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Inactivation of Sporeforming Spoilage Bacteria in Milk and Juice Using Ultra High Pressure Homogenization

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To the Graduate Council:

I am submitting herewith a thesis written by Julie Michelle Gidley entitled "Inactivation of Sporeforming Spoilage Bacteria in Milk and Juice Using Ultra High Pressure Homogenization." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

David A. Golden, Major Professor

We have read this thesis and recommend its acceptance:

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Accepted for the Council:
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Inactivation of Sporeforming Spoilage Bacteria in Milk and Juice Using Ultra High Pressure Homogenization

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Julie Michelle Gidley
May 2014

ACKNOWLEDGEMENTS

I would first and most importantly like to acknowledge and thank my major professor, Dr. David Golden. He has advised, encouraged, taught, and related to me on a personal level. For that I respect him and hold him in the highest regard. I would like to thank him for all of the times that he has stopped his busy schedule to help me with research, coursework, or life in general. I appreciate his ability to let me know that, as his student, I am important to him. Dr. Golden was willing to bring me into the University of Tennessee and serve as an advisor to me even before I had a thesis project lined up. Therefore, I owe him my deepest gratitude.

Secondly, I greatly thank Dr. Federico Harte and Dr. Irene Hanning for serving on my committee. Dr. Harte gave his unending guidance in my research and I have been blessed to spend my time at the University of Tennessee working with him. His innovation and intelligence have been an incredible resource for me as a student. Dr. Hanning has always made herself available when I needed her assistance and has graciously contributed her expertise to my project.

In addition to the above acknowledgements, I would also like to take this opportunity to acknowledge Wei Chen for being a role model and daily laboratory mentor. I thank my labmates, friends, colleagues, and department professors including Marta Corzo-Martinez, Maneesha Mohan, Avelina Vega-Francova and Vinay Mannam all of whom have spent many endless hours helping me with my research.

Lastly, I thank my family who gave me the most constant support. My parents, Marta and Gary, my sister, Kristen, and my fiancé, Jonathan have all taught me patience and wisdom and have served as the backbone of my education and life.

ABSTRACT

Inactivation of spores is essential for extending the shelf life of fluid milk and other food products. Three studies were conducted to evaluate the effects of ultra-high pressure homogenization (UHPH) on spores from three spore forming bacteria. The first experiment studied UHPH effects on *Geobacillus stearothermophilus, Paenibacillus lautus,* and *Bacillus licheniformis* in fluid milk. Homogenization pressures of 100 - 500 MPa were applied to spore-inoculated samples, and spore viability was determined by plating onto agar media. Heat shock treatments (80°C, 10 min) were applied to a portion of each sample prior to UHPH. UHPH treatment significantly reduced spore populations on the heat-shocked sample of *P. lautus* at 100 MPa ($P<0.05$, but had no significant effect on *G. stearothermophilus* and *B. licheniformis* ($P\geq0.05$).

The second experiment was conducted with a similar protocol to the first experiment but with the addition of a heat treatment at 95°C within the homogenizing system. Fluid milk (3.79 L) was inoculated with a10 ml spore *P. lautus* spore suspension and was treated with pressures of 100 - 500 MPa at 95ºC. A heat shock treatment of 80°C for 10 min was applied to a portion of the UHPH-treated samples. Generally, these treatments had no significant effect on spore reduction ($P\geq0.05$).

The third experiment evaluated the effect of UHPH on *P. lautus* spores under acidic conditions. Apple juice (3.79 L) was inoculated with 10 ml of a *P. lautus* spore suspension and was treated at pressures of 100 – 500 MPa. A heat shock treatment was applied to a portion of each UHPH treated juice sample.

Significant reductions ($P<0.05$) in spore concentrations were observed in non-heat shocked and heat shocked samples at all pressures.

This study indicates that UHPH is effective at inactivating *P. lautus* spores in apple juice and provides some beneficial reductions in spore numbers in milk. These findings have promising applications for the industry to extend shelf life of milk, apple juice, and similar products.

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INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Extending the shelf life of milk is difficult because quality attributes may be compromised due to harsh processing conditions (Beard et al., 1999). Endospore forming bacteria from the genera *Bacillus, Geobacillus,* and *Paenibacillus* are present in the dairy farm environment and may contaminate fluid milk. These bacteria are the primary microorganisms associated with spoilage of milk and other beverages. Because of their ability to withstand milk processing and survive refrigeration temperatures (Boor et al., 2011), it is important to continue developing innovative methods of inactivation.

Geobacillus stearothermophilus, Paenibacillus lautus, and *Bacillus licheniformis* are Gram-positive spore forming rods (Jay et al., 2005) that have been isolated from both raw and pasteurized milk (Scheldeman et al., 2004). Though *G. stearothermophilus* is the most heat resistant of the three spore-formers (Watanabe et al., 2003), all three are highly resistant to the heat involved in milk processing (Jay et al., 2005). Spores of specific species are well known for their resistance to hydrogen peroxide and heat, allowing them to survive the homogenization and cleaning procedures involved in dairy processing (Nieminen, et al., 2007).

According to Olsen et al. (2004), pasteurization is a heat treatment of milk that has been developed to “dramatically reduce” microbial populations in milk and milk products. Typically, milk pasteurization in the United States consists of heat treatment at 72ºC for 15 seconds while being pumped through the pasteurization system (Jay et al., 2005).

Homogenizers exerting high-pressure (>100MPa) and ultra-high pressure (>350MPa) on milk products are used for a more efficient emulsified end product (Floury et al., 2000; 2001). Homogenization reduces creaming in milk products and ultra-high pressure homogenization (UHPH) is known to produce even finer particles of fat globules (Sandra et al., 2004). Because of the benefits UHPH produces on the physical properties of milk, the possibility of the additional beneficial effect of spore inactivation was investigated.

The objectives of this research were:

1.) To investigate the inactivation effect of UHPH on bacterial spores of *Geobacillus stearothermophilus, Paenibacillus lautus,* and *Bacillus licheniformis.*

2.) To investigate the inactivation effect of UHPH and thermal processing on bacterial spores of *Paenibacillus lautus.*

3.) To investigate the inactivation effect of UHPH and acidic conditions on bacterial spores of *Paenibacillus lautus.*

LITERATURE REVIEW

History of Milk and Milk Processing

Milk is a nutritious and crucial part of the human diet. Many mammals act as a source of milk including goats, cows, and sheep among others. Typically, more liquid milk consumption has taken place in some non-tropical climates due to the ability to safely refrigerate the product. Some tropical climate nations consume their milk in different preserved forms such as boiling or immediate consumption. Over centuries, mankind has developed methods of using animal milk sources for mass production and commercialization. Due to continuing advances in milk studies, milk processing has transformed "from an art to a science." (Anonymous, 2009).

Microorganisms in Milk

Microorganisms that can be present in milk include lactic acid bacteria, coliforms, *Pseudomonas, Bacillus, Streptococcus,* and others. A few of the microorganisms present in milk, such as *Bacillus* spp., can also survive pasteurization temperatures and even grow at refrigeration temperatures. Upon growth at refrigeration temperatures, these microorganisms can cause spoilage problems in milk, therefore decreasing shelf life (Anonymous, 2009).

Raw milk poses the greatest threat for pathogenic bacterial contamination. Some pathogens present in raw milk may include *Campylobacter jejuni, Listeria monocytogenes, Salmonella*, and others (Anonymous, 2009).

There are several different methods by which milk may be contaminated. Milk is considered to be safe and sterile inside the animal until it is secreted from the udder, where it is still low in bacterial counts except in the cases of mastitis (Anonymous, 2009). Once the animal has been milked, sources of contamination are numerous. Feces, human handling, environmental bacteria, rodents, and dirt in general are just a few of the ways by which milk may become contaminated (CDC, 2013).

According to the CDC, 79% of outbreaks occurring between the years of 1998 to 2011 were due to consumption of raw milk or cheese. Among these outbreaks, there were 2 deaths and the majority of these illnesses occurred in children. This exemplifies the importance of feeding a family a healthy diet of pasteurized milk products (CDC, 2013).

When in their dormant state, bacterial spores are not only highly resistant to heat (Watanabe et al., 2003) but also to other physical stressors including radiation, pressure, and chemicals. Factors involved in this resistance include a thick spore coat and a core that contains low water content (Rogers, et al. 2005).

The main objective in food sterilization involves the inactivation of dormant bacterial spores. Often, a moist heat treatment below 100ºC is used for food, which does not inactivate bacterial spores but can destroy other harmful microorganisms. If the moist heat treatment is used at a temperature of 121ºC or higher, it can cause harmful effects on the sensory aspects of the food item (Watanabe et al., 2003).

Milk Pasteurization

Milk pasteurization is a thermal processing method in which liquid milk is heated to a specific temperature and held for a set time. The purpose of the pasteurization process is to destroy microorganisms that may cause disease through consumption (Jay et al., 2005). Heating is the minimal treatment that milk and milk products need to produce a safe product (CDC, 2013). Bacterial endospores are not destroyed during pasteurization. However, pathogens and other vegetative microorganisms can be completely destroyed through pasteurization (Jay et al., 2005).

There are multiple methods of thermal treatments for milk. Two of the most popular pasteurization methods include low temperature-long time (LTLT) and high-temperature-short time (HTST). Low temperature-long time pasteurization is a method where the milk reaches a temperature of 63ºC (145ºF) and is held for 30 minutes at that temperature. The HTST method is a method where the milk is heated to 72ºC (161ºF) and held for 15 seconds. According to Jay et al. (2005) other pasteurization methods include 89ºC for 1 second, 90ºC for 0.5 seconds, 94ºC for 0.1 second, and 100ºC for 0.01 second.

