CHANGES IN BAROTOLERANCE, THERMOTOLERANCE AND CELLULAR MORPHOLOGY OF *LISTERIA MONOCYTOGENES* THROUGHOUT THE LIFE CYCLE

A Thesis in

Food Science

by

Jia Wen

© 2008 Jia Wen

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

December 2008
The thesis of Jia Wen was reviewed and approved* by the following:

Stephen J. Knabel  
Professor of Food Science  
Thesis Co-Advisor

Ramaswamy C. Anantheswaran  
Professor of Food Science  
Thesis Co-Advisor

Allen T. Phillips  
Professor Emeritus of Biochemistry

John D. Floros  
Professor of Food Science  
Head of the Department of Food Science

*Signatures are on file in the Graduate School
ABSTRACT

Listeria monocytogenes is a psychrotrophic pathogenic bacterium that can grow in foods and cause a serious disease, listeriosis. It can be present in raw or Ready-To-Eat (RTE) foods and is one of the most pressure- and heat-resistant vegetative foodborne pathogens. High Pressure Processing (HPP) is a non-thermal process of increasing interest due to the needs for minimally processed foods in the market. HPP allows microbial inactivation while maintaining the fresh flavors of foods; however, non-linear inactivation (tailing) during HPP of L. monocytogenes, which is a potential danger to food safety, has been reported. My initial experiments focusing on the mechanism(s) producing tailing were unsuccessful. Therefore, the focus of this research turned to changes in barotolerance, thermotolerance and cellular morphology of L. monocytogenes throughout its life cycle. In part one, L. monocytogenes was grown to log, stationary, death and long-term survival phases at 35°C in tryptic soy broth with yeast extract (TSBYE). Cells were diluted in UHT whole milk and then high pressure processed at 400 MPa for 180 s or thermally processed at 62.8°C for 30 s. As cells transitioned from log to long-term survival phases, D_{400 MPa} and D_{62.8°C} increased 10 and 19 fold, respectively. Cells decreased in size as they transitioned from log to long-term survival phases, and rod-shaped cells transitioned to cocci as they entered late-death and long-term survival phases. In part two, cells in long-term survival phase were centrifuged, suspended in fresh TSBYE and incubated at 35°C. As cells transitioned from long-term survival to log and stationary phases, they increased in size and log reductions increased
following HPP or heat treatment. In part three, cells in long-term survival phase were centrifuged, suspended in UHT whole milk and incubated at 4°C. After HPP or heat treatment similar results were observed as in part two. These results demonstrate that cells of *L. monocytogenes* are most barotolerant and thermotolerant in long-term survival phase, which needs to be considered when developing food processing or preservation methods to eliminate *L. monocytogenes*. 
TABLE OF CONTENTS

LIST OF FIGURES........................................................................................................... viii
LIST OF ABBREVIATIONS ............................................................................................. x
ACKNOWLEDGEMENTS................................................................................................. xii
CHAPTER ONE. Statement of the problem................................................................. 1
CHAPTER TWO. Literature review............................................................................. 2
  2.1 Listeria monocytogenes ...................................................................................... 2
     2.1.1 Taxonomy........................................................................................................ 2
     2.1.2 Morphology..................................................................................................... 2
     2.1.3 L. monocytogenes in natural environments, food processing plants and foods .................................................................................................................. 3
     2.1.4 Listeriosis........................................................................................................ 3
  2.2 High Pressure Processing................................................................................... 4
     2.2.1 High Pressure Processing in food industry................................................. 4
     2.2.2 Principles of HPP......................................................................................... 5
     2.2.3 Factors affecting barotolerance of microorganisms................................. 5
     2.2.4 Nonlinear inactivation (tailing) during HPP.............................................. 7
  2.3 Factors affecting thermotolerance of microorganisms.................................... 7
  2.4 Bacterial life cycle.............................................................................................. 8
2.4.1 The long-term survival phase ........................................ 8
2.4.2 Transition between phases ........................................... 9
2.5 Starvation of bacteria ...................................................... 10
  2.5.1 Starvation and dormancy ........................................... 10
  2.5.2 Effect of starvation on cellular morphology ...................... 11
  2.5.3 Cytoplasmic condensation induced by starvation .............. 12
  2.5.4 Effect of starvation on protein synthesis ....................... 12
2.6 Biofilms ........................................................................ 13
  2.6.1 Introduction .............................................................. 13
  2.6.2 Formation and detachment ........................................... 14
2.7 Bacterial endospores ...................................................... 14
  2.7.1 Barotolerance of endospores ........................................ 14
  2.7.2 Thermotolerance of endospores .................................... 15
  2.7.3 Dehydration in the cores of endospores ......................... 15
2.8 References ..................................................................... 16

CHAPTER THREE. Changes in barotolerance, thermotolerance and cellular morphology of Listeria monocytogenes throughout the life cycle ...................... 28
  3.1 Abstract ....................................................................... 29
  3.2 Introduction ................................................................... 30
  3.3 Materials and methods .................................................. 33
  3.4 Results ......................................................................... 38
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGURE 1</td>
<td>The growth curve of <em>L. monocytogenes</em> showing different growth phases.</td>
<td>43</td>
</tr>
<tr>
<td>FIGURE 2</td>
<td>Growth of <em>L. monocytogenes</em> at different phases of the life cycle and subsequent survival after HPP and heat treatments.</td>
<td>45</td>
</tr>
<tr>
<td>FIGURE 3</td>
<td>Barotolerance and thermostolerance of <em>L. monocytogenes</em> at different growth phases.</td>
<td>47</td>
</tr>
<tr>
<td>FIGURE 4</td>
<td>Light Micrographs (LMs), Scanning Electron Micrographs (SEMs) and Transmission Electron Micrographs (TEMs) of <em>L. monocytogenes</em> at different growth phases.</td>
<td>49</td>
</tr>
<tr>
<td>FIGURE 5</td>
<td>Growth of long-term survival phase cells of <em>L. monocytogenes</em> after inoculation into fresh TSBYE at 35°C and subsequent survival after HPP and heat treatments.</td>
<td>51</td>
</tr>
<tr>
<td>FIGURE 6</td>
<td>Scanning Electron Micrographs (SEMs) of <em>L. monocytogenes</em> at different growth phases after inoculation of cells in long-term survival phase into fresh, sterile TSBYE.</td>
<td>53</td>
</tr>
<tr>
<td>FIGURE 7</td>
<td>Phase-contrast photomicrographs of long-term survival phase cells of <em>L. monocytogenes</em> after inoculation into fresh TSAYE.</td>
<td>55</td>
</tr>
<tr>
<td>FIGURE 8</td>
<td>Growth of long-term survival phase cells of <em>L. monocytogenes</em> after inoculation into UHT whole milk at 4°C and subsequent survival after HPP and heat treatments.</td>
<td>57</td>
</tr>
</tbody>
</table>
FIGURE 9  Increase in baro- and thermo-tolerance of cells of \textit{L. monocytogenes} F5069 after inoculation into TSBYE and incubation at 35°C ............85

FIGURE 10  Increase in baro- and thermo-tolerance of cells of \textit{L. monocytogenes} Scott A after inoculation into TSBYE and incubation at 35°C ...............87

FIGURE 11  Gram stains of cells of \textit{L. monocytogenes} F5069 and Scott A with 13 h or 21 d incubation at 35°C in TSBYE.................................................89

FIGURE 12  Growth curves of cells in different phases after inoculation in fresh TSBYE.................................................................92

FIGURE 13  Growth of \textit{L. monocytogenes} within 14–28 h incubation at 35°C in TSBYE.................................................................99
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>D</td>
<td>Decimal Reduction Time</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HPP</td>
<td>High Pressure Processing</td>
</tr>
<tr>
<td>log</td>
<td>Logarithmic (when preceding numbers means ( \log_{10} ))</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>MPa</td>
<td>Mega Pascal</td>
</tr>
<tr>
<td>MOX</td>
<td>Modified Oxford Agar</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>RTE</td>
<td>Ready-to-Eat</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TSAYE</td>
<td>Tryptic Soy Agar with Yeast Extract</td>
</tr>
<tr>
<td>TSBYE</td>
<td>Tryptic Soy Broth with Yeast Extract</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra-High Temperature</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
</tbody>
</table>
I want to thank my parents, Shiyan Wen and Lijun Zhang, for their encouragement and support. They helped me to decide to study abroad although they knew we would miss each other. I also want to thank my girlfriend, Jing Guo, for her love. I am so lucky and happy to be with her.

I would like to give my sincere thanks to my advisors, Dr. Knabel and Dr. Anantheswaran, for guiding my research and helping me become a professional. Dr. Knabel taught me to question everything, to raise multiple hypotheses, and to “think like a microorganism”. Dr. Anantheswaran helped me with experimental designs, statistics, planning things, as well as my English. I gained so much from them not only about research but also about life. I want to thank Dr. Floros and Dr. Phillips for serving on my committee and for their advice and enthusiasm during my project.

I would like to thank my lab mates, Yi Chen, Melinda Hayman, Mei Lok, Sara Lomonaco, Michael Kalaras, Jabari Hawkins and Bek Yilma for their friendship and helping me with unfamiliar equipments. Also I would like to thank all the faculty, graduate students and staff in Food Science Department for their support.

I would like to thank USDA for providing financial support (Milk Safety Grant).
CHAPTER ONE

STATEMENT OF THE PROBLEM

Listeria monocytogenes is a psychrotrophic pathogenic bacterium that can cause listeriosis in immuno-compromised individuals. It is widespread in natural environments and can be present in raw or processed foods. It is one of the most pressure- and heat-resistant vegetative foodborne pathogens. This pathogen is generally rod-shaped, but has been reported to form cocci in broth cultures and saline. High Pressure Processing (HPP) is a non-thermal process of increasing interest due to the demand for minimally processed foods in the market. HPP allows microbial inactivation at moderate temperatures and thus can be used to eliminate L. monocytogenes in RTE foods. Thermal processing has been widely used as a traditional food process to inactivate L. monocytogenes in RTE foods. Barotolerance, thermotolerance and cellular morphology of L. monocytogenes at log or stationary phases have been well studied, but there is a lack of research on these aspects during long-term incubation. Therefore, this research focused on changes in barotolerance, thermotolerance and cellular morphology of L. monocytogenes throughout the life cycle. Such information is important for developing food processes that ensure complete destruction of L. monocytogenes.
CHAPTER TWO

LITERATURE REVIEW

2.1 Listeria monocytogenes

2.1.1 Taxonomy

Listeria is a genus of Gram-positive, non-spore-forming bacteria, which includes the species L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seelilgeri and L. grayi, based on numerical taxonomic and chemical studies, DNA homology and 16S rRNA cataloging analysis (Gray and Killinger, 1966; Farber and Peterkin, 1991). L. monocytogenes and L. ivanovii are pathogenic and the other four species in the genus Listeria are nonpathogenic (Kandler and Weiss, 1986).

