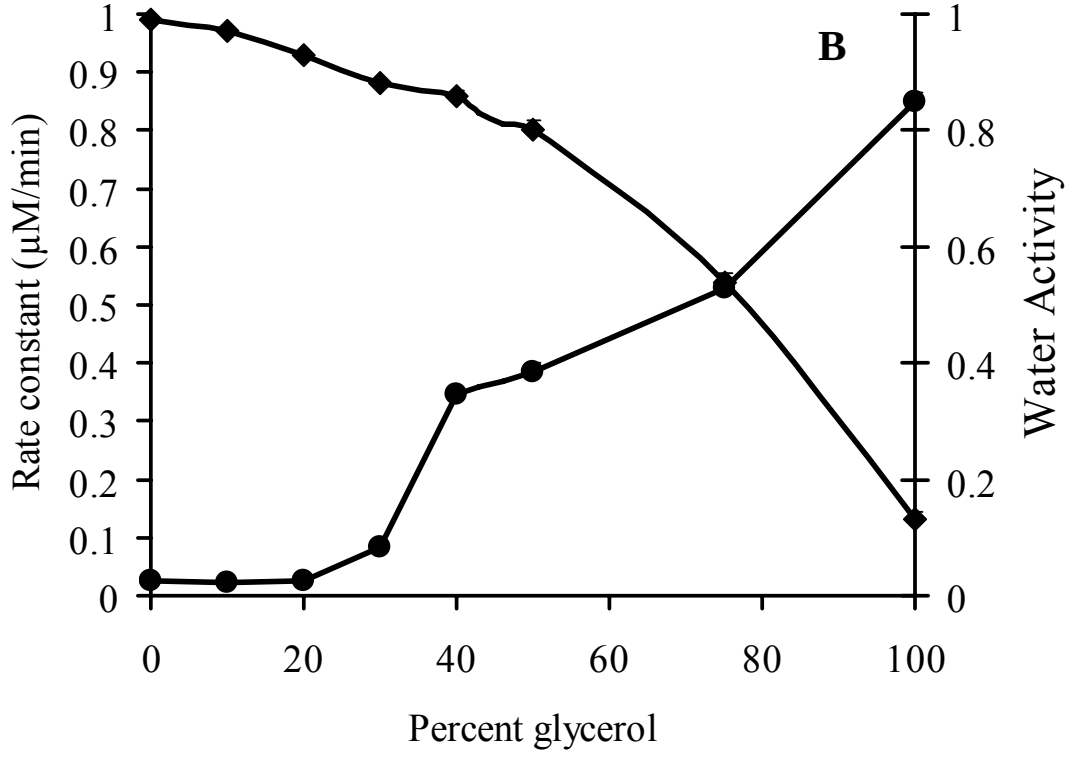
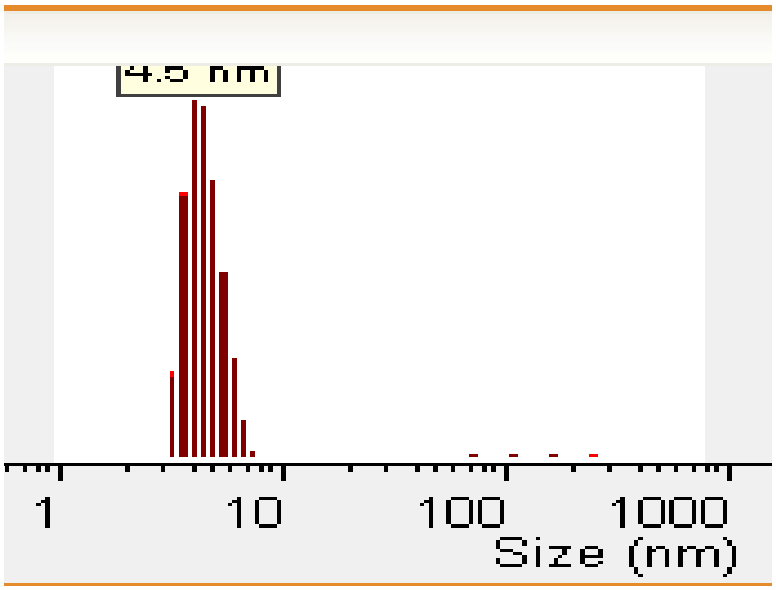
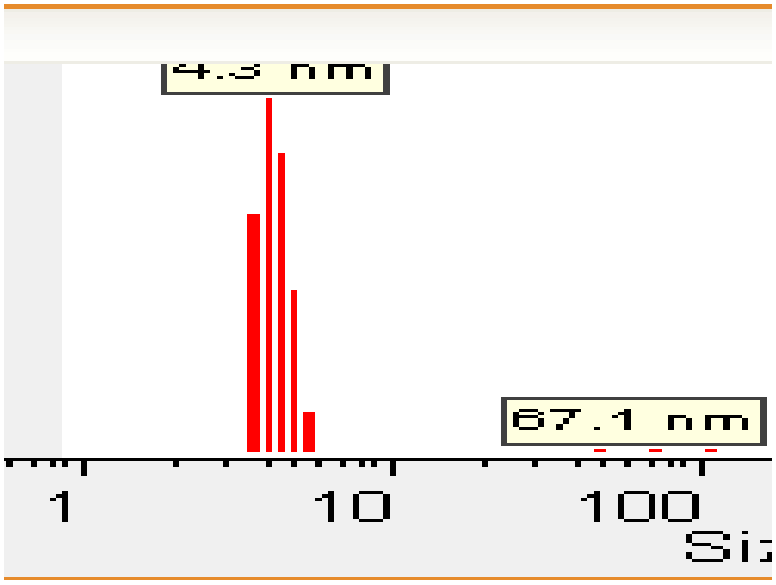

B
Rate constant (µM/min)
Water Activity
Percent glycerol
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
0.2
0.4
0.6
0.8
1
0
20
40
60
80
100

Figure 4.3 Mass distribution diagram of lactate dehydrogenase without high pressure processing treatment (A) and with treatment at 600 MPa for 5 min (B). Following HPP, the solutions were diluted to 0.2 mg/ml using 100 mM PSB. One ml of solution placed in 1.5 ml Eppendorf flex-tubes and centrifuged at 15,000 x *g* for 5 min. One hundred µl of supernatant was transferred to a clean tube and then filtered through a Whatman syringe filter, pore size 1 µm. Fifteen µl of filtrate was loaded into a 1 cm path quartz cuvette and placed into a Viscotek 802 DLS. The DLS was equipped with a class 3B 60 mW laser diode. Ten measurements were made for each sample at a wavelength of 830 nm.



A



B

Table 4.1 Dynamic light scattering analysis of lyophilized lactate dehydrogenase suspended in phosphate buffer at pH 7.4 with or without high pressure processing at 600 MPa for 5 min. Number of measurements = 10.

	% area	Hydrodynamic Radius (nm)	Molecular weight (kDa)
Untreated	100	4.6 ± 0.70	121.8
600 MPa-treated	99.6	4.3 ± 0.49	103.7
	0.4	67.1 ± 12.2	68487.58

Table 4.2 Inactivation rate constants of lyophilized lactate dehydrogenase high pressure processed at 600 MPa for 5 min suspended in glycerol/water solutions of varying concentration. Rate constants were obtained using a first-order kinetic model. The inactivation factor was calculated by dividing the untreated control rate constant by the rate constant of the treatment.

Treatment medium	Rate constant (min^{-1})	Decimal reduction time (min)	Inactivation factor	R^2
PBS pH 7.4	$4x10^{-4}$	5757.5	70.5	0.99
10% glycerol	$4x10^{-4}$	5757.5	70.5	0.97
20% glycerol	$5x10^{-4}$	4606	56.2	0.97
30% glycerol	$2.1x10^{-3}$	1096.8	13.4	0.96
40% glycerol	$2.3x10^{-3}$	1046.8	12.7	0.95
50%glycerol	$1.04x10^{-2}$	221.5	2.7	0.99
75% glycerol	$1.27x10^{-2}$	181.3	2.2	0.99
100% glycerol	$2.01x10^{-2}$	114.6	1.3	0.98
Dry enzyme	$2.70x10^{-2}$	85.3	1.04	0.98
No treatment	$2.81x10^{-2}$	81.9	1	0.98

4.5 Discussion

The objectives of this research were to: (1) Investigate the effect of lyophilization and (2) Examine the effect of reduced water activity on the inactivation of *L. monocytogenes* and LDH during HPP. The results show that HP resistance of *L. monocytogenes* and LDH was significantly enhanced by both lyophilization and reducing water activity using glycerol. Activity of lyophilized HP-treated LDH was similar to that of the native enzyme. As far as we are aware, no other information concerning HP resistance of lyophilized cells or enzymes has been published to date.

The extreme pressure resistance of bacterial endospores initially sparked our interest to examine the effect of pressure on lyophilized cells. The enhanced pressure resistance of lyophilized cells may be due to the same mechanism that protects bacterial spores from physical stresses such heat and high pressure. The water content of bacterial endospores is lower than that of vegetative cells, 0.4 to 0.65 g wet weight for spores, compared to 0.75 g for vegetative cells (Gould, 1985). However, water is distributed unevenly in the spore and hence the cytoplasm (core) may contain as little at 0.2 g water per g wet weight (Gould, 1985). The low a_w of the core is thought to protect the contents from being damaged by heat (Gould, 1985). Futhermore, as external a_w decreases the heat resistance of *Bacillus cereus* spores increases (Gaillard at al., 1998).

Our results also demonstrated that the HP-resistance of both *L. monocytogenes* and LDH dramatically increased with decreasing a_w. When a_w was reduced below 0.83, inactivation of *L. moncytogenes* by HPP was significantly reduced. Concurrently, inactivation of LDH by high pressure was significantly reduced at low a_ws. The protective effect of reduced a_w on inactivation of microbial cells by heat and HPP has

been observed by many authors. For example, Simpson and Gilmour (1997b) showed that HP-inactivation of *L. monocytogenes* was significantly reduced when cells were suspended in an olive oil mixture (30% v/v oil). Hayman et al. (2004) reported that the pressure resistance of *L. monocytogenes* was increased as the salt concentration of the medium was increased. Morales at al. (2006) showed that the a_w of cheese significantly affected the pressure resistance of *L. monocytogenes*. Moussa et al. (2006) investigated the inactivation of *Escherichia coli* by HPP using glycerol to depress a_w (to 0.85). They showed that reduced a_w significantly increased pressure resistance. They speculated that water may play a crucial role in pressure-induced denaturation of proteins. The results obtained here support this hypothesis. Koseki and Yamamoto (2007) showed that high concentrations of sodium chloride, sucrose and sodium phosphate all completely inhibited the inactivation of *L. monocytogenes* at 600 MPa. However, this effect was not directly related to a_w, but to solute concentration.

Cells were suspended in 100% glycerol to test the hypothesis that lack of inactivation in lyophilized cells was due to lack of direct contact with pressure transmitting fluid. Inactivation of *L. monocytogenes* and LDH in 100% glycerol was minimal, indicating that lack of inactivation was not due to lack of direct contact with a pressure-transmitting fluid. Glycerol is a permeant solute; hence it enters the cell by simple diffusion (Gould, 1985). Once diffusion is complete the water activity of the solution and the cytoplasm of the cell should be the same. Glycerol is known to have a protective effect on cells and proteins, which is why it is commonly used as a cryoprotectant. Glycerol also affects the dynamic and thermodynamic properties of protein solutions (Priev et al., 1996; Zancan and Sola-Penna, 2005). Suspension of a

protein in glycerol results in the displacement of water from the core of the protein, and hence decreases the volume and compressibility of the protein interior (Priev et al., 1996). Zancan and Sola-Penna (2005) demonstrated that glycerol significantly slowed unfolding of pyrophosphatase due to high temperature, which increased thermostability of the enzyme and aided in refolding of denatured enzymes. Ruan et al. (2003) studied the effect of glycerol on protecting a 33-kDa protein from spinach photosystem II from high pressure denaturation. They reported that addition of glycerol significantly decreased denaturation due to high pressure and caused a significant decrease in the standard volume change. Our results are also in agreement with these of Cioni and Strambini, (1995) and Di Primo et al. (1995) who reported that glycerol displayed a significant protection against pressure induced denaturation of monomeric proteins. The protective effect of glycerol is believed to be due to the ability of glycerol to alter the viscosity of the medium (Sola-Penna et al., 1998). This could also explain the resistance of LDH to denaturation in the presence of glycerol.