The HTST pasteurization is the most common treatment used for milk in the United States. In this process raw milk enters the pasteurizer at approximately 4ºC (40ºF) and is heated to approximately 57ºC (135ºF) from hot pasteurized milk flowing opposite of a thin stainless steel plate. At this point, the hot, pasteurized milk (which has reached at least 72ºC (161ºF)) is subsequently cooled to approximately 32ºC (90ºF). The warmed raw milk is moved further into

the HTST pasteurization system where it remains in a holding tube for at least 15 seconds. At this point in the system, the milk is at pasteurization temperatures and under pressure. The pasteurized milk then passes a flow diversion valve where thermometers record the milk temperature. The milk will move forward unless its temperature registers lower than 72°C, at which point the flow diversion valve diverts the milk back to the raw milk tank to be treated again (Anonymous, 2009).

Milk Processing

In addition to the necessary pasteurization steps for milk safety, homogenization is also a beneficial milk process. For the purpose of milk storage, homogenization is used to reduce the fat globule size, increase particle surface area, and evenly distribute the fat particles throughout the product. When smaller fat globules have been evenly dispersed, milk creaming is less likely to occur during storage.

Geobacillus stearothermophilus

Geobacillus stearothermophilus was formerly known as *Bacillus stearothermophilus*. Currently, *Geobacillus* is its own genus and was first isolated in 1917 by P.J. Donk from cream-style corn. It is a Gram-positive spore forming aerobic rod (Jay et al., 2005). Thermophilic spore-formers such as *G. stearothermophilus* have optimum growth in temperatures between 45 to 70ºC (Nazina et al., 2001).

Due to extreme resistance of heat in the spore form, *G. stearothermophilus* is often used as an indicator for evaluating the effectiveness of food sterilization processes. Killing *G. stearothermophilus* spores at a temperature of less than 100ºC is ideal for the preservation of food quality. Temperatures of 100ºC and below are assumed to not reduce the quality of the food product because these temperatures are considered a typical cooking temperature (Watanabe et al., 2003).

Research conducted on *Geobacillus stearothermophilus* with ultra-high pressure homogenization is minimal. However, studies have been conducted regarding *Geobacillus stearothermophilus* deactivation and high hydrostatic pressure (HHP).

In research conducted by Estrada-Giron and others (2007), the effect of HHP on *G. stearothermophilus* spores was evaluated. The purpose of this research was to evaluate the inactivation of *G. stearothermophilus* spores with combined treatments of HHP, various temperatures, and holding times. The methods conducted in this research were as follows. The spores were harvested and soymilk was prepared through sterilization at 121ºC for 15 minutes. Before inoculation, soymilk was acclimated to room temperature and subsequently inoculated with 10^6 CFU/ml of *G. stearothermophilus*. The HHP treatment was applied at pressure levels of 550, 585, and 620MPa. Each pressure was combined with temperatures of 70, 80, and 90ºC and held for time periods ranging from 2 seconds – 15 minutes. A standard plate count method was used for the enumeration of *G. stearothermophilus* survivors.

The results of this study demonstrated that the best combination of pressure and temperature for the inactivation of *G. stearothermophilus* occurred at 90ºC and 620MPa. The minimum amount of time required with these treatments for inactivation to less than 10CFU/ml was 7 minutes (Estrada-Giron, et al., 2007).

Ultra-high pressure homogenization utilizes even more forces than HHP. These forces include cavitation and shearing and result in the potential to reduce thermal treatments and unwanted sensory effects.

Paenibacillus lautus

Paenibacillus lautus is a species within the genus *Paenibacillus*, which has been moved from the previous taxonomic grouping with the genera *Bacillus* and *Clostridium*. As a Gram-positive spore forming rod, *P. lautus* can be categorized similarly to other *Bacillus* bacteria. Members within the genus *Paenibacillus* will typically produce a negative Gram-stain even though they have the cell wall structure of Gram-positive bacteria (Montes et al., 2004). Even though *P. lautus* is nonpathogenic, it does cause spoilage and can be problematic in foods. Sources of this bacteria include soil and water, plants, utensils, air, and dust (Jay et al., 2005). Dairy cattle feed and silage are sources of *Paenibacillus* contamination (Scheldeman et al., 2004). *Paenibacillus* can be found on fresh meats and poultry (Jay et al., 2005) but has also been isolated from raw and pasteurized milk though it is not the predominantly isolated bacteria in these products (Scheldeman et al., 2004). The bacterium is typically

problematic starting at the dairy farm, but can be found at many points including the processing plant environment (Durak et al., 2006).

Most studies on *Paenibacillus* spp. in fluid milk and dairy products have been conducted to identify which species are causing spoilage issues and at which points (from farm to consumer) they are contaminating milk (Durak et al., 2006; Vaerewijck et. al., 2001; Montes et al., 2004; Suominen et al., 2003; Ivy et al., 2012).

Paenibacillus and Psychrotolerance

Though it is of minor importance as a psychrotroph, *Paenibacillus* has been demonstrated to grow at or below 7ºC (Jay et al., 2005). However, *Paenibacillus* is relatively heat resistant and can withstand at least 120ºC when in a spore form (Scheldeman et al., 2004). Both *Bacillus* spp. and *Paenibacillus* spp. have been demonstrated to grow at low temperatures causing the dairy industry difficulty in developing fluid milk products with a shelf life of greater than 14 days (Durak et al., 2006).

Bacillus licheniformis

Bacillus licheniformis spores are known as a commonly isolated microorganism from all steps of milk processing. Similar to *G. stearothermophilus* and *P. lautus*, *B. licheniformis* is a heat resistant spore-former with similar strong resistance to other physical stressors. However, unlike *G. stearothermophilus*,

B. licheniformis is a mesophilic organism (Beard et al., 1999) and is a facultatively anaerobic bacteria used in the biotechnology industry. *B. licheniformis* is a bacterium that is used in both commercial and agricultural applications and can be isolated from the soil. According to recent taxonomic studies it is also closely related to *B. subtilis*, which has been well studied. *Escherichia coli* is the only organism that has been studied more extensively than *B. subtilis*. Agricultural functions of *B. licheniformis* include use in maize, grasses, and vegetable crops for reducing fungal pathogenic effects. Because of its sporeforming property and ability to survive harsh conditions, *B. licheniformis* has natural agricultural pest control properties (Rey et al., 2004).

***Bacillus licheniformis* and Toxigenicity**

In a study conducted by Salkinoja-Salonen et al. (1999), it was demonstrated that raw milk and baby food, which had been industrially produced, were contaminated with toxin-producing isolates of *B. licheniformis*. Though cooked meats and vegetables are the typical source of food-borne outbreaks involving *B. licheniformis*, dairy products are often contaminated with this bacterium. The authors (Salkinoja-Salonen et al., 1999) investigating these incidents conducted biological analyses, physiological tests, and toxicity tests to confirm that the strain of *B. licheniformis* isolated was toxin-producing. The toxic isolates that were found had all grown anaerobically and originated from a variety of sources including udders of cows that had previously had severe mastitis. The cows had since clinically recovered. Infant formula that led to one infant fatality

also consisted of two strains of toxic *B. licheniformis*. The authors believed their research to be the first of any done on human disease associated with toxin-producing *B. licheniformis* (Salkinoja-Salonen et al., 1999).

Another study conducted by Nieminen, et al. (2007) surveyed the milk samples for toxin-producing *B. licheniformis* strains. Because *B. licheniformis* spores can survive pasteurization, the authors wanted to assess the food safety risk of toxinogenic *Bacillus* spp.. Mastitic cows in Finland were used to collect 100 samples of milk and two of these samples were contaminated with heat stable, toxin-producing strains of *B. licheniformis* (Nieminen, et al., 2007).

Ultra-High Pressure Homogenization Effects on *Bacillus licheniformis*

There is an increasing popularity of studies using UHPH to inactivate spores because of the reduced quality of dairy foods that results when extreme heating is used for the destruction of spore-formers. In a study conducted by Feijoo et al. (1997) forces of shear, cavitation, and ultra-high pressure were evaluated during homogenization of ice cream inoculated with *B. licheniformis*. The authors began with an initial population of 2.0×10^4 *B.* licheniformis spores/ml of ice cream mix. Four different batches of ice cream mix were heated to temperatures of 33ºC, 36ºC, 44ºC, or 50ºC. The outlet temperatures of the samples were also measured. Microfluidization technology was used for the application of UHPH at levels of up to 200,000kPa. The largest spore reduction occurred at 68% and there was an increase in outlet temperature as pressure increased. The work resulted in reduced spore counts but not complete

destruction. The authors hypothesized this may have occurred due to the forces causing increased temperature during UHPH (Feijoo et al.,1997).

Homogenization and High Pressure Homogenization

Homogenization

Typical homogenization in the milk industry is used to produce a milk product (of desired fat content) that contains homogeneous fat globules that do not cream during the shelf life of the milk (Anonymous, 2009). The purpose of reducing the fat globules to a small, homogenous mixture is for increased stability (Sandra et al., 2004). Conventional homogenization methods involve a pressure of around 20MPa and a milk temperature of 45 to 50ºC (Hayes et al., 2004). Fluid is forced through a valve seat and the valve is used to control the pressure in the system by the amount of applied force occurring in the valve (Chavez-Lopez et al., 2009).