2.1.2 Morphology

Cells of L. monocytogenes are generally short rods with rounded ends, ~0.5 micron in diameter and 1–2 microns in length (Kandler and Weiss, 1986). L. monocytogenes has also been reported to form coccoid cells in broth cultures (Gray and Killinger, 1966) or saline (Nannapaneni et al., 2008). Cells from 12–24 h old colonies may be arranged in V or Y forms (Gray and Killinger, 1966). The formation of long cell chains has been reported in p60 mutants of L. monocytogenes, and cell chains
disaggregated into normal sized single bacteria after p60 (extracellular protein) treatment (Kuhn and Goebel, 1989). Cells grown in tryptic phosphate broth (TPB) containing 1.5 M NaCl formed ~50 μm-long filaments, and an osmotic downshift induced the formation of septa in these long cells within 2–3 h (Jørgensen et al., 1995). Isom et al. (1995) also reported the formation of filament-type cells of *L. monocytogenes* when NaCl concentration > 1 M or pH > 9. Cells incubated at room temperature (~20°C) are motile with peritrichous flagella (Farber and Peterkin, 1991).

2.1.3 *L. monocytogenes* in natural environments, food processing plants and foods

*L. monocytogenes* is widespread in natural environments, including water, soil, dust, vegetation, etc (Sutherland et al., 2003). Due to its ubiquitous nature, this pathogen is commonly found on the surfaces of equipment in food processing plants (Wong, 1998; Thevenot et al., 2006). It is often present in raw or processed foods, such as shrimp, fish, shellfish, lettuce, tomatoes, raw milk, cheese, coleslaw and other Ready-To-Eat (RTE) foods (Beckers et al., 1987; Gitter et al., 1980; Rocourt and Cossart, 1997; Farber and Peterkin, 1991). *L. monocytogenes* is able to grow at refrigeration temperatures in various foods, such as milk or RTE foods (Brackett, 1988; Lovett, 1989).

2.1.4 Listeriosis

*L. monocytogenes* can cause the serious disease, listeriosis, in birds and mammals by invading and colonizing multiple host tissues (Gaillard et al., 1987; Tilney and
Portnoy, 1989). In most cases, listeriosis occurs in immuno-compromised individuals and shows similar symptoms in animals and humans (Wemekamp-Kamphuis et al., 2004). Listeriosis is responsible for nearly 35 percent of deaths in the United States due to known foodborne bacterial pathogens (Mead, et al., 1999). Concerns about listeriosis were raised to a higher level in the 1980s, due to many food-related outbreaks with high fatality rates (Farber and Peterkin, 1991). Large outbreaks of listeriosis have been reported due to the consumption of RTE dairy products (Bunning et al., 1988) and RTE meat and poultry products (Farber and Peterkin, 1991). Thus, a zero tolerance policy has been established for this foodborne pathogen in RTE foods by USDA and FDA (Gilbert, 1996).

2.2 High Pressure Processing

2.2.1 High Pressure Processing in food industry

High Pressure Processing (HPP), or Ultra High Pressure (UHP) processing or High Hydrostatic Pressure (HHP), is a non-thermal food process of increasing interest due to the demand for minimally processed foods in the market. High pressure was applied in the production of ceramics, plastics and other materials long before its application in the food industry (Smelt, 1998). During HPP liquid or solid foods, which can be packaged or unpackaged, are subjected to 50–1000 MPa for seconds to minutes (Williams, 1994). HPP allows inactivation of viruses, bacteria, yeasts and molds inside foods at moderate temperatures, while some valuable food attributes remain largely
unaffected, such as color, flavor, texture, or vitamin content (Knorr, 1999; Smelt, 1998; Chen et al., 2006). The market for HPP foods keeps growing, and includes foods such as guacamole in the U.S., fruit preparations in France, cooked ham in Spain and the U.S., and oysters in England (Eisenbrand, 2005).

2.2.2 Principles of HPP

HPP is based on the following three concepts: (1) covalent bonds are unaffected by pressure and thus the primary structures of proteins are not disrupted by pressure (Knorr, 1999). (2) HPP affects non-covalent bonds (Knorr, 1993; Patterson, 2005) by causing changes to secondary or tertiary structures of large molecules, such as proteins (Knorr, 1999). These changes in protein conformation caused by HPP can affect protein functionality, such as enzyme activity (Knorr, 1999). (3) Isostatic pressure is pressure that is evenly transmitted within a medium (i.e. foodstuff), independent of the shape or volume (Smelt, 1998). Therefore solid foods subjected to HPP will not be destroyed since the pressure is applied to all sides (Smelt, 1998).

2.2.3 Factors affecting barotolerance of microorganisms

Growth phase has been reported to affect the resistance of bacteria to high pressure (Hayman et al., 2007; McClements et al., 2001; Mackey et al., 1995). At a growth temperature of 15°C, cells of *L. monocytogenes* in mid-stationary phase were significantly more resistant to high pressure than log phase cells (*P* < 0.05) (Hayman et
al., 2007). Similar results have been reported with *Bacillus cereus* and *Pseudomonas fluorescens* (McClements et al., 2001).

Growth temperature can also affect barotolerance. Cells of *L. monocytogenes* in mid-stationary phase grown at 35 or 43°C were significantly more pressure resistant than cells grown at 4, 15 or 25°C ($P < 0.05$) (Hayman et al., 2007). McClements et al. (2001) reported that log phase cells of *L. monocytogenes*, *B. cereus* and *P. fluorescens* grown at 8°C were more barotolerant than those grown at 30°C, but stationary phase cells showed the opposite results.

Starvation has been reported to enhance survival of a barophile, *Thermococcus peptonophilus*, at 45 or 30 MPa (Canganella et al., 1997), and induce pressure resistance in a marine vibrio (Novitsky and Morita, 1978b).

Strains of *L. monocytogenes* differ in barotolerance. Nine strains of *L. monocytogenes* were subjected to 400 or 500 MPa and significant ($P < 0.05$) variability in barotolerance was observed (Tay et al., 2003). *L. monocytogenes* OSY-8578 showed the highest resistance to pressure while *L. monocytogenes* Scott A was the most sensitive, therefore Tay et al. (2003) suggested that *L. monocytogenes* OSY-8578 should be the target strain for HPP efficacy studies.

Heat shock has been reported to enhance barotolerance of *L. monocytogenes* (Hayman et al., 2008b). Cells of *L. monocytogenes* at stationary phase grown at 15°C were heat shocked at 48°C for 10 min and then subjected to 400 MPa for 150 s. $D_{400\text{MPa}}$
of heat-shocked cells was significantly higher than the non-heat-shocked cells \((P < 0.05)\) (Hayman et al., 2008b).

Water activity is another factor affecting barotolerance. When water activity decreased below ~0.8 in glycerol/peptone water solutions, survival of \(L.\) monocytogenes following pressure treatment significantly increased \((P < 0.05)\) (Hayman et al., 2008a).

### 2.2.4 Nonlinear inactivation (tailing) during HPP

Nonlinear inactivation (tailing) during HPP of microorganisms has been reported (Hayman et al., 2007; Tay et al., 2003; Karatzas and Bennik, 2002). Tailing is a potential danger to food safety, and various mechanisms have been hypothesized. Tailing might be due to the survival of a genetically pressure-resistant subpopulation (Karatzas and Bennik, 2002), or due to a pressure-adaptation response induced by HPP (Tay et al., 2003). However, the mechanism(s) causing tailing is(are) still unclear.

### 2.3 Factors affecting thermotolerance of microorganisms

Growth phase can affect thermotolerance of bacteria. In \(L.\) monocytogenes Scott A, \(D_{56^\circ C}\) of stationary phase cells was significantly higher than log phase cells \((P < 0.05)\) (Lou and Yousef, 1996). Similar results were reported with \(Escherichia\) coli (Kaur et al., 1998).
Starvation enhanced thermotolerance of a marine vibrio and two *E. coli* strains (Jouper-Jaan et al., 1992). Jenkins et al. (1988) reported similar results in *E. coli*. Glucose starvation for 9 days enhanced thermotolerance of *L. monocytogenes* EGD (Herbert and Foster, 2001). Lou and Yousef (1996) reported similar results in *L. monocytogenes* Scott A. Starvation significantly (*P* < 0.05) enhanced thermotolerance of *A. globiformis*, 67% of starved cells survived 80°C for 10 min (Demkina et al., 2000). Hartke et al. (1994) reported similar results in *Lactococcus lactis*.

The intracellular position of *L. monocytogenes* in bovine phagocytes has been reported not to enhance thermotolerance (Bunning et al., 1988). Bunning et al. (1988) compared the thermotolerance of cells of *L. monocytogenes* within bovine milk phagocytes and those freely suspended in milk. Results of intracellular and extracellular experiments did not show a significant difference in thermotolerance (*P* > 0.05).

Other factors affecting thermotolerance of bacteria include growth temperature (Luedecke and Harmon, 1966; Hayashidani et al., 2005), acid shock, heat shock or osmotic shock (Skandamis et al., 2008). García et al. (2001) reported that cold shock enhanced thermotolerance in cells of *Clostridium perfringens*.

### 2.4 Bacterial life cycle

#### 2.4.1 The long-term survival phase

The textbook view of the bacterial growth curve traditionally was thought to be comprised of four phases: lag, exponential, stationary, and death phases (Madigan, 2000).
However, there are many studies reporting survival during long-term starvation, both in batch culture in the laboratory and in natural environments. In soil cultures of low nutrients, *Rhizobium meliloti* was able to survive over thirty years (Jensen, 1961). A psychrophilic marine vibrio, Ant-300, isolated from the Antarctic was able to survive in “organic-free” artificial seawater for 70 weeks (Novitsky and Morita, 1978). A variety of bacterial pathogens, including *L. monocytogenes, E. coli, Salmonella* spp., were able to survive in sterile distilled water from several months to sixteen years (Liao and Shollenger, 2003). Lappin-Scott and Costerton (1990) also stated that bacterial cells in soils and rocks were not dead after the death phase. A similar phenomenon was found in *E. coli*, which remained at ~$10^6$ CFU/ml for more than five years without any addition of nutrients after the death phase (Finkel, 2006). After a one to two log drop, the number of viable cells of *E. coli* remained constant for weeks or months (Kolter et al., 1993). Such survival was also reported for *Serratia* and *Sarcinia* spp. (Steinhaus, 1939). Finkel (2006) named this phase after death “the long-term stationary phase”.

### 2.4.2 Transition between phases

Bacteria exit log phase and enter the stationary phase at the point where growth rate decreases (Kolter et al., 1993). The transition between log and stationary phases might be due to exhaustion of one or more essential nutrient(s) (Madigan, 2000; Kolter et al., 1993), or due to accumulation of waste products to an inhibitory level (Madigan,
The death of some cells and the reproduction of others balance each other, thus the cell concentration at stationary phase is constant (Madigan, 2000).

The mechanism(s) of the transition from stationary and death phases is(are) not well understood. Finkel (2006) postulated that cells die randomly depending on how many cells can be supported by the limited available nutrients, thus the timing of death phase might also be random (stochastic). Another explanation is that cell death is programmed (apoptosis) and the population transitions to death phase when the cell concentration is too high at stationary phase. After a proportion of the population die, the survivors can exit the death program and then continue reproduction, thus apoptosis might actually represent a way to preserve the surviving population (Finkel, 2006).

Bacterial populations transition from death to long-term survival phase when survivors exit the death program (Finkel, 2006). Survivors (1–10% of the population) respond to the “surviving” signal and then live on the debris of dead cells (Finkel, 2006). This phenomenon is called cryptic growth (Kolter et al., 1993; Finkel, 2006; Postgate and Hunter, 1963).