Changes in enzyme structure, including dissociation of subunits, denaturation, and aggregation, affect enzyme activity (Yang et al., 1994). The absence of aggregation in the untreated LDH suggested the enzyme remained stable. When the enzyme was subjected to 600 MPa in buffer, aggregates were formed (Fig. 3), indicating that the stability of the enzyme was altered. Denaturation of proteins by pressure involves the disruption of hydrophobic and electrostatic interactions (Mozhaev et al., 1994). HPP does not involve the breakage of covalent bonds such as peptide bonds. When a protein is denatured by high pressure it undergoes an increase in hydration due partly to the electrostriction of water molecules around newly exposed charged groups (Mozhaev et

al., 1994). Our results show that the inactivation of *L. monocytogenes* and LHD increased as a_w increased. This observation highlights the importance of a_w as a determining factor in the inactivation of *L. monocytogenes* and LDH by HPP and also indicated that HPP has no effect on the conformation and folding of the enzyme in a dry medium. It has been known since ancient times that dehydration is one of the best methods for preservation of biological systems. This also appears be the basis for the resistance of *L. monocytogenes* and LDH to HPP in this study. It is generally accepted that protein hydration is essential for enzyme catalysis to occur and dry enzymes are inactive (Daniel et al., 2003), because a conformational flexibility is required for the enzyme to be in a state that allows it to bind and convert substrate to product to product (Schinkel et al., 1985, Zheng et al., 1999). Such a conformational flexibility in the presence of water may expose the hydrophobic moieties of the enzyme to the effects of HPP leading to alteration of the active sites (Schinkel et al., 1985). HPP may not cause enzyme inactivation in the absence of water because in such a condition its hydrophobic moieties are conformationally rigid and thus buried and not exposed to the stress generated by HPP. It is likely that LDH treated in 100% glycerol (very low a_w) experienced similar conditions. The increased a_w at lower glycerol concentrations may have allowed a conformation that exposed LDH to misfolding and aggregation during HPP. Since HPP induced misfolding and aggregation of LDH, it is probable that HPP has the same effect on other proteins, including proteins within *Listeria monocytogenes.* Hence, enzymes, membrane proteins and ribosomal proteins in the bacterial cells may become misfolded during HPP, and therefore were rendered incapable of assuming their vital functions within the cells. Transmission electron microscopy images of HP-

inactivated *L. monocytogenes* show circular regions of reduced density in the cytoplasm (Mackey et al., 1994, Hayman 2001). These regions may represent areas of protein aggregation in the cytoplasm.

In conclusion, lyophilization and reduced a_w exerted a significant protective effect on the inactivation of *L. monocytogenes* and LDH by HPP. Thus, water plays a critical role in the inactivation of bacteria and enzymes by HPP. The strong correlations between a_w and bacterial survival (r^2=0.84) and a_w and LDH activity (r^2 = 0.98) indicates a relationship exists between protein denaturation and bacterial inactivation duirng HPP. Hence, we believe that protein denaturation may be the primary mechanism by which bacterial cells are inactivated by HPP. The results show that application of HPP to dry foods, or foods with regions of low a_w, may not be practical due to the lack of sensitivity of microorganisms and enzymes to pressure in these circumstances.

4.6 Acknowledgements

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CHAPTER FIVE

HEAT SHOCK INDUCES BAROTOLERANCE IN *LISTERIA MONOCYTOGENES*

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5.1 Abstract

High pressure processing (HPP) is a non thermal method of food preservation that can inactivate microorganisms while retaining the fresh-like qualities of many foods. The aim of this study was to investigate the effect of heat shock on the resistance of *Listeria monocytogenes* to HPP. *L. monocytogenes* was grown to stationary-phase at 15°C and inoculated into whole UHT milk at ~10^7 CFU/ml. Five-ml samples of milk were placed into plastic transfer pipettes, which were heat sealed and then heat shocked at 48°C for 10 min. Immediately after heat shock the milk was cooled in tap water (20°C) for 25 min, and then placed on ice. The samples were high pressure processed at ambient temperature (~23°C) at 400 MPa for various times up to 150 s. Following HPP the samples were spread-plated on Tryptic Soy Agar supplemented with Yeast Extract. Heat shock significantly increased the $D_{400\ MPa}$ value of *L. monocytogenes* from 35 s in non-heat-shocked cells to 127 s in heat-shocked cells ($P < 0.05$). Addition of chloramphenicol prior to heat shock eliminated the protective effect of heat shock ($P < 0.05$). Heat shock for 5, 10, 15 or 30 min at 48°C resulted in maximal barotolerance ($P < 0.05$) ; increasing the time to 60 min significantly decreased survival compared to 5, 10, 15 or 30 min ($P < 0.05$). These results show that heat shock significantly increases the barotolerance of *L. monocytogenes* and that de novo protein synthesis during heat shock is required for enhanced barotolerance. The results also highlighted the need to consider the food safety implications of combining low heat and high pressure treatments.

Keywords: High pressure processing; *Listeria monocytogenes*; Heat shock

5.2 Introduction

High pressure processing (HPP) is a non-thermal method of food preservation which utilizes high hydrostatic pressures of 300-700 MPa (Stewart and Cole, 2001). Pressure is transmitted instantaneously and uniformly through foods, regardless of the volume or geometry (Smelt, 1998). High pressure inactivates microorganisms in foods, although sensitivity to pressure varies widely between cell type and species (Casedei, 2000; Chen, 2007). HPP can increase the shelf life and safety of foods, while maintaining many of the fresh-like qualities, such as flavor and texture. A range of pressure-treated food products are available in the US, Europe and Japan. The mechanism of microbial inactivation remains uncertain and many targets within the cell may be involved, including the cell wall (Brul et al., 2000), cytoplasmic membrane (Pagan and Mackay, 2000; Ritz et al., 2002), nucleic acids (Mackey et al., 1994), ribosomes (Niven et al., 1999) and various proteins (Simpson and Gilmour, 1997a; Wouters et al., 1998; Ritz et al., 2000a).

Listeria monocytogenes is a Gram-positive bacterium that causes the foodborne disease listeriosis. Approximately 2,500 human cases of listeriosis occur annually in the U.S. and approximately 20-30% of these cases lead to death (Farber and Peterkin, 1991; Mead et al., 1999). The elderly, immuncompromised people, and pregnant women are particularly susceptible to the disease, which can manifest as meningitis, septicemia or miscarriage. *L. monocytogenes* is ubiquitous in the environment and frequently colonizes food processing plants (Fenlon, 1999). Once it contaminates foods it can be hard to control, as the organism is psychrotrophic, tolerates low water activity and grows over a wide range of pH (Lou and Yousef, 1999). Listeriosis is commonly associated with

ready-to-eat foods, such as deli meats, hot dogs and soft cheeses (Farber and Peterkin, 1991; FDA/CFSAN, 2001).

Increased barotolerance resulting from sublethal heat shock has been reported in the yeast *Saccharomyces cerevisiae* (Iwahashi et al., 1991; Iwahashi et al., 1997) and the Gram-negative bacterium *Escherichia coli* (Aertsen et al., 2004b). Moreover, stress proteins are synthesized in response to sublethal pressure shock (Welch et al., 1993; Wemekamp-Kamphuis et al., 2002; Fernandes et al., 2004). *E coli* expresses about 55 proteins upon exposure to sublethal pressure (55 MPa); 11 of these were heat shock proteins (HSPs) (Welch et al., 1993). *S. cerevisiae* has also been reported to express HSPs in response to mild pressure treatments (Fernandes et al., 2004). Cold shock proteins (CSPs) are also induced by pressure (Wemekamp-Kamphuis et al., 2002).

The aim of this research was to investigate the effect of heat shock at 48°C on the pressure resistance of *L. monocytogenes* ATCC 19115 in whole UHT milk. We also investigated effect of time of heat shock on barotolerance of *L. monocytogenes*. Finally, we investigated whether *de novo* protein synthesis during heat shock was required for increased barotolerance.

5.3 Materials and Methods

A strain of *L. monocytogenes* serotype 4b (ATCC 19115) was maintained in glycerol stock at -80ºC. All media were obtained from Difco (Becton, Dickinson and Company, Sparks, MD, USA) unless otherwise stated. A loopful of glycerol stock was streaked onto Tryptic Soy Agar supplemented with 0.6% Yeast Extract (TSYEA). The plate was incubated at 35°C overnight, and then one colony was inoculated into 10 ml of

Tryptic Soy Broth supplemented with 0.6% Yeast Extract (TSYEB), which was then incubated at 35°C for 18-24 hours. The resulting culture was diluted to ~1 x 10^8 CFU/ml in TSYEB, and 100 µl of diluted culture was then added to 100 ml of TSYEB to yield ~1 x 10^5 CFU/ml. The cultures were incubated at 15°C for 72 h to yield mid-stationary-phase cells.

One hundred ml of chilled Paramlat whole UHT milk was poured into two sterile 250 ml flasks. Chloramphenicol (Sigma, St Louis, MO) was added to one flask to a final concentration of 100 µg/ml. One ml of stationary-phase culture was added to each of the flasks and swirled 20 times by hand to yield ~1.75 x 10^7 CFU/ml. Tips were aseptically cut off 3.1 ml sterile plastic transfer pipettes (Sigma-Aldrich, Milwaukee, WI, USA) and 5 ml of inoculated milk was aseptically added to each plastic pipette bulb using a sterile syringe. Pipette bulbs containing the inoculated milk were heat sealed by first melting the neck of the bulbs in a Bunsen flame and then pressing the melted neck with a heat sealer (MP-12, Impulse; Midwest Pacific, St. Louis, MO, USA). Sealed pipette bulbs were placed in 4" x 6" stomacher bags (VWR, West Chester, PA, USA) containing 20 ml of 1% chlorine and the stomacher bags were sealed using the same heat sealer.

Samples were heat shocked by completely submerging the stomacher bags for 10 min in a water bath at 48°C. The come up time for the milk at the center of the pipette bulb to 48°C was ~6 min. Following heat shock the stomacher bags were placed in tap water (20°C) for 25 min after which they were transferred to ice. For samples heat shocked at various times the same procedure was followed, except the stomacher bags were immersed in the 48°C water bath for 1 s, 5, 10, 15, 30 or 60 min.

Samples in the stomacher bags were processed in a 2-L HPP unit (Avure Technologies, Kent, WA, USA) at 400 MPa at room temperature (22-24°C), with water as the pressure-transmitting fluid. It took ~100 s to reach 400 MPa and decompression occurred in < 10 s. Samples heat shocked for various times were HP processed at 400 MPa for 90 s only, samples heat shocked for 10 min were processed at 400 MPa for various times up to 150 s. Samples were kept on ice before and after processing and were plated within four hours of processing. Samples were spread on plates of TSYEA, which where incubated at 35°C for 48 h. Samples were diluted with 0.1% peptone water before plating. Preliminary experiments confirmed that plating 100 µL of sample onto TSYEA relieved inhibition from chloramphenicol (Appendix B). The limit of detection was 10 CFU/ml. Each experiment was replicated 3 or 4 times.