High Pressure Homogenization (HPH)

Many studies have been conducted evaluating the effects of HPH on skim milk. The use of HPH on milk is attractive because bacteria can be destroyed through HPH and fat globule sizes can be easily reduced, ultimately increasing the shelf life of milk and milk products (Hayes et al., 2004). Studies using HPH up to 150ºC have indicated significant microbial reduction (Chaves-Lopez et al.,

2009). Though vegetative cells are easily inactivated by HPH, it has been reported that spores are highly resistant to HPH (Chaves-Lopez et al., 2009).

Ultra High Pressure Homogenization (UHPH)

Ultra-high pressure homogenization involves pressures above 300MPa. Forces occur during UHPH include shearing, cavitation, friction, high velocity, and turbulence (Zamora et al., 2007, Hayes et al., 2004). Furthermore, UHPH can produce even finer particle sizes when compared to traditional homogenization (Sandra et al., 2004). However, it can be difficult to apply such high pressures in the food industry (Watanabe et al., 2003).

The ultimate purpose of homogenizing milk products is to create a higher quality consumer product as well as increase the safety by killing pathogenic microorganisms (Hayes et al., 2004). Additionally, homogenization does not typically involve thermal processing that may have an effect on sensory aspects of milk products.

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CHAPTER II

EXPERIMENTS USING ULTRA HIGH PRESSURE HOMOGENIZATION FOR THE INACTIVATION OF BACTERIAL SPORES

ABSTRACT

Inactivation of spores is essential for extending the shelf life of fluid milk and other food products. Three studies were conducted to evaluate the effects of ultra-high pressure homogenization (UHPH) on spores from three spore forming bacteria. The first experiment studied UHPH effects on *Geobacillus stearothermophilus, Paenibacillus lautus,* and *Bacillus licheniformis* in fluid milk. Homogenization pressures of 100 - 500 MPa were applied to spore-inoculated samples, and spore viability was determined by plating onto agar media. Heat shock treatments (80°C, 10 min) were applied to a portion of each sample prior to UHPH. UHPH treatment significantly reduced spore populations on the heat-shocked sample of *P. lautus* at 100 MPa ($P<0.05$, but had no significant effect on *G. stearothermophilus* and *B. licheniformis* ($P\geq0.05$).

The second experiment was conducted with a similar protocol to the first experiment but with the addition of a heat treatment at 95°C within the homogenizing system. Fluid milk (3.79 L) was inoculated with a10 ml spore *P. lautus* spore suspension and was treated with pressures of 100 - 500 MPa at 95ºC. A heat shock treatment of 80°C for 10 min was applied to a portion of the UHPH-treated samples. Generally, these treatments had no significant effect on spore reduction ($P\geq0.05$).

The third experiment evaluated the effect of UHPH on *P. lautus* spores under acidic conditions. Apple juice (3.79 L) was inoculated with 10 ml of a *P. lautus* spore suspension and was treated at pressures of 100 – 500 MPa. A heat

shock treatment was applied to a portion of each UHPH treated juice sample. Significant reductions ($P<0.05$) in spore concentrations were observed in non-heat shocked and heat shocked samples at all pressures.

This study indicates that UHPH is effective at inactivating *P. lautus* spores in apple juice and provides some beneficial reductions in spore numbers in milk. These findings have promising applications for the industry to extend shelf life of milk, apple juice, and similar products.

INTRODUCTION

Spore forming bacteria from the genus *Bacillus* often occur in raw milk. These spores affect extended shelf-lives of milk products because they can spoil the milk. *Geobacillus stearothermophilus, Bacillus licheniformis, Bacillus subtilis, and Paenibacillus lautus* can be found in nature and are resistant to typical heat treatment processes. These spores have been isolated from milk products which had been pasteurized or otherwise heat-treated (Faille et al., 2001). Thermal processes alone may not be enough to sufficiently destroy the spores produced by these spoilage bacteria. In turn, this can cause a decrease in the shelf life of milk products (Jagannath et al., 2003).

The primary process for pasteurization of milk occurs at 72°C for 15 seconds. This is considered a high temperature, short time (HTST) method. Treatments such as HTST pasteurization can sufficiently destroy vegetative cells but do not have an effect on the survival of thermophilic organisms studied in this experiment (Jay et al., 2005).

Currently, consumers are demanding minimally processed products (Roig-Sagués et al., 2009**)**. For this reason, developing non-thermal methods for food processing is needed, and it is expected that products processed by such technologies retain more nutrients, keep sensory characteristics, and be safe food (Tribst et al., 2009).

As a promising alternative to heat treatments, there are new preservation techniques including high-pressure homogenization (HPH), high hydrostatic pressure (HHP), pulsed electric fields, ionizing irradiation, ultrasound, among

others. These ‘new techniques without heat’ can inactivate micro-organisms and do not induce thermal damage to the product (Bevilacqua et al., 2009)

High pressure technologies are gaining popularity in the food industry because they are considered to be the most promising emerging food processing technologies. This is due to recent advances in high-pressure machinery and the successful introduction of pressure-processed foods (Gervilla et al., 2000). The high hydrostatic pressure is the most widely studied and has been shown to be effective in inactivating most vegetative microorganisms, enzymes, and some yeasts and molds. Some bacterial spores are found to resist pressures as high as 1,000 MPa at room temperatures. This type of pressure treatment alone (at ambient temperatures) is not sufficient for spore inactivation (Balasubramanian and Balasubramanian, 2009).

Ultra-high-pressure homogenization (UHPH; also called dynamic high-pressure) is based on the same design principles as the conventional homogenization process used in the dairy industry for reducing the size of fat globules but also works at significantly higher pressures (>200 MPa), resulting in the destruction of microorganisms (Thiebaud et al., 2003). The inactivation capacity of this technology has been proven against vegetative cells, but the effects on bacterial spores are not yet well known.

Processing methods are used in the dairy industry to ensure the safety, quality, and consistency of products including liquid milk. Thermal treatments like pasteurization are utilized to kill pathogenic bacteria that could harm consumers. Homogenization processing is applied to stabilize emulsions in

liquid milk and to help reduce the risk of creaming during the shelf life. High pressures used during homogenization are attractive to the dairy industry because of the potential for inactivation of spore forming spoilage bacteria as well as the creation of finer particles for reduced creaming (Diels and Michiels, 2006).

Gram-positive spore-formers including *Paenibacillus lautus* have been demonstrated to have a negative impact on the shelf life of liquid milk. There are many possibilities for contamination sources because *P. lautus* has been isolated from the feed and silage at dairy farms as well as all throughout all processing steps (Vaerewijck M. et al. 2001). *P. lautus* is a relatively heat resistant spore-former but is also a psychrotolerant organism. The capability of *P. lautus* to survive refrigeration temperatures makes it an important organism to study in the dairy industry. Furthermore, liquid milk provides an optimal atmosphere for these spores to activate, resulting in spoilage problems (Durak et al., 2006).

The ability to inactivate *P. lautus* spores in liquid milk with thermal application and ultra-high pressure homogenization would allow the dairy industry to make progress in extending the shelf life of milk and milk products. Thus, one objective of this study was to analyze the effects of UHPH with the addition of a thermal treatment on *P. lautus* bacterial spore populations in liquid milk.

Juices treated with ultra-high pressure homogenization (UHPH) may be kept at a shelf life of up to 40 days when stored at refrigeration temperatures and possess reduced microbial activity. Should commercially acceptable shelf stability be achieved through UHPH, the pasteurization step in juices could

potentially be eliminated, reducing the negative sensory effects of high temperature applications. Typically, homogenization on juice is done by forcing the liquid between a valve seat and valve plate. This causes the juice to undergo "extremely rapid acceleration" resulting in completely broken down globules and a homogenized product (Clark, et al., 1993).

Studying the inactivation of bacteria such as *P. lautus* in juices can indicate whether acidic conditions enhances the effect of UHPH. Thus a second objective of this work was to evaluate the effect of UHPH on the survival of *P. lautus* bacterial spore populations in apple juice.

MATERIALS AND METHODS

Bacterial Strains and Culturing Conditions. *Paenibacillus lautus* ATCC 43898*, Geobacillus stearothermophilus* ATCC 29609*, and Bacillus licheniformis* ATCC 14594 were used. 2x Schaeffer's Sporulating Media consisted of 16g nutrient broth, 6.0g beef extract, 10.0g peptone, 15.0g agar, 2.0g KCl, 0.5g $MgSO_4 \bullet 7H_2O$ suspended in 1 liter of deionized water. After autoclaving the media was cooled to 55°C and sterile supplements were added: (1ml 1M $CaCl_2 \bullet 2H_2O$, 1ml 0.1M $MnSO_4 \bullet H_2O$, 1ml $FeSO_4 \bullet 7H_2O$, 2ml 50% (w/v) Glucose).

Spore induction from vegetative cells was conducted for each strain using the method of Murray et al. (2007) as a guideline for spore production. One "loop" of each culture was inoculated into 10ml of tryptic soy broth (Bacto, Sparks, MD). Inoculated broths were incubated at 55°C (*Geobacillus stearothermophilus*) or 37°C (*Paenibacillus lautus and Bacillus licheniformis*) for 48h. After incubation, 0.3 ml of each of the strains was inoculated onto 2x Schaeffer's Sporulating media plates. Plates were incubated at 37 or 55ºC (as appropriate) for seven to ten days.