2.5 Starvation of bacteria

2.5.1 Starvation and dormancy

It is claimed that after the death phase, bacteria can enter a “dormant but viable state” and survive for an indefinite period, until available nutrients are restored (Lappin-Scott and Costerton, 1990). Oliver and Stringer (1984) stated that starvation would result in dormancy, which might be the typical state of marine bacteria. For a psychrophilic
marine vibrio, after one week of starvation respiration had decreased to only 0.0071% of total carbon respired and remained constant thereafter (Novitsky and Morita, 1977), and ATP per cell decreased by 59% during three weeks of starvation (Oliver and Stringer, 1984). Another strain of vibrio showed declined synthesis of total RNA and proteins after several hours of starvation (Nyström et al., 1990). However, Kaprelyants et al. (1993) believed that starved cells may or may not be dormant, and defined dormancy as “unable to divide or to form a colony on an agar plate without a preceding resuscitation phase” and a state of “low metabolic activity” in which cells could survive for prolonged periods. A third possibility is that the population during long-term starvation is highly dynamic because newly created mutants are more competitive than older strains, with the former finally taking over the whole population under the selective pressure imposed by starvation (Finkel, 2006, Zambrano et al., 1993; Kolter et al., 1993; Martínez-Rodriguez et al., 2004).

### 2.5.2 Effect of starvation on cellular morphology

During starvation, bacterial cells decrease in size and/or form cocci. Glucose starvation resulted in reduced cell size of *L. monocytogenes* (Herbert and Foster, 2001). Starved bacteria in soils and rocks may decrease cell size to improve their penetration into the substratum (Lappin-Scott and Costerton, 1990). *A. globiformis* transitioned from rods to cocci in carbon-deficient media (Demkina et al., 2000). After starvation a marine vibrio was able to decrease size and change shape from a rod to a coccus, which then
regained its original size and shape without a significant lag when placed in fresh media (Novitsky and Morita, 1976). The rod shape of *E. coli* changed to a smaller coccoid shape during starvation, which might be due to cell divisions without enlarging cell volume (Lange and Hengge-Aronis, 1991). Such “reductive division” may enhance the survival of a population by increasing cell numbers (Morita, 1986). Coccoid cells of *E. coli* reverted to rod shape immediately after addition of fresh medium (Kolter, 1993).

### 2.5.3 Cytoplasmic condensation induced by starvation

Reeve et al. (1984) reported that glucose starvation for 170 h caused cytoplasmic condensation in *E. coli* K-12. Electron photomicrographs revealed that the cytoplasmic contents had shrunken away from cell wall after starvation, while the cell wall remained unchanged in size and shape. Reeve et al. (1984) found that the decrease in cytoplasm size was correlated with the extrusion of small molecules from the cellular material into the starvation medium. After prolonged starvation for up to 117 h, more $^{14}$C-labeled materials in the labeled cells were extruded into medium than those absorbed from medium into cells (Reeve et al., 1984).

### 2.5.4 Effect of starvation on protein synthesis

Jouper-Jaan et al. (1992) subjected a marine vibrio and two *E. coli* strains K165 and Sc122 to starvation stress in saline, and then used chloramphenicol to study the role of protein synthesis on heat resistance at different times of starvation. They postulated
that de novo protein synthesis might be responsible for enhanced thermotolerance at the initial stage of starvation; however, the continuous thermotolerance during long-term starvation was not due to de novo protein synthesis (Jouper-Jaan et al., 1992).

Reeve et al. (1984) studied the effect of chloramphenicol on culture viability during glucose starvation in *E. coli* K-12. They found that the more chloramphenicol was added or the earlier it was added to the starvation medium, the fewer the number of viable cells were observed upon extended incubation. The proteins most important for survival were those synthesized at the first 9 h of starvation (Reeve et al., 1984).

### 2.6 Biofilms

#### 2.6.1 Introduction

A biofilm is an accumulation of microorganisms on organic or inorganic surfaces by way of adhesive polysaccharides secreted by the cells (Hunt et al., 2004). Biofilms are commonly found in natural environments such as soil, plants or marine sediments (Gandhi and Chikindas, 2007); biofilms are also present on the surfaces of equipment in food processing plants (Wong, 1998; Thévenot et al., 2006). Cells in biofilms have different characteristics from those freely suspended, including enhanced resistance to stresses such as heat or sanitizers (Frank and Koffi, 1990), cell wall structure or enzyme activity (Gandhi and Chikindas, 2007).
2.6.2 Formation and detachment

The formation of biofilms is postulated to proceed in several stages: (1) reversible adsorption on surfaces, (2) irreversible attachment on surfaces, (3) growth of bacteria, and (4) formation of exopolymers composed of adhesive polysaccharides (Gandhi and Chikindas, 2007).

Biofilm detachment was triggered by nutrient starvation in *Aeromonas hydrophila* (Sawyer and Hermanowicz, 1998) and *Pseudomonas aeruginosa* (Hunt et al., 2004). Biofilms of *A. hydrophila* showed an increased detachment rate as the nutrient supply decreased (Sawyer and Hermanowicz, 1998). *P. aeruginosa* formed biofilms under continuous-flow conditions and biofilms detached after the flow stopped. However, after biofilms were nutrient starved, the detachment occurred under continuous-flow conditions (Hunt et al., 2004).

2.7 Bacterial endospores

2.7.1 Barotolerance of endospores

Bacterial endospores are highly barotolerant even to 1000 MPa (Smelt, 1998; Gould, 2006). However, low pressure at 50–200 MPa could cause spore germination into pressure-sensitive vegetative cells. Thus a two-step pressure treatment might be a possible way to inactivate spores: the first step of treatment at low pressure triggers the spore germination, and the second high pressure treatment kills germinated spores (Gould, 2006). Another effective way to kill spores is to combine heat treatment along with HPP (Smelt, 1998). The barotolerance of endospores can be significantly different
between species or strains within a species. The spores of *Clostridium botulinum* are some of the most barotolerant (Patterson, 2005). However, there is a lack of reports on the mechanism of high barotolerance of spores.

### 2.7.2 Thermotolerance of endospores

Endospores are extremely thermotolerant (Gould, 2006), and the mechanism of the high resistance has been extensively studied (Beaman et al., 1982 and 1984; Nakashio and Gerhardt, 1985). A widely accepted mechanism is that low water content (only 10–30% of the water content of vegetative cells) in the spore core contributes to the thermotolerance (Madigan, 2000). Beaman et al. (1982) reported that thermotolerance correlated inversely with water content in five types of *Bacillus* spores. Beaman et al. (1984) reported that core dehydration was sufficient to explain the high thermotolerance in *Bacillus megaterium*. Nakashio and Gerhardt (1985) reported similar results in *B. megaterium*. In addition, mineralization of calcium dipicolinic acid (DPA) contributes to heat resistance (Gould, 2006). Thirdly, small acid-soluble spore proteins (SASPs) in spore cores have been reported to bind and protect DNA, and thus enhance heat resistance (Gould, 2006).

### 2.7.3 Dehydration in the cores of endospores

During the formation of endospores, the first step of dehydration in the cores occurs when exosporiums appear and cortexes between the two membranes of forespores are formed. Then further dehydration occurs when dipicolinic acid and small acid-
soluble spore proteins are produced in the cores (Madigan, 2000). Dehydration inside the spore cores is thought to be maintained by the contraction and expansion of surrounding cortexes (Gould, 2006).

2.8 References


61. **Nannapaneni, R., R. Story, K. C. Wiggins, and M. G. Johnson.** 2008. Invasiveness of non-starved and up-to-24-month starvation-stressed cells of *Listeria monocytogenes* Scott A serotype 4b in the human Caco-2 cell model,


CHAPTER THREE

CHANGES IN BAROTOLERANCE, THERMOTOLERANCE AND CELLULAR MORPHOLOGY OF LISTERIA MONOCYTOGENES THROUGHOUT THE LIFE CYCLE

Jia Wen, Ramaswamy C. Anantheswaran, and Stephen J. Knabel*

Department of Food Science, The Pennsylvania State University, University Park, Pennsylvania 16802

* Corresponding author. Mailing address: Department of Food Science, The Pennsylvania State University, University Park, PA 16802. Phone: (814) 863-1372. Fax: (814) 863-6132. E-mail: sjk9@psu.edu.
3.1 Abstract

Changes in barotolerance, thermodtolerance and cellular morphology of *Listeria monocytogenes* throughout the life cycle were investigated. In part one, *L. monocytogenes* ATCC 19115 was grown to log, stationary, death and long-term survival phases at 35°C in tryptic soy broth with yeast extract (TSBYE). Cells were diluted in UHT whole milk and then high pressure processed (HPP) at 400 MPa for 180 s or thermally processed at 62.8°C for 30 s. As cells transitioned from log to long-term survival phases, D\textsubscript{400 MPa} and D\textsubscript{62.8°C} increased 10 and 19 fold, respectively. Cells decreased in size as they transitioned from log to long-term survival phases, and rod-shaped cells transitioned to cocci as they entered late-death and long-term survival phases. *L. monocytogenes* strains F5069 and Scott A showed similar results. In part two, cells in long-term survival phase were centrifuged, suspended in fresh TSBYE and incubated at 35°C. As cells transitioned from long-term survival to log and stationary phases, they increased in size and log reductions in CFU/ml increased following HPP or heat treatment. In part three, cells in long-term survival phase were centrifuged, suspended in UHT whole milk and incubated at 4°C. After HPP or heat treatment similar results were observed as in part two. We hypothesize that cells of *L. monocytogenes* enter the long-term survival phase due to starvation and become more barotolerant and thermodtolerant due to a decrease in water activity when they transition from rods to cocci and undergo cytoplasmic condensation.
3.2 Introduction

*Listeria monocytogenes* is a Gram-positive, nonsporing, facultatively anaerobic and pathogenic bacterium (Lovett, 1989). It is generally rod-shaped, but has been reported to form coccoid cells in broth cultures (Gray and Killinger, 1966). This pathogen is widespread in the environment, including water, soil, vegetation, feces, etc (Farber and Peterkin, 1991). It is often present in food processing plants and in raw or processed foods, such as raw milk and many ready-to-eat (RTE) foods (Beckers et al., 1987; Gitter et al., 1980; Rocourt and Cossart, 1997). It is able to grow at refrigeration temperatures (Brackett, 1988; Lovett, 1989) and can cause listeriosis in humans and animals, due to its ability to invade and grow inside host cells (Gaillard et al., 1987; Tilney and Portnoy, 1989). Listeriosis can occur in immuno-compromised individuals, as well as in infants and the elderly (Wemekamp-Kamphuis et al., 2004), and is responsible for nearly 35 percent of deaths in the United States due to known foodborne bacterial pathogens (Mead et al., 1999). Large outbreaks of listeriosis have been reported due to the consumption of RTE dairy products (Bunning et al., 1988) and RTE meat and poultry products (Farber and Peterkin, 1991). Thus, a zero tolerance policy was established for this pathogen in RTE foods by FDA and USDA (Gilbert, 1996).