Data were analyzed by ANOVA using Minitab (State College, PA, USA). For samples heat shocked for various times data were not normally distributed and hence were transformed by taking the natural log prior to ANOVA. Fisher's test was used to make pairwise comparisons. For samples heat shocked for 10 min and pressure treated for various times Microsoft Excel was used to perform regression analysis. Tukey's test was used to make pairwise comparisons between D values (using a 95% confidence interval).

5.4 Results

Heat shocking *L. monocytogenes* in whole milk at 48°C did not result in any loss of viability (data not shown). Data showing the effect of heat shock time at 48°C on barotolerance of *L. monocytogenes* are presented in Fig. 5.1. Time of heat shock

significantly affected survival of *L. monocytogenes* upon subsequent exposure to 400 MPa for 90 s ($P < 0.001$), with heat shock for approximately 5 min yielding maximal barotolerance (Fig. 5.1). Pairwise comparisons revealed that there was no difference in CFU/ml in samples heat shocked for 5, 10, 15 or 30 min ($P < 0.05$), with less than 1-$\log_{10}$ reduction in CFU/ml. Heat shock for 2 or 60 min also increased barotolerance ($P < 0.05$), with no difference in pressure resistance between the two times ($P > 0.05$). However, heat shock at 2 or 60 min did not increase barotolerance as much as heat shock at 5-30 min ($P < 0.05$). Heat shock for 1 s did not increase pressure resistance compared to the non-heat-shocked control ($P > 0.05$).

When cells were heat shocked at 48°C for 10 min prior to pressure treatment at 400 MPa they were significantly more pressure resistant than the non-heat-shocked cells (Fig. 5.2, $P < 0.05$). $D_{400\ MPa}$ values were 35.3 s for the control and 126.8 s for heat-shocked cells. Adding chloramphenicol prior to heat shock resulted in a $D_{400\ MPa}$ value of 32.1 s, which was not significantly different from the control ($P > 0.05$). There was also no significant difference between the control and non-heated-shocked cells with chloramphenicol (Fig. 5.2, $P > 0.05$).

Data from Figs. 5.1 and 5.2 were obtained using cells suspended in whole milk during both heat shock and HPP. When cells were heat shocked and pressure treated in TSYEB there was no increase in pressure resistance at 400 MPa. Survivors were not detected following 15 s of pressure treatment (data not shown).

Figure 5.1 Effect of heat shock time at 48°C on the barotolerance of *Listeria monocytogenes*. *L. monocytogenes* was grown at 15°C to mid-stationary phase in Tryptic Soy Yeast Extract Broth. Whole UHT milk was inoculated with *L. monocytogenes* to ~1.0 x 10^7 CFU/ml and heat shocked at 48°C for various times. Following heat shock samples were high pressure processed at 400 MPa for 90 s at ambient temperature. After HPP milk samples were spread on plates of Tryptic Soy Yeast Extract Agar which were incubated at 35°C for 48 h. The experiment was replicated four times. Bars with different letters are significantly different ($P < 0.05$).

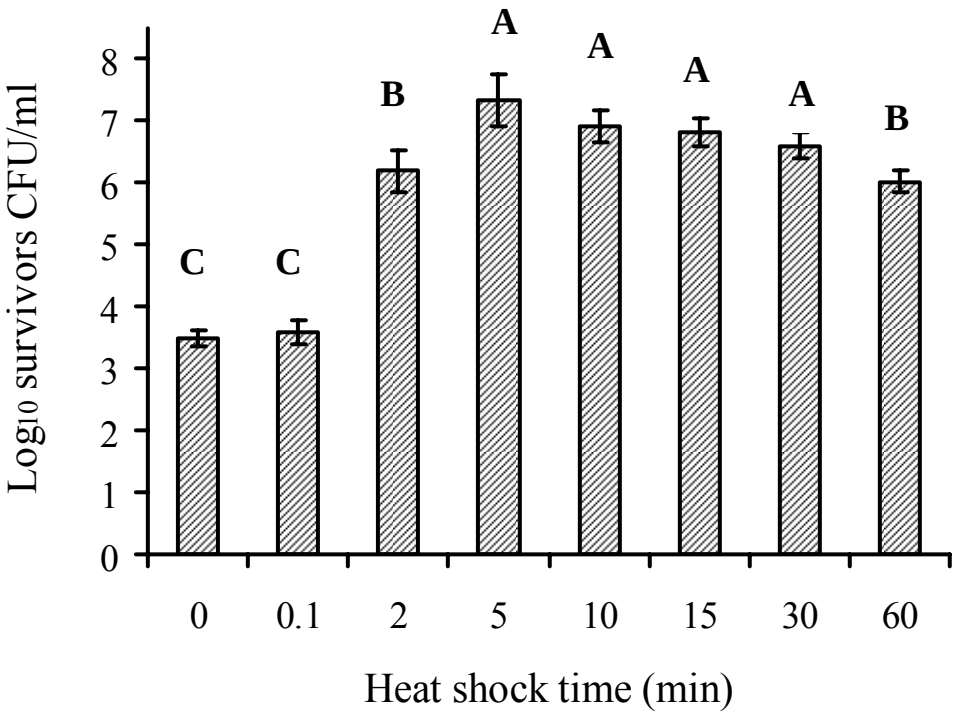
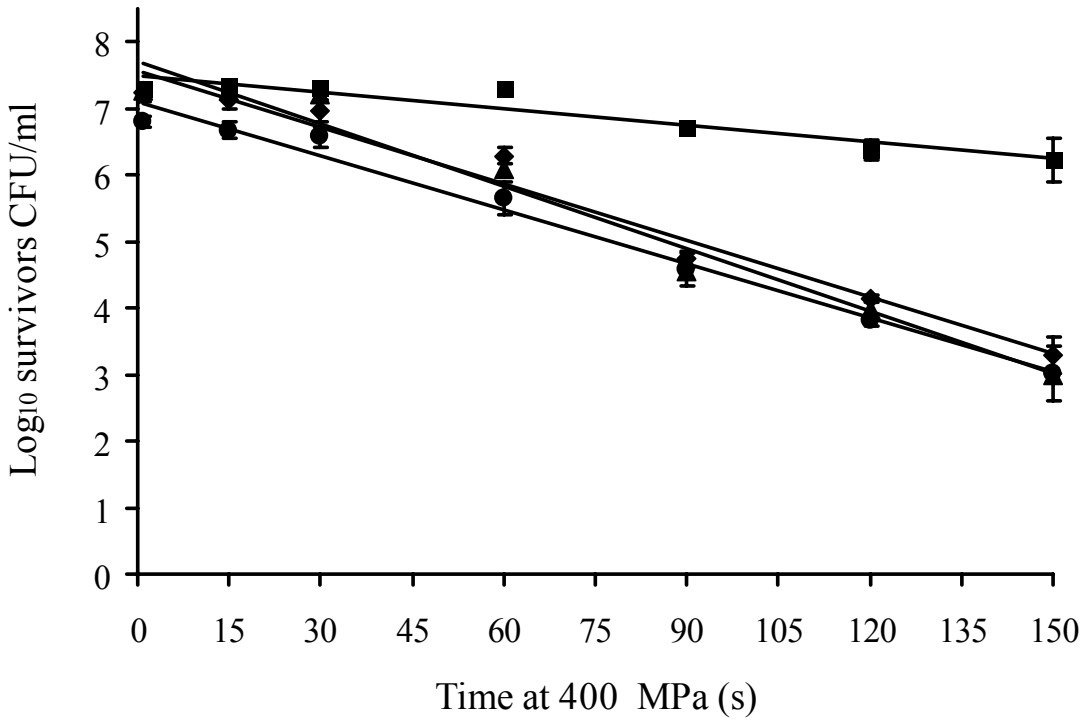

Log10 survivors CFU/ml
0
1
2
3
4
5
6
7
8
C
C
B
A
A
A
A
B
0
0.1
2
5
10
15
30
60
Heat shock time (min)

Figure 5.2 Effect of heat shock on barotolerance of *Listeria monocytogenes*. *L. monocytogenes* was grown at 15°C to mid-stationary phase in Tryptic Soy Yeast Extract Broth. Whole UHT milk was inoculated with *L. monocytogenes* to ~1.75 x 10^7 CFU/ml and heat shocked at 48°C for 10 min. Following heat shock samples were high pressure processed at 400 MPa for various times at ambient temperature. After HPP milk samples were spread on plates of Tryptic Soy Yeast Extract Agar which were incubated at 35°C for 48 h. The first data point represents 1 s at 400 MPa. The experiment was replicated three times. Key: Control (no-heat-shock) (♦); Control + Chloramphenicol (●); Heat Shock (■); Heat Shock + Chloramphenicol (▲).


Log10 survivors CFU/ml
0
1
2
3
4
5
6
7
8
0
15
30
45
60
75
90
105
120
135
150
Time at 400 MPa (s)

5.5 Discussion

Preliminary data indicated that heat shock at 48°C increased barotolerance of *L. monocytogenes*, hence the first aim was to determine the optimal heat shock time to achieve maximum barotolerance. Cells heat shocked for 5, 10, 15 or 30 min produced maximum barotolerance. It took samples ~3 min to reach 43°C and an additional ~3 min to reach 48°C. The times reported here are the total times the samples were immersed in the waterbath; therefore samples heat shocked for 5 min did not reach 48°C (final internal temperature of the tubes heat shocked for 5 min was 47°C). The results show that gain of barotolerance is rapid, in contrast to increased thermotolerance due to heat shock (Pagán et al., 1997). Knabel at al. (1990) reported that heat shock (43°C) of *L. monocytogenes* for 30 min increased thermotolerance compared to cells heat shocked at 5 min and Pagan et al. (1997) reported that thermotolerance of *L. monocytogenes* increased with the duration of heat shock up to 120 min. However Bunning et al. (1990) reported that heat shock at 48°C for 5 or 15 min slightly increased thermotolerance of *L. monocytogenes* in comparison to heat shock for 30 or 60 min. The effect of heat shock time on barotolerance of *S. cerevisiae* was reported by Iwahashi et al. (1991). In contrast to our results they reported that heat shock for 30 min at 43°C provided maximal tolerance to 150 MPa.

The results demonstrated that heat shock at 48°C for 10 min in milk significantly increased the $D_{400\ MPa}$ of *L. monocytogenes*. Other investigators have also reported increased barotolerance of microorganisms due to heat shock, although no D values were reported (Iwahashi et al., 1991; Iwahashi et al., 1997; Aertsen et al., 2004b). Aertsen et al. (2004b) reported that heat shock (50°C for 15 min) increased barotolerance of *E. coli*.

Iwahashi et al. (1991; 1997) reported that heat shock (43°C for 60 min) increased the barotolerance of *S. cerevisiae*. The results presented here show that heat shock did not result in barotolerance when chloramphenicol was added to the cells prior to heat shock. These finding are in agreement with Iwahashi et al. (1991), who reported that the addition of cycloheximide (an inhibitor of protein synthesis in eukaryotic cells) prior to heat shock attenuated the induction of barotolerance and thermotolerance in *S. cerevisiae*. Therefore, we conclude that *de novo* protein synthesis during heat shock is also required for enhanced pressure resistance in bacteria.