When at least 85% of the cells had sporulated (as determined by phase contrast microscopic examination), 1-2 ml of sterile, deionized water was added to each plate to collect the spores. Spores were collected by using a hockey stick and gently rubbing the plate surface. This suspension was subsequently collected using a pipette, filtered through sterile glass wool, and collected in 50 ml centrifuge tubes. The spore suspensions of each strain were centrifuged at

8000 x g for 10 min. The supernatants were discarded and the pellets were resuspended in 20 ml of sterile deionized water and mixed well. This procedure was repeated twice to wash the spores. Each of the final pellets were resuspended in 40 ml of sterile deionized water and divided into 1ml aliquots in sterile centrifuge tubes. The tubes of each strain were stored at a temperature of -20°C until use in the experiments.

To determine the number of spores in each sample, 1ml of spore stock was added to 9ml of sterile 0.1% peptone water (Difco). The mixture was heat shocked at 80°C for 10 min then serially diluted in 0.1% peptone water (Difco) and surface plated in duplicate on tryptic soy agar (Bacto, Sparks, MD). After 24 h of incubation at the appropriate temperature, spore populations were determined.

Sample Preparation. Thirty ml of thawed spore stocks (approximately 7 log CFU/ml) of each strain was inoculated into 11.4 L (3 gal) of ultra-pasteurized organic skim milk or apple juice purchased from a local grocery store.

High Pressure Homogenization Processing Treatments. Three separate experiments were conducted. In the first experiment, survival of *G. stearothermophilus*, *P. lautus*, and *B. licheniformis* spores in milk after treatment with UHPH was evaluated. In the second experiment, a heat exchanger set at 95ºC was included, and survival of *P. lautus* spores in milk after UHPH treatment at 95ºC was evaluated. In the last experiment, survival of *P. lautus* in apple juice

after UHPH treatment was evaluated. Samples were processed through an UHPH system for experiments 1, 2, and 3. The system used a waterjet cutting device that was capable of obtaining pressures of up to 600 MPa (Flow International, Kent, WA). All samples were treated with pressures of 0 (control), 100, 200, 300, 400, and 500MPa. A sample was collected in sterile tubes after each pressure treatment. A time period of one minute was allowed between each change in pressure treatment and sample collection. Once each sample was collected it was immediately placed on ice. A water bath set at 0°C was connected to a heat exchanger at the terminal end of the homogenizer to control the outlet temperature.

In each of the three experiments, a portion of each sample at each pressure level received a heat shock treatment to determine whether spore germination would be affected by heat shock. This treatment consisted of applying heat at 80°C for 10 min by using a hot water bath.

Enumeration of Survivors. Processed samples were serially diluted in 0.1% peptone (Difco) water and surface plated in duplicate onto TSA plates (Difco) and subsequently incubated for 24 hours. After incubation, colonies were enumerated and CFU/ml was calculated for all the samples.

Statistical Analysis. SAS 9.3 was used to analyze the data. Tukey's grouping was conducted in the analysis to demonstrate that there was a significant difference from the pressure treatment.

RESULTS

Experiment #1

There were no statistically significant differences in the numbers of surviving spores for all high pressure treatments applied to the milk samples artificially inoculated with *G. stearothermophilus* spores (P≥0.05). In addition, the application of heat shock to the samples did not reduce the number of spores (Figures 2.1 and 2.2).

As shown in Figure 2.4, the treatment at each pressure level had no significant effect on the inactivation of *P. lautus* spores artificially inoculated into organic skim milk (P≥0.05). However, when a thermal treatment was applied to the acquired sample (Figure 2.3) a significant reduction of *P. lautus* effect was observed (P≤0.05) between pressures of 0MPa and 100MPa, 0MPa and 200MPa, 0MPa and 300MPa, 0MPa and 400MPa, and 0MPa and 500MPa. There was no significant reduction of *P. lautus* when pressure levels from 100MPa to 500MPa were applied.

Treatment at each pressure level shows no significant effect on the inactivation of *B. licheniformis* spores in organic skim milk (P≥0.05). This was the case for all samples inoculated with *B. licheniformis* with and without a heat shock treatment (Figures 2.5 and 2.6).

Experiment #2

Using UHPH there was no significant (P≥0.05) reduction of *P. lautus* spores at any of the pressure levels (100-500MPa, Figure 2.7). Figure 3.1 shows the pressure treatment combined with the application of heat caused no reduction in spore count at any pressure level. There was a reduction of 0.42 log CFU/ml at 200MPa, although this

was not statistically significant (P>00.05). The addition of a heat exchanger set at 95°C did not further reduce the numbers of spores.

Similar to our experiments conducted on the effect of ultra-high pressure homogenization on spore-formers without an additional thermal application, the heat-shocked resulted in a significant reduction of *P. lautus* (P<0.05) from pressure values of 0MPa to 100MPa. Furthermore, Figure 2.8 shows that all pressure levels (100-500MPa) combined with the thermal treatment (95°C) reduced the spore populations ranging from 0.79 to 0.90 log CFU/ml. The greatest reduction occurred at a pressure level of 500MPa with a hot water bath heat exchanger (0.9 log CFU/ml reduction).

Experiment #3

There was a significant reduction of *P. lautus* spores when pressures of 0MPa and all other pressure levels (100-500MPa) were applied (P<0.05). The greatest reduction occurred at 500MPa accomplished without a heat shock treatment resulting in a 2.16 log CFU/ml reduction (Figure 2.9). Grouping with statistical analysis showed a mean difference from pressures of 0 MPa to 100 MPa of -1.65 log CFU/ml. The differences for the remaining pressure levels were not significant, ranging from -1.65 to -2.16 log CFU/ml.

DISCUSSION

Limited previous research has been conducted using UHPH up to 500MPa for spore reduction in dairy. Somewhat similar studies from Cruz et al (2007) in soymilk showed spore inactivation counts of approximately 2 logs at 200MPa. However, the same study showed only an inactivation of approximately .25log at 300MPa and the statistical significance of these reductions was not discussed (Cruz et al., 2007).

In our second experiment we hypothesized that the addition of a heat exchanger set at 95°C would work synergistically with the UHPH to reduce the number of *P. lautus* spores. However, no reduction was achieved. In our system there was not an accurate method of recording the output temperature but it was assumed the samples were reaching a temperature of 95°C while the pressure treatments were being applied.

In a study conducted to test the effect of high pressure homogenization and heat on spores in ice cream mix, the highest level of destruction was 68% at a pressure of 200MPa and 50°C (Feijoo, et al., 1997). Our results in liquid milk are similar to the minimal inactivation found by the research done on ice cream mix.

Some authors have studied the impact of high pressure and temperature on bacterial spores. It was confirmed in these experiments that spores treated with high pressure germinated but, similarly to our own experiments, no inactivation was observed when the bacterial spores were treated with >550MPa at 37°C. The authors noted that treatment temperature plays a primary role and detected an increase in lethal effect with a rising temperature and pressure (Reineke et al., 2011). As the industry continues to search for sterilization methods of food systems, temperature will be a major contributor to studies using UHPH.

For our third experiment we hypothesized that UHPH on an artificially inoculated acidic system would significantly reduce bacterial spores. Our previous studies on the inactivation of *P. lautus* using ultra-high pressure homogenization indicated potential for reduction of bacterial spores. The analysis conducted on the juice data (SAS 9.3) from heat-shocked samples (Figure 2.10) indicates significant effects ($P<0.05$). Furthermore, Tukey's grouping shows that there was a significant difference between pressures of 0 MPa and 100 MPa, 0 MPa and 300 to 500 MPa, and 100 MPa and 300 to 500 MPa.

The results of our research conducted on *P. lautus* reduction in apple juice concur with a study conducted by Saldo et al., (2009). The group investigated the preservation of apple juice by the application of UHPH. Pressures up to 300MPa were applied to the samples. The study reported a significant decrease in microbial counts at a pressure of at least 200 MPa. Though the main objective of the Saldo et al. (2009) study was to evaluate browning of the juices after being treated with UHPH, a microbial test was included in the research (Saldo et al., 2009). However, in the current research conducted with apple juice, only 100MPa of UHPH was required to produce a significant effect on the microbial spores.

Overall, we are unsure as to why there were some slight numerical reductions observed across heat-shocked samples in the experiments. This could be the result of the pressure treatment causing spore germination and consequently the heat-shock treatment causing reduction. Nonetheless, the reductions observed are not of sufficient significance for claiming these protocols as a method of sterilizing milk or juice.

Based on literature, we knew that the bacterial spores used in these experiments would be highly resistant to environmental stresses. *G. stearothermophilus* is known as

one of the most resistant bacterial spores in nature which may be why we saw more promise of reduction using *P. lautus*. Overall, we cannot statistically say that we were able to inactivate the sporeforming bacteria. Although the treatment didn't reduce spore formers, it is very effective against pathogenic bacteria. In a study done on the effects of UHPH on the pathogens *Listeria monocytogenes* and *Salmonella enterica* in juices, the authors found complete inactivation of *S. enterica* at a pressure of 400MPa. *L. monocytogenes* proved to be slightly more resistant to the treatment but viable counts became undetectable after the juice had been stored at 4°C (Velazquez-Estrada et al., 2011).

CONCLUSION

These studies conclude that low temperature ultra-high pressure homogenization is not an effective method for the inactivation of bacterial spores in non-fat organic milk and store-bought apple juice. Based on all of these experiments, future work involving bacterial spores such as *G. stearothermophilus*, *P. lautus*, and *B. licheniformis* might include investigating the use of higher levels of applied heat in addition to the ultra-high pressure homogenization treatment. Also, future studies could be conducted in model acidic systems to better understand the effect of acid from a store-bought juice.