High pressure processing (HPP) is a non-thermal process of increasing interest, which allows microbial inactivation at moderate temperatures. Solid or liquid foods are normally subjected to 50–1000 MPa for seconds to minutes (Williams, 1994). HPP can significantly reduce the number of vegetative microbial cells inside foods (Knorr, 1999;
Smelt, 1998; Chen et al., 2006), while some valuable food attributes remain largely unaffected, such as color, texture, or vitamin content. As a result, high pressure processed foods such as fruit preparations in France, cooked ham in Spain and the U.S., and oysters in England have become popular in the marketplace (Eisenbrand, 2005).

The bacterial growth curve typically consists of four phases: lag, log, stationary and death (Madigan, 2000). The effect of growth phase on barotolerance, thermotolerance and cellular morphology of bacteria has been widely studied. Cells of *L. monocytogenes* in the stationary phase are more barotolerant than log phase cells (Hayman et al., 2007). Thermotolerance of stationary phase cells is also higher than log phase cells of *Escherichia coli* (Kaur et al., 1998) and *L. monocytogenes* (Lou and Yousef, 1996). Rod-shaped cells are known to change from rods to cocci when *E. coli* and *Arthrobacter* cultures transition from log to stationary phases (Kolter et al., 1993; Hamilton et al., 1977; Forni and Grilli Caiola, 1992).

While most microbiology textbooks discuss only four phases of the bacterial life cycle (lag, log, stationary and death), some reports have discussed a fifth phase: the long-term survival phase. Bacteria are often not all dead after the death phase; instead they can enter a “starved” state and survive for long periods, until starvation is relieved (Lappin-Scott and Costerton, 1990). This fifth phase has been found in *E. coli* (Finkel, 2006) and in *Serratia* and *Sarcinia* spp. (Steinhaus, 1939).

Unfortunately, research describing this long-term survival phase in *L. monocytogenes* is nonexistent. However, there is well-developed general knowledge
regarding the effect of starvation on barotolerance, thermotolerance and cellular morphology of *L. monocytogenes* and other bacteria. Regarding barotolerance, starvation enhanced survival at 45 or 30 MPa in a barophile, *Thermococcus peptonophilus* (Canganella et al., 1997), and induced pressure resistance to ~25 MPa in a marine vibrio (Novitsky and Morita, 1978b). Thermotolerance is enhanced by starvation in many microorganisms, such as *L. monocytogenes* (Lou and Yousef, 1996; Herbert and Foster, 2001), *E. coli* (Jenkins et al., 1988), *Arthrobacter globiformis* (Demkina et al., 2000) and *Lactococcus lactis* (Hartke et al., 1994). Starvation is known to induce a change in cell shape from rods to cocci in *Arthrobacter crystallopoietes* (Hamilton et al., 1977), *A. globiformis* (Demkina et al., 2000), a marine vibrio, Ant-300 (Novitsky and Morita, 1976), and *Rhizobium leguminosarum* (Thorne and Williams, 1997). Starvation also induced the formation of smaller coccoid cells of *Staphylococcus aureus* (Watson et al., 1998). After glucose starvation, cells of *L. monocytogenes* were reportedly shorter and wider than log phase cells (Herbert and Foster, 2001).

Therefore, the purpose of the present study was to investigate changes in barotolerance, thermotolerance and cellular morphology of *L. monocytogenes* throughout the life cycle of this pathogen. Such information may not only expand our knowledge of the long-term survival phase in this important foodborne pathogen, but also provide insights into its resistance to pressure and heat throughout its life cycle. This information is important when developing food processing methods that ensure complete destruction of *L. monocytogenes*. Furthermore, to simulate real-world food processing conditions,
the effect of storage time in milk at 4°C on barotolerance and thermodurability of cells in
the long-term survival phase was also investigated.

3.3 Materials and Methods

**Preparation of microorganisms.** *L. monocytogenes* ATCC 19115, serotype 4b, which was isolated from human cerebrospinal fluid (Begot et al., 1997) was obtained from the American Type Culture Collection (ATCC) (ATCC, Manassas, VA, USA). *L. monocytogenes* F5069, serotype 4b, which was isolated from raw milk (Fleming et al., 1985) was obtained from Lewis Graves (Foodborne and Diarrheal Disease Branch, Centers for Disease Control and Prevent, USA). *L. monocytogenes* Scott A, serotype 4b, which was isolated from a patient in the 1983 Massachusetts milk outbreak (Fleming et al., 1985) was obtained from Food Science Department (the Pennsylvania State University, PA, USA). Glycerol stocks of these strains were maintained at -80°C and streaked on tryptic soy agar with 0.6% yeast extract (TSAYE) (Becton, Dickinson and Company, Sparks, MD, USA) with incubation at 35°C for 48 h. For each strain, one colony on the plate was inoculated into 10 ml of tryptic soy broth with 0.6% yeast extract (TSBYE), with incubation at 35°C for 24 h. A 0.1 ml aliquot of the resulting culture was then diluted with 9.9 ml 0.1% peptone water (Becton, Dickinson and Company, Sparks, MD, USA) to achieve ~10⁷ CFU/ml, and 0.1 ml of the diluted culture was inoculated into 100 ml of TSBYE.

**Part I. From log to the long-term survival phase.** *L. monocytogenes* ATCC 19115 was incubated in TSBYE at 35°C for 12 h, 16 h, 22 h, 28 h, 41 h and 2–30 d to
obtain cells in late-log, stationary, early-death, mid-death, late-death and long-term survival phases, respectively. _L. monocytogenes_ strains F5069 and Scott A were incubated in TSBYE at 35°C for up to 21 d (these two strains were used only in high pressure, thermal processing and Gram stain experiments). Cells were then pressure or heat treated and observed following the procedures described below.

**(i) High pressure processing.** To measure survival after high pressure processing at 400 MPa for 180 s, bottles containing 99 ml of sterile UHT whole milk were inoculated with 1 ml of cell cultures in different phases and shaken well. To determine D$_{400}$ MPa, cell cultures at different phases were diluted in sterile UHT whole milk to achieve a similar starting concentration of 10$^6$–10$^7$ CFU/ml.

Samples were prepared and pressure treated following the procedure described by Hayman et al. (2007). Samples in sealed plastic vials were high pressure processed at 400 MPa for various times at room temperature (~22°C). The come-up time to reach 400 MPa was ~90 s and come-down time was ~9 s. After high pressure processing the sealed plastic vials containing the samples were put in ice water until plating (~20 min). The experiment was replicated three times.

**(ii) Thermal processing.** To measure survival after thermal processing at 62.8°C for 30 s, bottles containing 99 ml of sterile UHT whole milk were inoculated with 1 ml of cell cultures in different phases and shaken. To determine D$_{62.8}$°C, cell cultures at different phases were diluted in UHT whole milk to achieve a similar starting concentration of 10$^4$–10$^5$ CFU/ml.
Samples were transferred to thermal death time (TDT) tubes which were sealed using a Type 3 blowpipe, and then heat treated following the procedure described by Knabel et al. (1990). After heating at 62.8°C for various times TDT tubes were removed from the water bath and placed in ice water for 10 min before plating. The come-up time to reach 62.8°C from room temperature (~22°C) was 87 s and the come-down time was 130 s (from 62.8°C to 4°C). The experiment was replicated three times.

(iii) Enumeration after high pressure or thermal processing. Pressure or heat treated samples were plated on TSAYE with subsequent incubation at 30°C for 48 h before colony enumeration. Samples were diluted in 0.1% peptone water before plating when necessary. The limit of detection was 10 CFU/ml.

(iv) Gram stains. Gram stains of cell cultures were examined using a BX40F4 light microscope (Olympus optical, Japan) equipped with a DP71 digital camera (Olympus optical, Japan) using a 100× oil immersion objective lens. The experiment was replicated twice.

(v) Scanning Electron Microscopy (SEM). Cells of *L. monocytogenes* ATCC 19115 in different growth phases were collected on a 0.22 μm filter, then fixed with the primary fixative (1.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1 M cacodylate buffer [pH 7.4]) at 4°C overnight, and refixed with the secondary fixative (1% osmium tetroxide in 0.1 M cacodylate buffer [pH 7.4]) at room temperature (~22°C) for 1 h. Fixed cells were dehydrated by washing with 50%, 70%, 85%, 95% and 100% ethanol sequentially, dried using a CPD 030 Critical Point Dryer (Bal-Tec, Brookline, NH, USA)
using liquid CO₂ as the transitional fluid, sputter coated with Au/Pt, and then examined using a JSM 5400 Scanning Electron Microscope (JEOL, Tokyo, Japan) at an instrument magnification of 10,000–20,000×. The experiment was replicated twice.

(vi) Transmission Electron Microscopy (TEM). 1.5 ml of cell cultures of *L. monocytogenes* ATCC 19115 in different growth phases were centrifuged at 13,000 × g for 5 min at room temperature (~22°C). Pellets were fixed with the same primary fixative as used in the SEM preparation at 4°C for 3 d, and refixed with the same secondary fixative as used in the SEM preparation at room temperature (~22°C) for 1 h. Fixed cells were dehydrated by washing with 50%, 70%, 90%, 100% ethanol and 100% acetone sequentially. Dehydrated cells were infiltrated at room temperature (~22°C) using eponate resin and embedded at 70°C overnight for polymerization. Polymerized resin blocks were sectioned into thin slices, which were examined using a JEM 1200EX II Transmission Electron Microscope (JEOL, Tokyo, Japan) at an instrument magnification of 6,000–25,000×. The experiment was replicated twice.

Part II. Reentering the log phase from the long-term survival phase in TSBYE at 35°C. *L. monocytogenes* ATCC 19115 was incubated in TSBYE at 35°C for 30 d (long-term survival), and then 10 ml of the culture was centrifuged at 13,000 × g for 15 min at 20°C using an Avanti J-26 XPI centrifuge (Beckman Coulter, Fullerton, CA, USA). Pellets were suspended in 10 ml of fresh TSBYE to achieve a starting concentration of ~10^8 CFU/ml and then incubated at 35°C for 0.3–1, 2–4 or 5.5–9 h to
obtain cells in lag, log or stationary phases, respectively. Cells were then pressure or heat treated and observed using SEM following the same procedures described in part I, and then observed using slide cultures, as described below.

(i) Slide cultures. A 0.1 ml aliquot of a 30-d-old cell culture of \textit{L. monocytogenes} ATCC 19115 in the long-term survival phase was mixed with 10 ml molten TSAYE at ~39°C. One drop of the mixture was then added onto a sterile microscope slide and covered by a sterile cover slip. After the slide culture solidified, it was examined using a BX40F4 phase-contrast microscope (Olympus optical, Japan) using a 100× oil immersion objective lens at room temperature (~22°C) every 1–1.5 h. The light source was shut off between observations to avoid excessive heat that might kill cells on the slide. The experiment was replicated twice.

Part III. Reentering the log phase from the long-term survival phase in milk at 4°C. 10 ml cultures of \textit{L. monocytogenes} ATCC 19115 in the long-term survival phase were harvested and centrifuged as described in part two. Pellets were suspended in 10 ml of sterile UHT whole milk and then incubated at 4°C for 2–4 or 6–29 d to obtain cells in log or stationary phases. Cells were then pressure or heat treated following the same procedures described in part I.