As *de novo* protein synthesis during heat shock was required for increased barotolerance it is probable that HSPs play a role in increasing barotolerance of *L. monocytogenes*. HSPs are a large family of ubiquitous proteins that are highly conserved among prokaryotic and eukaryotic organisms. Researchers have suggested that HSPs are not necessarily the major agents responsible for the development of thermotolerance, but are required for recovery from heat stress (Johnson, 2003). Many HSPs are molecular chaperones or ATP-dependent proteases and play major roles in protein folding, repair, and degradation under normal and stress conditions (Yura et al., 2000). Molecular chaperones constitute 15 to 20% of the total cellular protein in response to elevated temperatures (Yousef and Courtney, 2003). Iwahashi et al. (1997) reported that HSP104 (related to the Clp proteins in prokaryotes) contributed to barotolerance in yeast, but that the mechanism of barotolerance and thermotolerance in yeast may be different. They suggested that trehalose, which the formation of is induced by heat shock in yeast, may play an important role in heat-shock-related barotolerance in yeast.

Induction of HSPs and other stress proteins, such as cold shock proteins (CSPs), by pressure has been reported by several research groups. Welsh et al. (1993) found 55 pressure induced proteins when they exposed *E. coli* to 55 MPa. Of these 11 were HSPs (ClpB, ClpP, Lon, RpoH, DnaK, GroEL, GroES, Grp, E, G21.0 and F10.1) and 4 were CSPs (G41.2, RecA, HNS and F10.6). The synthesis of many proteins was inhibited during heat shock and the overall rate of synthesis was 16% of that of the control.

Aertson et al. (2004b) reported that three heat shock genes, *lon, dnaK*, *clpPX* and the sigma factor *rpoH* were induced after exposure to pressure (150 MPa). The genes are regulated by σ^{32} in Gram-negative bacteria. *dnaK* transcription paralleled the development of increased pressure resistance. They found that pressure shock did not increase heat or pressure resistance, supporting Iwahashi et al.s' (1991) hypothesis that the mechanisms of pressure resistance may be different to that of thermal resistance. They found that the heat shock genes, but not *rpoH,* were expressed in basal levels in pressure resistant mutants. They also found that some heat shock proteins (DnaK, GroEl, GroES, GrpE, ClpB and HtpG) were more strongly expressed in pressure-resistant mutants during growth at 37°C.

Fernandes at al. (2004) investigated the effect of HPP on the gene expression of *S. cerevisiae*. They found that 5% of known genes were affected by pressure treatment. 131 genes were induced >2-fold while 143 suffered a < 2-fold downregulation. Most (~45%) of the pressure-regulated mRNAs corresponded to uncharacterized open reading frames, and even amongst the induced characterized genes, almost half had unknown function. The three most upregulated genes were an uncharacterized open reading frame, followed by two genes that code for small HSPs, HSP30 and HSP12. HSP 12 is activated

by heat shock and a wide range of environmental stresses, while HSP30 is more specifically regulated. For genes with known functions they reported that most of the upregulated genes were involved in metabolism (particularly carbon metabolism), whilst many of the downregulated genes were involved in protein synthesis.

In addition, Wemekamp-Kamphuis et al. (2002) applied a low level HPP treatment (200 MPa for 10 min) to exponentially growing *L. monocytogenes* and measured levels of CSPs. They reported that the levels of CSP1 and CSP2 were 3.5 and 2 fold higher, respectively, than in controls following HPP. In contrast to Aertsen et al. (2004b) they reported that cold shocked cells (10°C for 4 hours) were more pressure resistant than cells grown at 37°C.

Barotolerance of heat shocked cells increased when cells were HP processed in milk, but not when they were heat shocked and pressure-treated in TSYEB. This is in contrast to Iwahashi et al. (1991), who heat shocked *S. cervisiae* in YM medium and still reported increased barotolerance. However, Iwahashi et al. (1991) pressure treated at 150 MPa. Perhaps reducing the pressure below 400 MPa in this study would have produced a similar effect. Components in milk, such as minerals, lactose, fat or protein, may have a protective effect on *L. monocytogenes* during or after high pressure processing. Lactose may help to stabilize proteins and the cell membrane and milk protein and fat may also play a role in stabilizing the cell membrane. Black et al. (2007) reported that minerals present in milk, namely calcium, citrate and phosphate, contribute significantly to the baroprotective effect of milk, perhaps by acting as buffers or stabilizing the cell membrane. Further research is needed in this area to determine why cells heat shocked

and HP processed in milk were more barotolerant than cells heat shocked and/or HP processed in TSYEB.

A main function of heat shock proteins is to repair damage to proteins via chaperone activity. Heat shock proteins are responsible for the refolding or degradation of damaged proteins and they also prevent aggregation of damaged proteins (Yura et al., 2000). Increased barotolerance due to heat shock proteins that prevent or repair protein denaturation indicates that protein denaturation plays an important role in the inactivation of bacteria by high hydrostatic pressure. This is consistent with reports that enzymes are rapidly inactivated by HPP (Smelt et al., 1994; Simpson and Gilmour, 1997a). It has also been reported that growth of *L. monocytogenes* at 35 and 43°C resulted in tailing inactivation kinetics when exposed to 400 MPa, yet growth at 4, 15, or 25°C resulted in log-linear inactivation kinetics (Hayman et al., 2007a). As *L. monocytogenes* is a psychrotroph, growth at 35 or 43°C may result in a stress response. Therefore, a possible explanation for tailing in cells grown at higher temperatures could be low level expression of stress proteins, including HSPs. This hypothesis is consistent with the results reported here, but needs to be confirmed by additional research.

Bacterial endospores are not inactivated by pressures up to 1000 MPa; therefore combination treatments are required to control them in high pressure treated foods (Patterson, 2005). Options include refrigeration, low pH or combined heat and pressure treatment (Anonymous, 2005). Generally it is assumed that pressure combined with low-level thermal treatments will increase the rate of destruction of foodborne microorganisms, including spores. The results presented here show that heat shock significantly increased the $D_{400\ MPa}$ value of *L. monocytogenes*. Therefore, care must be

taken in developing HP processes in the food industry, as treatment times predicted to inactivate *L. monocytogenes* may not do so if the cells are subjected to sublethal heat shock prior to HPP.

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CHAPTER SIX

INVESTIGATING THE EFFECTS OF HIGH PRESSURE PROCESSING ON *LISTERIA MONOCYTOGENES* USING MICRO DIFFERENTIAL SCANNING CALORIMETRY

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6.1 Abstract

High pressure processing (HPP) is a non-thermal method of food preservation that can inactivate microorganisms while retaining the fresh-like qualities of many foods. The mechanism of bacterial inactivation by HPP has not been clearly elucidated. The aim of this study was to investigate the effects of HPP on whole cells of *Listeria monocytogenes* using micro-differential scanning calorimetry (mDSC). *L. monocytogenes* was grown to stationary phase at 15°C and then suspended in potassium phosphate buffer at ~10^{11} CFU/ml. Cells were high pressure (HP) processed at 200, 400 or 600 MPa at ambient temperature for 150 s. Samples were scanned from 10°C to 110°C, with a scan rate of 60°C h^{-1}, in a microcalorimeter. Following the first scan samples were rapidly cooled to 10°C and then rescanned under the same conditions. Thermograms of all samples showed endothermic transitions only. Thermograms of control (No HPP) cells in buffer showed five peaks in the first scan, one of which was reversible and hence detected in the rescan. The two largest transitions in control cells had maximum temperatures (T_ms) of 68.4°C and 87.8°C, and these peaks were attributed to cellular proteins and DNA, respectively. Cells HP-processed in buffer at 200 MPa had thermograms similar to those of the control. The thermograms of cells HP-processed at 400 MPa showed four peaks (T_ms = 57.5, 61.4, 71.1, 78 and 88.5°C), and the peak with T_m = 68.4°C was absent. Thermograms of cells HP-processed at 600 MPa contained only one peak with a T_m = 89.4°C, which is associated with DNA. The rescan of all samples showed one transition, with a T_m of ~86°C, that did not appear to be affected by pressure. HPP did not appear to affect the enthalpy associated with DNA, but did eliminate the enthalpy associated with proteins. Hence, we conclude that a majority of proteins in the

cell are denaturated by HPP and that protein denaturation may be the main mechanism by which vegetative Gram-positive bacteria are inactivated by HPP.

Keywords: High pressure processing; *Listeria monocytogenes*; Differential scanning calorimetry, Protein denaturation

6.2 Introduction

High pressure processing (HPP) is a non-thermal method of food preservation which utilizes pressures of 300-700 MPa (Stewart and Cole, 2001). HPP has garnered much attention in the last couple of decades because of its ability to preserve foods while maintaining their fresh-like qualities. Pressure is transmitted instantaneously and uniformly through foods, regardless of the volume or geometry (Smelt, 1998) and reactions that lead to a decrease in volume are enhanced by pressure (Gross and Jaenicke, 1994). Unlike heat, high pressure does not affect covalent bonds (Tewari et al., 1999). Therefore many HP-processed foods have superior flavor, texture and nutritional quality in comparison to their thermally treated counterparts (Farr, 1990). High pressure inactivates microorganisms in foods, thereby increasing their shelf life and safety (Hoover et al., 1989; Patterson, 2005). Many factors influence microbial barotolerance, including cell type and species (Casedei, 2000; Chen, 2007), growth phase (McClements et al., 2001; Casadei et al., 2002; Hayman et al., 2007a), the food in which the cells are suspended (Patterson et al., 1995; Simpson and Gilmour, 1997b) and the temperature of treatment (Gervilla et al., 1997; Alpas et al., 2000). The mechanism of microbial inactivation remains uncertain and multiple targets within the cell may be involved,

including the cell wall (Brul et al., 2000), cytoplasmic membrane (Pagan and Mackay, 2000; Ritz et al., 2002), nucleic acids (Mackey et al., 1994), ribosomes (Niven et al., 1999; Kaletunç et al., 2004) and various proteins (Simpson and Gilmour, 1997a; Wouters et al., 1998; Ritz et al., 2000a).

Listeria monocytogenes is a ubiquitous, Gram-positive, rod-shaped bacterium. *L. monocytogenes* is psychrotrophic, can tolerate a wide pH range, and can grow at a water activity as low as 0.9 (Seelinger and Jones, 1986). *L. monocytogenes* is the etiological agent of the foodborne disease listeriosis. Although listeriosis is a fairly rare disease, with ~2,500 cases reported annually in the U.S., it is of concern to the food industry due to the systemic nature of the disease and the high fatality rate (20-30%) (Farber and Peterkin, 1991; Mead et al., 1999). Listeriosis is commonly associated with ready-to-eat foods, such as soft milk cheeses, hot dogs and deli meats (FDA/CFSAN, 2001; Sutherland et al., 2003). Populations at high risk of contracting listeriosis include the elderly, the immunocompromised and pregnant women (Farber and Peterkin, 1991).

Micro-Differential Scanning Calorimetry (mDSC) is a very sensitive method that can measure very small changes in specific heat (Cp). DSC measures the production (exothermic) or absorption (endothermic) of heat that occurs in a sample during a fixed rate of temperature increase or decrease. When the sample undergoes a physical transformation, such as a phase transition, more or less heat will need to flow to the sample in comparison to a reference in order to maintain both at the same temperature. DSC can be used to investigate the folding and stability of biomolecules, including proteins, nucleic acids and lipids (Belliveau et al., 1992). DSC is normally used to characterize isolated biopolymers, but has been successfully applied to whole bacterial

cells (Anderson et al., 1991; Mackey et al., 1991; Belliveau et al., 1992; Teixeira et al., 1997; Lee and Kaletunç, 2002b). Unlike many techniques in microbiology, DSC gives instantaneous information about the physiology of bacterial cells (Vine and Bishop, 2005). DSC allows cell death and the denaturation of components in the cell to be superimposed so that the components responsible for cell can be identified (Miles, 2006).