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APPENDIX

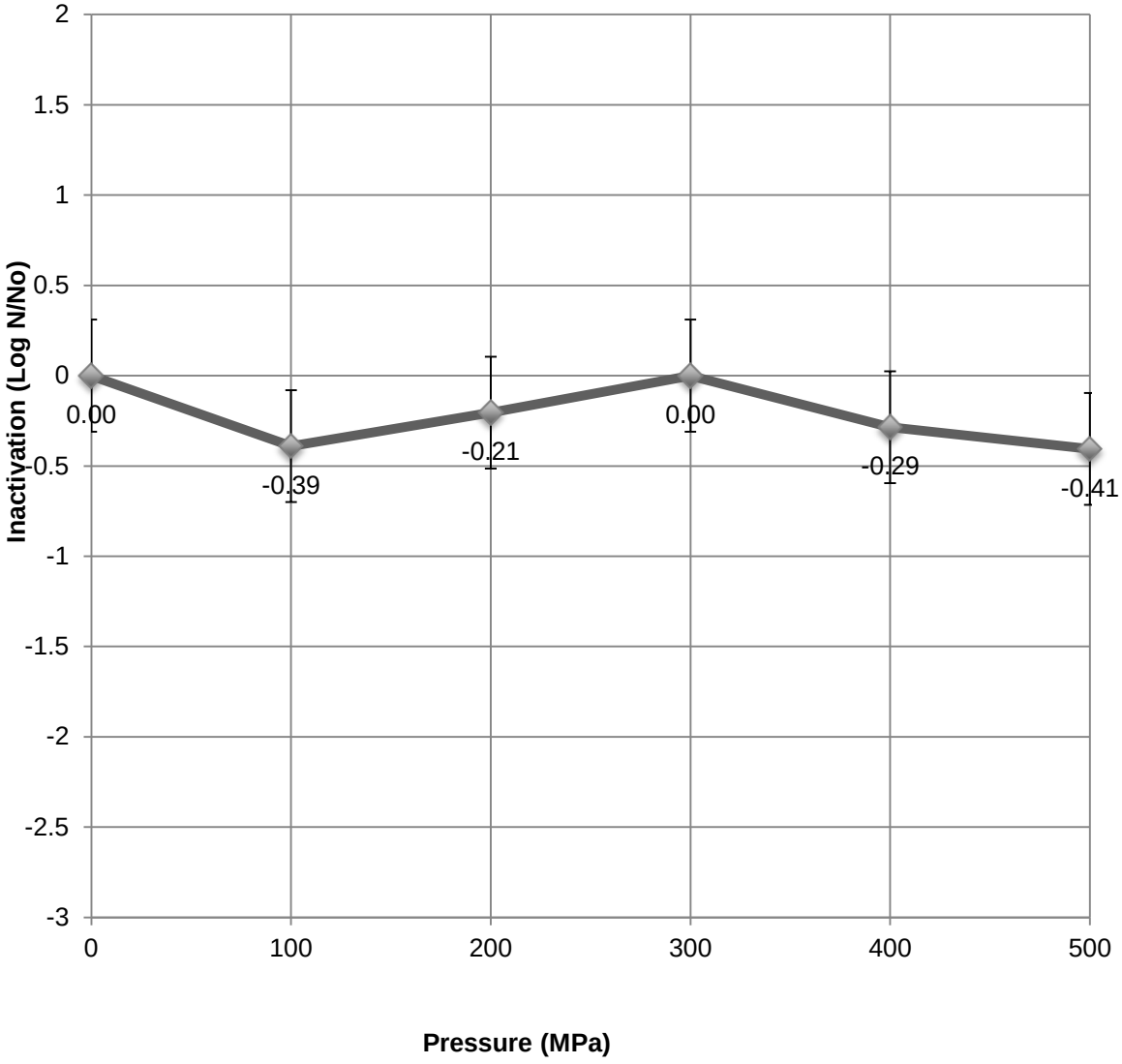

Figure 2.1. Inactivation of *Geobacillus stearothermophilus* spores by ultra-high pressure homogenization with heat shock at 80ºC for 10 min.

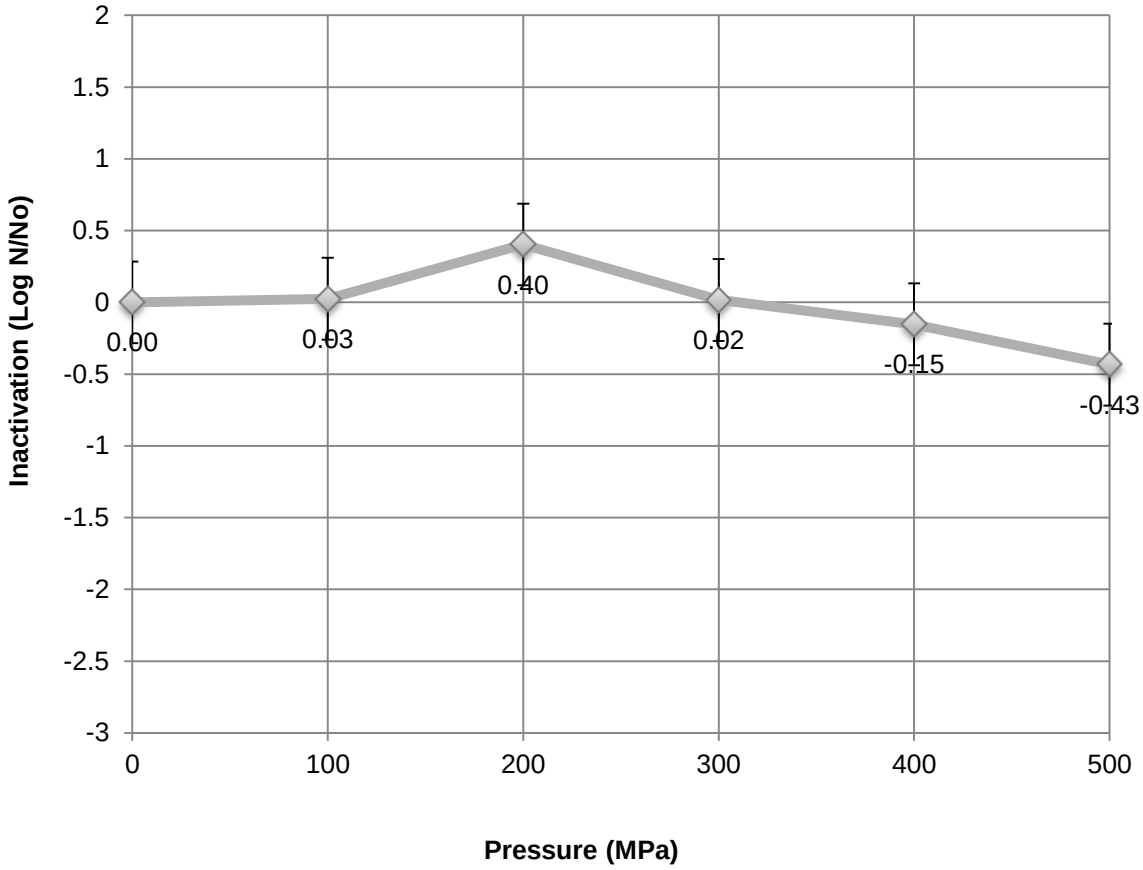

Figure 2.2. Inactivation of *Geobacillus stearothermophilus* spores by ultra-high pressure homogenization without heat shock.