Statistical analysis. Enumeration and cellular morphology data were analyzed using ANOVA (general linear model) using Minitab software (version 14.20; Minitab, State College, PA, USA). Pairwise comparisons were made using Tukey’s least significant difference test (\(\alpha = 0.05\)). To calculate D-values, linear regressions of
enumeration data from high pressure and thermal inactivation experiments were performed using Microsoft Excel (version 2003; Microsoft, Redmond, WA, USA). Coefficient of determination (R^2) of the linear regression of log reductions versus cell length was calculated to evaluate the correlation between cell length and barotolerance or thermotolerance using Microsoft Excel.

3.4 Results

Part I. From log to the long-term survival phases. Growth curves at 35°C (Fig. 1) demonstrated that once cells of L. monocytogenes ATCC 19115 were inoculated into fresh TSBYE, there was a log phase (Fig. 1A) until the population reached ~3×10^9 CFU/ml (stationary phase; Fig. 1B). After a ~2 h stationary phase (Appendix E) cells rapidly entered the death phase (Fig. 1C), where approximately 90% of the cells died within 24 h. However, after this rapid death, ~10^8 CFU/ml survivors remained viable for at least one month without any addition of nutrients, indicating that the cells had entered a long-term survival phase (Fig. 1D). Pairwise comparison of mean CFU/ml at each growth time revealed that there was no significant difference in CFU/ml within the long-term survival phase at 70, 214, 502 and 718 h (Fig. 1D) (P > 0.05).

ANOVA revealed that log reductions after high pressure or thermal processing were significantly affected by growth phase (P < 0.001) (Fig. 2). Log reductions after pressure treatment at 400 MPa for 180 s decreased significantly (P < 0.001) from 5.1 in late-log and stationary phases, to 2.1 in death phase to 0.3 in long-term survival phase.
Log reductions after pressure treatment of late-death phase cells at 41 h and long-term survival phase cells at 70, 214, 502 and 718 h were significantly lower than cells at all previous sampling times \((P < 0.05)\). Log reductions after heat treatment at 62.8°C for 30 s decreased significantly \((P < 0.001)\) from 5.7 in stationary phase, to 3.8 in death phase to 1.6 in long-term survival phase. Log reductions after heat treatment of long-term survival phase cells at 70, 214, 502 and 718 h were significantly lower than cells at all previous sampling times \((P < 0.05)\). Similar patterns were observed using \textit{L. monocytogenes} strain F5069 and strain Scott A (Appendix A).

Both barotolerance and thermotolerance significantly increased as cells of \textit{L. monocytogenes} ATCC 19115 progressed from late-log to long-term survival phases \((P < 0.001)\) (Fig. 3). To calculate \(D_{400 \text{ MPa}}\), linear regression was applied to the linear portions (the part without a shoulder or a tail) of the inactivation curves of cells at different phases. ANOVA revealed that \(D_{400 \text{ MPa}}\) was significantly affected by growth phase \((P < 0.001)\). Pairwise comparison of mean \(D_{400 \text{ MPa}}\) showed that \(D_{400 \text{ MPa}}\) of cells in late-log phase \((23.2 \text{ s})\) < early-death \((64.5 \text{ s})\) < late-death \((242.0 \text{ s})\) = long-term survival \((230.1 \text{ s})\) \((P < 0.05)\). \(D_{400 \text{ MPa}}\) of cells in stationary phase \((30.2 \text{ s})\) was not significantly different from late-log or early-death phases \((P > 0.05)\). For thermal processing (Fig. 3B), all the curves exhibited log-linear kinetics, thus linear regression of all data points was used to calculate D-values at 62.8°C. ANOVA revealed that \(D_{62.8{}^\circ \text{C}}\) was significantly affected by growth phase \((P < 0.001)\). Pairwise comparison of mean \(D_{62.8{}^\circ \text{C}}\) showed that \(D_{62.8{}^\circ \text{C}}\) of cells in late-log \((3.3 \text{ s})\) < late-death \((19.6 \text{ s})\) < long-term survival \((61.4 \text{ s})\) \((P < 0.05)\).
D$_{62.8}^{\circ}C$ of cells in stationary (9.4 s) and early-death (10.8 s) phases were not significantly different from late-log or late-death phases ($P > 0.05$).

Representative photomicrographs of cells of *L. monocytogenes* ATCC 19115 in different phases are shown in Fig. 4. Gram stains and SEM photomicrographs showed that some percentage of rods became cocci as the population entered the long-term survival phase and that cells decreased in size as they transitioned from the log phase to the long-term survival phase (Fig. 4, LM and SEM). SEM results showed that all cells in late-log, stationary and early-death phases were rod-shaped, and that cocci started to appear in the late-death phase. Pairwise comparison of percentages of cocci (% cocci) in late-death phase at 41 h and long-term survival phase at 9, 21 and 30 days revealed that % cocci in Gram stain at 41 h (4%) < at 30 days (26%) ($P < 0.05$). There was no significant difference between % cocci in Gram stain at 9 days (16%) or 21 days (22%) and at 41 h (4%) or 30 days (26%) ($P > 0.05$). Also, % cocci in SEM at 41 h (2%) = at 9 days (13%) < at 21 days (79%) = at 30 days (85%) ($P < 0.05$). Similar results were observed using *L. monocytogenes* strains F5069 and Scott A (Appendix A).

ANOVA of SEM data revealed that cell length was significantly affected by growth phase ($P < 0.001$). Pairwise comparison of mean cell length at each phase revealed that cell length at late-log phase (1.25 μm) > stationary phase (1.00 μm) = early-death phase (0.97 μm) = late-death phase (0.92 μm) > long-term survival phase at 9 days (0.81 μm) > long-term survival phase at 21 days (0.64 μm) = long-term survival phase at 30 days (0.58 μm) ($P < 0.05$) (Fig. 4, SEM). There was a correlation between cell length before treatment and log reductions after pressure ($R^2 = 0.73$) or after heat treatment ($R^2$
= 0.82). Some cocci exhibited wrinkled surfaces (Fig. 4G, SEM). TEM revealed cells in the long-term survival phase at 30 days with a dark thin cell wall and a network in the cytoplasm, which appeared condensed and attached to the cell wall (Fig. 4G, TEM).

**Part II. Reentering the log phase from the long-term survival phase in TSBYE at 35°C.** When cells of *L. monocytogenes* ATCC 19115 in the long-term survival phase after 30 days of incubation were inoculated into fresh, sterile TSBYE and incubated at 35°C, cells transitioned from lag to log and to stationary phases (Fig. 5).

ANOVA revealed that log reductions after pressure or thermal processing were both significantly affected by growth phase (*P* < 0.001) (Fig. 5). Pairwise comparison of mean log reductions after pressure treatment demonstrated that mean log reductions of long-term survival phase cells (0.4) = lag phase (0.2) < log phase (1.2) < stationary phase (2.2) (*P* < 0.001). Pairwise comparison of mean log reductions after heat treatment showed that mean log reductions of lag phase cells (1.4) < long-term survival phase (1.8) < log phase (2.8) < stationary phase (4.9) (*P* < 0.001).

After inoculation of long-term survival cells into fresh, sterile TSBYE, mean length of cells and proportion of rod-shaped cells significantly increased (*P* < 0.05), as cells rapidly transitioned from lag phase to stationary phase (Fig. 6). Slide culture results showed the process of cocci converting to rod-shaped cells, and then forming microcolonies (Fig. 7). During this transition there was a strong correlation between cell length before treatment and log reductions after pressure (R^2 = 0.96) or heat treatment (R^2 = 0.93).
Part III. Reentering the log phase from the long-term survival phase in milk at 4°C. After inoculation into UHT whole milk and incubation at 4°C, cells of *L. monocytogenes* ATCC 19115 in the long-term survival phase transitioned to log phase and remained there for 6 d. After 6 d incubation cells transitioned into the stationary phase and remained there for at least 29 d (Fig. 8). ANOVA again revealed that log reductions after pressure or heat treatment were significantly affected by growth phase (*P* < 0.001). Pairwise comparison of mean log reductions after pressure treatment revealed that mean log reductions of long-term survival phase cells (0.4) < log phase (0.9) < stationary phase (1.3) (*P* < 0.001). Pairwise comparison of mean log reductions after heat treatment showed that mean log reductions of long-term survival phase cells (1.7) < log phase (2.0) < stationary phase (3.2) (*P* < 0.001).

Pairwise comparison of mean log reductions after pressure treatment at 400 MPa for 180 s revealed that log reductions of log phase cells in part I > part II = part III, and that log reductions of stationary phase cells in part I > part II > part III (*P* < 0.001). Similarly, log reductions after heat treatment at 62.8°C for 30 s of log phase cells in part I > part II > part III (*P* < 0.001), and log reductions after heat treatment of stationary phase cells in part I > part II > part III (*P* < 0.05).
FIG. 1. Growth of *L. monocytogenes* ATCC 19115 in TSBYE at 35°C for different times to yield log (A), stationary (B), death (C), or long-term survival (D) phase cells. Cells were enumerated by plating on TSAYE with incubation at 30°C for 48 h. Data points and error bars represent means and standard deviations based on three replications of the experiment.
Growth time (h) at 35°C in TSBYE

CFU/ml TSBYE
FIG. 2. Growth of *L. monocytogenes* at different phases of the life cycle and subsequent survival after HPP and heat treatments. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for different times to yield log (A), stationary (B), death (C), or long-term survival (D) phase cells. Cells in different phases were then diluted 1:100 in UHT whole milk. Milk inoculated with *L. monocytogenes* was pressure treated at 400 MPa for 180 s at room temperature (~22°C) or heat treated at 62.8°C for 30 s. Cells were enumerated before and after pressure or heat treatment by plating on TSAYE with incubation at 30°C for 48 h. Data points and error bars represent means and standard deviations based on three replications of the experiment.
Growth time (h) at 35°C in TSBYE prior to pressure or heat treatment

CFU/ml whole milk

Before treatments
After pressure treatment
After heat treatment

0 100 200 300 400 500 600 700 800

Growth time (h) at 35°C in TSBYE prior to pressure or heat treatment
FIG. 3. Barotolerance (A) and thermotolerance (B) of *L. monocytogenes* at different growth phases. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for 12 h, 16 h, 22 h, 41 h, or 30 d to yield late-log, stationary, early-death, late-death, or long-term survival phase cells. Then cells in different phases were diluted in UHT whole milk. Milk inoculated with *L. monocytogenes* was pressure treated at 400 MPa for different times at room temperature (~22°C) or heat treated at 62.8°C for different times. Cells were enumerated before and after pressure or heat treatment by plating on TSAYE with incubation at 30°C for 48 h. Data points and error bars represent means and standard deviations based on three replications of the experiment.
FIG. 4. Light Micrographs (LMs), Scanning Electron Micrographs (SEMs) and Transmission Electron Micrographs (TEMs) of *L. monocytogenes* at different growth phases. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for various times to yield cells at different phases, and then observed using LM (Gram stain), SEM and TEM. (LM) Bars = 10 μm. (SEM) Bars = 1 μm. (TEM) Bars = 0.5 μm.
FIG. 5. Growth of long-term survival phase cells of *L. monocytogenes* after inoculation into fresh TSBYE at 35°C and subsequent survival after HPP and heat treatments. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for 30 d to yield long-term survival phase cells and 30 ml cultures were centrifuged. Pellets were suspended in 30 ml fresh TSBYE and then incubated at 35°C for various times before being diluted 1:100 in UHT whole milk. Milk inoculated with *L. monocytogenes* was pressure treated at 400 MPa for 180 s at room temperature (~22°C), or heat treated at 62.8°C for 30 s. Cells were enumerated before and after pressure or heat treatment by plating on TSAYE with incubation at 30°C for 48 h. Data points and error bars represent means and standard deviations based on three replications of the experiment.
Growth time (h) at 35°C in TSBYE prior to pressure or heat treatment