Previous research conducted in our laboratory indicated that protein denaturation may be the primary mechanism by which cells of *L. monocytogenes* are inactivated by high pressure (Hayman et al., 2007b). Therefore, the aim of the present study was to directly investigate the effect of HPP on the proteins of whole cells of *L. monocytogenes* using mDSC.

6.3 Materials and Methods

A strain of *L. monocytogenes* serotype 4b (ATCC 19115) was maintained in glycerol stock at -80 ºC. All media were obtained from Difco (Becton, Dickinson and Company, Sparks, MD) unless otherwise stated. A loopful of glycerol stock was streaked onto Tryptic Soy Agar supplemented with 0.6% Yeast Extract (TSYEA). The plate was incubated at 35°C overnight, and then one colony was inoculated into 10 ml of Tryptic Soy Broth supplemented with 0.6% Yeast Extract (TSYEB), which was then incubated at 35°C for 18-24 hours. The resulting culture was diluted to ~10^8 CFU/ml in TYSEB, and 100 µL of diluted culture was added to 100 ml of TSYEB (~10^5 CFU/ml). The resulting broth was incubated at 15°C for 72 h to obtain mid-stationary-phase cells. Note: Growth curves were determined in advance by both spread plating and absorbance at 600 nm (data not shown). Two bottles of broth were used for each pressure treatment. The entire

100 ml of broth was emptied into a 250 ml centrifuge tube (Cole-Parmer, Quebec, Canada). The tubes were centrifuged in an Avanti J-26 XP1 High-Speed Centrifuge (Beckman Coulter, Fullerton, CA) at 13,000 g for 15 min, the supernant was discarded and the pellets were washed in 20 ml of sterile deionized water, and then the tubes were centrifuged again. Supernates were discarded and the pellets from two centrifuge tubes were re-suspended in 1 ml of 0.1 M potassium phosphate buffer (KH_2PO_4; pH 6.6; Sigma, St Louis, MO) to obtain ~10^{11} CFU/ml.

Tips were aseptically cut off 3.1 ml plastic transfer pipettes (Sigma-Aldrich, Milwaukee, WI) and the sample was added to the pipette bulb using a 30 ml sterile syringe (Becton Dickinson). Pipette bulbs were heat sealed by melting in a Bunsen flame and then pressing with an Impulse MP-12 heat sealer (Midwest Pacific, St. Louis, MO). Sealed pipette bulbs were placed in 4"x 6" stomacher bags (VWR, West Chester, PA) containing 1% chlorine, which were then heat sealed. Samples were processed in a 2-L HPP unit (Avure Technologies, Kent, WA) at 200, 400 or 600 MPa for 150 s at room temperature (20-25 °C), with water as the pressure-transmitting fluid. The ramp rate was approximately 25s/100 MPa and decompression occurred in < 10 s. Cells were enumerated following HPP by serially diluting in 0.1% peptone diluent and spread-plating on TYSEA. Plates were then incubated at 37°C for 48 h.

The samples were transferred into test tubes and degassed at 10°C for 8 min using a ThermoVac (MicroCal LLC, Northampton, MA). Approximately 0.5 ml of sample was injected into a VP-DSC MicroCalorimeter (MicroCal LLC). The samples were then scanned from 10 to 110°C, with a scan rate of 60°C h^{-1}. Samples were cooled rapidly to 10°C and rescanned once more at 10 to 110°C with a scan rate of 60°C h^{-1}. Temperature

and specific heat were recorded by the VP-DSC software (MicroCal LLC) every ten seconds. Data was graphed using the average of two or three replicates in Excel (Microsoft, Redmond, WA). The T_ms (maximum temperatures) for all of the peaks were identified by viewing plots of the thermograms in Excel to gain the approximate location of the peak, and then using the raw data to find the exact temperature value.

6.4 Results

All of the transitions observed in all of the samples were endothermic. Although samples were scanned from 10°C to 110°C, there were no transitions below 40°C; therefore only the data from 40 to 110°C are presented in the thermograms (Figs. 6.1 to 6.4). Thermograms of control (No HPP) *L. monocytogenes* showed five peaks on the first scan, these were labeled as Peaks 1, 2, 3, 4, and 5a (Fig. 6.1). One of these peaks, Peak 5a, was reversible and therefore apparent when the sample was rescanned. The peak in the rescan was labeled Peak 5b (Fig. 6.1). The T_ms for all of these peaks are shown in Table 6.1. The two largest peaks in the first scan were Peaks 2 and 5a, and the corresponding T_ms were 68.4°C and 87.8°C respectively (Fig. 6.1, Table 6.1).

Cells HP-processed in buffer at 200 MPa were not inactivated (as determined by spread-plating) and the thermograms contained five peaks in the first scan and one in the second (Fig. 6.2). The position and size of the peaks in the thermograms of cells processed at 200 MPa were similar to those of the control (Fig. 6.1, Table 6.1). The thermogram of the second scan of cells HP-processed at 200 MPa appeared to be the same as the second scan in the control (Fig. 6.1 & 6.2).

Cells HP-processed in buffer at 400 or 600 MPa were completely inactivated. The thermograms of the first scan of cells HP-processed at 400 MPa (Fig. 6.3) showed five transitions and three of these, Peaks 1, 4 and 5a, corresponded to transitions in the control (Fig. 6.1). Two transitions had T_ms which did not correspond to a peak in the control; these peaks were labeled Peaks 1.5 and 2.5 as they occurred between Peaks 1 and 2 and between Peaks 2 and 3. Peak 2, present in control cells, was absent in cells HP-processed at 400 MPa (Fig. 6.3). The thermogram of the second scan of cells HP-processed at 400 MPa appeared to be the same as the second scan in the control (Fig. 6.1 & 6.3). The thermogram of the first scan of cells processed at 600 MPa contained only one peak, Peak 5a (Fig. 6.4). The thermogram of the second scan of cells HP-processed at 600 MPa appeared to be the same as the second scan in the control (Fig. 6.1 & 6.4).

Other, very minor peaks may exist in the control cells and cells HP-processed at 200 MPa (Figs. 6.1 & 6.2). The broad shoulder around peak 5a appears to contain a smaller peak on either side. Cp values were viewed in Excel to determine if a peak existed. However, the Cp values in the raw data did not reveal a clear peak.

Figure 6.1 Thermogram of control (No HPP) *Listeria monocytogenes* ATCC 19115. Cells were grown at 15°C for 72 h and then centrifuged. Pellets were suspended in potassium phosphate buffer to obtain ~10^{11} CFU/ml. Five hundred µl of sample was injected into a microcalorimeter and scanned from 10 to 110°C at a rate of 60°C h^{-1} (black line). Samples were rapidly cooled to 10°C and rescanned (grey line). Lines show average of two replicates.

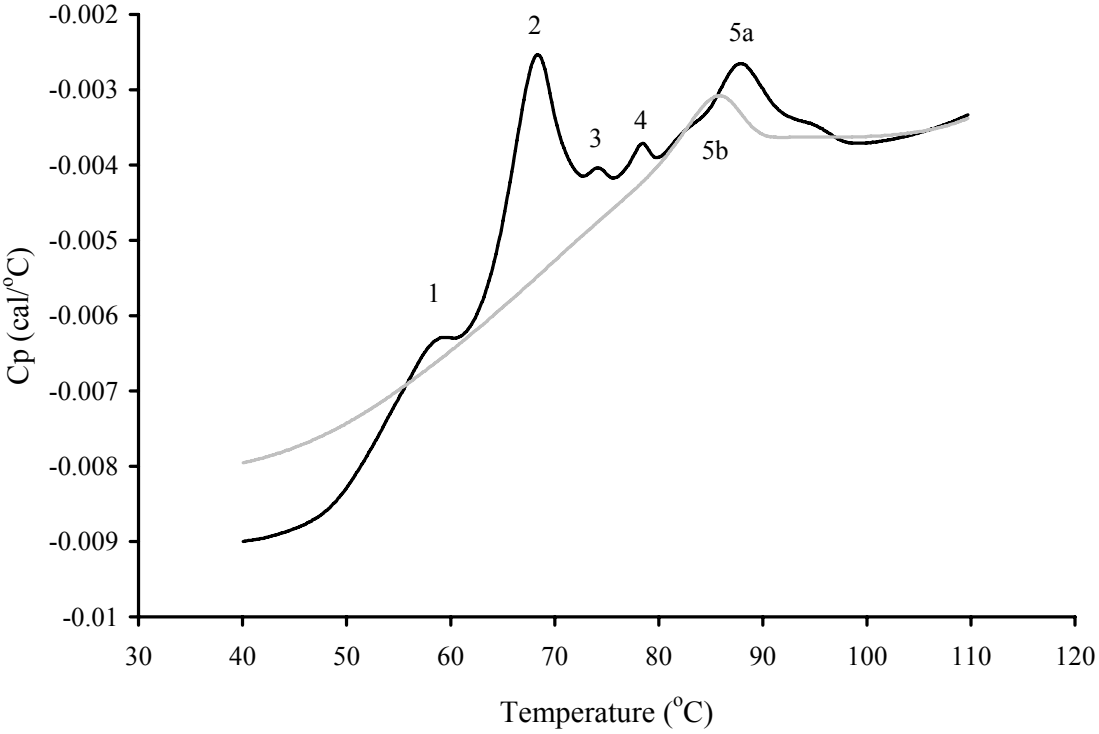
-0.002
-0.003
-0.004
-0.005
-0.006
-0.007
-0.008
-0.009
-0.01
Cp (cal/°C)
30
40
50
60
70
80
90
100
110
120
Temperature (°C)
1
2
3
4
5a
5b

Figure 6.2 Thermogram of *Listeria monocytogenes* ATCC 19115 following high pressure processing at 200 MPa. Cells were grown at 15°C for 72 h and then centrifuged. Pellets were suspended in potassium phosphate buffer to obtain ~10^{11} CFU/ml. Cells were HPP at ambient temperature at 200 MPa for 150 s. Five hundred µl of sample was injected into a microcalorimeter and scanned from 10 to 110°C at a rate of 60°C h^{-1} (black line). Samples were rapidly cooled to 10°C and rescanned (grey line). Lines show average of two replicates.