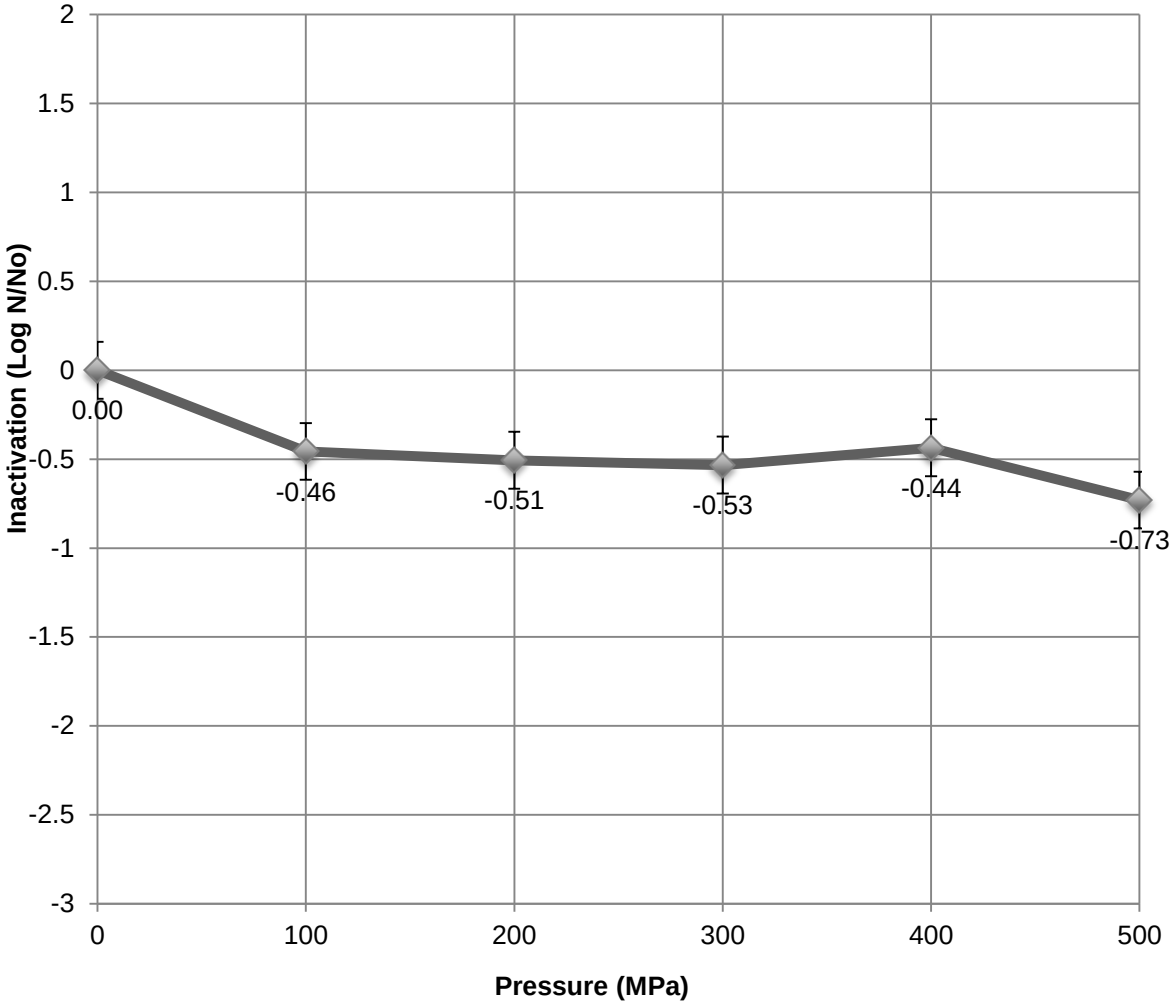

Figure 2.3. Inactivation of *Paenibacillus lautus* spores by ultra-high pressure homogenization with heat shock at 80ºC for 10 min.

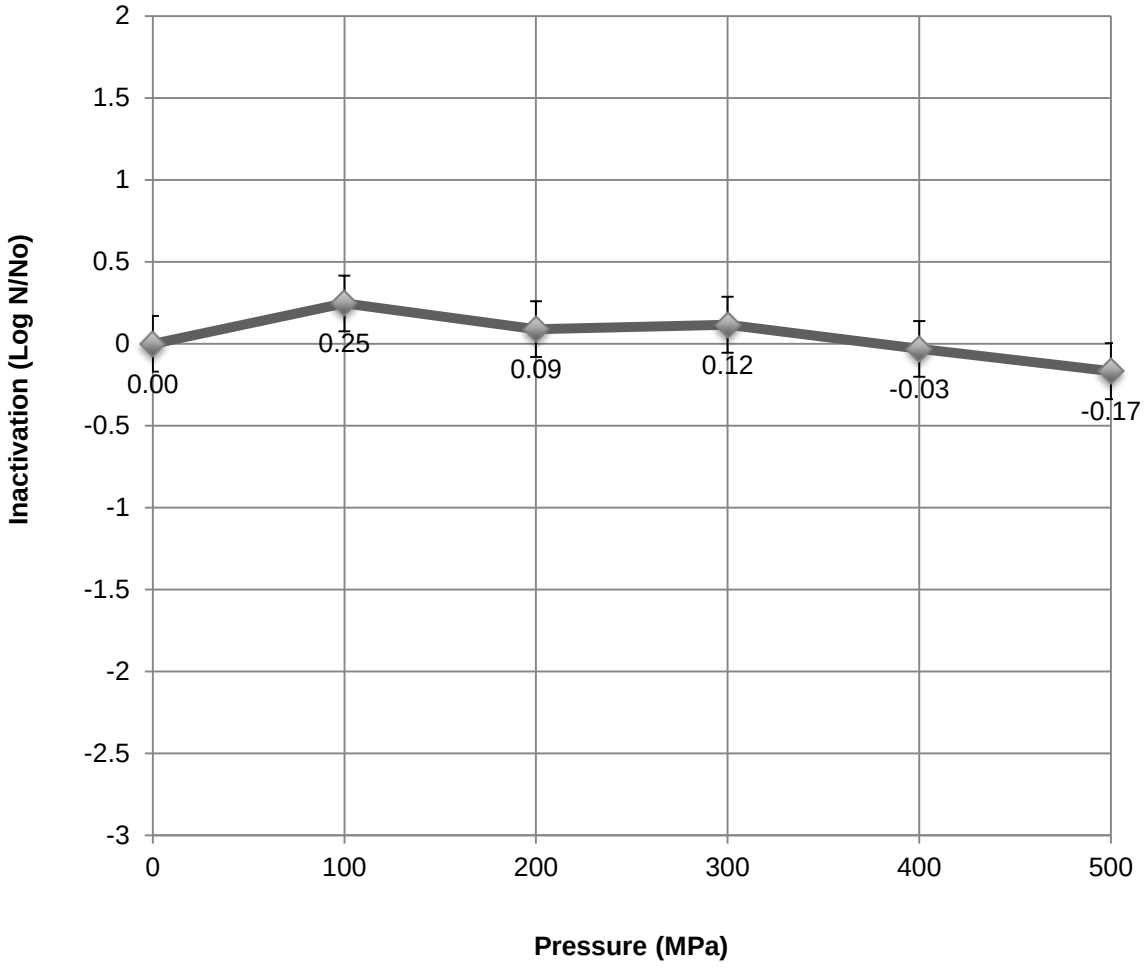

Figure 2.4. Inactivation of *Paenibacillus lautus* spores by ultra-high pressure homogenization without heat shock.

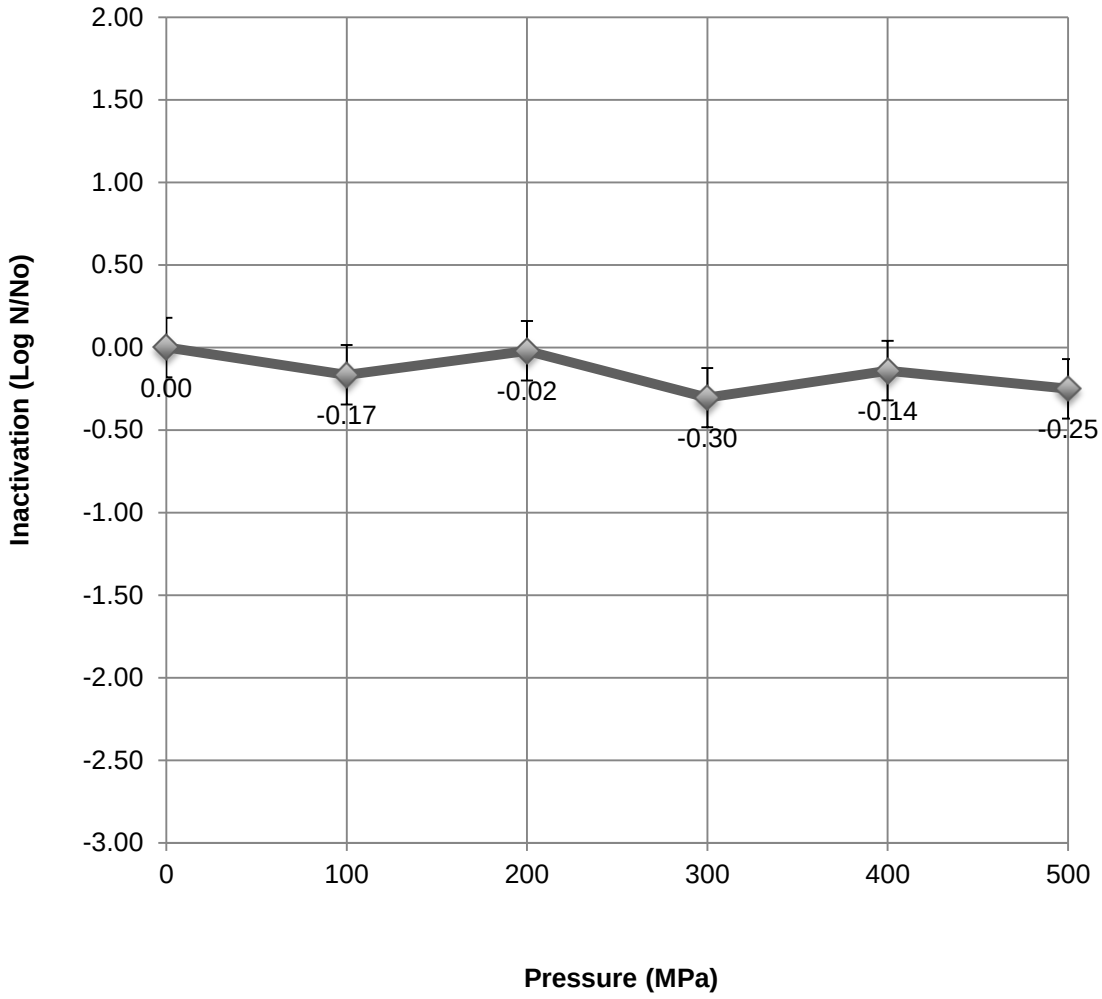

Figure 2.5. Inactivation of *Bacillus licheniformis* spores by ultra-high pressure homogenization with heat shock at 80ºC for 10 min.