CFU/ml whole milk

- Before treatments
- After pressure treatment
- After heat treatment

Growth time (h) at 35°C in TSBYE prior to pressure or heat treatment
FIG. 6. Scanning Electron Micrographs (SEMs) of *L. monocytogenes* at different growth phases after inoculation of cells in long-term survival phase into fresh, sterile TSBYE. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for 30 d to yield long-term survival phase cells and 30 ml cultures were centrifuged. Pellets were suspended in 30 ml fresh TSBYE and then incubated at 35°C for 0, 1, 4 or 7 h to yield cells at long-term survival (A), lag (B), log (C) or stationary (D) phases, respectively. Cells were then observed using SEM. Bars = 1μm.
FIG. 7. Phase-contrast photomicrographs of long-term survival phase cells of *L. monocytogenes* after inoculation into fresh TSAYE. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for 30 d to yield long-term survival phase cells. A 0.1 ml aliquot of this cell culture was then mixed with 10 ml molten TSAYE to make a slide culture. After the slide culture solidified, it was examined using a phase-contrast microscope at room temperature (~22°C). Photomicrographs were taken at 0 h (A), 1.5 h (B), 2.5 h (C), 3.5 h (D), 5 h (E) and 15 h (F) of incubation. Arrows indicate the transition from a coccoid cell to a rod-shaped cell that formed a microcolony. Bars = 10 μm.
FIG. 8. Growth of long-term survival phase cells of *L. monocytogenes* after inoculation into UHT whole milk at 4°C and subsequent survival after HPP and heat treatments. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for 30 d to yield long-term survival phase cells and 30 ml cultures were centrifuged. Pellets were suspended in 30 ml UHT whole milk and then incubated at 4°C for various times before being diluted 1:100 in UHT whole milk. Milk inoculated with *L. monocytogenes* was pressure treated at 400 MPa for 180 s at room temperature (~22°C), or heat treated at 62.8°C for 30 s. Cells were enumerated before and after pressure or heat treatment by plating on TSAYE with incubation at 30°C for 48 h. Data points and error bars represent means and standard deviations based on three replications of the experiment.
Growth time (d) at 4°C in whole milk prior to pressure or heat treatment.

- **Before treatments**: 
  - **CFU/ml whole milk**
  - Values range from $1.0 \times 10^0$ to $1.0 \times 10^7$.

- **After pressure treatment**: 
  - **CFU/ml whole milk**
  - Values range from $1.0 \times 10^0$ to $1.0 \times 10^4$.

- **After heat treatment**: 
  - **CFU/ml whole milk**
  - Values range from $1.0 \times 10^0$ to $1.0 \times 10^4$.
3.5 Discussion

The life cycle of many microorganisms contains five phases: lag, log, stationary, death and long-term survival (Lappin-Scott and Costerton, 1990; Finkel, 2006; Steinhaus, 1939). However, the growth curve only containing the first four phases has become a dogma found in microbiology textbooks (Madigan, 2000). The concept of life cycle is critical for many microorganisms including \textit{L. monocytogenes}: cells in the long-term survival phase are preserved for next round of growth; once nutrients are restored, long-term survival phase cells resume growth and continue their life cycle.

Bacteria exit log phase and enter the stationary phase at the point where growth rate decreases (Kolter et al., 1993). Bacteria are believed to reach the stationary phase when: (1) one or more essential nutrient(s) is (are) exhausted (Madigan, 2000; Kolter et al., 1993) or (2) waste products are accumulated to an inhibitory level; or both (Madigan, 2000). In the stationary phase some cells in the population die while other cells reproduce, which can balance each other and thus maintain a constant cell concentration (Madigan, 2000; Kolter et al., 1993). In the present study, the stationary phase was relatively short (~2 h) (Appendix E). The rapid transition from stationary to death phases may be regulated by available nutrients or quorum sensing described below.

After remaining at high density in the stationary phase, bacterial cells enter the death phase (Finkel, 2006). However, the mechanism(s) of the transition from stationary phase to death phase is (are) not clear. It is possible that entry to death phase is a random (or stochastic) event. In this passive model, when cells enter the death phase only depends on the level of available nutrients. On the other hand, cell death could be a
programmed event (apoptosis) regulated by quorum sensing (Bassler, 2002). In this latter active model, bacteria sense that there is (or will be) limited nutrients or their population is too high and they then communicate a suicide command to other cells via signaling molecules (Finkel, 2006). By dying and lysing, dead bacteria release nutrients into the medium, which serve as food for survivors. This might partially explain the long-term survival of those cells that survive the death phase. Lysed dead cells are known to release another signal to let survivors exit apoptosis (Finkel, 2006), but this signal might not be effective until it reaches a threshold concentration when 90–99% of cells are dead. Survivors (1–10% of the population) can respond to the “surviving” signal and live on the debris of dead cells. This phenomenon has been termed cryptic growth (Kolter et al., 1993; Finkel, 2006; Postgate and Hunter, 1963). Such surviving cells might still be metabolically active and exhibit long-term survival (Kolter et al., 1993). Although cell concentrations in the long-term survival phase at 502 and 718 h were not significantly different ($P > 0.05$) (Fig. 1D), there was a slight increase in cell numbers after 502 h incubation. This increase might have been significant if the culture had been incubated longer than 718 h. Further research is needed to test whether this increase is significant and if so what might cause it.

In the long-term survival phase, bacteria might be in a dormant state. Dormancy induced by starvation has been reported in soil and rock microorganisms (Lappin-Scott and Costerton, 1990) and marine bacteria (Oliver and Stringer, 1984; Novitsky and Morita, 1977). The dormant state is based on very low carbon respiration (Novitsky and Morita, 1977), decreased ATP level (Oliver and Stringer, 1984) and/or declined RNA and
protein synthesis (Nyström et al., 1990) after starvation. However, Kaprelyants et al. (1993) claimed that starved cells may or may not be dormant. Assays to measure the above parameters might be able to determine whether or not the long-term survival phase cells in the present study are truly dormant. A third possibility is that the long-term survival phase is highly metabolically dynamic with populations changing over time (Finkel, 2006; Martínez-Rodriguez et al., 2004). In this scenario newly created mutants that are more competitive than the parent culture finally take over the whole population, which occurs reproducibly in the long-term survival phase of E. coli (Finkel, 2006, Zambrano et al., 1993; Kolter et al., 1993).

The bacterial life cycle in batch cultures is akin to that in the natural environment. Nutrients in an environment are typically available in a feast-or-famine scenario, and starvation-survival is thought to be the most common state for most bacteria (Kolter et al., 1993; Finkel, 2006). Starved bacteria in nature are analogous to cells in the long-term survival phase in the laboratory (Finkel, 2006). The life cycle of bacteria is controlled by available nutrients and bacteria have to adapt their metabolism to maintain long-term starvation-survival (Finkel, 2006). Such long-term survival would increase fitness by preserving bacteria for later reproduction when conditions become more favorable for growth. Long-term starvation-survival has been characterized in Gram positive bacteria including L. monocytogenes (Herbert and Foster, 2001; Liao and Shollenberger, 2003; Nannapaneni et al., 2008), A. crystallopoietes (Hamilton et al., 1977), A. globiformis (Demkina et al., 2000) and S. aureus (Watson et al., 1998) and Gram negative bacteria
including *E. coli* (Kolter et al., 1993), a marine vibrio (Novitsky and Morita, 1978a), *R. meliloti* (Jensen, 1961) and *R. leguminosarum* (Thorne and Williams, 1997).

Previous research on barotolerance in the long-term survival phase is lacking. The results in this study showed that barotolerance significantly increased from log to long-term survival phases (Fig. 2 and 3A). Similar to the findings in this study, Novitsky and Morita (1978b) found that starvation for one week enhanced the barotolerance of a marine vibrio, which could also change cellular morphology from rods to cocci during starvation (Novitsky and Morita, 1976). It is speculated that the enhanced barotolerance of cells in long-term survival phase is due to cytoplasmic condensation (Reeve et al., 1984), which might lower water activity. Lowered water activity was shown to dramatically enhance the barotolerance of *L. monocytogenes* (Hayman et al., 2008).

Nonlinear inactivation, or tailing, of microorganisms by high pressure has been reported by other researchers (Hayman et al., 2007). In this study, tailing was evident in early-death or long-term survival phase cells (Fig. 3A). The causative mechanism of tailing is still unclear, but it is possible that it is due to the presence of pressure-resistant cocci. The linear part of the inactivation curve before tailing might be due to the inactivation of more sensitive rods present in the population at these latter phases (Fig. 4). Further research is needed to clarify the cause of tailing during HPP.

The finding in this study that cells in stationary phase were not significantly more thermotolerant than cells in log phase disagrees with a previous study with *E. coli* (Kaur et al., 1998), perhaps because stationary phase cells in the present study still had not
transitioned to resistant cocci (Fig. 4). The finding that cells in long-term survival phase were the most thermotolerant in the life cycle is consistent with other reports that starvation enhances heat resistance in many microorganisms, including *L. monocytogenes* (Lou and Yousef, 1996; Herbert and Foster, 2001), *E. coli* (Jenkins et al., 1988), *A. globiformis* (Demkina et al., 2000) and *L. lactis* (Hartke et al., 1994). It is possible that the high thermotolerance in the long-term survival phase in *L. monocytogenes* results from a lowered water activity in the cocci-shaped cells, similar to the core of endospores (Beaman et al., 1982 and 1984; Nakashio and Gerhardt, 1985). This would make sense, as non-spore-forming cells also would benefit from a fitness strategy that produces long-term survival in natural environments. It is also possible that the starvation stress prior to or during the long-term survival phase caused the synthesis of stress proteins, which also helped to protect cells against heat. However, Jouper-Jaan et al. (1992) demonstrated that was the case only at the beginning of starvation, and that after a longer period of starvation the continuous protection was not due to de novo protein synthesis.