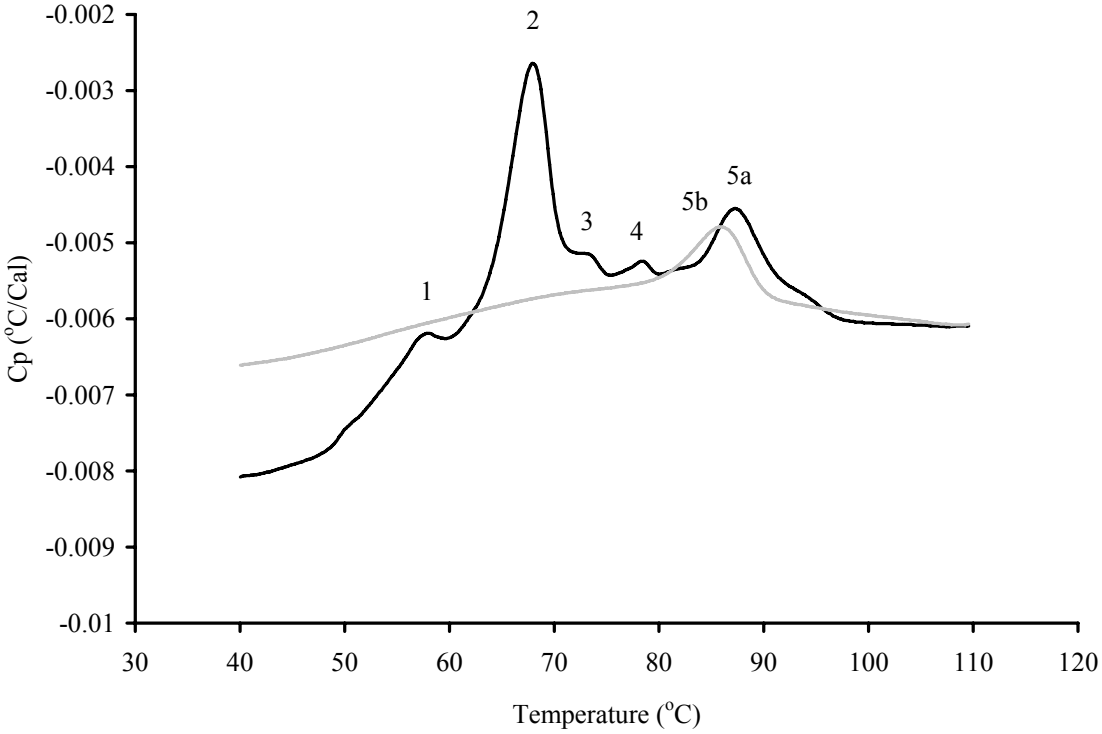
-0.002
-0.003
-0.004
-0.005
-0.006
-0.007
-0.008
-0.009
-0.01
Cp (°C/Cal)
30
40
50
60
70
80
90
100
110
120
Temperature (°C)
1
2
3
4
5b
5a

Figure 6.3 Thermogram of *Listeria monocytogenes* ATCC 19115 following high pressure processing at 400 MPa. Cells were grown at 15°C for 72 h and then centrifuged. Pellets were suspended in potassium phosphate buffer to obtain ~10^{11} CFU/ml. Cells were HPP at ambient temperature at 400 MPa for 150 s. Five hundred µl of sample was injected into a microcalorimeter and scanned from 10 to 110°C at a rate of 60°C h^{-1} (black line). Samples were rapidly cooled to 10°C and rescanned (grey line). Lines show average of three replicates.

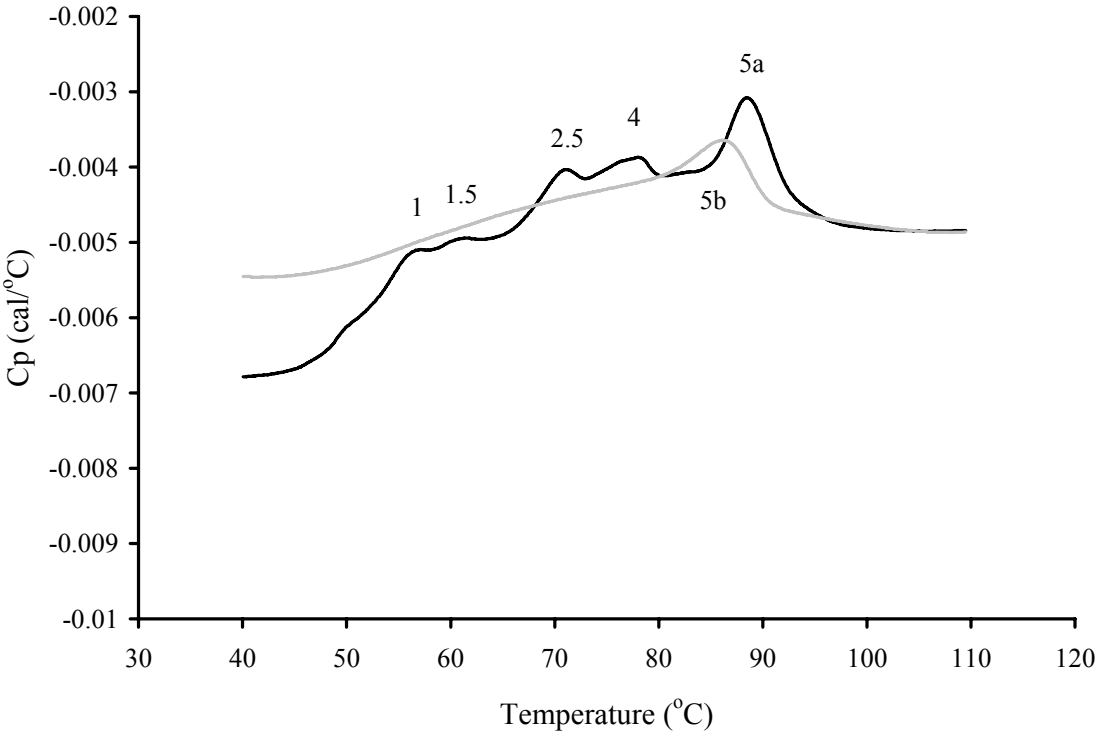
-0.002
-0.003
-0.004
-0.005
-0.006
-0.007
-0.008
-0.009
-0.01
Cp (cal/°C)
30
40
50
60
70
80
90
100
110
120
Temperature (°C)
1
1.5
2.5
4
5a
5b

Figure 6.4 Thermogram of *Listeria monocytogenes* ATCC 19115 following high pressure processing at 600 MPa. Cells were grown at 15°C for 72 h and then centrifuged. Pellets were suspended in potassium phosphate buffer to obtain ~10^{11} CFU/ml. Cells were HPP at ambient temperature at 600 MPa for 150 s. Five hundred µl of sample was injected into a microcalorimeter and scanned from 10 to 110°C at a rate of 60°C h^{-1} (black line). Samples were rapidly cooled to 10°C and rescanned (grey line). Lines show average of two replicates.

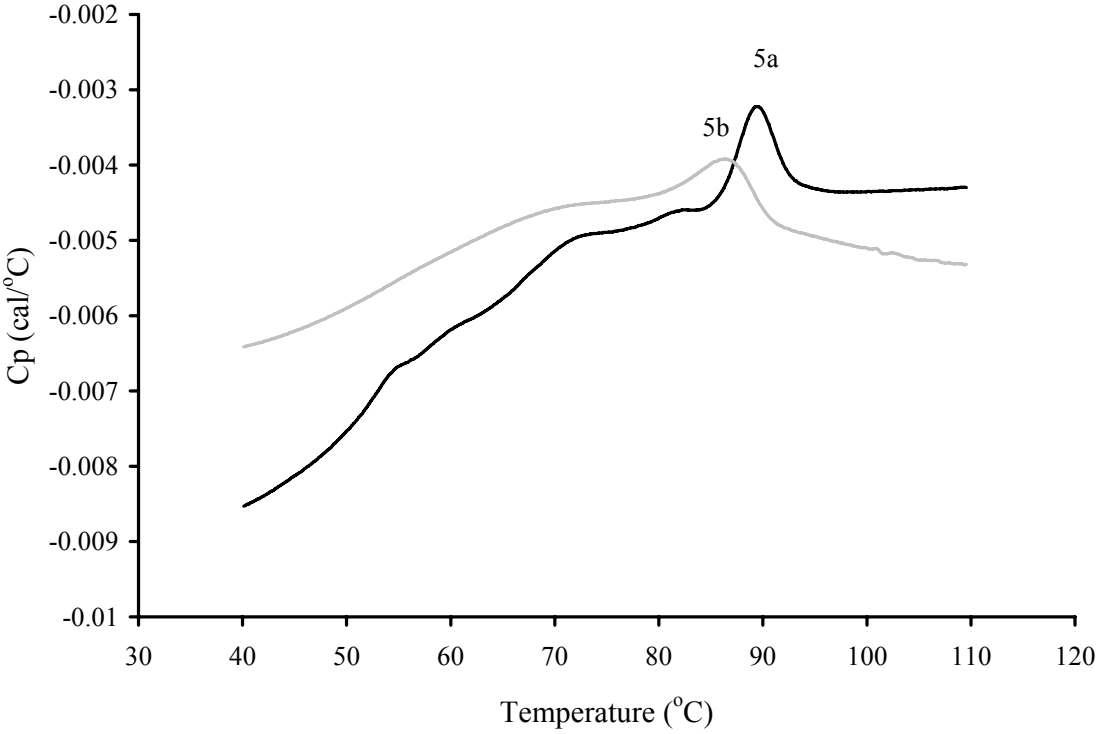
-0.002
-0.003
-0.004
-0.005
-0.006
-0.007
-0.008
-0.009
-0.01
Cp (cal/°C)
5a
5b
30
40
50
60
70
80
90
100
110
120
Temperature (°C)

Table 6.1 Maximum temperatures (T_ms) of endothermic peaks in control and high pressure processed *Listeria monocytogenes*.

Peak Number	Control	200 MPa	400 MPa	600 MPa
1	59.4	57.9	57.5	NP
1.5	NP∗	NP	61.4	NP
2	68.4	68.0	NP	NP
2.5	NP	NP	71.1	NP
3	74.1	73.22	NP	NP
4	78.4	78.4	78	NP
5a	87.8	87.2	88.5	89.4
5b	85.9	85.9	86.2	86.4

∗NP = Not present

6.5 Discussion

Miles (2006) recently proposed the critical target theory, which relates microbial inactivation to irreversible damage to a critical target of the cell. This target must be critical to cell function, and the number of copies of the target may influence microbial inactivation curves. Numerous sites within the cell have been reported to be involved in HPP inactivation of bacteria; however the critical targets remain unknown. Previous research has investigated the effect of HPP on the cell wall of *Saccharomyces cerevisiae* (Brul et al., 2000) and the cytoplasmic membrane of Gram-negative bacteria (Pagan and Mackay, 2000; Ritz et al., 2002). Nucleic acids have been shown to condense following HPP using transmission electron microscopy (Mackey et al., 1994; Hayman, 2001). Various proteins have been shown to be denatured by HPP (Simpson and Gilmour, 1997a; Wouters et al., 1998; Ritz et al., 2000a). However, the relative importance of each target in the inactivation of microorganisms by HPP is not known. Furthermore, it does not appear that the cell wall or the cell membrane are primary targets in the inactivation of *L. monocytogenes* by HPP (Hayman, 2007).

Many factors affect the barotolerance of microbial cells. Increased growth temperature increases barotolerance of *L. monocytogenes* (McClements et al., 2001; Bull et al., 2005; Hayman et al., 2007a), and stationary-phase cells are more barotolerant than mid-exponential phase cells (Casadei et al., 2002; Hayman et al., 2007a). Reduced water activity (Simpson and Gilmour, 1997c; Hayman et al., 2007b) and sublethal heat shock (Hayman, 2007) also increase the barotolerance of *L. monocytogenes*. Protein stability may lead to increased barotolerance in all of these situations and thus protein denaturation may play a major role in the inactivation of *L. monocytogenes* by HPP.

Therefore, the aim of this study was to determine the effect of HPP on protein denaturation in whole cells of *L. monocytogenes*.

A number of studies have employed DSC to investigate thermal injury or inactivation of various microorganisms. For example, Anderson et al. (1991) and Teixeira et al. (1997) used DSC to investigate the effect of salt on bacterial thermotolerance. DSC has also been used to investigate the effect of cold shock on the thermotolerance *L. monocytogenes* (Bayles et al., 2000). DSC has also been used to estimate D-values for microbial inactivation (Lee and Kaletunç, 2002a). Lepock at al (1990) used DSC to investigate the relationship between maximum growth temperature and thermal transitions in *Bacillus* spp. Mackey et al (1991) used DSC to investigate whole cells and cell components of *Escherichia coli*. They were able to elucidate ten peaks in whole cells and identify the cellular structures associated with some of these peaks by generating thermograms of isolated cell components.