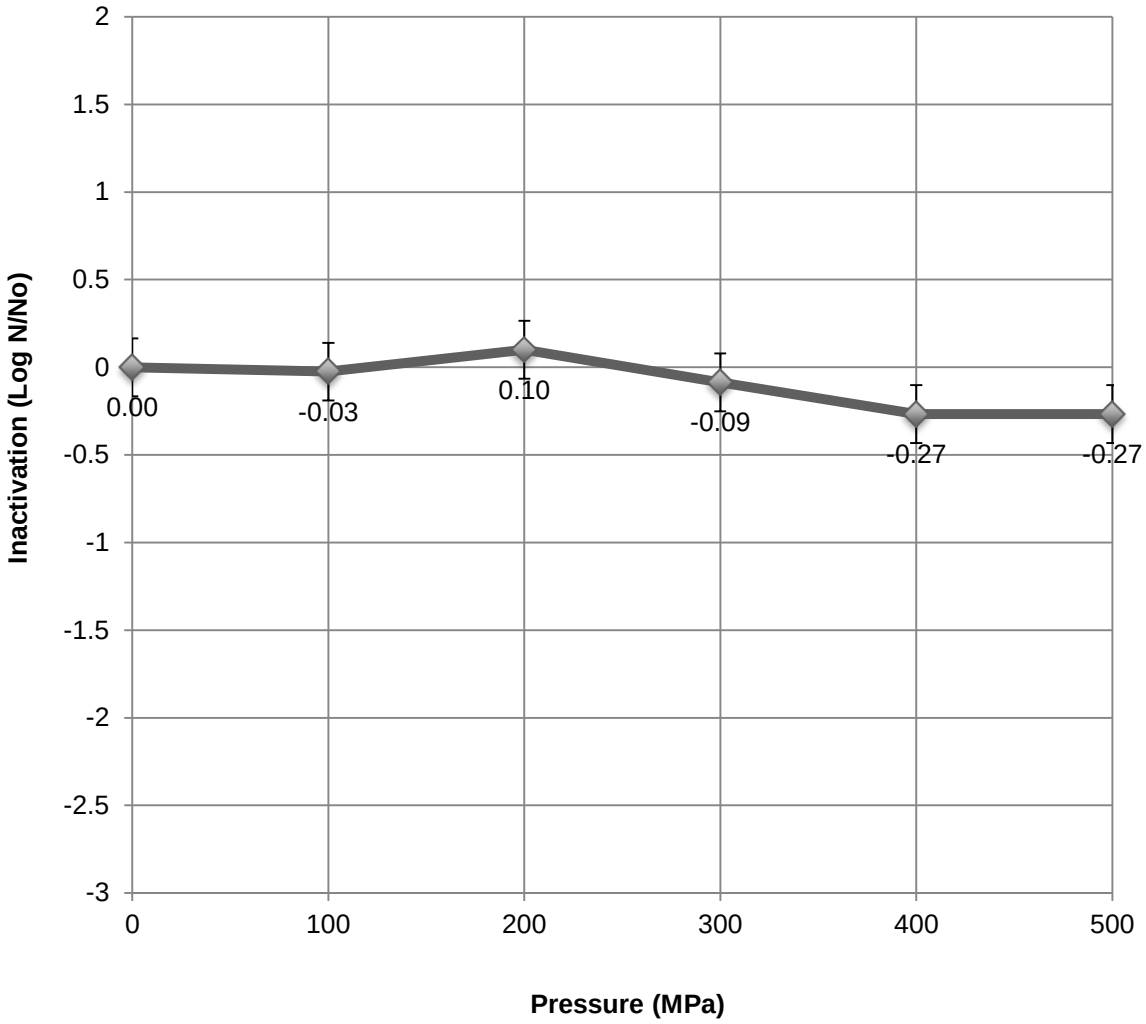

Figure 2.6. Inactivation of *Bacillus licheniformis* spores by ultra-high pressure homogenization without heat shock.

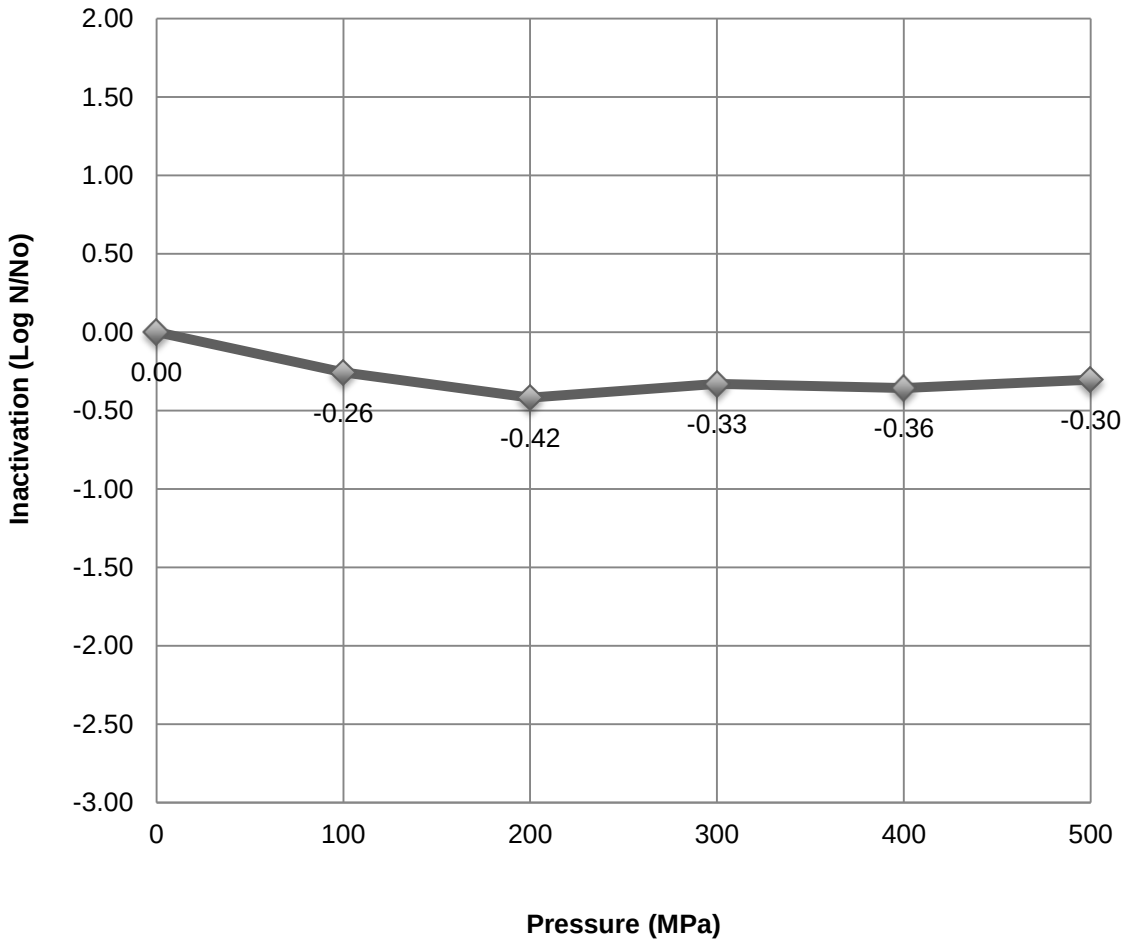

Figure 2.7. Inactivation of *Paenibacillus lautus* spores in non-fat organic milk using ultra-high pressure homogenization at 95ºC and a post treatment heat shock at 80ºC for 10 min.

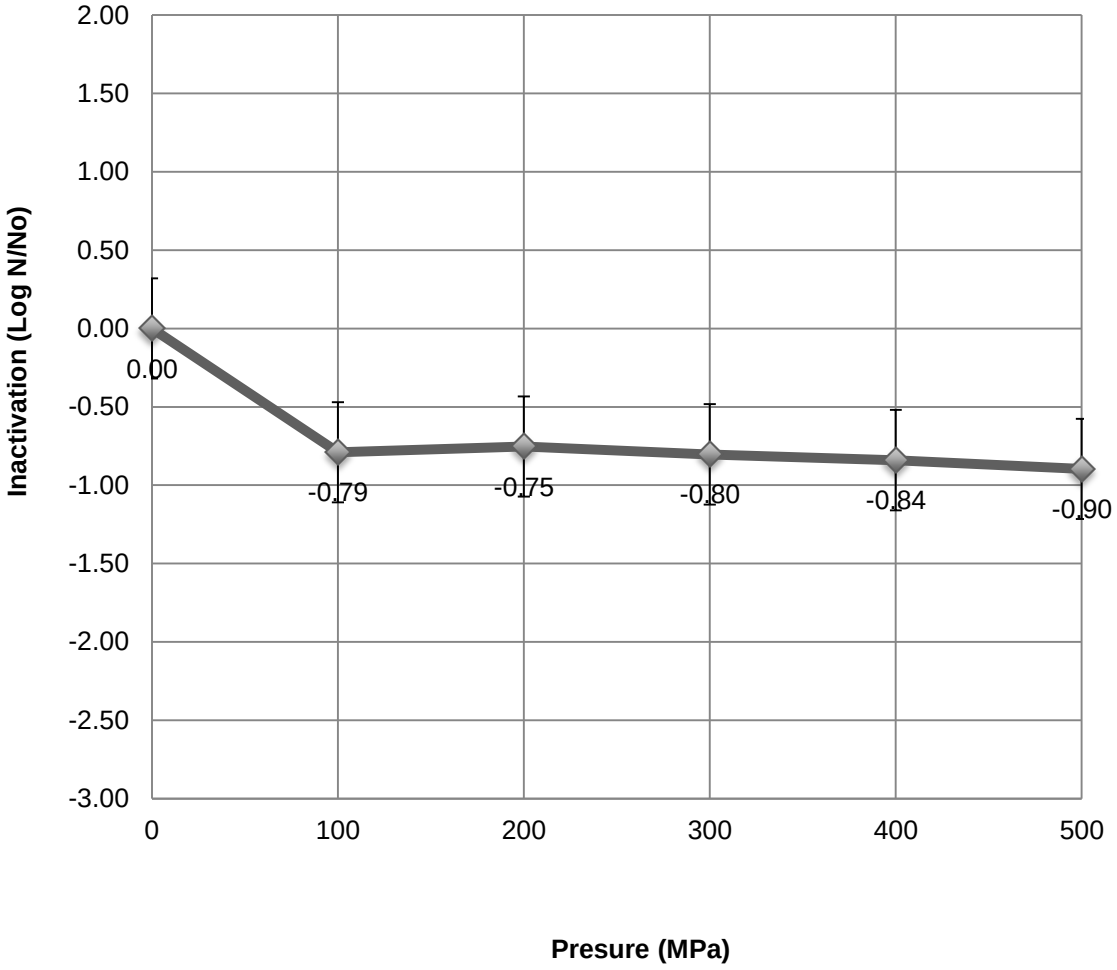

Figure 2.8. Inactivation of *Paenibacillus lautus* spores in non-fat organic milk using ultra-high pressure homogenization at 95ºC and no post treatment heat shock.