Previous studies have demonstrated that cells of *E. coli* (Kolter, 1993) and *Arthrobacter* species (Hamilton et al., 1977; Forni et al., 1992) changed from rods to cocci when the cultures exited log phase and entered stationary phase. The finding that *L. monocytogenes* formed cocci agrees with earlier reports (Gray and Killinger, 1966; Pomanskaya, 1961). The formation of cocci may be due to cell division without an increase in cell size or total biomass, as a response to starvation stress in long-term survival phase (Novitsky and Morita, 1978; Thorne and Williams, 1997; Kolter et al.,
Cocci might also be formed by cell shrinkage and cytoplasmic condensation (Kolter et al., 1993), which might have produced the textured surface of cocci seen in the present study (Fig. 4G, SEM). Compared to *S. aureus*, the SEM images of coccoid cells of *L. monocytogenes* appear more wrinkled on the surface (Amako and Umeda, 1977). Reeve et al. (1984) demonstrated that starvation led to cytoplasmic condensation in *E. coli*. Cytoplasmic condensation might lead to lowered water activity by decreasing cell volume, decreasing water content, and/or increasing solute concentration. Cytoplasmic condensation in non-spore-forming bacteria might be akin to the dehydrated core in a spore (Beaman et al., 1984), which is supported by the correlation between cell length and log reductions after pressure ($R^2 = 0.73$) or heat treatment ($R^2 = 0.82$). The percentage of cocci in the long-term survival phase at 30 days (85%) as determined by SEM was significantly higher than by Gram stain (26%) ($P < 0.05$) (Fig. 4G), which might be due to the serial dehydration utilized in the SEM preparation. The occurrence of cocci (41–718 h) coincided with the occurrence of maximum barotolerance (41–718 h) and with the occurrence of maximum thermotolerance (70–718 h) (Fig. 2 and 3). Smaller cocci have a larger surface-to-volume ratio than rods, which can enhance simple or facilitated diffusion for nutrient uptake, which reduces the need for energy in nutrient transportation during starvation (Erlebach et al., 2000). If the batch cultures in the present study had been incubated longer, it is possible that the coccoid cells of *L. monocytogenes* could have become even smaller and more resistant. Further research is needed to determine if this occurs.
In part II, after inoculation into fresh TSAYE cells in the long-term survival phase significantly decreased in barotolerance and thermotolerance as they reentered the log phase of the life cycle ($P < 0.05$) (Fig. 5). The decrease in resistance could be largely explained by the increase in cell length ($R^2 = 0.96$ for barotolerance and 0.93 for thermotolerance), which supports the hypothesis that resistance is due to cytoplasmic condensation. The correlation between cell length and resistance is higher when cells transition from cocci to rods (part II), possibly because resistance of cocci (part I) is due to both stress proteins induced by starvation (Jouper-Jaan et al., 1992) and cytoplasmic condensation, while loss of resistance of rods is only due to rehydration of the cytoplasm (part II). Compared with part I, log reductions following either pressure or heat treatments of cells in either log or stationary phases were significantly lower ($P < 0.05$) in part II (Fig. 2 and 5). Perhaps this higher resistance is due to the presence of highly resistant cocci in the log and stationary phases in part II.

Cocci were able to quickly enlarge to regain the rod shape and thereafter start cell division after inoculation into fresh TSAYE (Fig. 7). Similar observations were reported in *A. crystallopoietes* (Hamilton et al., 1977) and marine microorganisms (Kjelleberg et al., 1982). Therefore, the rod-coccus-rod life cycle of *L. monocytogenes* is similar to that of other microorganisms that inhabit natural environments.

In part III, after inoculation of long-term survival phase cells into milk, barotolerance and thermotolerance decreased significantly as cells reentered the log phase of the life cycle ($P < 0.05$) (Fig. 8). Both log and stationary phase cells in part III showed
the highest barotolerance and thermotolerance \((P < 0.05)\), which might be due to the presence of cocci in log and stationary phases, or the higher protective effect of milk than TSBYE, or cold shock at \(4^\circ\)C, or some combinations of the three. García et al. (2001) reported that cold shock enhanced thermotolerance in cells of \textit{Clostridium perfringens}.

Although \textit{L. monocytogenes} does not form spores, the mechanism(s) by which cells in the long-term survival phase become resistant to high pressure and heat may be analogous to that of bacterial endospores. Bacterial endospores are highly barotolerant (Smelt, 1998; Gould, 2006) and thermotolerant (Beaman et al., 1982 and 1984; Nakashio and Gerhardt, 1985). Thermotolerance of spores is thought to be due to dehydration of the core of the spore (Beaman et al., 1982 and 1984; Nakashio and Gerhardt, 1985). The formation of endospores is thought to be a strategy for long-term survival in adverse conditions, and the longevity of spores is compatible with their dormancy (Gould, 2006). The same may well be true for cells of \textit{L. monocytogenes} in the long-term survival phase.

\textit{L. monocytogenes} is ubiquitous in nature and could be a common contaminant in various foods such as milk and meats (Rocourt and Cossart, 1997). Since starvation-survival might be the most common state for bacteria (Kolter et al., 1993; Finkel, 2006), it is logical that most cells of \textit{L. monocytogenes} in the environment such as soil, water and dust are in the long-term survival phase, and thus food contamination is often caused by cells in this phase. \textit{L. monocytogenes} has also been found on the surfaces of equipment in dairy or meat processing plants (Wong, 1998; Thévenot et al., 2006), where this pathogen can potentially form biofilms and exhibit long-term survival (Wong, 1998; Blackman and Frank, 1996). Cells of \textit{L. monocytogenes} were shown to be more resistant
to heat and sanitizers once they formed biofilms (Frank and Koffi, 1990). On the other hand, biofilm detachment was triggered by nutrient starvation in *Aeromonas hydrophila* (Sawyer and Hermanowicz, 1998) and *Pseudomonas aeruginosa* (Hunt et al., 2004). In *L. monocytogenes*, cells in the long-term survival phase within biofilms might also be released due to nutrient limitation, which might enhance post-processing contamination of foods with cells that are highly resistant to pressure and heat.

In conclusion, this study reveals that cells of *L. monocytogenes* form cocci in the long-term survival phase, where they also become most barotolerant and thermotolerant. It is possible that cells in this phase are also the most resistant to other kinds of stresses and remain virulent (Nannapaneni et al., 2008). Food safety studies on the resistance of microorganisms are usually based on the results of log or stationary phase cells, which might not be the most resistant. Therefore, the food processing or preservation methods based on those processes might be inadequate, and thus their effectiveness overestimated. Further research is needed to address these important questions.

### 3.6 Acknowledgements

This research was funded by a U.S. Department of Agriculture Special Grant on Milk Safety to the Pennsylvania State University.

We would like to thank Melinda Hayman for her technical assistance with the High Pressure Processing unit, and Lewis Graves at the Centers for Disease Control for
sending us *L. monocytogenes* strain F5069. We also appreciate the assistance of Mei Lok in confirming the identity of isolates as *L. monocytogenes*.

### 3.7 References


CONCLUSIONS

This study demonstrated significant changes in barotolerance, thermotolerance and cellular morphology of Listeria monocytogenes throughout the life cycle. In the life cycle a brief stationary phase was followed by a rapid death phase, after which the cell concentration remained stable at ~10^8 CFU/ml for ~30 days in a long-term survival phase. As cells transitioned from log to stationary, to death, to long-term survival phases in TSBYE at 35°C (part I), barotolerance and thermotolerance significantly increased \((P < 0.05)\), cell size significantly decreased \((P < 0.05)\). Coccoid cells appeared during and after late-death phase, and the percentage of cocci increased significantly \((P < 0.05)\). There was a correlation between cell size and barotolerance \((R^2 = 0.73)\) and thermotolerance \((R^2 = 0.82)\) as cells transitioned from late-log phase to long-term survival phase. The high barotolerance and thermotolerance in long-term survival phase might be due to a decrease in water activity when cells transitioned from rods to cocci.

After inoculation of cells in the long-term survival phase into fresh sterile TSBYE at 35°C (part II), cells rapidly reentered log phase and then transitioned to stationary phase. During this transition they significantly decreased in barotolerance and thermotolerance \((P < 0.05)\), cell size significantly increased \((P < 0.05)\), and percentage of cocci significantly decreased \((P < 0.05)\). Cocci were shown to regain the rod shape after inoculation into fresh TSAYE. There was a high correlation between cell size and barotolerance \((R^2 = 0.96)\) or thermotolerance \((R^2 = 0.93)\). Results of part I and II
together showed the low-high-low cycle of barotolerance and thermotolerance, which correlated with the rod-coccus-rod cycle of cellular morphology throughout the life cycle.

After inoculation of cells in long-term survival phase into UHT whole milk at 4°C (part III), cells reentered log and then stationary phases, and showed a significant decrease in barotolerance and thermotolerance ($P < 0.05$). Cells in log or stationary phases in part III showed the highest barotolerance and thermotolerance compared to those in parts I and II ($P < 0.05$).

Cells of *L. monocytogenes* are most barotolerant and thermotolerant in the long-term survival phase, and they may also be most resistant to other kinds of stresses. However, food safety studies typically use log or stationary phase cells to study resistance, thus these studies may underestimate the actual resistance of this pathogen. The above findings need to be considered when developing food processes to ensure complete destruction of *L. monocytogenes* in RTE foods.
QUESTIONS FOR FUTURE RESEARCH

- What is(are) the mechanism(s) of phase transition, especially the transition from death phase to long-term survival phase? The transition might be due to the expression of specific genes. cDNA microarrays and protein 2-D gels could be utilized to study gene expression at different phases and then compare the differences. The transition might be regulated by bacterial quorum sensing which is controlled by genes. Bacteria may communicate commands to transit to another phase via signaling molecules. These molecules might be separated and identified using High Performance Liquid Chromatography (HPLC) or other methods. Understanding the mechanism(s) of phase transition may help to explain the changes in barotolerance, thermotolerance and morphology throughout the life cycle observed in the present study.

- Is there a significant increase in cell numbers in the long-term survival phase after 30 days of incubation? In the present study the incubation time was limited to 30 days. There was a slight increase in cell numbers after 21 days of incubation, but it was not significant \( (P > 0.05) \). However, this increase might have been significant if the culture had been incubated longer than 30 days. Thus, samples should be collected after 30 days incubation to answer the above question. If there actually is a significant increase, it might be due to reductive division of cells, however, this hypothesis needs to be tested by further studies.
Is there a change in barotolerance, thermotolerance and morphology in the long-term survival phase after 30 days of incubation? Cells may become more resistant to pressure or heat after incubation longer than one month (even up to several years), and may form smaller cocci similar to the ultramicrobacteria (diameter = 0.2–0.3 μm) found in natural environments.

Are cells in the long-term survival phase dormant? According to previous reports on dormancy of microorganisms, dormancy is based on low levels of carbon respiration, ATP, RNA and/or protein synthesis. Assays to measure these parameters might answer the question.

Is the water activity of cells in the long-term survival phase lower than other phases? We hypothesized that the enhanced barotolerance and thermotolerance of cells at the long-term survival phase might be due to cytoplasmic condensation and a decrease in water activity. Water activity of cells could be measured using a water activity meter. After water activity is measured, correlations between cell size, water activity, barotolerance and thermotolerance could be further studied.

Is the high resistance of cells at long-term survival phase due to the resistance of cocci? To answer this question, we can separate cocci from rods in long-term survival cultures to compare their resistances. The separation of cocci from rods might be done using flow cytometry or density gradient centrifugation.

Are cells in the long-term survival phase more resistant to other kinds of stresses than other phases? The highly barotolerant and thermotolerant cells in long-term
survival phase may also be highly resistant to other stresses, such as UV, drying, sanitizers, bacteriocins, etc. Further study is needed to test this hypothesis.

- What is the expression pattern of stress proteins in the long-term survival phase? 2-D protein gels of long-term survival phase cells might answer the question.