Thermograms of control cells of *L. monocytogenes* in this study revealed five prominent endothermic peaks. Peaks may not represent a distinct transition in a single component of the cell, but rather a sum of many components (Lepock et al., 1990). The identity of Peak 1, T_m = 59.4°C, is not known, but it has been associated with metabolism in *Bacillus* spp. (Lepock et al., 1990) and the 30S ribosomal subunit in *E. coli* (Mackey et al., 1991; Lee and Kaletunç, 2002b). The identity of Peak 2, T_m = 68.4°C, has been identified as proteins and ribosomes in whole bacterial cells (Lepock et al., 1990; Mackey et al., 1991; Teixeira et al., 1997; Benito et al., 1999; Mohácsi-Farkas et al., 1999; Niven et al., 1999). Mackey et al. (1991) identified Peak 2 as ribosomes in an early publication that subsequent authors have referenced when identifying this peak as ribosomes.

However this peak was present in the thermograms of ribosome-free samples (Mackey et al., 1991), which indicates that this peak may have been incorrectly identified and may be due to cellular proteins. As proteins comprise approximately 55% of the dry weight of bacterial cells, it makes sense that the largest peak in the cell is associated with proteins. Also, many isolated proteins have T_ms between 60 to 70°C (Giancolaa et al., 1997; Grinberg et al., 2000; Dàvilaa et al., 2007). The enthalpy associated with ribosomes is a more complex issue, as ribosomes are comprised of two subunits: 30S (1 molecule of 16S rRNA and 21 proteins) and 50S (1 molecule each of 5S and 23S rRNA and 32 different proteins), and potential transitions may exist for the 30S and 50S subunits and the entire 70S particle (Neidhardt et al., 1990; Mackey et al., 1991). However, the ribosome is comprised of protein (~38%) and rRNA (~62%), and it is possible that transitions may exist for the denaturation of protein and rRNA separately, and the dissociation of rRNA from the protein may result in another transition. The identity of Peak 3, T_m = 74.1°C, is also uncertain, but may be associated with melting of RNA (Mackey et al., 1991), protein (Lepock et al., 1990) or ribosomes (Mohácsi-Farkas et al., 1999). The identity of Peak 4, T_m = 78.4°C, is also uncertain, but may be associated with protein (Lepock et al., 1990) or ribosomes (Teixeira et al., 1997; Mohácsi-Farkas et al., 1999; Niven et al., 1999). The Peak 5a, T_m = 87.8°C, is due to the reversible melting of DNA (Lepock et al., 1990; Anderson et al., 1991; Mackey et al., 1991; Belliveau et al., 1992; Teixeira et al., 1997). Peak 5b is also associated with DNA. Peak 5b has a slightly lower T_m and enthalpy than Peak 5a, which suggests that DNA does not re-anneal completely following the first scan.

HPP at 200 MPa, a non-lethal pressure treatment, produced thermograms that were not different than the control. Lethal HP-treatments of 400 and 600 MPa produced

DSC thermograms that were dramatically different from the control. HP-treatment at 400 MPa appeared to eliminate Peaks 2 and 3, and two new peaks (Peak 1.5 and 2.5) were observed. It is possible that the two new peaks were present in the control thermogram, but could not be resolved due the presence of Peak 2. HP-treatment at 600 MPa eliminated all of the peaks, except Peak 5a. The size and T_m of Peak 5a was the same in all treatments, as compared to the control, therefore DNA associated enthalpy was not affected by HPP. In contrast to these results, Kalentunç et al. (2004) reported that DNA-associated enthalpy and T_m was slightly reduced in *Leuconostoc mesenteroides* following lethal HP treatment at 500 MPa. Protein-associated enthalpy, represented by Peak 2, was eliminated following lethal pressure treatments, indicating that irreversible protein denaturation occurred during HPP. Niven et al (1999) found that a peak occurring at 68°C (i.e., corresponding to Peak 2 in this study) in *E. coli* was eliminated in DSC thermograms following HPP at 250 MPa. They concluded that changes in ribosome conformation due to HPP leads to cell death. Kalentunç et al. (2004) also reported that a peak occurring at 62°C was reduced following HPP of *L. mesenteroides* at 250 and 500 MPa, and also attribute this to ribosome denaturation. Peak 2 is also associated with loss of cell viability due to heat inactivation (Anderson et al., 1991; Lee and Kaletunç, 2002b). We conclude that Peak 2 represents the critical target in the inactivation of *L. monocytogenes* by HPP. In support of this conclusion, the inactivation volume (ΔV_{act}) values obtained for the inactivation of vegetative bacteria by HPP fall within the range for protein denaturation (Prehoda et al., 1998; Klotz at al., 2007). While Peak 2 can be attributed to cellular proteins it could be due to ribosomal proteins specifically, however

this needs to be verified. It is also possible that Peak 2 represents both ribosomal and and other critical cellular proteins.

DSC thermograms presented in this study show that protein-associated enthalpy, but not DNA-associated enthalpy, is eliminated in *L. monocytogenes* following HPP. Therefore, we conclude that protein denaturation occurs in HP-inactivated cells and is the main mechanism of inactivation of *L. monocytogenes* by HPP.

6.6 Acknowledgements

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CHAPTER SEVEN

CONCLUSIONS AND FUTURE RESEARCH

7.1 Conclusions

Results presented in this thesis show that many factors can increase the barotolerance of *L. monocytogenes*. These factors included entering the stationary growth phase, elevated growth temperature, heat shock, reduced water activity and lyophilization. All of these conditions may lead to increased protein stability. The most convincing evidence that protein stability contributes to barotolerance came from investigating the effect of HPP on lyophilized *L. monocytogenes*. Lyophilized *L. monocytogenes* and lactate dehydrogenase (LDH) were not inactivated by extreme HP (600 MPa, 5 min). The most plausible explanation for this result is that dehydrated proteins are in a rigid conformation, and therefore are not compressed by HP. Lack of compressibility may protect proteins from denaturation, and hence prevent loss of cell viability. In the other cases increased protein stability may arise from protein-protein interactions. Heat shock proteins are known to increase thermotolerance by repairing or degrading damaged proteins. It is probable that they increase barotolerance by performing the same function, i.e. repairing or degrading pressure-damaged proteins. As cells approach stationary-phase, transcription factors, including σ^B, induce cells to produce many new proteins. Many of these proteins are also involved in stress response, and may act as protein chaperones. Cells grown at elevated temperature may also produce stress proteins as *L. monocytogenes* is a psychrotrophic organism and thus cells may be mildly stressed when grown at 35 or 43°C.

The above findings, combined with the findings from preliminary experiments that indicated that the cell wall and cell membrane of *L. monocytogenes* are not damaged by HPP (summarized in Appendix A), lead to the conclusion that protein denaturation is the main mechanism by which Gram-positive organisms are inactivated by HPP. This hypothesis was supported by the finding that HPP caused aggregation of LDH. TEM images also indicated that protein aggregation may occur in HP-inactivated cells. DSC results show that protein-associated enthalpy in whole cells was eliminated following HPP, which also supports the hypothesis that cellular proteins are denatured by HPP.

While the critical target for inactivation of microorganisms by high pressure seems to be protein, the specific proteins involved are unknown. There may be one critical protein that leads to cell inactivation by high pressure. If this is the case, it must be a protein critical for the function of the cell. Many proteins are critical to the cell, but ribosomal proteins and proteins involved in respiration are amongst the most crucial, and may also be sensitive to pressure. For example, if ribosomal proteins were denatured by pressure and could not be repaired the cell would not be able to synthesize new proteins and would no longer be able to divide.

However, many proteins are inactivated by pressure, so perhaps there is no one critical protein target. I believe that cell inactivation is due to the inactivation of many types of protein within the cell. Therefore, the proposed mechanism of inactivation of *L. monocytogenes* by HPP is as follows;

1. Cellular proteins are compressed during pressurization.
2. There are permanent changes to protein conformation following decompression. These changes may include dissociation of the subunits

of multimeric proteins, modification to active sites and exposure of side chains.

3. Denatured proteins aggregate together and possibly with non-damaged proteins.
4. Massive protein aggregation cannot be repaired by the cell, and the cell cannot resume normal function or cell division.

7.2 Applications and future research

My main reason for undertaking this project was curiosity; I wanted to understand what changes occurred in bacteria upon exposure to pressure and what lead to their inactivation. However, this information can also have many practical implications. Understanding the factors that influence barotolerance can help processors develop strategies to optimize processing. For example, cells grown at lower temperatures were more pressure-sensitive in milk than cells grown at higher temperatures. Therefore, it may be desirable to hold raw milk at refrigeration temperatures before HPP. Whether this would actually decrease barotolerance would need to be verified. We have shown that microbial inactivation by HPP is highly dependant on water activity; therefore HPP is not suitable to treat dry or powdered foods. Knowing the mechanism of inactivation can also help to optimize processing and aid in the development of hurdle technologies. Understanding the mechanism of inactivation can also aid in the development of mathematical models to predict processing pressures and times.

There are several interesting questions that have arisen from this research. Protein denaturation has been concluded to be the primary mechanism of inactivation of *L. monocytogenes* by HPP; however it is not known which cellular proteins are involved in this process. Proteins are known to vary in their resistance to HP; therefore certain proteins in the cell may not be affected by pressure, while others are. It may be that certain classes of proteins may be more sensitive to pressure than others. For example, membrane bound proteins and multimeric proteins have been reported to be inactivated by HPP.

The tailing inactivation kinetics of microorganisms by HPP is also another fascinating issue. This phenomenon has been reported by many researchers, but the mechanism is unknown. It is possible that elevated expression of stress or stationary-phase proteins in a small part of the population may lead to tailing. This would be an interesting problem to investigate and solve.

APPENDIX A

Preliminary physiology experiments

Several experiments were undertaken to assess cell wall, cell membrane and DNA damage in HP-treated *Listeria monocytogenes*. They are briefly outlined here.

Cell membrane

Spectroscopy

- **Null Hypothesis (Ho)**: HPP inactivated cells do not leak UV absorbing material, detected at 260 mn.
- **Method**: Cells were grown at 15°C and 43°C to mid-exponential and mid-stationary phase. Cells were centrifuged and resuspended in TSYEB to obtain ~10^9 CFU/ml. Samples were HPP at 400 or 600 MPa for 5 to completely inactivate cells. Following HPP, the solution was filtered through 0.2 μm filter to remove the cells. The absorbance of the supernatant was measured at 260 nm.
- **Result**: No UV absorbing material was detected.
- **Conclusions**:
    1. Cells are not leaking UV absorbing material
    2. The cell membrane may not be the target of inactivation.

Flow Cytometery

- **Ho**: There will be no difference in cell color when HPP inactivated and non-HPP cells are stained with the BacLight Kit (Molecular Probes) and detected by flow cytometry.
- **Method:** Cells were grown to mid-exponential and mid-stationary-phase at 37°C and inactivated by HPP at 600 MPa for 5 min. Following HPP cells were suspending in water at 10^6 CFU/ml. Cells were stained with the BacLight Kit, using 3 µl of Syto green and 3 µl of propidium iodide. Live cells should stain green and dead cells (with damaged membranes) should stain red.
- **Results:** HPP-inactivated cells stained green.
- **Conclusion:** The cell membrane may not be affected by HPP.

Cell wall

Gram staining

- **Ho**: HP processed cells will stain Gram-positive and have identical morphology to non- HPP cells at 100 x magnification.
- **Method:** Cells were grown to mid-stationary-phase at 37°C and inactivated by HPP at 600 MPa for 5 min. Cells were Gram stained as normal, with ~1 min for each dye.
- **Results:** HPP and non-HPP cells looked exactly the same (very short, Gram-positive rods, many cells occurred in pairs).
- **Conclusion:** The cell wall may not be affected by HPP.