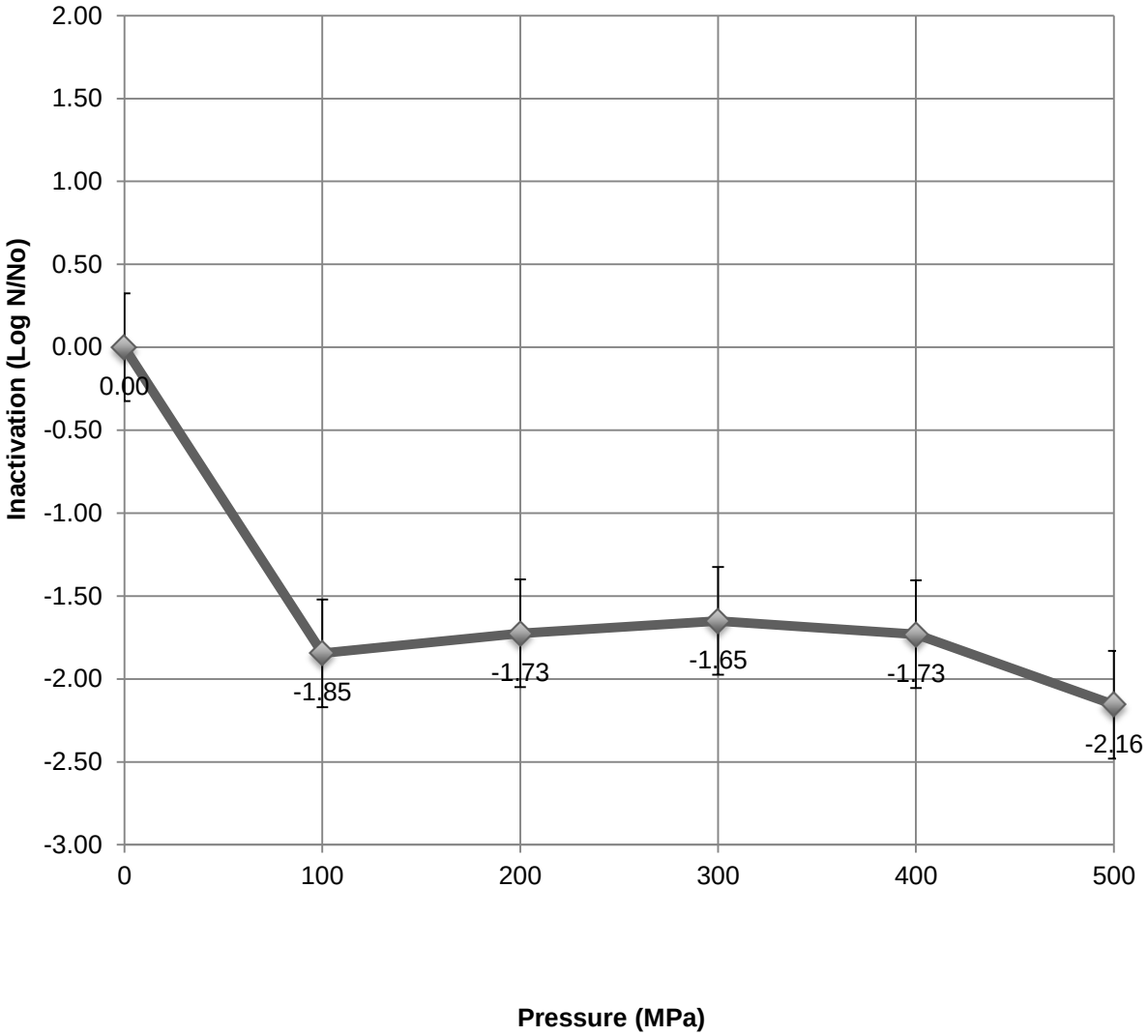

Figure 2.9. Inactivation of *Paenibacillus lautus* spores in apple juice using ultra-high pressure homogenization and a post treatment heat shock at 80ºC for 10 min.

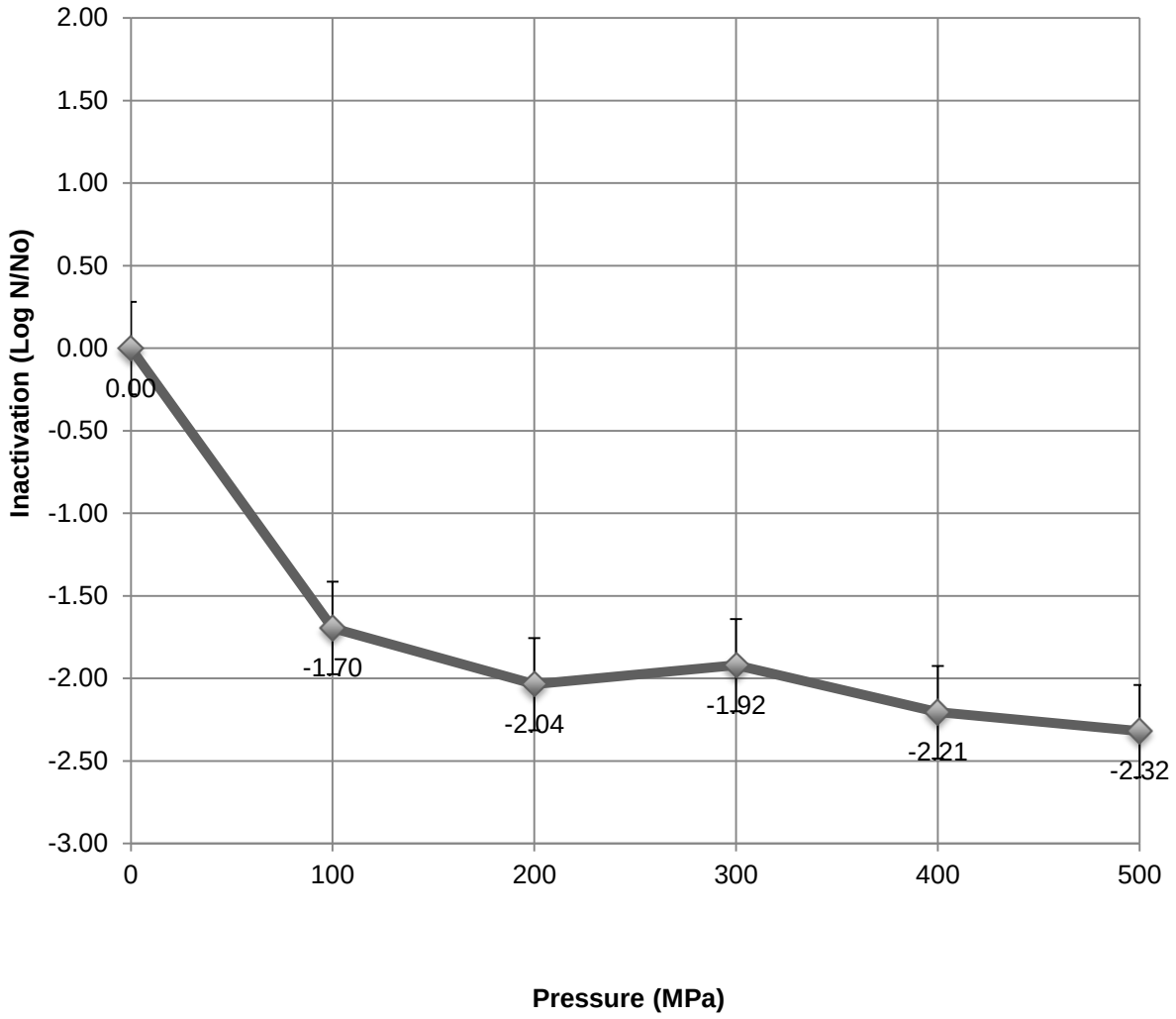

Figure 2.10. Inactivation of *Paenibacillus lautus* spores in apple juice using ultra-high pressure homogenization and no post treatment heat shock.

VITA

Julie Gidley was born on August 19, 1986 in Wabash, Indiana. She graduated from Northfield High School in May 2005. She then attended Indiana University where she received a Bachelor's Degree in Nutrition Science in May 2009. Under the direction of Dr. David Golden and Dr. Federico Harte, Julie began her Master of Science studies at the University of Tennessee in August 2012. She received her graduate degree in Food Science and Technology in May 2014 and plans to continue learning in the field of Food Science through working in the industry.