- Is there any water pumped out of cells when they transition from death to long-term survival phases? Pumping water out of cells might be a way to decrease water activity.
APPENDIX A

CHANGES IN SURVIVAL AFTER PRESSURE OR THERMAL PROCESSING AND MORPHOLOGY OF *LISTERIA MONOCYTOGENES* STRAINS F5069 AND SCOTT A

**Purpose:** To confirm that the findings with *L. monocytogenes* ATCC 19115 apply to other *L. monocytogenes* strains.

**Ho:** Incubation time does not affect log reductions after high pressure or thermal processing and cellular morphology of *L. monocytogenes* F5069 and Scott A.

**Methods:** Cells of *L. monocytogenes* F5069 and Scott A were grown in TSBYE at 35°C for 13 h or 19–21 d. Then cells were diluted 1:100 in UHT whole milk. Milk inoculated with *L. monocytogenes* was pressurized at 400 MPa for 180 s at room temperature (~22°C), or heated at 62.8°C for 30 s. Cells were enumerated before and after high pressure and thermal processing by plating on TSAYE with incubation at 30°C for 48 h. Cells were also Gram stained to observe cellular morphology. The experiment was replicated once.

**Results:** Both *L. monocytogenes* F5069 and Scott A showed similar results as ATCC 19115. Cells incubated for 19 d showed less log reductions after high pressure or thermal processing than incubated for 13 h (Fig. 9 and 10). Cells appeared to change shape from rods to cocci after long-term incubation (Fig. 11).
Conclusion: The changes in barotolerance, thermotolerance and morphology throughout the life cycle appeared common in *L. monocytogenes* strains.
FIG. 9. Increase in baro- and thermo-tolerance of cells of *L. monocytogenes* after inoculation into TSBYE and incubation at 35°C. Cells of *L. monocytogenes* F5069 were grown in TSBYE at 35°C 13 h or 19 d. Then cells were 1:100 diluted in UHT whole milk. Milk inoculated with *L. monocytogenes* was pressurized at 400 MPa for 180 s at room temperature (~22°C), or heated at 62.8°C for 30 s. Cells were enumerated before and after HPP or heating by plating on TSAYE with incubation at 30°C for 48 h. Data were based on one replication.
Growth time (h) at 35°C in TSBYE prior to thermal or pressure treatment

- Before HPP or heating
- After HPP at 400 MPa for 180 s
- After heating at 62.8°C for 30 s
FIG. 10. Increase in baro- and thermo-tolerance of cells of *L. monocytogenes* after inoculation into TSBYE and incubation at 35°C. Cells of *L. monocytogenes* Scott A were grown in TSBYE at 35°C 13 h or 19 d. Then cells were 1:100 diluted in UHT whole milk. Milk inoculated with *L. monocytogenes* was pressurized at 400 MPa for 180 s at room temperature (~22°C), or heated at 62.8°C for 30 s. Cells were enumerated before and after HPP or heating by plating on TSAYE with incubation at 30°C for 48 h. Data were based on one replication.
Growth time (h) at 35°C in TSBYE prior to thermal or pressure treatment

CFU/ml whole milk

- Blue diamond: Before HPP or heating
- Red triangle: After HPP at 400 MPa for 180 s
- Pink square: After heating at 62.8°C for 30 s

Growth time (h) at 35°C in TSBYE prior to thermal or pressure treatment
FIG. 11. Gram stains of cells of *L. monocytogenes* strains F5069 and Scott A with 13 h or 21 d incubation at 35°C in TSBYE. Cells were Gram stained before being observed using LM (Model BX40F4, Olympus optical, Japan) at a magnification of 1,000×. Bars represent fifty microns.
13 h incubation

21 d incubation

F5069

Scott A
APPENDIX B

COMPARISON OF LAG TIMES AFTER INOCULATION IN FRESH TSBYE WITH CELLS OF \textit{LISTERIA MONOCYTOGENES} AT DIFFERENT PHASES

\textbf{Purpose:} To determine whether the lag time after inoculation in fresh TSBYE with cells in long-term survival phase is longer than cells in other phases.

\textbf{Ho:} There is no difference between lag times after inoculation in fresh TSBYE with cells in different phases.

\textbf{Methods:} Cells of \textit{L. monocytogenes} ATCC 19115 were incubated in TSBYE at 35°C for 12 h, 22 h and 16 days to yield cells in late-log, early-death and long-term survival phases, respectively. Cells at different phases were then inoculated into fresh sterile TSBYE and incubated at 35°C for 5 h. Growth curves were measured by plating cultures on TSAYE with incubation at 30°C for 48 h. The experiment was replicated three times.

\textbf{Results:} The lag time after inoculation in fresh TSBYE with cells in late-log phase was 20 min, while cells in early-death and long-term survival phases showed a lag time of 1 h (Fig. 12).

\textbf{Conclusion:} There was a significant difference (40 min) between lag times after inoculation in TSBYE with cells in late-log and in long-term survival phases ($P < 0.05$), but no significant difference between the lag times of cells in early-death and long-term survival phases ($P > 0.05$).
FIG. 12. Growth curves of cells in different phases after inoculation in fresh TSBYE. 

Cells of *L. monocytogenes* ATCC 19115 were incubated in TSBYE at 35°C for 12 h, 22 h and 16 days to yield cells in late-log, early-death and long-term survival phases, respectively. Cells at different phases were then inoculated into fresh sterile TSBYE and incubated at 35°C for 5 h. Growth curves were measured by plating cultures on TSAYE with incubation at 30°C for 48 h. Data points and error bars represent means and standard deviations based on three replications of the experiment.
Growth time (h) in TSBYE at 35°C after inoculation

- Inoculated with cells in late-log phase at 12 h
- Inoculated with cells in early-death phase at 22 h
- Inoculated with cells in long-term survival phase at 16 d
APPENDIX C
MECHANISM OF NONLINEAR INACTIVATION OF *LISTERIA MONOCYTOGENES* BY HIGH PRESSURE PROCESSING

**Purpose:** To determine whether a genetically pressure-resistant subpopulation or the protective protein(s) synthesized during HPP is the mechanism of nonlinear inactivation (tailing) of *L. monocytogenes* by High Pressure Processing (HPP).

**Experiment 1**

**Ho:** Tailing is due to the survival of a genetically pressure-resistant subpopulation (mutant) after HPP.

**Methods:** Cells of *L. monocytogenes* ATCC 19115 were incubated in TSBYE at 35°C to stationary phase, then were diluted 1:100 in sterile UHT whole milk and pressure treated at 400 MPa for various times at room temperature (~22°C). Cells were then enumerated by plating on TSAYE with incubation at 35°C for 48 h. The survivors (11 colonies) that caused tailing were inoculated in TSBYE at 35°C, grown to stationary phase, diluted in milk and pressure treated, and survivors were enumerated as described above. The experiment was replicated once.

**Results:** All the 11 daughter cultures showed similar tailings as the parent culture.

**Conclusion:** Tailing is not due to the survival of a genetically resistant subpopulation.
Experiment 2

**Ho:** Tailing is due to the protein(s) synthesized during HPP that can protect cells from pressure damage.

**Methods:** Cells of *L. monocytogenes* ATCC 19115 were incubated in TSBYE at 35°C to stationary phase, and then were diluted 1:100 in sterile UHT whole milk with 0.01% chloramphenicol as treatment or without chloramphenicol as control. Both treatment and control were high pressure processed at 400 MPa for various times at room temperature (~22°C). Cells were then enumerated by plating on TSAYE with incubation at 35°C for 48 h. The experiment was replicated once.

**Results:** Cells in both treatment and control showed similar tailings.

**Conclusion:** Tailing is not due to the protective protein(s) synthesized during HPP.
APPENDIX D

EFFECTS OF PRESSURE SHOCK AND INITIAL CELL CONCENTRATION ON THE BAROTOLERANCE OF *LISTERIA MONOCYTOGENES*

**Purpose:** To investigate the effects of pressure shock and initial cell concentration prior to HPP on the barotolerance of *L. monocytogenes*.

**Experiment 1**

**Ho:** There is no effect of pressure shock at 50 MPa for 7 min before HPP on the barotolerance of *L. monocytogenes*.

**Methods:** Cells of *L. monocytogenes* ATCC 19115 were incubated in TSBYE at 35°C to stationary phase, and then were diluted 1:100 in sterile UHT whole milk. Cells were then pressure shocked at 50 MPa for 7 min (treatment) before HPP at 400 MPa for various times, or directly HPP without pressure shock (control). Cells were then enumerated by plating on TSAYE with incubation at 35°C for 48 h. The experiment was replicated once.

**Results:** The slopes of inactivation curves of treatment and control appeared similar.

**Conclusion:** Pressure shock at 50 MPa for 7 min may not affect the barotolerance of *L. monocytogenes*. 
Experiment 2

**Ho:** There is no effect of initial cell concentration prior to HPP on the barotolerance of *L. monocytogenes*.

**Methods:** Cells of *L. monocytogenes* ATCC 19115 were incubated in TSBYE at 35°C to stationary phase (~10⁹ CFU/ml), and then were diluted 1:10⁷, 1:10⁴, 1:10² or 1:1 in TSBYE to achieve starting concentrations of ~10², ~10⁵, ~10⁷ or ~10⁹ CFU/ml. Cells were then high pressure processed at 400 MPa for various times at room temperature (~22°C). Survivors were enumerated by plating on TSAYE with incubation at 35°C for 48 h. The experiment was replicated once.

**Results:** The slopes of inactivation curves with different starting concentrations appeared similar.

**Conclusion:** Starting concentration prior to HPP may not affect the barotolerance of *L. monocytogenes*.
APPENDIX E

GROWTH CURVE OF *LISTERIA MONOCYTOGENES* SHOWING A SHORT STATIONARY PHASE

**Purpose:** To measure the length of the stationary phase in the life cycle of *L. monocytogenes* at 35°C in TSBYE.

**Ho:** There is not a stationary phase in the life cycle of *L. monocytogenes*.

**Methods:** Glycerol stock of *L. monocytogenes* ATCC 19115 was maintained at -80°C and streaked on TSAYE with incubation at 35°C for 48 h. One colony on the plate was inoculated into 10 ml of TSBYE with incubation at 35°C for 24 h. A 0.1 ml aliquot of the resulting culture was then diluted with 9.9 ml 0.1% peptone water to achieve ~10^7 CFU/ml, and 0.1 ml of the diluted culture was inoculated into 100 ml of TSBYE, which was then incubated at 35°C for up to 28 h. The culture at 14–28 h was plated onto TSAYE with incubation at 30°C for 48 h to enumerate cells. The experiment was replicated three times.

**Results:** Tukey multiple comparison revealed that cell concentration was the highest at 16 h (2.6 × 10^9 CFU/ml) and 18 h (2.2 × 10^9 CFU/ml), and there was no significant difference between the cell concentrations at 16 h and 18 h (*P > 0.05*) (Fig. 13).

**Conclusion:** There was a ~2 h stationary phase (within the growth from 16 to 18 h) between log (before 16 h) and death phases (after 18 h).
Fig 13. Growth of *L. monocytogenes* within 14–28 h incubation at 35°C in TSBYE. Data points and error bars represent means and standard deviations based on three replications. Data points with different letters are significantly different in cell concentrations ($P < 0.05$).
Growth time (h) at 35°C in TSBYE

CFU/ml TSBYE

C         A         A          B         C         BC       C         C

Growth time (h) at 35°C in TSBYE