Transmission Electron Microscopy

- **Ho**: HP processed cells will have identical morphology to non- HPP cells when viewed by TEM.
- **Method:** Grew cells to mid-exponential-phase at 15°C and inactivated by HPP in TSYEB at 400 MPa or 600 MPa for 5 min. Cells were prepared for TEM as recommended by the PSU TEM facility.
- **Results:** The cell walls of HPP and non-HPP cells looked the same. DNA condensation was observed in HPP cells. Circular patches of less density occurred in the cytoplasm HPP cells.
- **Conclusions:** The cell wall and cell membrane may not be affected by HPP. However, there are significant changes in cytoplasmic morphology. DNA condensation occurs. Protein and/or ribosome aggregation may occur. The role these play in inactivation is unclear from the pictures.

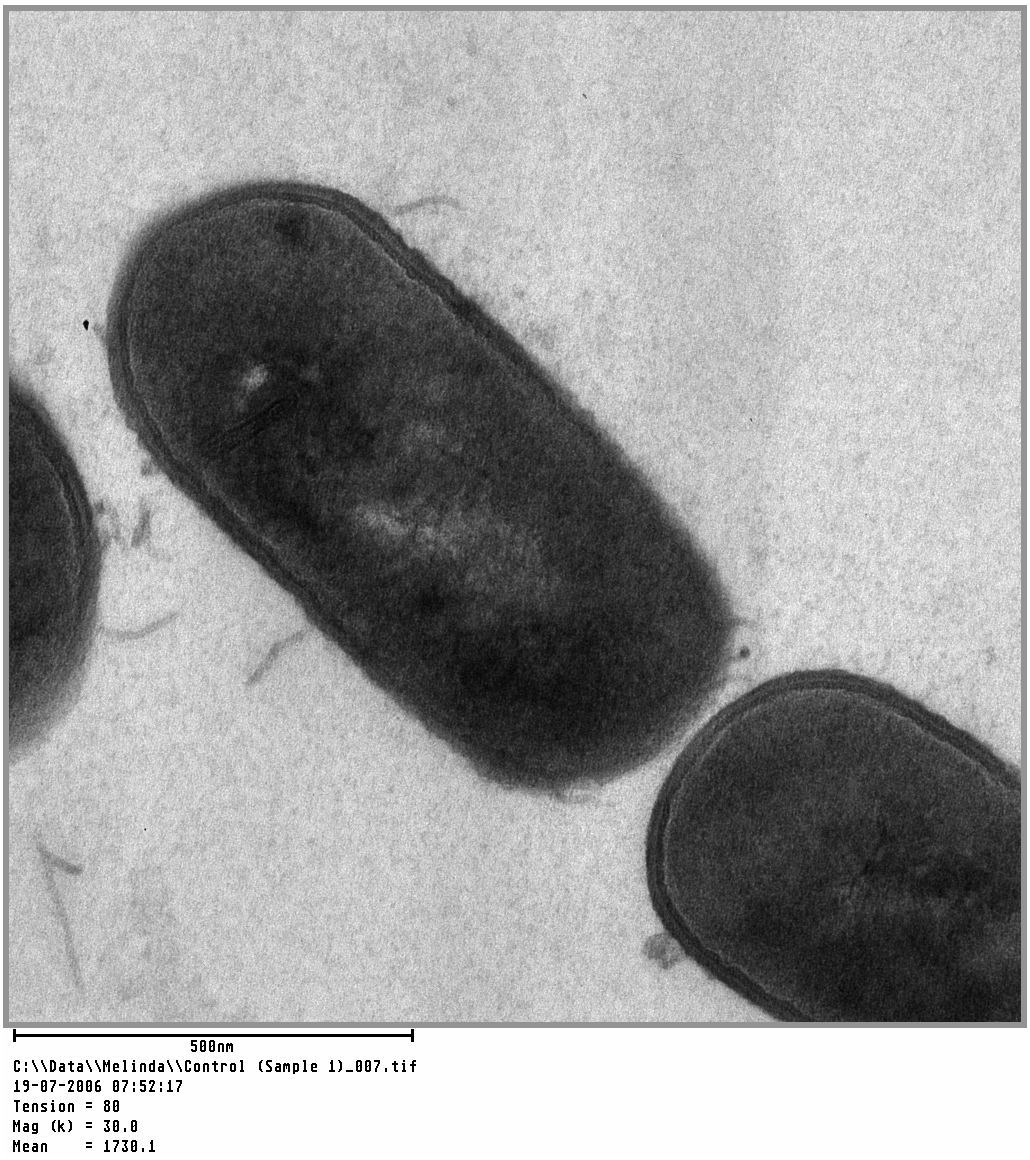

Figure A.1 Transmission Electron Microscopy image of control (No high pressure processing) *Listeria monocytogenes*. Cells were grown to mid-exponential phase at 15°C.

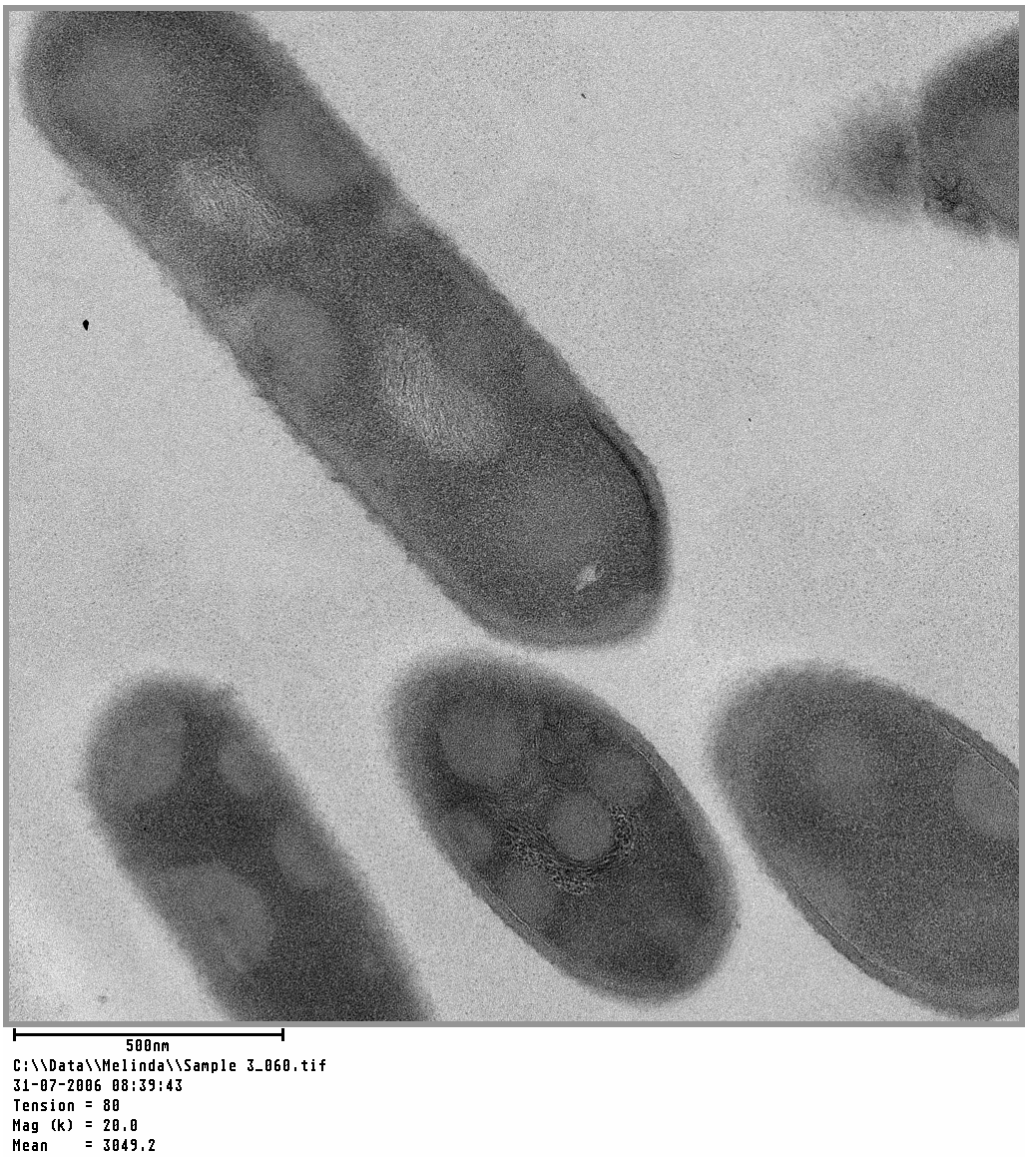

Figure A.2 Transmission Electron Microscopy image of *Listeria monocytogenes* inactivated by high pressure processing (600 MPa, 5 min). Cells were grown to mid-exponential phase at 15°C.

APPENDIX B

Using chloramphenicol to inhibit protein synthesis

Chloramphenicol is a bacteriostatic antibiotic that inhibits proteins synthesis by binding to the A-site on the 50S ribosomal subunit (Pestka, 1971). Review of the literature showed that 100 μg/ml is the standard concentration of chloramphenicol used to inhibit protein synthesis in Gram-positive and Gram-negative bacteria (Iandolo and Ordal, 1966; Black and Subjeck, 1989; Amoroso, 1995; Aersten et al., 2004; Noma et al., 2006). Amoroso (1995) confirmed that concentrations of 15-150 μg/ml will inhibit replication of *L. monocytogenes* in TSYEB.

Typically it may take three or four hours to run an experiment, including diluting and plating the samples. The aim was investigate whether chloramphenicol would reduce cell numbers over four hours and whether dilutions and spread plating would stop inhibition of protein synthesis. *L. monocytogenes* ATCC was grown overnight in 10 ml TSYEA at 35°C. Ten mg of chloramphenicol was added to a sterile flask and 100 g of UHT milk was added (final chloramphenicol concentration = 100 μg/ml). The flask was swirled for 60 s. Either 1 ml of overnight culture was added to yield ~10^7 CFU/ml, or the overnight culture was diluted in 0.1% peptone to result in ~10^2 CFU/ml in the flask. The flasks were incubated on the bench (~21°C) for four hours, with samples taken periodically for counting. In the flask with ~10^2 CFU/ml 100 μl and 1 ml of milk was directly plated onto TSYEA. In the flasks with ~10^7 CFU/ml milk was diluted in 0.1% peptone before spread-plating. All plates were incubated at 35°C for 48 h.

Table A.2. Effect of chloramphenicol on *Listeria monocytogenes* viability over four hours.

Sample	Time (h)	CFU/ml
No Chloramphenicol Initial inoculum ~10^7 CFU/ml	0	1.55 x 10^7
	0.5	1.95 x 10^7
	1	1.53 x 10^7
	2	2.04 x 10^7
	3	2.18 x 10^7
	4	2.50 x 10^7
Chloramphenicol Initial inoculum ~10^7 CFU/ml	0	1.55 x 10^7
	0.5	1.60 x 10^7
	1	2.09 x 10^7
	2	2.24 x 10^7
	3	1.57 x 10^7
	4	2.00 x 10^7
Chloramphenicol Initial inoculum ~10^2 CFU/ml	0	140
	0.5	330
	1	350
	2	270
	3	100
	4	190
No Chloramphenicol Initial inoculum ~10^2 CFU/ml	0	163

The data above show that chloramphenicol was not lethal to a high or low inoculum of *L. monocytogenes* during four hours of incubation in UHT milk. Dilution in peptone diluent, or even spreading 100 µl directly on TSYEA was sufficient to alleviate inhibition. When 1 ml of milk containing chloramphenicol, inoculated with ~10^2 CFU/ml, was spread on TSYEA no colonies grew after incubation for 48 h. Therefore milk did not interfere with chloramphenicol inhibition.

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